DIFFERENTIATION PROPENSITY, SAFETY, AND THERAPEUTIC POTENTIAL OF MESENCHYMAL STEM CELLS OF DENTAL ORIGIN

SABRI BIN MUSA

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

20**20**

University of Malaya

DIFFERENTIATION PROPENSITY, SAFETY, AND THERAPEUTIC POTENTIAL OF MESENCHYMAL STEM CELLS OF DENTAL ORIGIN

SABRI BIN MUSA

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF PhD BY PRIOR PUBLICATION

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

University of Malaya

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Sabri Bin Musa

Registration/Matric No: HHR160001

Name of Degree: PhD by Prior Publication

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): Differentiation propensity, safety, and therapeutic potential of Mesenchymal stem cells of dental origin

Field of Study: Dental Studies (Regenerative Medicine)

I do solemnly and sincerely declare that:

(1)I am the sole author/writer of this Work;
(2)This Work is original;
(3)Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4)I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5)I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6)I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

University of Malaya

ABSTRACT

Introduction: A new area in medical research has emerged with the discovery of mesenchymal stem cells (MSCs) and their ability to differentiate into other cell types. Specifically, these cells are found in various tissues and have been utilized in the field of regenerative medicine to support tissue repair as well as in cell replacement. Lately, MSCs of dental origin have been the main players used for regenerative purposes although subtle differences based on their tissue source separate them. **Objectives:** This study aims to determine the differential propensity, safety, and potential therapeutic applications of dental-derived MSCs. This involves examining various aspects of the cells ranging from their basic characteristics, dynamics of miRNA, differential potential, and immunomodulatory effects. **Methods:** The first investigation compared the basic characteristics of deciduous and permanent teeth in terms of their proliferation rates, gene expressions profile, and lineage-specific proclivity over 5 passages. The second study looks into the differentiation ability of dental stem cells in particular, into hepatic lineage cells so as to examine the efficacy of human platelet lysates by substituting fetal bovine serum. The third research work deals with immunologic properties of dental stem cells originated from deciduous and permanent teeth, and periodontal ligament cells. All these cells were subjected into a long-term culture condition. The proteins were validated using Luminex platform. The fourth research work compared the immunomodulatory properties of dental pulp from healthy and inflamed teeth. Their immunosuppressive effects were compared on phytohemagglutinin-induced T cell proliferation. The fifth research work profiled the miRNA regulations in dental pulp stem cells in comparison to bone marrow stem cells by subjecting them to ingenuity analysis. Finally, the sixth and seventh works reviewed the use of dental pulp stem cells for cardiac regeneration and wound healing in skin, respectively. **Results:** The dental pulp stem cells from deciduous teeth showed higher proliferation rates over the 5 passages while retaining their plasticity compared to those from permanent teeth which tended towards neuronal lineage. As for substitution of human platelet lysates, the efficacy of dental pulp stem cells compared to fetal bovine serum was reflected in higher proliferation rates and the ability to differentiate into hepatic lineage. The work on immunomodulatory genes established that the dental pulp stem cells of deciduous teeth surpassed other cells in terms of expressing immune genes at a late passage despite being cultured under identical conditions. In comparison to their healthy counterparts, inflamed dental pulp stem cells showed minimal pro-inflammatory tendencies and in stimulating anti-inflammatory responses. In addition, miRNA expression in dental pulp stem cells showed integration as a group rather than having a solitary role in the basic regulation of the stem cells. Finally, the two review papers noted the capability of dental stem cells for cardiac regeneration via the PI3-Kinase/Akt signaling pathway while providing greater understanding of the growth factors and cytokines in stem cells for the management of skin wound healing. **Conclusions:** The results strongly suggest that stem cells of dental origin may be good candidates for regenerative therapy purposes. However, further work is required mainly on *in vitro* and *in vivo* aspects to determine their safety and efficacy for use in regenerative medicine.

University of Malaya

ABSTRAK

Pengenalan: Era baru dalam penyelidikan perubatan telah muncul dengan penemuan sel stem mesenchymal (MSC) dan keupayaannya untuk membeza kepada jenis sel lain. Khususnya, sel-sel ini terdapat dalam pelbagai tisu dan telah digunakan dalam bidang perubatan regeneratif untuk menyokong pembaikan tisu serta penggantian sel. Akhir-akhir ini, MSCs berasal dari gigi telah menjadi peneraju utama yang digunakan untuk tujuan regenerative. Walau bagaimana pun perbedaan halus yang wujud telah memisahkan ia berdasarkan sumber tisu tersebut. **Objektif:** Kajian ini bertujuan untuk menentukan kecenderungan perbezaan, keselamatan, dan potensi aplikasi terapeutik MSCs yang berasal dari kegigian. Dengan itu, pelbagai aspek MSCs yang berasal dari kegigian ini diperiksa termasuk ciri-ciri asas mereka, dinamik miRNA, kepelbagaian potensi pembezaan, dan kesan imunomodulator mereka. **Kaedah:** Penyiasatan pertama membandingkan ciri-ciri asas gigi desidus dan gigi kekal dari segi kadar proliferasi, ekspresi gen, dan keterlibatan spesifik keturunan meliputi 5 peringkat tapisan. Kajian kedua melihat akan kebolehan sel stem pergigian untuk membeza kepada sel keturunan hepatik dan mengkaji keberkesanan lysate platelet manusia yang menggantikan serum janin lembu. Kajian ketiga adalah berkenaan sifat imunologi sel stem pergigian yang berasal dari gigi susu dan kekal dan dari ligament periodontal. Semua sel ini adalah berkaitan dengan keadaan kultur jangkamasa panjang. Protein disahkan menggunakan platform Luminex. Kerja penyelidikan keempat merupakan perbandingan sifat imunomodulator antara pulpa gigi sihat dan radang. Kesan imunosupresif mereka juga dibandingkan dengan phytohemagglutinin terindus oleh proliferasi sel T. Kerja penyelidikan kelima berkaitan regulasi miRNA yang diprofilkan dalam sel stem pulpa gigi dan dibandingkan dengan sel stem sumsum tulang dalam aspek analisis keaslian. Manakala, karya keenam dan ketujuh melihat akan kajian semula penggunaan sel stem pulpa gigi untuk regenerasi jantung dan pengurusan penyembuhan kulit yang luka. **Keputusan:** Sel stem pulpa pergigian dari gigi desidus menunjukkan kadar proliferasi yang lebih tinggi berikutan pemeringkatan dan dalam masa yang sama masih mengekalkan plastisiti mereka. Manakala sel stem pulpa dari gigi kekal lebih cenderung ke arah keturunan neuron. Bagi penggantian lysate platelet manusia, keberkesanan ditunjukkan terhadap kadar proliferasi yang tinggi dan kebolehan untuk membeza kepada keturunan hepatik berbanding dengan serum janin lembu. Kajian terhadap gen imunomodulator menunjukkan bahawa sel-sel stem pulpa gigi desidus mengatasi sel-sel lain dari segi mengekspresikan gen-gen imun pada pemeringkatan lewat walau pun dikultur dalam keadaan yang sama. Berbanding dengan sel stem dari gigi yang sihat, sel stem dari gigi yang radang menunjukkan kecenderungan pro-radang yang rendah dan merangsang tindakbalas anti-inflamasi. Di samping itu, ekspresi miRNA dalam sel-sel stem pulpa gigi menunjukkan integrasi sebagai satu kumpulan jika dibanding dengan peranan bersendiri dalam regulasi asas sel-sel stem. Kajian kedua-dua kertas kerja terakhir telah mencadangkan keupayaan sel stem pergigian terhadap regenerasi jantung menerusi laluan signal PI3-Kinase/Akt dan dalam masa yang sama menambah baik pemahaman mengenai faktor pertumbuhan dan sitokin dalam sel stem untuk pengurusan penyembuhan luka kulit. **Kesimpulan:** Keputusan kajian mencadangkan dengan kuat bahawa sel stem yang berasal dari kegigian mungkin calon yang berpotensi untuk tujuan terapi regeneratif. Walau bagaimanapun kajian lebih lanjut diperlukan terutamanya untuk aspek in vitro dan in vivo untuk menentukan keselamatan dan keberkesanan sel-sel tersebut untuk digunakan dalam perubatan regeneratif.

University of Malaya

ACKNOWLEDGEMENTS

First of all, I would like to express my humble gratitude to Almighty Allah, Most Merciful and Most Gracious for giving me the opportunity to complete this thesis.

The research presented in this thesis was supported by grants from University of Malaya (UMRG/RG073/09/HTM, UM.C/625/HIR/032 and UM.C/HIR/MOHE/DENT/02). The thesis and the series of publications presented would not have been possible without the help of many.

I would like to thank especially Professor Dr. Noor Hayaty Abu Kasim, Dr. Vijayendran Govindasamy, Wijenthiran Kunasekaran, Punitha Vasanthan, Pukana Jeyaraman, Loo Zhang Xin and others under Regenerative Dentistry Research Group, Faculty of Dentistry, University of Malaya for their kind assistance, hard work, support and collaboration.

My sincere thanks to my supervisors Professor Dr. Mohammad Tariqur Rahman and Associate Professor Dr. Mariam Abdullah for their guidance, continuous support and patience throughout the duration of completing this thesis.

Special thanks to my wife, Nur Aireen and my six children who gave me all their love, understanding and courage throughout this journey.

.

University of Malaya

TABLE OF CONTENTS

University of Malaya

University of Malaya

CONCLUSION

University of Malaya

LIST OF TABLES

University of Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

APC	:	Antigen-presenting Cell
ASC	:	Adult Stem Cells
BAFF	:	B-cell Activating Factor
bFGF	:	Basic Fibroblast Growth Factor
BM-MSC	:	Bone Marrow Mesenchymal Stem Cell
BMSC	:	Bone Marrow Stem Cell
CCR	:	C Chemokine Receptor
CD	:	Cluster of Differentiation
CFU	:	Colony-forming Units
CK	:	Cytokeratin
CXCL	:	Chemokine Ligand
CXCR	:	Chemokine Receptor
DC	:	Dendritic Cell
DFSC	:	Dental Follicle Stem Cell
DPSC	:	Dental Pulp Stem Cell
DSC	:	Dental Stem Cell
EGF	:	Epidermal Growth Factor
EGFR	:	Epidermal Growth Factor Receptor
ESC	:	Embryonic Stem Cells
EPT	:	Electric Pulp Test
FBS	:	Fetal Bovine Serum
FGF	:	Fibroblast Growth Factor
GABA	:	Gamma-Aminobutyric Acid

University of Malaya

GMP	:	Good Manufacturing Practice
GTP	:	Good Tissue Practices
GvHD	:	Graft versus Host Disease
HGF	:	Hepatocyte Growth Factor
HLA	:	Human Leukocyte Antigen
HPL	:	Human Platelet Lysate
ICA	:	Islet Cell Aggregate
IFN	:	Interferon
Ig	:	Immunoglobulin
IGF	:	Insulin Growth Factor
IL	:	Interleukin
iPSC	:	Induced Pluripotent Stem Cell
ISCT	:	International Society for Cellular Therapy
KOA	:	Knee Osteoarthritis
MR	:	Magnetic Resonance Imaging
mRNA	:	Messenger RNA
miRNA	:	Micro RNA
MSC	:	Mesenchymal Stem Cell
NK	:	Natural Killer
OA	:	Osteoarthritis
PCR	:	Polymerase Chain Reaction
PDGF	:	Platelet Derived Growth Factor
PDL	:	Periodontal Ligament
PDLSC	:	Periodontal Ligament Stem Cell

University of Malaya

qRT-PCR	:	Quantitative Reverse Transcription Polymerase Chain Reaction
SCAP	:	Stem Cells from the Apical Papilla
SCD	:	Deciduous Stem Cell
SCDIP	:	Inflamed Pulp Deciduous Stem Cell
SGSC	:	Salivary Gland-derived Stem Cell
SHED	:	Stem Cells from Human Exfoliated Deciduous Teeth)
TGF	:	Transforming Growth Factor
VEGF	:	Vascular Endothelial Growth Factor

University of Malaya

SYNOPSIS OF PUBLISHED WORKS

The discovery and characterisation of multipotent mesenchymal stem cells (MSCs) from the bone marrow has led to extensive studies conducted on similar populations from other tissues including dental pulp. More attentions were focused on MSCs due to their therapeutic potential in the repair and replacement of damaged, infected, and aged tissues and organs. Following the isolation of the first MSC-like cells from human dental pulp by Grontos and his co-worker in 2000, different types of dental stem cells were identified namely exfoliated primary teeth (SHED), dental pulp from permanent teeth (DPSCs), buccal mucosa, apical papilla and periodontal ligament (PDLSCs). These cells are easier to obtain, employ less invasive procedures and are less ethical concerns compared to MSCs which are subjected to invasive bone marrow isolation procedures. Nevertheless, numerous issues remain to be resolved for therapeutic applications of stem cells obtained from dental pulp and periodontal ligaments. The following research and review articles provide more insight of the properties, safety and potential roles of dental origin stem cells.

The first published work in the list is 'Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth' (Govindasamy V, Abdullah AN, Sainik Ronald V, **Musa S**, Che Ab. Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH, Journal of Endodontics. 2010, 36(9):1504-1515). In this paper, we compared proliferation rates, gene expression profiles, and the lineage-specific propensity of stem cells derived from human deciduous (SHED/SCD) and permanent teeth (DPSCs) over 5 passages. The proliferation rate for the SHED/SCD was higher (cell number, 25 106 cells/mL; percent of colony-forming units (CFUs), 151.67 10.5; percent of cells in S/G2 phase, 12.4 1.48) than that of the DPSCs (cell number, 21 106 cells/mL; percent of CFUs, 133 17.62; percent of cells in S/G2 phase,

University of Malaya

10.4 1.18). It was observed that fold expressions of several pluripotent markers such as OCT4, SOX2, NANOG, and REX1 were higher (>2) in the SHED/SCD compared to the DPSCs. However, the DPSCs showed higher expressions of neuroectodermal markers PAX6, GBX2, and nestin (fold expression >100). Similarly, higher neurosphere formations and neuronal marker expressions (NF, GFAP) were found in the DPSCs differentiated into neuron-like cells compared with SCD. This study thus demonstrates that both SHED/SCD and DPSCs exhibit specific gene expression profiles, with a clear-cut inclination of DPSCs toward neuronal lineage.

Organ scarcity is a major obstacle in organ transplantations, thus, regenerative medicine such as generating hepatocytes may provide an alternative in therapeutic applications. At present, dental pulp stem cells (SHED/SCD) have emerged as a source for regenerative medicine applications. However, existing protocols for cell culture require fetal bovine serum (FBS) as a nutritional supplement although it may carry the risk of disease transmission. This second original research work titled 'Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells' (Vasanthan P, Gnanasegaran N, Govindasamy V, Abdullah AN, Jayaraman P, Ronald VS, **Musa S**, Kasim NHA, Biochemical Engineering Journal. 2014, 88:142-153). The study was undertaken to examine the efficacy of human platelet lysate (HPL) as a substitute for FBS in terms of proliferation and differentiation of SHED/SCD into hepatic lineage cells. The results show that HPL has a superior effect on the proliferation of SHED/SCD than FBS. In addition, we induced SHED/SCD into hepatic lineage cells which thrived by initiation followed by maturation into functional hepatocytes for a total of 21 days. We observed that during this differentiation process, the gene, protein and its functional profile reiterated *in vivo* liver development thus demonstrating a steady down-regulation of early endoderm markers (GATA4, GATA6, SOX17, HNF4, HNF3, and AFP) with the up-regulation of

University of Malaya

hepatic specific markers (TDO, TO, TAT, ALB, AAT, CK18). We also noticed the presence of CK19 suggesting a progenitor population. Confirmation was done via the expression of pluripotent markers and it was observed that they remained unchanged throughout the experiment period. Our results provide new insights on the ability of SHED/SCD to differentiate into hepatic lineage cells and, most importantly, this can be done in autologous settings where both cell source and HPL are derived from the same donor thus reducing the risk of disease transmission.

In general, although cell viability and functionality are the most common aspects taken into consideration in culturing cells over the long term, they may not truly represent their biological state. Accordingly, in the third original work titled 'Expression patterns of immune genes in long-term cultured dental stem cells' (Jayaraman P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Vasanthan P, **Musa S**, Kasim NH, Clin Oral Investig. 2016, 20(1):109-116) we explored the immune properties in long-term cultured cells. Dental pulp stem cells from deciduous (SHED/SCD; n=3) and permanent (DPSCs; n=3) teeth as well as periodontal ligament stem cells (PDLSCs; n=3) were cultured under identical culture conditions. The immune properties of each cell lines were profiled at passage 2 [P2] and passage 9 [P9] as early and late passages, respectively. This was further validated at the protein level using the Luminex platform. A major shift of genes was noticed at P9 with SHED/SCD being the highest. SHED/SCD cultured at P9 displayed many genes representing pathogen recognition ($P < 0.001$), immune signalling ($P < 0.001$, pro-inflammatory ($P < 0.001$), anti-inflammatory ($P < 0.001$), and immune-related growth and stimulation factor ($P < 0.001$) compared to DPSCs and PDLSCs. Surprisingly, SHED/SCD also expressed many cytotoxicity genes ($P < 0.001$). Communally, the instability of the immune genes in our findings suggests that long-term cultured cells may not be feasible for transplantation purposes. We recommend that a complete biological characterization

University of Malaya

covering all major aspects including immune properties should be made a prerequisite prior to the use of the long-term cultured stem cells in clinical settings.

The fourth original research work in this series of published works titled 'Comparison of immunomodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth' (Yazid FB, Gnanasegaran N, Kunasekaran W, Govindasamy V, **Musa S,** Clinical Oral Investig. 2014, 18:2103-2112) investigated the immunomodulatory properties of dental pulp stem cells derived from healthy SHED/SCD and inflamed pulp deciduous (SCDIP) tissues. The overall hypothesis was that SCDIP possess equal immune properties as SHED/SCD and could be used as an alternative tissue source in regenerative medicine. Assessment was carried out to determine the status of the pulp tissues either healthy or inflamed. Primary cells were established from SHED/SCD and SCDIP, and basic mesenchymal stem cell (MSC) characterizations were conducted. The expression of human leukocyte antigen (HLA), namely HLA-G, HLA-DR, and HLA-ABC were examined in both cell lines using flow cytometry. We further compared the immunosuppressive effects of SHED/SCD and SCDIP on phytohemagglutinin-induced T cell proliferation. Supernatants were tested for cytokine profiling using multiplex array. While SHED/SCD exhibited typical MSC characteristics, SCDIP, on the other hand, did not. Compared with SCDIP, SHED/SCD effectively suppressed mitogen-induced T cells proliferation in a dose-dependent manner, as well as expressed a higher percentage of HLA-ABC and HLA-G. In addition, levels of several cytokines, such as TNF-α, TNF-β, and IL-2, were drastically suppressed in SHED/SCD rather than SCDIP. Further, a high level of IL-10, an important anti-inflammatory cytokine, was present in SHED/SCD compared with SCDIP. These findings suggest that SCDIP are highly dysfunctional in terms of their stemness and immunomodulatory properties.

University of Malaya

MicroRNAs (miRNAs) are small non-coding RNAs that regulate the translation of mRNA into protein and have a crucial role in almost all biological activities. However, their identification from mesenchymal stem cells (MSCs) especially from dental pulp is poorly understood. The fifth paper titled 'Differential expression of basal microRNAs' patterns in human dental pulp stem cells' (Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, **Musa S**, Abu Kasim NH, J Cell Mol Med. 2015, 19(3):566-580). A total of 104 known mature miRNAs were profiled using real-time PCR. Notably, we observed 19 up-regulated miRNAs and 29 significantly down-regulated miRNAs in DPSCs, respectively compared to the bone marrow MSCs (BM-MSCs). The former was subjected to ingenuity pathway analysis and composed into 25 functional networks from which the top 2 were chosen comprising 10 miRNA (hsa-miR-516a-3p, hsamiR-125b-1-3p, hsa-miR-221-5p, hsa-miR-7, hsa-miR-584-5p, hsa-miR-190a, hsa-miR-106a-5p, hsa-mir-376a-5p, hsa-mir-377-5p, and hsalet-7f-2-3p). A prediction was carried out on the target mRNAs and associated biological pathways regulated by each of the miRNA. We paid special attention to hsa-miR-516a-3p and hsa-miR-7-5p as these miRNAs were highly expressed upon validation with qRT-PCR analysis. We further proceeded with loss-of-function analysis of these miRNAs and observed that hsa-miR-516a-3p knockdown induced a significant increase in the expression of WNT5A. Likewise, the knockdown of hsa-miR-7-5p increased the expression of EGFR. Nevertheless, further validation revealed the role of WNT5A as an indirect target of hsa-miR-516a-3p. These results provide new insights into the dynamic role of miRNA expression in DPSCs. In conclusion, using miRNA signatures in humans as a prediction tool will enable us to elucidate the biological processes occurring in DPSCs.

The sixth article included in this thesis titled 'Dental stem cells as an alternative source for cardiac regeneration' (Xin LZ, Govindasamy V, **Musa S**, Abu Kasim NH,

University of Malaya

Medical Hypotheses. 2013, 81(4):704-706) is based on published work in relation to available data suggesting that there is a coherent idea to hypothesize that DSCs as an alternative source for cardiac regeneration. As we all know, dental tissues contain stem cells or progenitors which have a high proliferative capacity, are clonogenic *in vitro,* and can differentiate into multiple type cells involving neurons, bone, cartilage, fat, and smooth muscle. Numerous experiments have demonstrated that multipotent stem cells are not rejected by immune systems making it possible to use them in allogeneic settings. In addition, these remarkable cells are easily and abundantly available and require less invasive isolating procedures compared to bone marrow aspiration. Here we propose dental stem cells as likely candidates for cardiac regeneration based on their immature characteristics and propensity towards cardiac lineage via the PI3-Kinase/Akt signalling pathway.

In the seventh published work which is included in the thesis titled 'Stem cells conditioned medium: a new approach to skin wound healing management' (Pukana Jayaraman, Prakash Nathan, Punitha Vasanthan, **Sabri Musa** and Vijayendran Govindasamy, Cell Biol Int 37 (2013) 1122–1128) we proposed the possibility of using stem cells conditioned medium as a novel and promising alternative in healing treatments for skin wounds. Stem cell biology has gained remarkable interest in recent years, driven by the hope of finding cures for numerous diseases including skin wound healing through transplantation medicine. Initially upon transplantation, these cells home to and differentiate within the injured tissue into specialised cells. Conversely, it is now appeared that only a small percentage of transplanted cells integrate and survive in host tissues. Thus, the foremost mechanism by which stem cells participate in tissue repair seems to be related to their trophic factors. Indeed, stem cells provide the microenvironment for a wide range of growth factors, cytokines and chemokines, which can be broadly defined as the stem cells secretome. Under *in vitro* condition, these

University of Malaya

molecules can be traced from the conditioned medium or spent media harvested from cultured cells. Conditioned medium now serves as a new treatment modality in regenerative medicine and has shown a successful outcome in some diseases. With the emergence of this approach, we described the possibility of using stem cells conditioned medium as a novel and promising alternative in the treatment of skin wounds. Numerous pre-clinical data have shown the possibility and efficacy of this treatment. Despite this, significant challenges need to be addressed before translating this technology to the bedside.

In summary, we have presented a synopsis of five original research works and two review papers in this body of work on differentiation propensity, safety, and therapeutic potential of mesenchymal stem cells of dental origin. The candidate is the primary author, if not also the corresponding author in four of the seven publications. The work presented here was conducted and published over a period of seven years (2010-2016) under three research grants (UMRG/RG073/09/HTM, UM.C/625/HIR/032 and UM.C/HIR/MOHE/DENT/02) in which the candidate was the principal investigator. The candidate is the primary researcher directly involved in the study design concept, acquisition of data and analysis as well as writing and revision of manuscripts. However, the contribution from other authors and collaborators are acknowledged as the publications would not have been possible without their research expertise, contributions, and input.

University of Malaya

CHAPTER 1: INTRODUCTION

1.1 Research issues

The discovery of Mesenchymal Stem Cells (MSCs) and their multipotency attributes have opened up an entirely new field of medical research due to the range of different cells that multipotent stem cells can differentiate into (Pittenger et al., 1999). This has allowed the cells to be used in regenerative medicine as they are able to both support tissue repair as well as differentiate and to form and replace permanently lost tissue (Bussolani, 2011). To be defined as MSCs, the cells must have the ability to at least form osteoblasts, chondroblasts, and adipocytes (Dominici et al., 2006). MSCs can be derived from bone marrow, placenta, umbilical cords, adipose tissue, adult muscle, corneal stroma, and dental sources (Prockop, 1997). While several types of MSCs exist, their source tissue provides subtle differences in terms of character and differentiation potential. For example, umbilical cord stem cells have a lower ability to form adipocytes but greater osteogenic capacity (Kern et al., 2006; Han et al., 2017). Recent advances in regenerative medicine have brought dental-derived MSCs to the fore as suitable tool for tissue regeneration. This is also due to the less controversial ethical concerns involving their usage and convenience of harvesting. The first MSCs isolated from dental origin were derived from the third molar dental pulp (Gronthos et al., 2000) and subsequently from deciduous and periodontal ligaments (Miura et al., 2003, Seo et al., 2004). The basic outcomes of MSCs of dental origin have been minimal and has led to the cultivation and scaling-up of MSCs using human platelet lysates in providing autologous settings for cell-based regenerative medicine (Govindasamy et al., 2011). As such, long term cultivation has been considered in order to obtain sufficient amounts of cells for the purpose while retaining their plasticity for therapeutic reasons. Investigations have revealed the ability of human PDLSCs to differentiate into hepatic cells and insulin-producing cells indicating their propensity to differentiate into

University of Malaya

endodermal lineage (Kawanabe et al., 2010; Lee et al., 2014). On this note, MSCs of dental origin need not be limited to tooth regeneration purposes but also beyond that. Nevertheless, many unanswered questions regarding this relatively untapped resource remain in terms of their isolation, expansion, parameters for monitoring their clinical efficacy, and the proper biological source of dental MSCs in sufficient numbers for therapeutic purposes. This research work seeks to address some of these questions and the proposals made based on results from several publications from around the world. In addition, it also explores several main issues related to the differential propensity, safety efficacy, and potential therapeutic applications of dental-derived mesenchymal stem cells.

1.2 Basic characteristics of MSCs of dental origin

This section describes the basic characteristics of MSCs of dental origin. Although stem cells originate from similar anatomical sites, it must be noted that dental pulp is present in donors of all ages (Abatangelo et al., 2011) and may have some degree of differences in their basic characteristics. That entails the identification and evaluation of the key factors such as their growth kinetics and cell cycles. This study compares such factors between sources of these stem cells involving deciduous and permanent teeth and from patients of different ages to determine which have the optimal basic characteristics.

1.3 Optimising the cultivation of MSCs

This study also explores various aspects involved in the cultivation of MSCs as an optimal culturing technique is essential for their fully efficacious growth and

University of Malaya

function (van der Sanden et al., 2010). While specific culture media for MSCs of dental origin already exist, they are not without inherent limitations. For example, fetal bovine serum (FBS) can lead to phenotypical variations due to their varied composition (van der Valk et al., 2010). Thus, this study looked at growth rates and the concentration and exposure times of various possible culture media to determine an optimal culture medium for the cells.

In addition to the culture media, another aspect of cultivation examined involved establishing the best source of the dental pulp to be used. While dental pulp has proven to be a good source of MSCs, healthy pulp is fairly difficult to obtain under normal circumstances without extracting the teeth. Moreover, the extraction of otherwise healthy teeth remains an ethical challenge (Béry, 2014). Thus, alternative sources of these cells had to be considered. This work covers studies that test the efficacy of these alternative sources of cells such as those from inflamed rather than healthy dental pulp.

1.4 Immunomodulatory effects and mRNA of MSCs of dental origin

Another cultivation aspect leading into the next objective of this study as well as providing an understanding of the molecular aspects of MSCs of dental origin was also sought and involves illustrating the mechanisms of their immunomodulation. It is hoped that stem cells, being capable of repeated cell division, can be a renewable resource especially if they possess adequate viability and functionality. However, this gives rise to the issue of how long such viability and functionality will remain at a given standard of the cultures. Thus, this study examines this aspect and the effects of long term cultures of the cells including their immunity properties.

In addition, this study investigates the micro RNA of the stem cells. These molecules have a key role in gene expression and the signaling network of all cells

University of Malaya

(Bartel, 2004). Further, they have a significant impact on almost every biological process, including those relating to the main features of stem cells such as self-renewal and differentiation (Li et al., 2017; Mathieu & Ruohola-Baker, 2013). However, such identification is poorly understood in the case of MSCs of dental origin. Thus, this work explores the profiling of miRNA in such cells in attempting to understand their appropriate biological progressions.

1.5 Potential applications of MSCs of dental origin

This study then explored the various ways in which such knowledge of MSCs can potentially be applied in modern day medicine. While the multipotency of the cells offers a range of possible treatments some merit greater attention than others. Thus, this work investigates several major organs and their disorders including post myocardial infarction of the heart and liver failure. Current management of these issues mainly involves lifelong preventive measures (Rosenwasser et al., 2013), medication (Chatterjee & Davies, 2015) or even invasive methods like transplantation which, in turn, have issues of their own such as side effects, organ shortage (Saidi & Hejazii Kenari, 2014), or the need for immunosuppressions (Beyar, 2011). The theoretical ability of stem cells to address such issues and their advantages are thus studied in this work.

In addition, this study also examines possible way of treating skin wounds where the methods used to culture this particular type of cell can potentially be applied in treating certain conditions. While there are limitations to what the cells themselves can do, it should be remembered that the culture media used is replete with growth factors and cytokines which stimulated the growth of the cells in the first place (van der Sanden

University of Malaya

et al., 2010). This study thus examines whether or not the presence of such trophic factors can succeed where the cells cannot.

1.6 Research questions

1. What is the proliferation rate, gene expression profiles and lineage propensities of SDC and DPSCs?
2. Can HPL substitute FBS in terms of proliferation and differentiation of SCDs into hepatic lineage cells?
3. Are the immunity genes of long-term cultured cells of SHED, DPSC, and PDLSC stable and feasible for transplant purposed?
4. Does SCDIP function in terms of stemness and immunomodulatory properties?
5. What is the expression profile of miRNAs in DPSCs as compared to BM-MSCs?
6. Can dental stem cells be used for cardiac regeneration?
7. Can the stem cells conditioned medium be an alternative treatment in healing skin wounds?

1.7 The aim of the study

The aim of this study was to determine the differential propensity, safety efficacy, and potential therapeutic applications of dental-derived mesenchymal stem cells.

1.8 The objectives of the study

MSCs of dental origins were investigated to:-

(1) compare cellular, morphological, and cultural characteristics (Publications 1-3)
(2) determine the dynamics of miRNA expression (Publications 4 & 5)
(3) determine the differential potential (Publications 6 & 7)
(4) analyse the immunomodulatory effects and therapeutic potentials (Publications 4-7)

University of Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 Stem Cells

Stem cells exist in almost all tissues in the body, including in neural (Gage, 2000), gastrointestinal (Potten, 1998), and adipose tissue (Cawthorn et al., 2012), and in hepatic (Forbes et al., 2002), hematopoietic (Weissman, 2000), epidermal (Watt, 1998) and mesenchymal stem cells (Pittenger et al., 1999). They are immature and unspecialized cells that have potential to develop into many different cell lineages via differentiation (Slack, 2008). All stem cells differ from other cells in terms of three general properties: they are unspecialized; capable of dividing and renewing themselves; and can become specialized cells through differentiation (Telles et al., 2011).

2.1.1 Classification of stem cells

Stem cells are naturally present in the human body and can be divided into two broad groups which are naturally present in the human body, namely, adult stem cells and embryonic stem cells (Rimondini et al., 2009). This classification is based on their residency (Guleria et al., 2014). Besides these two types of naturally present human stem cells there are also induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006).

2.1.1.1 Adult stem cells (ASCs)

ASCs can be defined as the stem cells found in any developed tissues in the organisms (Bongso & Richards, 2004) and are also known as somatic stem cells or postnatal stem cells (Egusa et al., 2012). They are undifferentiated multipotent or

University of Malaya

unipotent cells that can be found in most adult tissues and organs, and can only differentiate into a limited number of cell types. These cells can be harvested from different kinds of tissues in the body such as bone marrow, umbilical cords, amniotic fluids, brain tissue, liver, pancreas cornea, dental pulp, and adipose tissue (Guleria et al., 2014). The most recognized ASCs are hematopoietic stem cells and mesenchymal stem cells (Pittenger et al., 1999). ASCs are believed to reside in "stem cell niches", a specific area of each tissue. Although few cells are present in adult tissue compared to embryonic tissues, these adult stem cells will undergo self-renewal and differentiation to maintain healthy tissues and repair injured ones (Egusa et al., 2012).

2.1.1.2 Embryonic stem cells (ESCs)

ESCs are the cells derived from the inner cell mass of the blastocyst during early embryonic (morula stage) development. They are pluripotent and capable of differentiating into all cell types from three germ layers (Thomson et al., 1998). The inner cell mast is selected as the source of ESCs and they are harvested through immunosurgery procedures (Heins et al., 2004). Two advantages of ESCs are the capacity to differentiate into any cell type in the body and the ability to self-replicate for numerous generations, while their disadvantages of these cells are the ethical issues involved and their virtually unlimited proliferation and differentiation capacity. Clinically observed teratoma is an example of ESCs growing into wrong tissues (Guleria et al., 2014).

University of Malaya

2.1.1.3 Induced pluripotent stem cells (iPSCs)

iPSCs are cells which were generated via the genetic manipulation of somatic cells. Human iPSCs cells are similar to ESCs in terms of their morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. In addition they are pluripotent, able to differentiate into all cell types from three germ layers *in vitro,* and capable of forming teratomas. Somatic cells that have been reprogrammed to a pluripotent state by the introduction of a group of transcription factors namely, Sry-related high- mobility-group (Sox)-2, Lin-28 homolog (Lin)-28, Cellular-myelocytomatosis viral oncogene homolog (c-Myc), Octamer (Oct)-3/4, Kruppel-like factors (Klf)-4 and Nanog (Takahashi & Yamanaka, 2006) eventually generate iPSCs. The advantage of the iPSCs technology is that it can generate the specific stem cells of a patient, a classic example of which was demonstrated in a study by Freund et al. (2010) who successfully generated cardiomyocytes from the skin fibroblast of a patient with hereditary haemorrhagic telangiectasia. There are a few other breakthroughs in this field. Ieda et al. (2010) used a combination of cardiac transcription factors to directly reprogrammed cardiac fibroblast into cardiomyocytes. Yamanaka and Yoshida (2011) proposed iPSCs as a potential source for cardiac regeneration if the reprogramming efficiency can be improved and safety issue are addressed before progressing to clinical application.

2.2 Mesenchymal Stem Cells (MSCs)

MSCs are adult multipotent stem cells that have two unique properties. The first is their capacity for self-renewal beyond Hayflick's limit, a property shared by embryonic stem cells and the second is their ability to differentiate into mesenchymal

University of Malaya

and non-mesenchymal mature cell lines such as fat, bone, cartilage and neural cells (Caplan, 1991). Since then, extensive studies have been done on MSCs from various sources.

In dentistry, several MSCs have been discovered since a few decades ago such as bone marrow stem cells (BMSCs) from orofacial bones, dental tissue-derived stem cells, oral mucosa-derived stem cells, periosteum-derived stem cells and salivary gland-derived stem cells (SGSCs) (Egusa et al., 2012).

2.2.1 MSCs of Dental Origin

The first mesenchymal stem cells (MSCs) of dental origin were discovered from dental pulp tissues of adult human impacted third molars teeth in year 2000. These cells were found to be clonogenic, highly proliferative, and able to produce tissue. They were termed Dental Pulp Stem Cells (DPSCs) (Gronthos et al., 2000). Stem cells can be obtained from various tissues in the oral and maxillofacial regions (Chalisserry et al., 2017). To date, different types of MSCs of dental origin have been discovered and were named according to the site where they were harvested. The inner tooth pulp of adult molars (DPSCs), the pulp of deciduous exfoliated teeth (SHED), and the apical papilla (SCAP) are locations for MSCs in teeth. From the supporting tissues, dental MSCs can be derived from the dental follicle (DFSCs), the periodontal ligament (PDLSCs), the gingiva (GFSCs), and from tissue taken during dental implants (DISCs) (Sharpe, 2016). The expression markers and differentiation propensity characteristics of these different MSCs are not identical despite all of them being located in tooth-related structures (Park, 2015).

University of Malaya

2.2.1.1 Dental pulp stem cells (DPSCs)

DPSCs reside in the dental pulp. Dental pulp is a dentine-covered loose connective tissue (Hosoya & Nakamura, 2015). The first population of DPSCs was originated from the neural crest-mesenchymal while another one is derived from the dental laminae (Suchanek et al., 2007) and they can either regenerate new stem cells or can undergo differentiation processes depending on the specific signals from their environment. Studies demonstrate that adult dental pulps contain precursors that are capable of developing into odontoblasts under appropriate signals (About et al., 2000; Alliot-Licht et al., 2005). DPSCs are acknowledged to have a high proliferating potential and can differentiate into osteoblasts, adipocytes, chondrocytes, hepatocytes and neurons *in vitro* as well as form dentin-like structures *in vivo* (Saito et al., 2015). DPSCs have been shown to be positive for cell surface markers as expressed by BMMSCs such as CD44, CD73, CD105 and STRO-1, while being negative for CD45, CD34, and CD14 (Huang et al., 2009). DPSCs and BMMSCs were shown to have similar morphologies and flow cytometry profiles, with the former showing a higher proliferation rates and numbers of cells and better multipotency compared to BMMSCs (Alge et al., 2010).

2.2.1.2 Stem cells from human exfoliated deciduous teeth (SHED)

SHED are MSCs from human exfoliated deciduous teeth and have the ability of differentiating into various cell types including neural cells, adipocytes, and odontoblasts (Huang et al., 2010). Moreover, they have been reported to have potential for generating significant amounts of alveolar and orofacial bone for tissue generation (Grontos et al., 2000; Miura et al, 2003). What differentiates SHED from DPSCs besides being isolated from the pulp tissue of the crown of exfoliated deciduous teeth is

University of Malaya

their higher proliferation rate, higher cell-population doublings, formation of sphere-like cell-cluster, *in vivo* osteogenic differentiation capacity, and inability to form a dentin–pulp-like complex. SHED forms sphere-like clusters but can grow as individual fibroblastic cells after separation (Miura et al., 2003). Compared to other sources of MSCs of dental origin, SHED are found to proliferate faster due to their less matured nature (Kabir et al., 2014). SHED has been found to express CD146, CD73, CD90, CD105, and CD29 surface markers and were negative for CD14, CD45, CD34, CD25, and CD28 markers (Yildirim et al., 2016).

2.2.1.3 Periodontal ligament stem cells (PDLSCs)

Stem cells from the periodontal ligaments were first isolated from extracted human third molars (Seo et al., 2004). The periodontal ligament is a specialized tissue located between the cementum and the alveolar bone where it functions to maintain and support the teeth (Chalisserry et al., 2017). PDLSCs are able to differentiate into osteoblasts, chondrocytes, and adipocytes (Gay et al., 2007) and can form collagen fibers, similar to Sharpey's fibers, connecting to the cementum-like tissue, suggesting their potential to regenerate PDL attachments *in vivo* (Seo et al., 2004). PDLSCs have been shown to express MSC markers such as CD9O, CD29, CD166, CD105, and CD44, while being negative for HLA-DR, HLAB27, COl5, CD33, CD34, CD45, CDI4, CD2, CD7, CD3, COl17, but weakly positive for CD13 (Trubiani et al., 2005). The expressions of specific cell surface markers of PDLSCs have also been positive towards the STRO-1 and CD146. Furthermore, these cells can be used to develop roots or the periodontal complex to support normal tooth functions (Sonoyama et al., 2006).

University of Malaya

2.2.1.4 Stem cells from apical papilla (SCAP)

Dental papilla is derived from ectomesenchyme induced by the overlying dental lamina during tooth development and this organ plays an important role in tooth development as it will later convert to pulp tissues after being encased by the dentine. Apical papilla is the dental papilla tissue that is loosely attached to the apex of a developing root and can be easily detached with tweezers (Kumar, 2011). A new population of stem cells isolated from root apical papilla which is known as stem cells from apical papilla (SCAP) has been discovered in 2006 (Sonoyama et al., 2006). Two years later, the researchers further characterized the apical papilla tissue and stem cell properties of SCAP using histological, immunohistochemical, and immunocytofluorescent analyses. These two studies conclude that SCAP is similar to DPSCs and offer a distinct source for potent dental stem/progenitor cells (Sonoyama et al., 2008).

2.2.1.5 Dental follicle stem cells (DFSCs)

The dental follicle contains the precursors of the periodontium and as such has a crucial role in the development of teeth. Cells of the dental sac develop into a mature periodontium consisting of alveolar bone, cementum and the periodontal ligaments (PDL) (Morsczeck et al., 2005). Animal studies showed that the removal of dental follicle prior to the onset of eruption prevents tooth eruption. (Wise et al., 2002). Morsczeck et al. (2005) isolated precursor cells derived from the dental follicles of third molar teeth which are shown to have fibroblast-like cell characteristics and being colony forming and plastic adherent. DFSCs are known to express positive stem cell markers such as Notch1, STRO-1 and Nestin, and the ability to sustain this under *in vitro* environments for at least 15 passages (Morsczeck et al., 2005).

University of Malaya

2.3 Characteristics of MSCs

The International Society for Cellular Therapy (ISCT) has set three minimum criteria for characterizing mesenchymal stem cells. These criteria are the ability of the cells to adhere to plastic when cultured in the flasks, the expression of CD105, CD73, and CD90 markers accompanied by lack of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR class II markers expression (Table 2.1), and the ability to achieve trilineage differentiation to osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). Panel of surface markers are used to define human MSCs obtained from fresh and cryopreserved samples as there is no specific single surface marker (Patel et al., 2013).

Table 2.1 : Summary of criteria to identify MSCs
(Adapted from Dominici et al., 2006)

1. Adherence to plastic in standard culture conditions		
2. Phenotype	Positive (≥ 95% +)	Negative (≤ 2% +)
i.	CD105	CD45
ii.	CD73	CD34
iii.	CD90	CD14 or CD11b
iv.		CD79α or CD19
v.		HLA-DR
3. *In vitro* differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture)		

MSCs of dental origin differ from those originating from other parts of the body such as bone marrow or adipose tissue as they derive from the neural crest and have neurotropic properties (Stanko et al., 2018). Dental pulp is similar to bone marrow in that both contain MSCs which then can become cells that produce minerals to surround the soft tissues. These MSCs are known as osteoblasts in bone marrow and odontoblasts in dental pulp (Sharpe, 2016). All MSCs of dental origin express nestin which is a marker for neural stem cell, aside from other markers for neural crest stem cells

University of Malaya

including musashi-1, p75, snail-1,-2, slug, and Sox-9 thus proving their embryonic origin (Marei & Backly, 2018).

However, dental MSCs are similar to other types of MSCs in that they lose their potency over time and are significant throughout the sub-culturing processes. This is due to senescence and aging caused by the decrease in telomerase activity. *In vitro* culture causes considerable amounts of telomere shortening (Fernandez & Fernandez, 2016). In addition, it has been suggested that senescence of MSC is related to the shortening of telomere during *in vitro* expansion. BMMSCs experienced senescence in long-term culture as seen in the decrease in their differentiation potential, telomere length shortening and changes in morphology. It has been suggested that MSCs undergone senescence and almost undetectably lose their stem cell properties when *in vitro* culture begins. Thus, early stages of the cells are preferable in any type of therapy modalities (Bonab et al., 2006).

2.4 Immunomodulatory effect of MSCs

A special characteristic of MSCs is their ability to avoid the immune system thus preventing an immune response (Rasmusson, 2006). MSCs are found to have immune-suppressive activity, therefore, there is a potential for their use in transplant treatment. The immunomodulatory and anti-inflammatory effects of MSCs are useful in dealing with graft rejection and autoimmune diseases. Allogeneic MSCs transplantations have been shown to be possible but the xenogeneic model results in transplant rejection (Grinnemo et al., 2004). *In vitro* results of studies on the expression of human leucocyte antigens (HLA) and the immunologic properties of differentiated and undifferentiated MSCs show that both have immunosuppressive effects and may thus be able to be used in transplantations with minimal risk of rejection (Le Blanc et al., 2003). MSCs do

University of Malaya

not cause allogeneic or xenogeneic lymphocytes proliferation, and they interfere with B cell and dendritic cell development (Marti et al., 2011).

MSCs are capable of influencing both innate and adaptive immune response and proven to control various immune cells such as T-cells, B-cells, natural killer cells, dendritic cells and neutrophils, while stimulating T-regulatory cells (Wada et al., 2013). The effect of MSCs on innate and adaptive immunity involves the suppression of inflammatory markers such as interleukin-1β, tumor necrosis factor α and interleukin-6 together with an increase in protective cytokines such as interleukin-10, prostaglandin E2 and indoleamine 2, 3 dioxygenase (Moreira et al., 2017). These properties contribute to the low immunogenicity characteristics of MSCs. Recent findings suggested that MSCs have different immunomodulatory effects on the same types of immune cell and it was influenced by the microenvironment or the state of disease. In addition, it was shown that MSCs were capable to protect the body in dealing with diseases under different conditions (Gao et al., 2016).

As for dental MSCs, compared to BMMSCs, DPSCs show a higher rate of suppression for the T lymphocyte by 18%, thus demonstrating significant immunosuppression potential for MSCs of dental origin (Taşh et al., 2016). The immunomodulatory properties of SHED and BMMSCs were compared with the former considerably affecting Th17 *in vitro* (Yamaza et al., 2010). Kim et al. (2010) showed that there were immunomodulatory effects of PDLSCs from canine on allogenic and xenogenic peripheral blood mononuclear cells through the inhibition of cell divisions.

University of Malaya

2.4.1 MSCs and T-Cell

T-cells have the most important role in the immune system. Inhibition of T-cell proliferation and cytokine production are related to the anti-inflammatory and immunosuppressive properties of MSCs (Wada et al., 2013). MSCs were reported to block the response of naïve and memory T-cell towards their associated antigens and it has been suggested that MSCs physically inhibit T-cell and antigen-presenting cell (APC) contact in a non-cognate interaction (Krampera et al., 2003). MSCs are capable of controlling the immunity response by promoting the apoptosis of activated T-cells through cell-to-cell contact in the FAS ligand (FASL)/FAS-mediated death pathway and this increases the production of regulatory T-cells (Tregs) which lead to immune tolerance (Wang et al., 2012).

2.4.2 MSCs and Natural Killer Cells

Natural killer (NK) cells function in the innate immune system. It has been found that MSCs can change the phenotype and arrest the growth, cytokine production and also cytotoxicity against HLA-class I– expressing targets through cell-to-cell contact or soluble factors (Sotiropoulou et al., 2006). Human MSCs are known to reduce IFN-γ secretion from NK cells (Aggarwal & Pittenger, 2005). Furthermore, aside from inhibiting NK cells proliferation, MSCs also inhibit important NK cells effector functions such as cytolytic activity and cytokine production where the combined total effect would result in inhibition of NK cells activities (Spaggiari et al., 2008).

University of Malaya

2.4.3 MSCs and Dendritic Cells

In terms of functions, it is generally accepted that cells produced from dendritic cell progenitors lack the proper dendritic cell phenotype as well as have impaired functions in the presence of MSCs, but not in control dendritic cells (DCs) (Spaggiari & Moretta, 2013). Jiang et al. (2005) studied the effect of MSCs on monocyte-derived dendritic cells noting that mature DCs have reduced CD83 expression when treated with MSCs thus suggesting their immature status. This was accompanied by reduced expression of presentation molecules, costimulatory molecules and IL-12 secretion. It was concluded that the MSCs acted to suppress the differentiation of monocytes into DCs.

2.4.4 MSCs and B cells

B cells play a major role in facilitating humoral immunity in the adaptive immune system where their main function is to secrete antibodies in response to antigens. By blocking the IFN-γ production in T-cells, MSCs block the growth of B cells and the formation of plasma cells. This is augmented by the reduced production of the B-cell activating factor (BAFF) by dendritic cells (Fan et al., 2016). Corcione et al. (2006) showed the inhibition of human MSCs B cell proliferation, differentiation, and chemotaxis *in vitro*. B-cell proliferation was inhibited through an arrest in the G0/G1 phase of the cell cycle while inhibition of B-cell differentiation was seen in the impaired production of IgM, IgG, and IgA. The chemotactic properties were affected as evidenced by the CXCR4, CXCR5, and CCR7 B-cell expression and CXCL12, the CXCR4 ligand, and CXCL13, the CXCR5 ligand chemotaxis.

University of Malaya

2.5 Growth factors of MSCs

Growth factors are important for development, influencing stem cell differentiation, modulating the growth of tissues and organs during embryogenesis and involved in physiological tissue repair. In addition, they can be used to induce differentiation towards specific lineage and to control tissue engineering processes for therapeutics purposes (Casagrande et al., 2011). Tissue regeneration might not be achievable with regards to the low amount of MSCs that is obtainable from the donors and this is compounded by the issue of MSCs survival after transplantation. However, besides other trophic factors, the growth factors that control cell growth, motility, viability and morphogenesis might be involved in tissue regeneration. Thus, any *ex vivo* expansion of MSCs need to incorporate the use of growth factors. Aside from influencing cell proliferative and pro-survival ability, growth factors can also affect differentiation. However, it is preferable that differentiation at an early stage is not stimulated by the growth factor as it results in extremely low numbers of early-differentiating progenitors (Rodriguez et al., 2010).

The various functions of MSCs are attributed to the different factors secreted by them including the transforming growth factor (TGF) β, hepatocyte growth factor (HGF), basic fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) which promote cell proliferation and angiogenesis especially fibroblasts and, epithelial or endothelial cells. Insulin growth factor (IGF) 1, interleukin (IL) 6 and stanniocalcin-1 play a role in apoptotic reversal in fibroblasts while VEGF, HGF and TGFβ1 can protect endothelial cells from apoptosis (Pers et al., 2015). Furthermore, MSCs appear to express cytokines such as transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and various other molecules that can induce tissue repair (Freitag et al., 2016).

University of Malaya

Rodrigues et al. (2010) stated three important criteria in choosing growth factors for MSCs proliferation and expansion. First is the need of the growth factor to increase proliferation cycle i.e., population doublings in order to produce a significant number of MSCs prior to undergoing differentiation. Second is the ability of the growth factor to be a substitute serum of animal origin used i.e., removing the use of xenogenic substances and decreasing chances of variability. Third is the requirement of localized and controlled delivery to maintain the mitogenic and protective signals for preventing the uncontrolled proliferation of MSCs.

Expressions of various growth factors such as the platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) in normal state or as a response to toxic stimuli such as injury or hypoxia is related to paracrine-mediated angiogenesis of DPSCs (Marei & Backly, 2018). Osathanon et al. (2014) compared the effects of chemical induction protocol and growth factor induction protocol on the neurogenic differentiation of DPSCs. The chemical induction protocol failed to enhance the expression of neuronal mRNA. However, the growth factor induction protocol which incorporated FGF resulted in an increase in neurogenic markers for mRNA and protein, with increases in gamma-aminobutyric acid (GABA) receptor expression indicating neuronal-like function of the cells. The study concluded that the protocol using the growth factor is preferable compared to chemical induction for studying neuronal differentiation of DPSCs.

University of Malaya

2.6 Differentiation potential of MSCs

As multipotent stem cells, MSCs are capable of differentiating into various cell types including adipocytes, osteoblasts, chondrocytes, myoblasts and neuron-like cells (Wei, et al., 2013). In 2011, Govindasamy and his group studied the differentiation potential of DPSCs into islet-like cell aggregates (ICAs). The success in obtaining ICAs from DPSCs suggested possible use in autologous stem cell therapy for treating diabetes (Govindasamy et al., 2011). It is found that stem cells harvested from different sites have different gene expressions and growth factor profiles. The gene expression profiles, growth patterns and propensity of embryonic stem cells, BMMSCs, and umbilical cords are not identical. Stem cells from different tissues differ from each other having different propensity towards a specific lineage. It was also hypothesized that MSCs from different sources of the same group such as dental stem cells have different gene expressions which determine the developmental pathway (Govindasamy et al., 2010). DPSCs and PDLSCs have been found to be capable of differentiating into mineralized cells or to a neuronal fate under the influence of pharmacological stimuli thus proving their potential for neuroregenerative therapy aside for dental or bone reconstruction (Kadar et al., 2009). It was found that DPSCs can differentiate into neuronal and pancreatic cells of non-mesodermal lineage (Jeon et al., 2015).

A study comparing different types of dental MSCs from a single donor showed that DPSCs, DFSCs, and SCAP have identical cellular characteristics and were able to differentiate into neuronal cells with DPSCs having the highest neurogenic potential (Ullah et al., 2016). However, differentiated dental tissues do not undergone continuous remodeling such as bone tissue. Thus, MSCs of dental origin might be more restricted in terms of their differentiation propensity compared to BMMSCs, where they are more directed to carry out odontogenic rather than osteogenic differentiation (Huang et al.,

University of Malaya

2009). Following clonal expansion, a subset of MSCs clones maintained the potential to carry out multi-lineage differentiation (Halleux et al., 2001). In addition, MSCs of dental origin are able to differentiate into neural crest-derived cells like neuron and glia cells both *in vitro* and *in vivo* since they are originated from the neural crest (Xiao & Tsutsui, 2013).

A study by Tomasello et al. (2017) on the osteogenic potential of MSCs from dental pulp and the gingival mesenchymal of periodontally affected teeth noted that the stem cell properties were maintained while the osteogenic capability increased as seen in the increase in pro-inflammatory cytokine-dependent chaperones and stress response proteins expressions. Kolar et al. (2017) studied the neurotrophic effects of different human dental MSCs noting that the SCAP, DPSCs, and PDLSCs from the same donors greatly increased the regeneration of axons after 2 weeks accompanied with neuroprotective effects on the dorsal root ganglia neurons, with SCAP having the best outcome. This suggests the potential of these dental MSCs to substitute Schwann cells for treating peripheral nerve injuries.

2.7 Safety characterization of MSCs

The therapeutic potential of dental stem cells in regenerative medicine has been extensively studied in both preclinical and clinical levels. MSCs are not immunogenic, have no alloreactivity properties and can avoid T-cells and NK cells actions which offers the possibility of transplantation between HLA-mismatched individuals with no requirement for host immunosuppression. In addition, adult MSCs reduce alloreactivity and cytotoxic lymphocyte formation *in vitro* thus showing immunosuppressive properties (Le Blanc, 2003). These findings support the safety characterization of MSCs.

University of Malaya

To date, studies on the safety of MSCs conducted at different levels covering *in vitro* expansion, animal disease models, and clinical trials have concluded that MSCs are safe. Furthermore, Good Manufacturing Practice (GMP) and Good Tissue Practice (GTP) laboratories are required for MSC manipulation for use in clinical applications (Zhao et al., 2016). Dental stem cells have been shown to maintain their stem cell properties after cryopreservation which is important for their long term storage and high production (Hilkens et al., 2016).

The safety of MSC transplantation is acknowledged and has been much studied in clinical trials of cardiovascular, neurological, and immunological diseases with positive outcomes (Parekkadan & Milwid, 2010). A review and meta-analysis of clinical trials found no detected association with acute infusional toxicity, organ system complications, infection, death, or malignancy on participants receiving MSC therapy for different medical conditions such as ischemic stroke, Crohn's disease, cardiomyopathy, ischemic heart disease, and graft versus host disease, although transient fever was significantly observed. Based on the clinical trials, it was concluded that MSCs therapy is safe (Lalu et al., 2012).

Furthermore, a systemic review on the delivery of autologous expanded MSCs through intra-articular injections involving 844 procedures for a mean of 21 months follow-up period showed no adverse outcomes such as infection, death, or malignancy (Peeters et al., 2013). A pilot study by Nakashima et al. (2017) investigating pulp regeneration by transplantation of DPSCs in pulpitis, found no adverse effects or toxicity in clinical and laboratory evaluations. Positive results were observed in the electric pulp test (EPT) of the pulp after 4 weeks. The regenerated tissue in the root canal showed similar results to control dental pulp in terms of signal intensity from magnetic resonance imaging (MRI), accompanied by functional dentin formation

University of Malaya

observed in 3 out of 5 patients. It was concluded that human mobilized DPSCs are safe and effective for pulp regeneration in humans.

2.8 Therapeutic Advantages

Among the advantages of using MSCs for clinical use is their availability and ease of harvesting, capability of multilineage differentiation, immunomodulatory properties, absence of risk for malignant transformation in allogeneic transplantation as in embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), and absence of ethical issues like ESCs (Kim & Park, 2017). MSCs are also valuable candidates for cell therapy because of their accessibility, simple isolation method with ease of upscale for clinical use, low potency storage as well as the ability to be stored for delivery, and no adverse effects to allogeneic transplant, thus enabling a single isolation for the treatment of a large number of patients (Parekkadan & Milwid, 2010). Another important property of MSCs is their capacity to rescue cells from apoptosis induced by trauma, an oxidative environment, radiation or chemical injury (Pers et al., 2015). The attraction of using dental MSCs is also due to the disadvantages of stem cells from other origins such as bone marrow and adipose tissue which involve a painful harvesting method, decreasing cell numbers with age, the need for anesthesia use, low growth potential, and harmful risks to harvest site (Marei & Backly, 2018).

MSCs have promising potential as therapeutic agents for the treatment of various diseases and their use is of much interest due to their advantages over other types of stem cells. Many studies have been conducted which focus on the potential of MSCs to treat specific medical conditions. These findings showed future possibility of using MSCs to treat diseases.

University of Malaya

MSCs therapeutic potential in atherosclerosis has been studied and found to be able to control secretions of cytokine and chemokine, decrease endothelial dysfunction, enhance the action of regulatory T cell, reduce dyslipidemia and stabilize plaque during the development of atherosclerosis. Furthermore, MSCs might move to lesions and become functional cells by restoring the endothelium function in atherosclerotic lesions. However, further research is needed to establish if immunomodulation can control atheroprotective effects and if different factors can increase the effectiveness of MSC therapy (Li et al., 2017).

Studies on the therapeutic potential of MSCs in treating diabetes show their ability to mitigate fibrosis, regulate inflammation, and induce vascular growth thus suggesting their usefulness for treating endocrine disorders (Moreira et al., 2017). Furthermore, Monfrini et al. (2017) investigated the therapeutic potential of MSCs in the treatment of diabetic peripheral neuropathy. They demonstrated the *in vivo* ability of allogenic MSCs to decrease the number of pancreatic islets needed to reach glycaemic control in diabetic rats and the positive outcome on diabetic neuropathy accompanied with the decrease in signs of diseases. The study showed MSCs as a promising therapeutic agent to enhance the clinical feasibility of pancreatic islet transplantation for the treatment of diabetes.

MSCs have been proposed to treat Osteoarthritis (OA) due to their ability to differentiate into chondrogenic lineage. Osteoarthritis can be considered a mesenchymal disease where the activity, phenotype, or mobilization of MSC populations are affected, causing the absence of repair and increased degenerative changes. This was based on the idea that MSCs determine the development and homeostasis of the tissues in healthy joints (Barry & Murphy, 2013) suggesting that MSC therapy may be able to treat osteoarthritis. A meta-analysis on the efficacy of MSCs to treat osteoarthritis of the knee

University of Malaya

(KOA) showed improvement from pain and functionality relative to KOA basal evaluation, with sustained results for two years after treatment (Cui et al., 2016).

MSCs also have the possibility to treat cancer. The introduction of MSCs will cause their recruitment to the sites of tissue injury and inflammation. MSCs have been altered to produce cytokines, growth factor antagonists, antiangiogenic factors, prodrug-converting enzymes and pro-apoptotic proteins in some preclinical cancer models. The use of MSCs as oncolytic viruses has been applied in some cancer models such as colon cancer, pancreatic cancer, lung cancer, breast carcinoma, ovarian cancer, prostate cancer, hepatocellular carcinoma, glioma, melanoma, malignant mesothelioma, and lymphoma although extremely few clinical trials have been approved (Mohr & Zwacka, 2018).

University of Malaya

CHAPTER 3: PUBLISHED WORKS

3.1 Publication 1 (Basic Research)

Govindasamy V, Abdullah AN, Sainik Ronald V, **Musa S**, Che Ab. Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH. Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth. Journal of Endodontics. 2010, 36(9):1504-1515

3.1.1 Contributions of co-authors:

Design and concept of study	GV, ABNH, SM
Acquisition of data	GV, SM, AAN, SRV, TS
Analysis of data	GV, ABNH, SM, AAN, BRR
Drafting of manuscript	GV, ABNH
Revising manuscript for intellectual content	All authors

University of Malaya

UNIVERSITI MALAYA

اونيۏرسيتي مالايا

20 Feb 2019

Govindasamy V, Abdullah AN, Sainik Ronald V, Musa S, Che Ab. Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH. Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth. Journal of Endodontics. 2010, 36(9):1504-1515

As the corresponding author of the article named above, I consent to Sabri bin Musa including the published article above as part of his collection of published work to be submitted for his "PhD by prior publication" at University of Malaya, Kuala Lumpur.

Signed,

.....

Full Name: Dr. Noor Hayaty Abu Kasim

Designation: Professor

Full address: Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya

Email address: nhayaty@um.edu.my

Tel no: 03-7967 7089

Fax no: 03-7967 7813

University of Malaya

Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth

Vijayendran Govindasamy, PhD, Aimi Naim Abdullah, BSc,† Veronica Sainik Ronald, BSc,† Sabri Musa, BDS, MSc,† Zeti Adura Che Ab. Aziz, BDS, MSc,‡ Rosnah Binti Zain, BDS, MS,§ Satish Totey, PhD,* Ramesh R. Bhonde, PhD,* and Noor Hayaty Abu Kasim, PhD‡*

Abstract

Introduction: Lately, several new stem cell sources and their effective isolation have been reported that claim to have potential for therapeutic applications. However, it is not yet clear which type of stem cell sources are most potent and best for targeted therapy. Lack of understanding of nature of these cells and their lineage-specific propensity might hinder their full potential. Therefore, understanding the gene expression profile that indicates their lineage-specific proclivity is fundamental to the development of successful cell-based therapies. **Methods:** We compared proliferation rate, gene expression profile, and lineage-specific propensity of stem cells derived from human deciduous (SCD) and permanent teeth (DPSCs) over 5 passages. **Results:** The proliferation rate of SCD was higher (cell number, 25 $\times$ 10^6 cells/mL; percent colony-forming units [CFUs], 151.67 $\pm$ 10.5; percent cells in S/G2 phase, 12.4 $\pm$ 1.48) than that of DPSCs (cell number, 21 $\times$ 10^6 cells/mL; percent CFUs, 133 $\pm$ 17.62; percent cells in S/G2 phase, 10.4 $\pm$ 1.18). It was observed that fold expression of several pluripotent markers such as OCT4, SOX2, NANOG, and REX1 were higher (>2) in SCD as compared with DPSCs. However, DPSCs showed higher expression of neuroectodermal markers PAX6, GBX2, and nestin (fold expression >100). Similarly, higher neurosphere formation and neuronal marker expression (NF, GFAP) were found in the differentiated DPSCs into neuron-like cells as compared with SCD. **Conclusions:** This study thus demonstrates that both SCD and DPSCs exhibit specific gene expression profile, with clear-cut inclination of DPSCs toward neuronal lineage. *(J Endod 2010;36:1504–1515)*

Key Words

Deciduous teeth, dental pulp stem cells, inherent propensity, permanent teeth

The therapeutic potential of stem cells derived from human dental pulp since its discovery (1) in regenerative medicine has been extensively studied at several preclinical (2) and clinical levels (3). The dental pulp tissue (DPT) appears to be an excellent source for stem cells because it can be obtained from the deciduous dentition requiring extraction as part of a planned serial extraction for management of occlusion and is originated from migrating neural crest cells during early development of embryos (4). The DPT can be isolated from various age groups and teeth; for example, cells isolated from dental tissue of human impacted tooth germ are known as tooth germ progenitor cells (TGPCs) (5); stem cells from human exfoliated deciduous teeth are known as SHED (6), and stem cells can also be isolated from human permanent teeth (impacted molar) (DPSCs) (7) or from apical papilla (SCAP) (8).

Our present work is specifically focused on stem cells from extracted deciduous (SCD) and permanent teeth (DPSCs). Past studies showed that both these groups of cells are able to differentiate into osteogenic, chondrogenic, adipogenic, and myogenic cells in culture (9, 10). Moreover, recent studies have shown that both SHED and permanent teeth are able to break germ layer commitment and differentiate into cells expressing neurons (11) and hepatocytes (12). Furthermore, this group of cells has been reported to have potential for use in cell-based therapy for neurodegenerative and cardiac diseases (13, 14).

It has been established that gene expression and growth factor profile reflect the source of tissue from which the stem cells have been obtained. This indicates that stem cell heterogeneity is biologically relevant. We have demonstrated previously that gene expression profile, growth pattern, and propensity of human embryonic stem cells, bone marrow–derived mesenchymal stromal cells, and umbilical cord are different, and hence mesenchymal stem cells derived from various tissue sources are different from each other and indicate their propensity toward a specific lineage (15, 16). Therefore, it is logical that different tissue sources might generate stem cell products producing different cytokines and growth factors that might be more suited for specific clinical applications. Similarly, we hypothesized that gene expression varies within various sources of the same group such as in the case of stem cells of dental origin, which determines the development pathway of these cells.

Although during the past few years the interest in dental stem cells has risen markedly and several reports on characterization and differentiation of dental stem cells from different sources and age groups have been published (17), the gene expression profile–dependent propensity toward lineage specificity remains poorly understood. Therefore, we undertook this present study comparing proliferation rate, gene

From the *Stempeutics Research Malaysia Sdn Bhd, Kuala Lumpur, Malaysia; and †Department of Children's Dentistry and Orthodontics, ‡Department of Conservative Dentistry, and §Department of Oral Pathology, Oral Medicine & Periodontology/Oral Cancer Research and Coordinating Centre(OCRCC), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia.

Address requests for reprints to Noor Hayaty Abu Kasim, BDS, PhD, Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail address: nhayaty@um.edu.my.

0099-2399/$0 - see front matter

Copyright © 2010 American Association of Endodontists.

doi:10.1016/j.joen.2010.05.006

expression profile, and their lineage propensity of SCD and DPSCs to better understand their inherent therapeutic potential for specific clinical indications.

Materials and Methods

Isolation and Culture of SCD and DPSCs

Sound intact human third molars from adults (24–35 years of age) and deciduous teeth (5–8 years of age) were collected with informed consent from patients undergoing extraction at the Department of Children Dentistry and Orthodontics and Department of Oral and Maxillofacial Surgery, University of Malaya, respectively, under approved guidelines set by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number DF CD0907/0042(L). Root surfaces were cleaned with povidone-iodine (Sigma Aldrich, St Louis, MO), and the pulps were extirpated within 2 hours after extraction and processed. The pulp tissue was minced into small fragments before digestion in a solution of 3 mg/mL collagenase type I (Gibco, Grand Island, NY) for 40 minutes at 37°C. After neutralization with 10% of fetal bovine serum (FBS) (Hyclone; Thermo Fisher Scientific Inc, Waltham, MA), the cells were centrifuged and were seeded in culture flasks with culture medium containing α-MEM (Invitrogen, Carlsbad, CA), 0.5% 10,000 μg/mL penicillin/streptomycin (Invitrogen); 1% 1 × Glutamax (Invitrogen) and 10% FBS, with humidified atmosphere of 95% of air and 5% of CO_2 at 37°C. Nonadherent cells were removed 48 hours after initial plating. The medium was replaced every 3 days. When primary culture became subconfluent, after 10–14 days, cells were collected by trypsinization and processed for subsequent passages. All the experiments were done with pool of 5 dental pulp tissues. Therefore, 3 different pooled samples were used in 3 replicates. Human bone marrow samples were taken after written consent by using guidelines approved by the Ministry of Health, Malaysia. Bone marrow–derived mesenchymal stem cells (BM-MSCs) cultures were established from 3 donors (age range, 18–25 years) as previously described (18).

Colony-forming Units

The number of colony-forming units (CFUs) was determined by plating 100 cells in 35-mm dish. After 14 days in culture, the cells were fixed in 100% methanol for 20 minutes and stained by 3% crystal violet stain. Colonies more than 2 mm in diameter were counted. The CFU equals the total number of colonies divided by the initial number of cells multiplied by 100%.

Growth Kinetics

The proliferation rate was determined by plating 5000 cells/cm^2 from SCD and DPSCs per T25 cm^2 culture flask (BD Pharmingen, San Diego, CA). Three replicates were performed for each passage and time point. Cells were detached by trypsinization after reaching confluency of 90%. Cells were counted and assessed for viability by means of trypan blue dye exclusion before the next passage. Cells were replated for subsequent passages, and total of 5 passages were studied in this experiment. Growth kinetics was analyzed by calculating population doubling (PD) time. The PD time was obtained by the formula: TD = tplg2/(lgNH – lgNI), where NI is the inoculum cell number, NH is the cell harvest number, and t is the time of the culture (in hours).

Cell Cycle Analysis

Cells were seeded at 5000 cells/cm^2 on a 35-mm tissue culture dish (BD Pharmingen) and cultured until reaching a confluence of 90%. The cells were detached, fixed, and permeabilized in 70% ethanol overnight in the dark at 4°C. DNA was stained with propidium iodide/RNase staining buffer (BD Pharmingen) in a volume of 500 μL (containing 1 × 10^6 cells) for 15 minutes at room temperature and then washed in DPBS (Invitrogen). DNA content was analyzed on Guava Technologies (Millipore, Billerica, MA) flow cytometer by using Cytosoft, Version 5.2, Guava Technologies software.

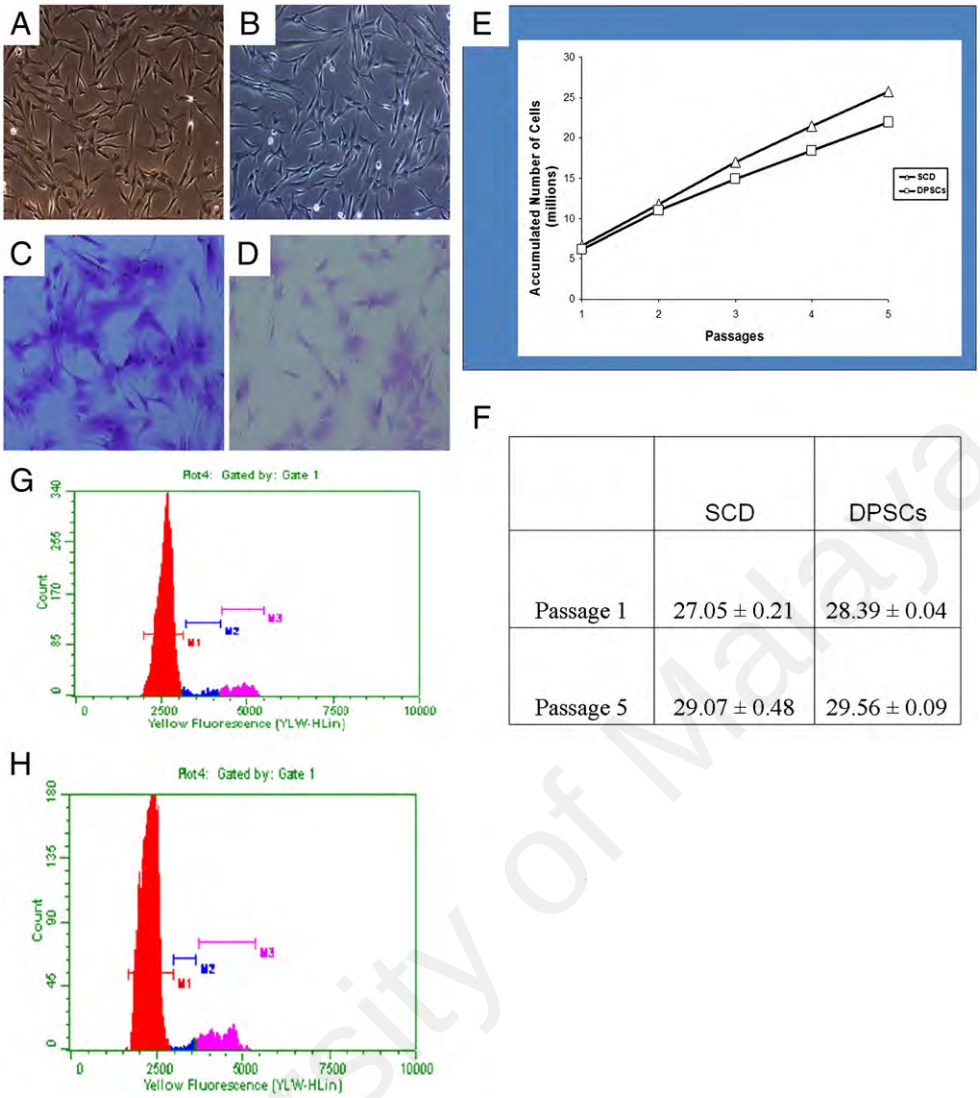
Flow Cytometric Analysis

The immunophenotyping was done by using flow cytometry at passage 5. On reaching 90% confluency, the cells were harvested

TABLE 1. List of Primers Used in this Study

Gene symbol / name	Forward	Reverse	Base pairs
18s RNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT	186
REX1	GCGTACGCAAATTAAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT	282
OCT 3/4 (POU5F1)	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA	572
SOX2	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT	447
NANOG	CCTCCTCCATGGATCTGCTTATTCA	TCGGCGCCGGGGAGATACAT	259
Osteocalcin	CATGAGAGCCCTCACA	AGAGCGACACCCTAGAC	314
Osterix	GCAGCTAGAAGGGAGTGGTG	GCAGGCAGGTGAATTCTTCC	358
ABCG2	GTTTATCCGTGGTGTGTCTGG	CTGAGCTATAGAGGCCTGGG	651
AFP	AGAACCTGTCACAAGCTGTG	GACAGCAAGCTGAGGATGTC	675
PAX6	ATGAACAGTCAGCCAATGGG	CACACCAGGGGAAATGAGTC	625
GATA2	AGCCGGCACCTGTTGTGCAA	TGACTTCTCCTGCATGCACT	243
β-III Tubulin	AACAGCACGGCCATCCAGG	CTTGGGGCCCTGGGCCTCCGA	242
MSX1	CCTTCCCTTTAACCCTCACAC	CCGATTTCTCTGCGCTTTTC	284
NF	ACGCCTGAGGAATGGTTCACG	GCCTCAATGGTTTCC	555
BMP 4	GTCCTGCTAGGAGGCGCGAG	GTTCTCCAGATGTTCTTCG	338
hTERT	AGCTATGCCCGGACCTCCAT	GCCTGCAGCAGGAGGATCTT	184
Nestin	CAGCGTTGGAACAGAGGTTGG	TGGCACAGGTGTCTCAAGGGTAG	388
HNF-3 Beta	GACAAGTGAGAGAGCAAGTG	ACAGTAGTGGAAACCGGAG	234
HAND 1	TGCCTGAGAAAGAGAACCAG	AGGATGAACAAACAC	254
GFAP	CGATCAACTCACCGCCAACA	GTGGCTTCATCTGCTTCCTGTC	158
KRT1-5	CACAGTCTGCTGAGGTTGGA	GAGCTGCTCCATCTGTAGGG	155
NUUR1	CGGACAGCAGTCCTCCATTAAGGT	CTGAAATCGGCAGTACTGACAGCG	711
TH	TCATCACCTGGTCACCAAGTT	GGTCGCCGTGCCTGTACT	124
KRT-8	TGAGGTCAAGGCACAGTACG	TGATGTTCCGGTTCATCTCA	160
TGF 1	GCCCGCTTCTCTTACAGTGTGATT	AGTACGTGCAGACGGTGGTAGTTT	497

University of Malaya



	SCD	DPSCs
Passage 1	27.05 ± 0.21	28.39 ± 0.04
Passage 5	29.07 ± 0.48	29.56 ± 0.09

Figure 1. Morphology, CFUs, DNA content, and growth kinetics of SCD and DP-MSC. (*A, B*) Phase contrast microscope; original magnification, ×10 of SCD and DPSCs, respectively, at passage 5; (*C, D*) CFUs of SCD and DPSCs, respectively, at passage 5; (*E*) long-term growth curves of SCD and DPSCs; (*F*) PD time of SCD and DPSCs at passages 1; and (*G, H*) assessment of DNA content in SCD and DPSCs, respectively, at passage 5. In all experiments, the results represent average of 5 culture replicates with SD, and a representative photomicrograph was given for each experiment. SD, standard deviation. Scale bar, 100 μmol/L. (This figure is available in color online at www.aae.org/joe/.)

with 0.05% trypsin (Invitrogen) and resuspended in phosphate-buffered saline (PBS) at a cell density of 1.5×10^6 cells/mL. Two hundred microliters of the cell suspension (1×10^5 cells) was incubated with the labeled antibodies in the dark for 1 hour at 37°C. The following antibodies were used to mark the cell surface epitopes: CD90-phycoerythrin (PE), CD44-PE, CD73-PE, CD166-PE and CD34-PE, CD45-fluoroisothiocyanate (FITC), and HLA-DR-FITC (all from BD Pharmingen). All analyses were standardized against negative control cells incubated with isotype-specific immunoglobulin (Ig) G1-PE and IgG1-FITC (BD Pharmingen). At least 10,000 events were acquired on Guava Technologies flow cytometer, and the results were analyzed by using Cytosoft, Version 5.2, Guava Technologies.

Differentiation of DPSCs

The cultures were initiated at a density of 1000 cells/cm^2 in 6-well plates and were grown to confluence and subjected to differentiation into adipogenic, chondrogenic, and osteogenic lineages as per the method described earlier (18).

Adipogenic lineage was stimulated by inducing the cells with 10% FBS, 200 μmol/L indomethacin, 0.5 mmol/L 3-isobutyl-1-methyxanthine (IBMX), 10 μg/mL insulin, and 1 μmol/L dexamethasone (all reagents from Sigma Aldrich). Lipid droplets were visualized by staining with oil red O staining (Sigma Aldrich). The percent of adipocytes was estimated by counting 500 total cells in multiple fields.

For chondrogenesis differentiation, briefly cells were cultured in media supplemented with ITS+1 (Sigma Aldrich), 50 μmol/L L-ascorbic acid-2 phosphates (Sigma Aldrich), 55 μmol/L sodium pyruvate (Invitrogen), 25 μmol/L L-proline (Sigma Aldrich), and 10 ng/mL of transforming growth factor-beta (TGF-β) (Sigma Aldrich). Assessment of proteoglycan accumulation was visualized by alcian blue staining (Sigma Adrich).

The osteogenic differentiation was stimulated in a 3-week culture in media supplemented with 10% FBS, 10^{-7} mol/L dexamethasone, 10 mmol/L β-glycerol phosphate (Fluka, Buchs, Switzerland), and 100 μmol/L of L-ascorbic acid-2 phosphates. Assessment of calcium

University of Malaya

TABLE 2. Phenotype Characterization, Colony-forming Ability, DNA Content and Differentiation Potential of SCD and DPSCs Cultured at Passage 5

Parameters	SCD	DPSCs
Phenotypic characterization (%)*		
CD34	0	0
CD44	94.21 ± 2.9	95.83 ± 1.8
CD45	0	0
CD73	99.88 ± 3.1	99.45 ±4.1
CD90	93.71 ± 0.9	99.49 ± 0.8
CD166	98.11 ± 0.8†	79.85 ± 6.8
HLA-DR	0	0
Colonies (CFU) (% cells seeded)‡	151.67 ± 10.5†	133 ± 17.62
Cell cycle analysis§		
% of G1/G0 phase	87.6 ± 1.48	89.6 ± 1.18
% of S/G2 phase	12.4 ± 1.48†	10.4 ± 1.18
In vitro adipogenic differentiation‖	58 ± 8†	45 ± 6
In vitro osteogenic differentiation¶	59 ± 5	64 ± 8

SCD, stem cells derived from human deciduous teeth; DPSCs, stem cells derived from human permanent teeth.

*Fluorescence-activated cell sorter analysis (FACS) of SCD and DPSCs: The result shows the average value of % positive cells ± standard deviation to the total number of cells analyzed ($n \geq 5$).

†$P < .05$.

‡Percent SCD and DPSCs colonies grown at passage 5. Data are expressed as average value of % of colonies ± standard deviation.

§Percent DNA content in SCD and DPSCs observed in G1/GO and S/G2 phase at passage 5.

‖Percent neutral oil droplet formation stained with red O cells out of 500 total cell counted.

¶Percent mineralized area.

accumulation was visualized by von Kossa staining. Percentage of calcium accumulation was analyzed by using Image ProPlus software (Media Cybernetics, San Diego, CA). Osteogenic differentiation is presented as percent of the mineralized area in the total culture area. Quantitative amplifications of osteogenic markers (osteocalcin and osterix) were carried out in duplicate by using SYBR green master mix (Applied Biosystems, Foster City, CA). Polymerase chain reactions were run on an ABI 7900HT RT-PCR system (Applied Biosystems), and SDS v2.1 software was used to analyze the results. All measurements were normalized by 18s rRNA. The sense and antisense primers used for each gene are shown in Table 1.

Human Taqman Low Density Array

Human Taqman Low Density Array (TLDA) (Applied Biosystems) containing a well-defined set of validated gene expression markers to characterize embryonic stem cell identity was used for analyzing the expression of a focused panel of pluripotent and stem cell markers. The 384 wells of each TLDA card were preloaded with fluorogenic probes and primers (Applied Bioystems). The pooled cDNAs were loaded on the microfluidic cards for thermal cycling on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Expression values for target genes were normalized to the expression of 18s rRNA. Transcriptional analysis was performed for BM-MSCs (early passage), SCD and DPSCs (both early and late passages). For data analysis, the ABI PRISM 7900HT sequence detection system software (SDS) calculated the levels of target (SCD and DPSCs) gene expression in samples relative to the level of expression in the calibrator (BM-MSCs) with comparative C_T method ($\Delta\Delta C_T$). For estimation of the fold change by TLDA when the initial transcript levels were undetectable, the initial cycle threshold (C_T) value was assigned to be 39, which would lead to a possible underestimation of the actual fold change.

Reverse Transcription Polymerase Chain Reaction and Real-time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted by using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA by using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA amplification was performed in a thermocycler by using *Taq* polymerase supplied with KCl buffer and 1.5 mmol/L $MgCl_2$ (Invitrogen) at 94°C/1 min, 58°C/30 sec, 72°C/1 min. Polymerase chain reaction (PCR) products were resolved on 1.5% agarose (Invitrogen) gel run in 1× Tris borate–ethylenediaminetetraacetic acid buffer. The primer sequences are tabulated in Table 1.

For the real-time PCR, the amplification reaction was performed by using Taqman Universal Master Mix and Assay-on-Demand Taqman primer/probe sets (Applied Biosystems) according to manufacturer's protocol using the ABI 7900HT RT-PCR system (Applied Biosystems). The assays for OCT 4, SOX 2, NANOG, and DNMT1 were Hs00742896_s1 (130 base pairs [bp]), Hs02387400_g1 (122 bp), Hs02387400_g1 (109 bp), and Hs00945900_g1 (100 bp). Eukaryotic 18S rRNA (assay ID Hs99999901_s1 [188 bp]) was used as an internal control in all assays. The relative quantification of gene expression was assessed by $\Delta\Delta C_T$ method. All PCRs were performed in duplicates. The expressions of some primers in the semiquantitative reverse transcription (RT)-PCR analysis were quantified in duplicate by using SYBR green master mix. PCR reactions were run on an ABI 7900HT RT-PCR system, and SDS v2.1 software was used to analyze the results. All measurements were normalized by 18s rRNA.

Neurogenic Differentiation

For neuronal differentiation, SCD and DPSCs were cultured at the rate of 10,000 cells/mL in a non-coated 35-mm dish containing Neurobasal-A medium (Invitrogen) supplemented with B-27 supplement (Invitrogen), penicillin-streptomycin, L-glutamine, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) (all from Invitrogen) for 15 days. Neurosphere-like bodies generated after 15 days were then counted. Briefly, a fixed area (10 mm^2) of the center of each well was converted into a digital image with a digital still camera (DSC-S70; Sony, Tokyo, Japan), and the number of neurospheres with a diameter of more than 60 μm was counted with a Scion Image Beta 4.02 (Scion Corporation, Frederick, MD).

Neurospheres that were generated after 15 days of incubation were triturated by using poliSCD glass pipettes, and single-cell suspension was obtained and seeded on gelatin-coated 35-mm dishes in neurodifferentiation medium one (Neurobasal A) containing 1 μg/mL laminin (Invitrogen), 5 μg/mL fibronectin (Nitta Gelatin, Osaka, Japan), 2 mmol/L L-glutamine, 10 μg/mL N2 supplement (Invitrogen), 20 ng/mL bFGF, and 40 ng/mL EGF. The medium was changed to neurodifferentiation medium 2 (Neurobasal A) containing 1 μg/mL laminin, 5 μg/mL fibronectin, 2 mmol/L L-glutamine, 10 μg/mL N2 supplement, 20 ng/mL neurotrophin-3 (Peprotech, Rocky Hill, NJ) after 24 hours of cultivation. The medium was changed every 3 days. Immunocytochemical analysis was performed 21 days after cultivation. The cells were fixed for 30 minutes in cold 4% paraformaldehyde in PBS, treated with 0.1% Triton-X for optimal penetration of cell membrane, and incubated at room temperature in a blocking solution (0.5% bovine serum albumin; Sigma Aldrich) for 30 minutes. The primary antibodies used were goat anti-human-OCT4 (1:400; ABCAM Inc, San Francisco, CA), mouse anti-human-GFAP (1:400; Chemicon, Temecula, CA), and mouse anti-human-β-III tubulin (1:400; Chemicon). Secondary antibodies used were FITC-conjugated rabbit anti-goat IgG (1:700;

University of Pune

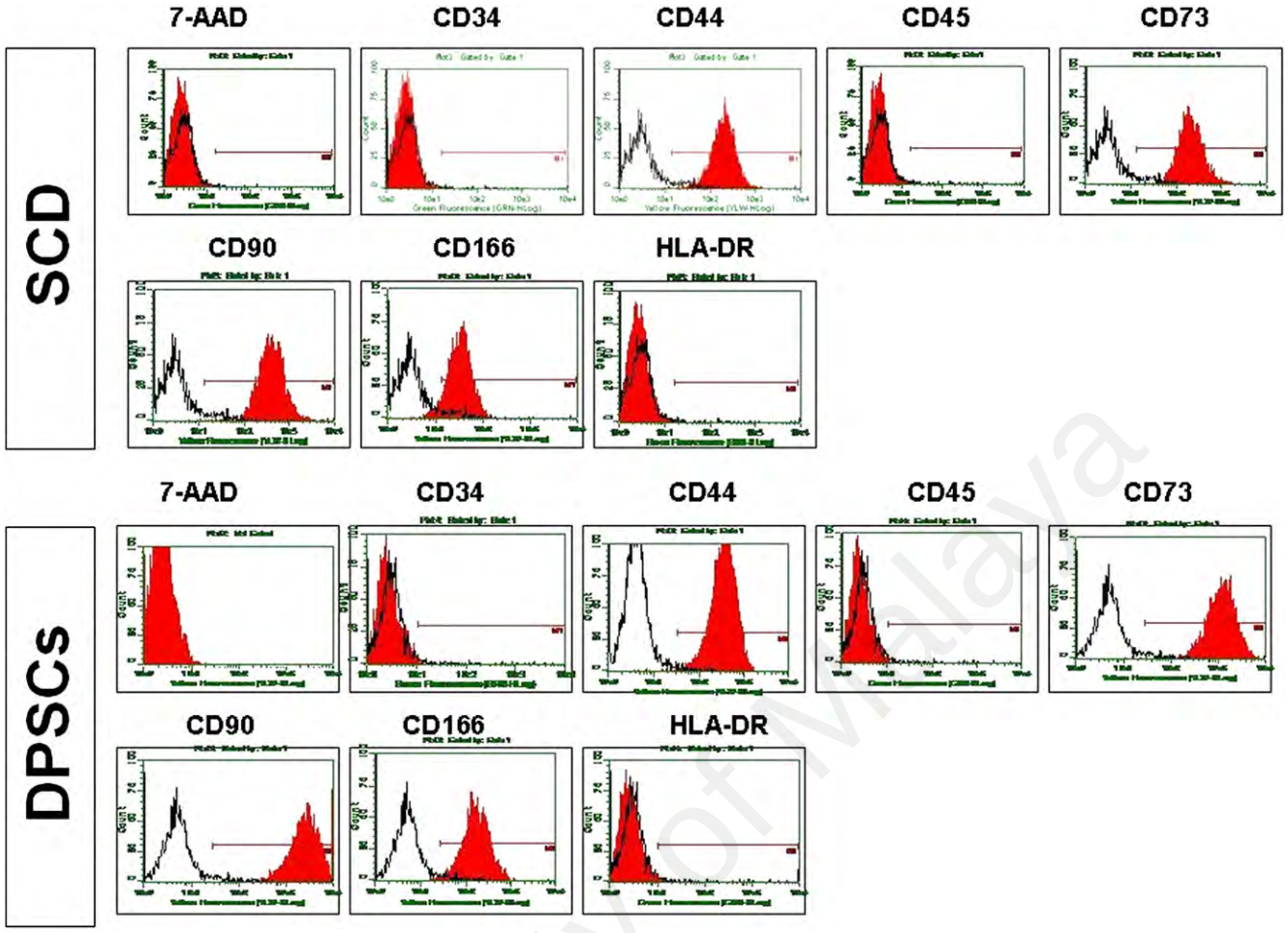


Figure 2. Immunophenotype analysis of SCD and DPSCs at passage 5. Cells were tested against human antigens CD34, CD44, CD45, CD73, CD90, CD166, and HLA-DR. 7-Amino-actinomycin D (7-AAD) was used to check the viability of the cells. SD, standard deviation; CD, cluster of differentiations. Scale bar, 100 μmol/L. (This figure is available in color online at www.aae.org/joe/.)

Chemicon) and rhodamine-conjugated anti-mouse IgG (1:500; Chemicon). Slides were counterstained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI; Chemicon) for 5 minutes. Fluorescent images were captured by using a Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan) and Image-Pro Express software (Media Cybernetics, Inc, Silver Spring, MD).

Karyotype Analysis

Cultures were treated with colcemid 2 hours before harvest, detached by trysinization, and treated with 0.5 mol/L hypotonic solution (KCl/water) before fixing with Conroy's solution (3:1 methanol/glacial acetic acid). The spreads were treated with 0.005% trypsin, stained with Giemsa (Sigma Aldrich), and 20–30 separate metaphase spreads were examined for each culture.

Data and Statistical Analysis

The descriptive statistical analyses were performed by using the software SPSS for Windows (Version 11.0; SPSS Predictive Analytics, Chicago, IL). The data were analyzed by using two-way analysis of variance (ANOVA). The significance level was set at $P = .05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between groups. To visualize the differences between SCD and DPSCs, we applied a novel approach based on principle component analysis (PCA) (Plymouth Routines In Multivariate Ecological Research version 6 [PRIMER 6] software [http://www.zen87707.zen.co.uk/primer-e]) on our pluripotent array results. PCA is a mathematical algorithm that describes the data on the basis of their dissimilarity, so that a greater distance corresponds with a greater dissimilarity. The main aim of this technique is to reduce the dimensionality and to display the nature of the variation present in the data. This is achieved by creating new variables, the" principle components" that are linear combinations of the observations (19). The first principle component explains as much of the variation as possible with a single statistic. The second principal component, which is uncorrelated with the first, accounts for as much as possible of the remaining variation, and so on. If there are P variables, then it is possible to calculate P different principle components, but the first few will normally account for most of the variation, and these might therefore be used to describe the data without undue loss of information (20).

Results

Isolation and Characterization of SCD and DPSCs

Morphologic characteristics of SCD and DPSCs displayed indistinguishable fibroblastic morphology resembling that of BM-MSCs (Fig. 1*A, B*). The colony-forming properties of SCD and DPSCs were assessed. The CFUs were higher in SCD as compared with those of DPSCs ($P < .05$) (Table 2). The number and size of colonies were more in SCD than in DPSCs, indicating that SCD has higher

University of Malaya

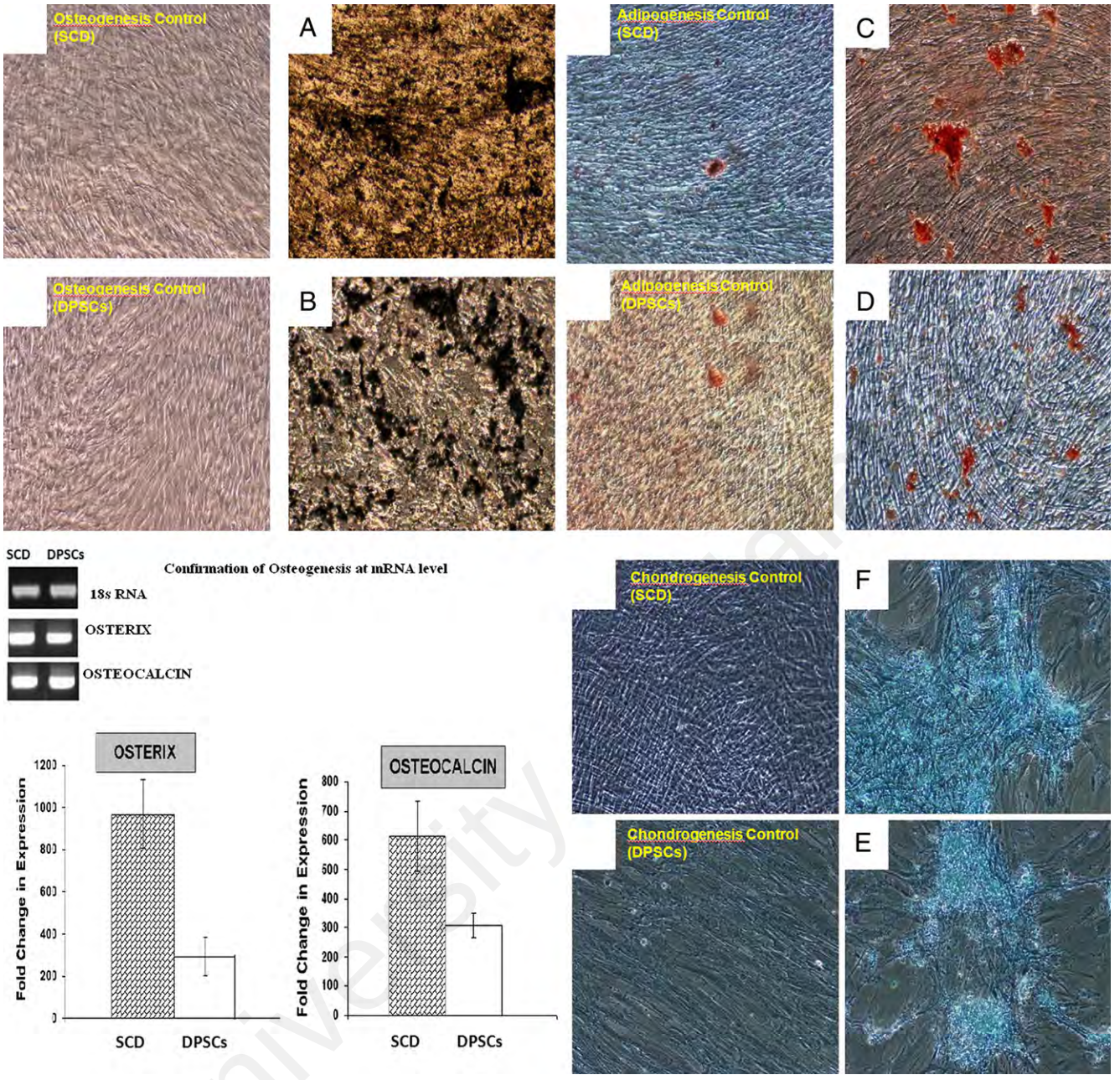


Figure 3. *In vitro* mesoderm differential potentiality of SCD and DPSCs. Osteogenesis was confirmed by mineralized matrix deposition stained with von Kossa staining at day 21 in SCD (*A*) and DPSCs (*B*) followed by confirmation of osteogenesis at mRNA level; mRNA expression of osterix and osteocalcin by RT-PCR by using SYBR green reagent, and values were normalized to 18sRNA. Adipogenesis was detected by neutral oil droplet formation stained with oil red O at day 21 in SCD (*C*) and DPSCs (*D*). Chondrogenesis was detected by the presence of proteoglycans stained with alcian blue at day 21 in SCD (*E*) and DPSCs (*F*). All experiments were conducted at passage 5. Results represent average of 5 culture replicates. (This figure is available in color online at www.aae.org/joe/.)

proliferation rate than DPSCs (Fig. 1*C, D*). This result reflected the growth kinetics of the cells. After the end of passage 5, overall cell yield was significantly higher in SCD (25×10^6 cells) in T25 cm^2 flask as compared with DPSCs (21×10^6 cells) (Fig. 1*E*) ($P < .05$). The time required for PD varied between approximately 27 hours (P1) and 29 hours (P5) for SCD, whereas approximately 28 (P1) and 29 hours (P5) were observed in DPSCs (Fig. 1*F*). We further analyzed cell cycle analysis of SCD and DPSCs at passage 5. Flow cytometry analysis revealed that the percentage of proliferation rate was higher in SCD (S + G2 + M = 12.4 ± 1.48) ($P < .05$) with approximately 85% of cells in phase G1/GO, as compared with that of DPSCs (S + G2 + M = 10.4 ± 1.18) with approximately 90% of the cells in phase G1/GO (Table 2; Fig. 1*G, H*).

Cell Surface Antigen Profile of SCD and DPSCs

Immunophenotyping of stem cells derived from SCD or DPSCs showed that the cells were negative for hematopoietic markers (21) CD34 and CD45 and HLA-DR, whereas more than 90% of cells were positive for mesenchymal stem cell markers (22) CD44, CD73, CD90, and CD166. Cells from SCD expressed higher percentage of CD166 (98.11 ± 0.8) ($P < .05$) than cells derived from DPSCs (79.85 ± 6.8) (Table 2; Fig. 2).

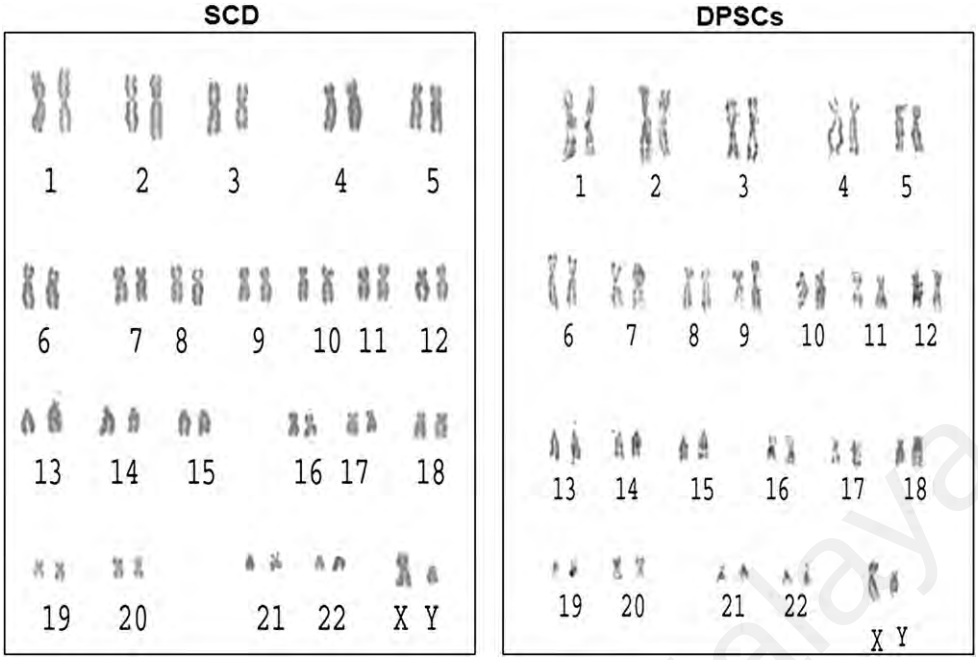


Figure 4. Karyotyping analysis of SCD and DPSCs. (*A*) Karyotype expanded in SCD and DPSCs at passage 5 revealed chromosomal stability. Results are representative of 3 experiments.

Differentiation of SCD and DPSCs Into Classic Lineages

Osteogenic differentiation was confirmed in both SCD and DPSCs by the deposition of a silver-stained mineralized matrix (Fig. 3*A, B*). Percent mineralization was 59 ± 5 for SCD and 64 ± 8 for DPSCs, respectively (Table 2). The mRNA expression of 2 osteoblast markers, osteocalcin and osterix (22, 23), was found to be higher in SCD as compared with DPSCs. Observations from the real-time PCR analyses were in line with the results from the staining assays. Adipogenic differentiation was confirmed in both SCD and DPSCs by the accumulation of neutral lipid vacuoles (Fig. 3*C, D*). After adipogenic induction, 58% ± 8% of SCD and 45% ± 6% of DPSCs possessed cells with an adipogenic phenotype (Table 2). Thus, a higher number of SCD ($P < .05$) than that of DPSCs featured an adipogenic differentiation capacity. Chondrogenic differentiation was confirmed by the formation of spheres in the micromass culture and the secretion of cartilage-specific proteoglycans stainable. Both SCD and DPSCs demonstrated a cartilage-like phenotype with chondrocyte-like lacunae (Fig. 3*E, F*).

Cytogenetic Stability of SCD and DPSCs

Both SCD and DPSCs showed normal karyotypes at passage 5. A representative ideogram is illustrated in Fig. 4.

Pluripotent Gene Array Analysis between SCD and DPSCs

Pluripotent marker profiles of SCD and DPSCs were compared with BM-MSCs as a calibrator to evaluate the stability of the gene expression profile over the course of culture. Forty-one genes were up-regulated, whereas 12 genes were down-regulated in either SCD or DPSCs as compared with BM-MSCs (Fig. 5*A–C*). On the whole, the pluripotent was highly maintained in SCD as compared with DPSCs and BM-MSCs. The expression profiles (ratio [DPSCs were kept at 1]) of some of the pluripotent markers (16, 23) between DPSCs and SCD at passage 5 were as follows: POU5F1 (OCT3/4) (18.54); TDGF1 (17.98); SOX2 (10.14); GABR3 (21.73); GAL (32.4); IFTM1 (28.40); and LIN28 (9.72). We also found that SCD expressed some of the endoderm and mesoderm markers such as GATA6, GATA4, SOX17, CDH5, FLT1, and DES (22, 23) as compared with DPSCs. Interestingly, DPSCs expressed higher neuron/ectoderm markers (24). The expression profiles (ratio [SCD was kept at 1]) at passage 5 were as follows: nestin (10.08); GBX2 (1.14); PAX6 (1.50); and TH (2.09). PCA (Fig. 5*D*), on the basis of up-regulated genes, showed 98.7% of all the variance between the experimental groups (SCD and DPSCs at passages 1 and 5) could be described by using the first (#PC1) and second principle (#PC2) component analysis. The remaining principle component (#PC3) had minor contributions to the total gene expression variance and produced no significant shifts between the experimental groups.

Confirmation of Pluripotent Array Analysis

The expression of OCT4, SOX2, and NANOG was significantly up-regulated ($P < .05$) in SCD as compared with DPSCs (Fig. 6*A, B*). On the contrary, the expression of some of the neuron markers (25) (PAX6, NUUR1, nestin, β-III tubulin) was up-regulated in DPSCs compared with in SCD. These results were consistent with the pluripotent array results (Fig. 6*A, C*).

Neuronal Differentiation in SCD and DPSCs

As shown in Fig. 7*A, B*, both SCD and DPSCs are capable of forming 3 distinct neurospheres. The neurospheres counted at day 15 revealed that DPSCs had a significantly increased number of neurites as compared with SCD. The total neurospheres counted per 10 mm^2 between SCD and DPSCs were as follows: 30 ± 7 and 39 ± 9, respectively, ($P < .001$) in the range of neurosphere sizes from 60–80 μmol/L; 29 ± 1 and 25 ± 5, respectively, in the range of neurosphere sizes from 81–100 μmol/L; and 9 ± 1 and 12 ± 2, respectively, in neurosphere sizes greater than 100 μmol/L (Fig. 7*C*). Once attached in coated dish at day 16, these neurospheres spontaneously show outgrowth and dendrite-like structure by day 5 (Fig. 7*D–I*). Real-time PCR showed a higher expression of neuronal markers (23) (βIII-tubulin, NF,

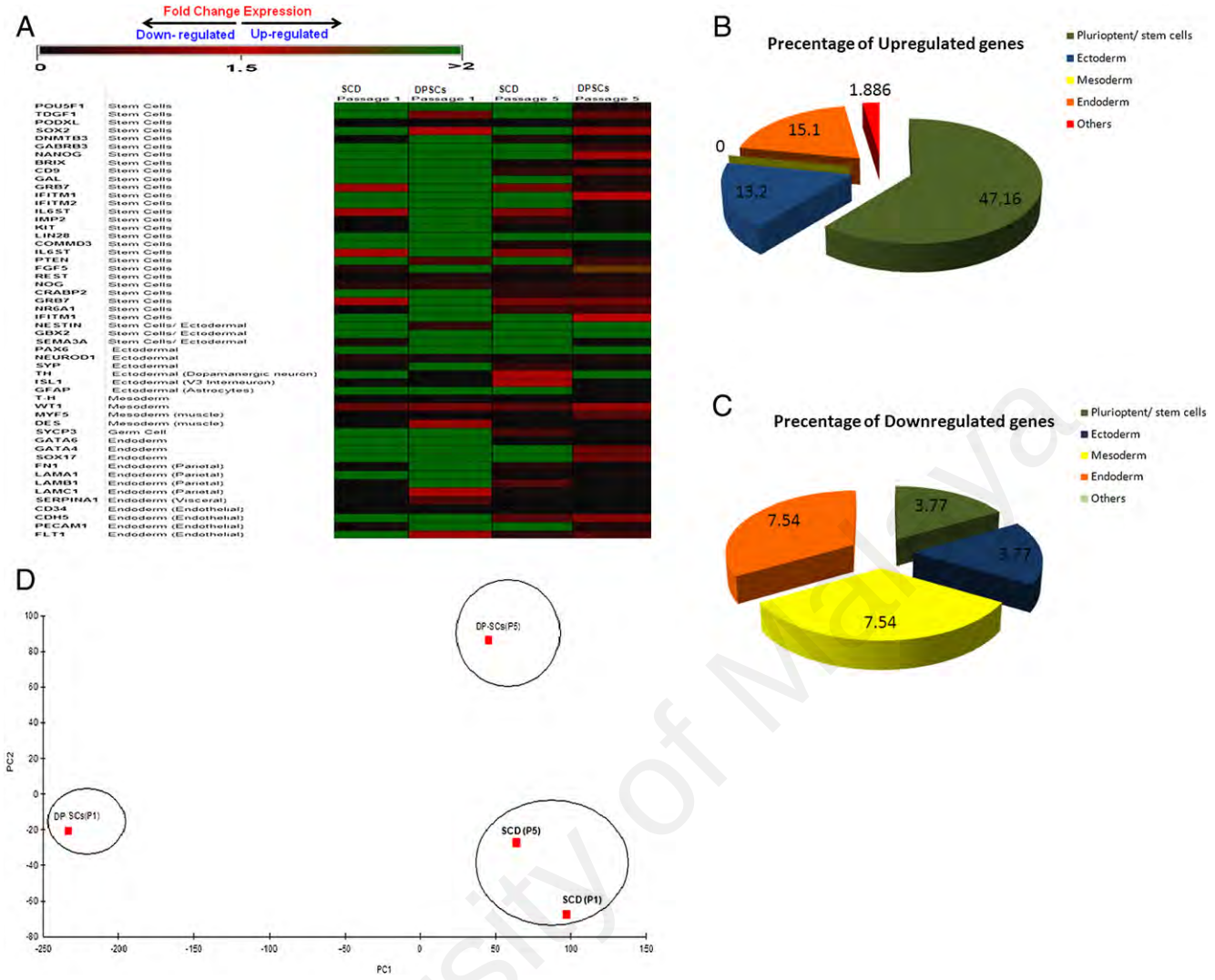


Figure 5. Expression profile of pluripotent/stem cells and lineage markers of SCD and DPSCs at passages 1 and 5. Expression levels of set of pluripotent/lineage marker genes. Gene levels were normalized against 18s, and mRNA from BM-MSCs was used as calibrator. (*A*) The genes were represented in the heat map (low expression in black and high expression in red); (*B*) percentage of up-regulated genes in SCD or DPSCs as compared with BM-MSCs; (*C*) percentage of down-regulated genes in SCD or DPSCs as compared with BM-MSCs; (*D*) PCA of pluripotent/stem cells and lineage markers of SCD and DPSCs. Cells were plotted according to their coordinates on PC1 and PC2. (This figure is available in color online at www.aae.org/joe/.)

GFAP; $P < .05$; $P < .001$) with the down-regulation of OCT4 in neuronal cells differentiated from DPSCs, as compared with neuronal cells differentiated from SCD (Fig. 7*J*). This was also established at protein level (Fig. 7*K–N*).

Discussion

The aim of this study was to characterize and to assess the propensity toward specific lineage of SCD and DPSCs. The growth kinetics results revealed that SCD possessed higher proliferation rate than DPSCs. Our results are concurrent with previous reports on the comparison between SCD and DPSCs (26). The use of SCD and DPSCs is easy for several reasons. The first and foremost reason is the ease of isolation, noninvasive collection with less or no ethical issues compared with BM-MSCs (26).

We further demonstrated that both SCD and DPSCs were able to differentiate into osteoblasts, adipocytes, and chondrocytes, thus qualifying the minimum requirement of MSCs (27). However, quantification results of osteogenic and adipogenic indicated that SCD exhibited better differentiation capability than DPSCs. SCD was regarded as a novel population of stem cells that is capable of differentiating into various cell types (at both *in vitro* and *in vivo*) into osteoblasts, odontoblasts, adipocytes, chondrocytes, and even hepatocytes (12, 28–30). On the other hand, DPSCs were considered to be more appropriate candidates for dental tissue regeneration (31) and neurodegenerative diseases (32). Nonetheless, most of the stem cells might differentiate *in vitro* into the desirable cells through well-defined transcriptional cascade, which can be initiated experimentally with xenobiotics such as IBMX, dexamethasone, and insulin (33–35).

In the current study we have investigated the inherent propensity of the stem cells to differentiate into their natural destiny on the basis of their origin. We found that SCD highly expressed many pluripotent markers as compared with DPSCs. Similar findings have been reported previously by our group (16) by using Wharton's jelly mesenchymal stem cells (WJ-MSCs), whereby higher expression of pluripotent markers was found in WJ-MSCs as compared with BM-MSCs. Hence,

University of Malaya

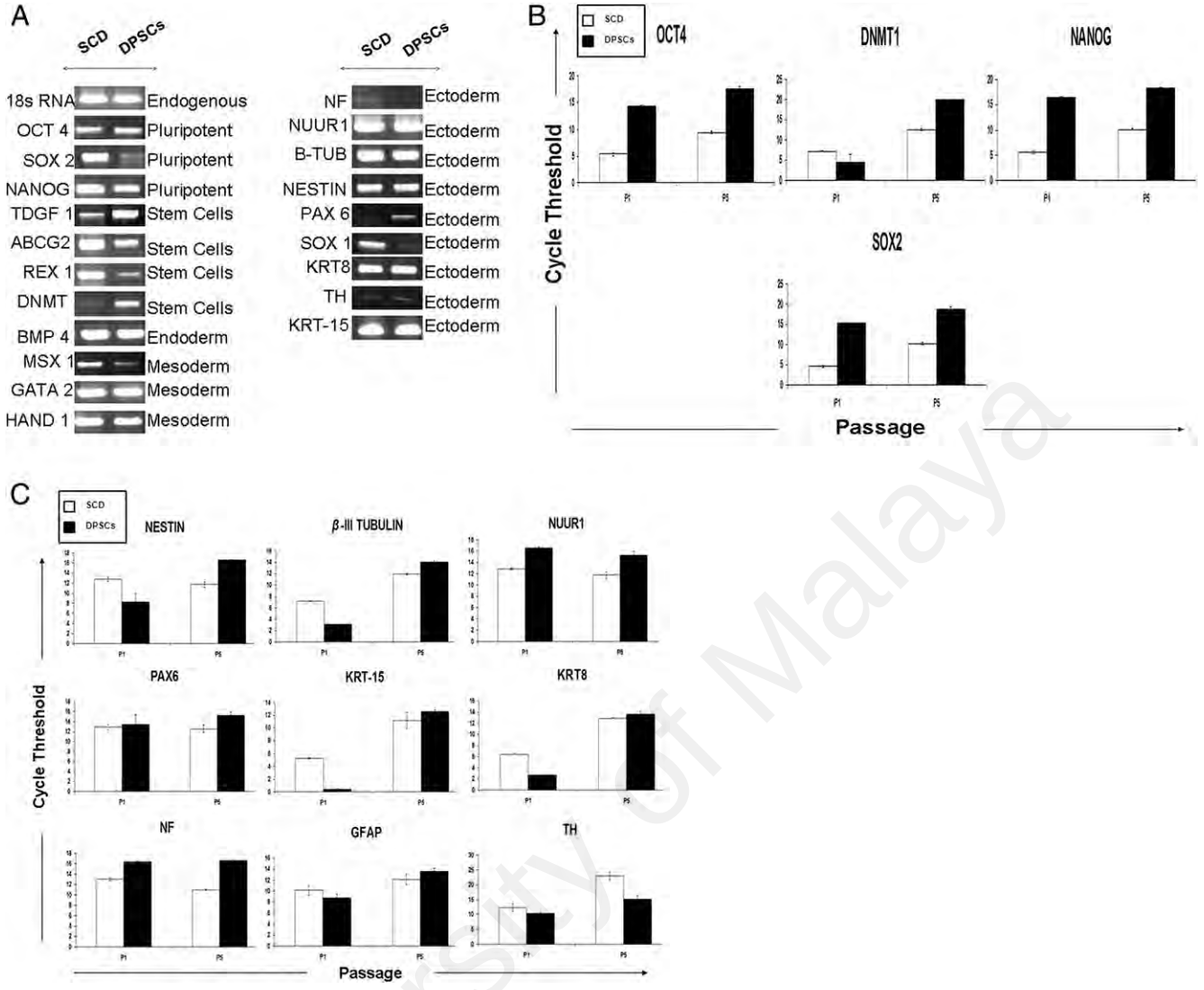


Figure 6. Expression profile of pluripotent and lineage-specific stem cell markers of SCD and DPSCs. (*A*) Semiquantitative RT-PCR of selected pluripotent/stem cells and lineage markers. (*B*) Relative levels of selected pluripotent/stem cells and lineage markers were performed by Taqman-based assay qRT-PCR. (*C*) Relative levels of selected pluripotent/stem cells and lineage markers were performed by SYBR green–based qRT-PCR. The lower the C_T value, the more copies are present in the specific sample. Values are presented after normalized to 18s RNA level. The average of 2 replicates is displayed.

these data support the notion that SCD is more primitive or pluripotent population of cells similar to WJ-MSCs. One of the striking features of this study is the overexpression of many transcription factors such as POU5F1 (also known as OCT3/4), SOX2, NANOG, and LIN28 that are responsible for the maintenance of pluripotency in early embryos and embryonic stem cells (36, 37). The abundant persistence of these markers leading to stemness status was very recently shown where limbal progenitor cells could be induced to pluripotent stem cells with the characteristics of embryonic stem cells under different culture conditions and without induction of exogenous transcription factors (38). Given that cells from the same organ or tissue will share some commonalities in gene expression and share the same microenvironmental niche (39), we hypothesize that the generation of ectoderm-specific cell type would be highly efficient as compared with BM-MSCs, because DPSCs are of neural crest origin (4). On the basis of our results, we predicted that SCD could also be forced to form desired cell type under the influence of appropriate microenvironment. PCA performed on the dataset by using the regulated genes showed that SCD and DPSCs exhibited a distinct expression pattern. Van de Waterbeemd et al (40) used this PCA method to study the optimal harvest point in bacterial vaccine production, and Liu et al (41) described the detection of endogenous signaling activation pathways by an oncogenic stimulus. Here we show yet another novel facet of PCA method to distinguish between gene expression pattern of SCD and DPSCs.

Dental cells are neural crest derived (4), and because neural crest stem cells can differentiate into neural cells *in vivo* (42, 43), dental pulp stem cells are likely to have a greater potential for neural differentiation than any other stem cells. However, our data revealed differential admixture of lineage proclivity between DPSCs and SCD, although both came from the same origin. The ectoderm lineage commitment of DPSCs was reflected by their ability to express higher neuronal mRNA in undifferentiated state. To test whether the variation of neuronal mRNA found in undifferentiated DPSCs and SCD will reflect their end point differentiation, we detoured undifferentiated DPSCs and SCD cells into neuronal cells and characterized them. A generally accepted experiment for the presence of neuronal cells is the induction of neurospheres (44–46)

University of Malaya

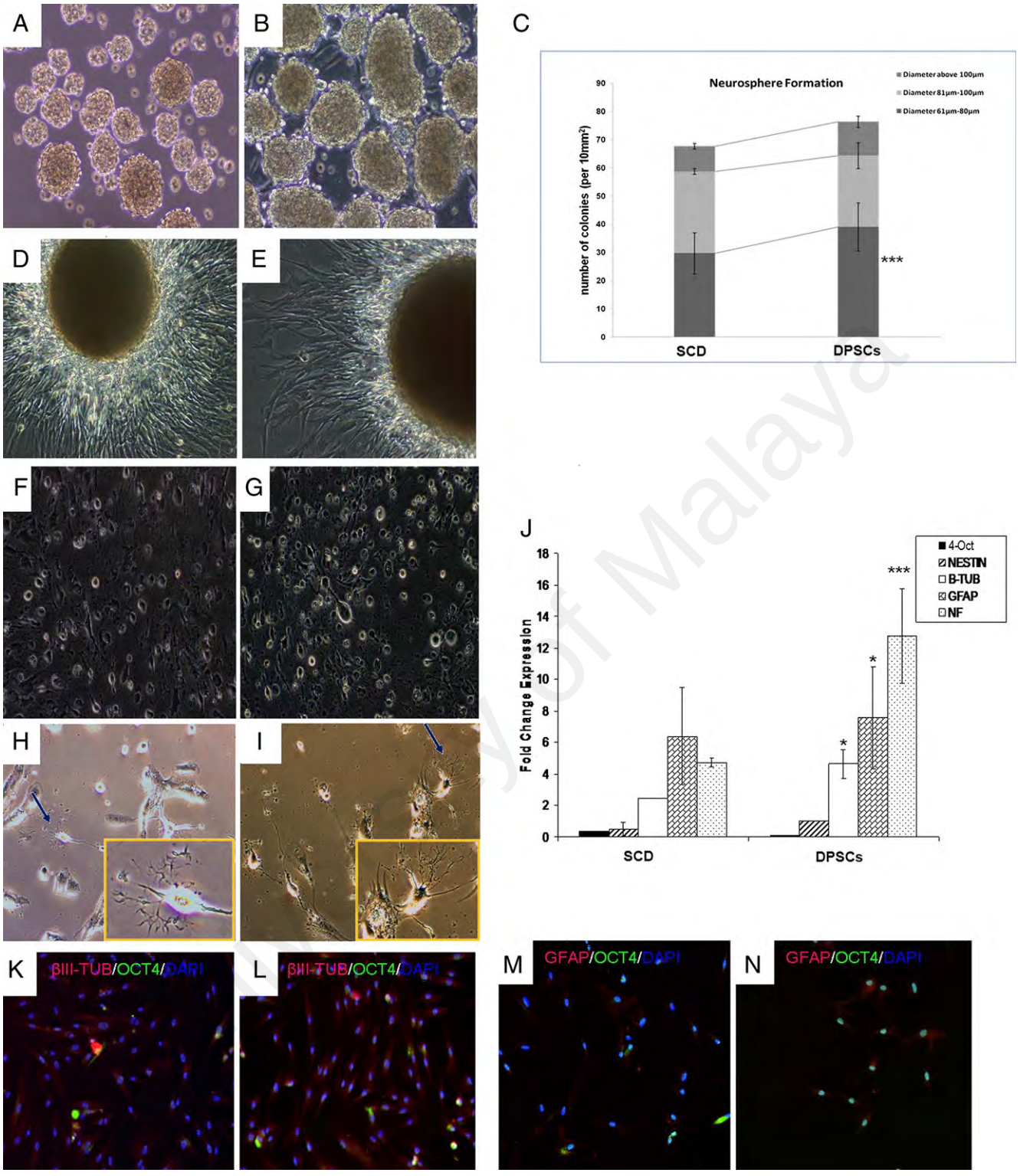


Figure 7. Schematic overview of the generation of neurospheres in SCD and DPSCs and gene expression profile of selected neuron markers. (*A, B*) Formation of neurosphere in non-coated dish for 15 days under neuron media in SCD and DPSCs, respectively; (*C*) the neurospheres formed under neuron-condition media at day 15. The number of neurosphere was divided with small (61–80 μmol/L), medium (81–100 μmol/L), and large diameters (>100 μmol/L). (*D, E*) Neurospheres were transferred into a coated dish at day 16 and were migrated radially out of the sphere in SCD and DPSCs, respectively. (*F, G*) After 2 days of maturation, cells had morphologic features typical of neuron in SCD and DPSCs, respectively. (*H, I*) After 5 days of maturation, dendrite-like outgrowth from SCD and DPSCs, respectively, showing complex neuronal processess (arrow and insert); (*J*) gene expression profile of OCT4, nestin, β-III tubulin, GFAP, and NF at day 20 in SCD and DPSCs. (*K–N*) Specific co-immunocytochemical staining of neurospheres indicated the presence of β-III tubulin and GFAP at day 20 in SCD and DPSCs, respectively. In both co-immunocytochemical pictures, nuclei were stained with DAPI (blue) and OCT4 (green); $*P < .05$, $**P < .01$, and $***P < .001$. (This figure is available in color online at www.aae.org/joe/.)

University of Malaya

in which cells aggregate to floating spheres. We found higher neurospheres in DPSCs as compared with SCD. One of the probable reasons could be the tremendous fold expression of nestin that was found in DPSCs as compared with SCD. Nestin, the marker for neuroepithelial stem cells (47, 48), seems to be essential for the induction of neurospheres (49). Wislet-Genedebien et al (50) generated neurospheres only from rat BM-MSCs after induction of nestin expression by supplementation with N2 and B27. This abundant expression of nestin could potentially enable DPSCs to differentiate more efficiently than SCD into neural cells and/or act neuroprotective after transplantation. Whether DPSCs indeed possess the ability to differentiate into neuronal committed cells is currently under investigation. We are aware that the expression of 1 or 2 markers like GFAP, NF, or βIII-tubulin is not sufficient proof for neuronal functionality. Nevertheless, our finding is a step forward in evaluating higher propensity of DPSCs toward neuronal lineage as compared with SCD, which is novel and unexpected.

In conclusion, our study showed that gene variations occurred within the different sources of the same stem cells, and these variations determine their lineage propensity toward a specific destination. From this study we infer that SCD retained their plasticity over the passages, whereas DPSCs lost their plasticity and were shown to be more committed toward neuronal lineage. Our results clearly demonstrated that both SCD and DPSCs could act as useful candidates for regenerative medicine in various diseases, emphasizing the usage of DPSCs for neurologic diseases.

Acknowledgments

This work was funded by University Malaya Research Grant (UMRG/RG073/09HTM) under Stempeutics Research Malaysia Sdn Bhd and University Malaya joint research collaboration. We are grateful to Ms Saratha Thevi Thrichelvam, Stempeutics Research Malaysia Sdn. Bhd, Kuala Lumpur, Malaysia for her assistance in FACS, karyotyping, cell cycle data analysis and Ms Srinda Nathan from Analisa Resources for helping with TLDA pluripotent microarray.

References

1. Gronthos S, Mankani M, Brahim J, et al. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 2000;97:13625–30.
2. Ikeda E, Yagi K, Kojima M, et al. Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease. Differentiation 2008;76:495–505.
3. D'Aquino R, De Rosa Alfredo, Lanza V. Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur Cell Mater 2009;18:75–83.
4. Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initation of the mammalian tooth germ. Development 1988;103:155–69.
5. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004;364:149–55.
6. Sonoyama W, Liu Y, Fang D, et al. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS ONE 2006;1:e79.
7. Gronthos S, Brahim J, Li W, et al. Stem cell properties of human dental pulp stem cells. J Dent Res 2002;81:531–5.
8. Laino G, Carinci F, Graziano A, et al. In vitro bone production using stem cells derived from human dental pulp. J Craniofac Surg 2006;17:511–5.
9. Iohara K, Zheng L, Ito M, et al. Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. Stem Cells 2006;24:2493–503.
10. Zhang W, Walboomers XF, Van Kuppevelt TH, et al. In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. J Tissues Eng Regen Med 2008;2:117–25.
11. Takeyasu M, Nozaki T, Daito M. Differentiations of dental pulp stem cells into neural lineage. Pediatric Den J 2006;16:154–62.
12. Ishkitiev N, Yaegaki K, Calenic B, et al. Deciduous and permanent dental pulp mesenchymal cells acquire hepatic morphologic and functional features in vitro. J Endod 2010;36:469–74.
13. Arthur A, Rychkov G, Shi S, et al. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 2008;26:1787–95.
14. Gandia C, Arminan A, Garcia-Verdugo JM, et al. Human dental pulp stem cells improve left ventricular function, induce angiogenesis and reduce infarct size in rats with acute myocardial infarction. Stem Cells 2008;26:638–45.
15. Pal R, Totey S, Mamidi MK, et al. Propensity of human embryonic stem cell lines during early stage of lineage specification controls their terminal differentiation into mature cell types. Exp Biol Med 2009;234:1230–43.
16. Nekanti U, Rao VB, Bahirvani AG, et al. Long-term expansion and pluripotent marker array analysis of Wharton's Jelly-derived mesenchymal stem cells. Stem Cells Dev 2010;19:117–30.
17. Lindroos B, Maenpaa K, Ylikomi T, et al. Characterization of human dental stem cells and buccal mucosa fibroblasts. Biochem Biophys Res Commun 2008;368:329–35.
18. Pal R, Hanwate M, Jan M, et al. Phenotypic and functional comparison of culture condition for upscaling of bone marrow derived mesenchymal stem cells. J Tissue Eng Regen Med 2009;3:163–74.
19. Afifi AA, Azen SP. Statistical analysis: a computer oriented approach. 2nd ed. New York: Academic Press; 1979.
20. Jollifte IT. Principle component analysis. 2nd ed. Aberdeen, UK: Springer Series in Statistics; 2002.
21. Nakamura S, Yamada Y, Baba S, et al. Culture medium study of human mesenchymal stem cells for pratical use of tissue engineering and regenerative medicine. J Bio-Medical Materials and Engineering 2008;18:129–36.
22. Karaoz E, Dogan BN, Aksov A, et al. Isolation and in vitro characterization of dental pulp stem cells from natal teeth. Histochem Cell Biol 2010;133:95–112.
23. Morsceck C, Vollner F, Saugspier M, et al. Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SCD) after neural differentiation in vitro [published online ahead of print July 10, 2009]. Clin Oral Invest doi:10.1007/s00784-009-0310-4.
24. Vescovi AL, Reynolds BA, Fraser DD, et al. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EFG-generated CNS progenitor cells. Neuron 1993;11:951–66.
25. Vollner F, Ernst W, Driemel O, et al. A two-step strategy for neuronal differentiation *in vitro* of human dental follicle cells. Differentiation 2009;77:433–41.
26. Nakamura S, Yamada Y, Katagiri W, et al. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J Endod 2009;35:1536–42.
27. Dominici M, Blanc KL, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells: the international society for cellular therapy position statement. Cytotherapy 2006;8:315–7.
28. Koyama N, Okubo Y, Nakao, et al. Evaluation of pluripotency in human dental pulp cells. J Oral Maxillofac Surg 2009;67:501–6.
29. Kerkis I, Kerkis A, Dozortsev, et al. Isolation and characterization of a population of immature dental pulp stem cells expressing Oct-4 and other embryonic stem cell markers. Cells Tissues Organs 2006;184:105–16.
30. Miura M, Gronthos S, Zhao M, et al. SCD: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 2003;100:5807–12.
31. Batouli S, Miura M, Brahim J, et al. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 2003;82:976–81.
32. Sasaki R, Aoki S, Yamato M, et al. Neurosphere generation from dental pulp of an adult rat incisor. Eur J Neurosci 2008;27:538–48.
33. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 1988;136:42–60.
34. Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomycte phenotype in the adult murine heart. Circulation 2002;105:93–8.
35. Dennis JE, Merriam A, Awadallah A, et al. A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. J Bone Miner Res 1999;14:700–9.
36. Niwa H. How is pluripotency determined and maintained? Development 2007;134:635–46.
37. Pesce M, Anastassiadis K, Scholer HR. Oct-4: lessons of totipotency from embryonic stem cells. Cells Tissues Organs 1999;165:144–52.
38. Balasubramanian S, Babai N, Chaundhuri A, et al. Non cell-autonomous reprogramming of adult ocular progenitors: generation of pluripotent stem cells without exogenous transcription factors. Stem Cells 2009;12:3053–62.
39. Ma D, Ma Z, Zhang X, et al. Effect of age and extrinsic microenvironment on the proliferation and osteogenic differentiation of rat dental pulp stem cell in vitro. J Endod 2009;35:1546–53.
40. Van de Waterbeemd B, Streefland M, Peenings J, et al. Gene-expression-based quality indicates optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnol Bioeng 2009;103:900–8.
41. Liu Z, Wang M, Alvarez JV, et al. Singular value decomposition-based regression activation of endogenous signaling pathways in vivo. Genome Biol 2008;9:R180.

42. Lasky JL, Wu H. Notch signaling, brain development and human disease. Pediatr Res 2005;57:104R–9.
43. Pardal R, Ortega-Saenz P, Duran R, et al. Glial-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. Cells 2007;131:364–77.
44. Cattaneo E, McKay R. Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 1990;347:762–5.
45. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–10.
46. Stroch A, Paul G, Csete M, et al. Long term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. Exp Neurol 2001;170: 317–25.
47. Dahlstrand J, Lardelli M, Lendahl U. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res Dev 1995;84:109–29.
48. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cells 1990;60:585–95.
49. Tropepe V, Hitoshi S, Sirard C, et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 2001;30:65–78.
50. Wislet-Gendebien S, Leprince P, Moonen G, et al. Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. J Cell Sci 2003; 116:3295–302.

University of Malaya

3.2 Publication 2 (Original Research)

Vasanthan P, Gnanasegaran N, Govindasamy V, Abdullah AN, Jayaraman P, Ronald VS, **Musa S**, Kasim NHA: Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. Biochemical Engineering Journal. 2014, 88:142-153

3.2.1 Contributions of co-authors:

Design and concept of study	GV, SM
Acquisition of data	VP, SM, JP, GN, RVS
Analysis of data	VP, SM, GV
Drafting of manuscript	VP, SM, GV
Revising manuscript for intellectual content	All authors

University of Malaya

Biochemical Engineering Journal 88 (2014) 142–153

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells

Punitha Vasanthan [a], Nareshwaran Gnanasegaran [b], Vijayendran Govindasamy [c,*], Aimi Naim Abdullah [a], Pukana Jayaraman [a], Veronica Sainik Ronald [a], Sabri Musa [a,**], Noor Hayaty Abu Kasim [d]

[a] Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
[b] Regenerative Dentistry Research Group (ReDreG), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
[c] cGMP Compliance Stem Cells Laboratory, Hygieia Innovation Sdn. Bhd, Lot 1G-2G, Lanai Complex, No. 2, Persiaran Seri Perdana, Persint 10, Federal Territory of Putrajaya, Malaysia
[d] Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 17 October 2013
Received in revised form 18 April 2014
Accepted 19 April 2014
Available online 26 April 2014

Keywords:
Endoderm
Progenitors
Autologous
Allogeneic
Cell transplantation
Liver failure

ABSTRACT

The scarcity of organs for liver transplant is a major pressure point of liver transplantation. Hence, generating hepatocytes may provide an alternative choice for therapeutic applications. At present, dental pulp stem cell (SCDs) is an emerging source in regenerative medicine. However, existing protocols for cell culture requires fetal bovine serum (FBS) as a nutritional supplement and may carry the risk of transmitting diseases. Therefore, the present study was undertaken to examine the efficacy of human platelet lysate (HPL) as a substitute for FBS in terms of proliferation and differentiation of SCDs into hepatic lineage cells. The result showed that HPL had displayed a superior effect on the proliferation of SCDs. Next, we induced SCDs into hepatic lineage cells which thrived by initiation and followed by maturation into functional hepatocytes for a total of 21 days. We observed that the gene, protein and its functional profile during this differentiation process reiterated in vivo liver development demonstrating a steady down-regulation of early endoderm markers (GATA4, GATA6, SOX17, HNF4α, HNF3β and AFP) with the up-regulation of hepatic specific markers (TDO, TO, TAT, ALB, AAT, CK18). We also noticed the presence of CK19 suggesting a progenitor population. To ascertain this, we checked for the expression of pluripotent markers and observed that it remained unchanged throughout the experiment period. Our results provide new insights on the ability of SCDs to differentiate into hepatic lineage cells and most remarkably, this can be done in autologous settings whereby both cell source and HPL can be derived from the same donor thus reducing the risk of disease transmission.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

In recent years, the general human population faced a wide range of chronic liver diseases that have led to the deficiency of liver functions. Hence, there is a great necessity for therapeutic intervention to overcome this situation. Previously, allogeneic liver transplantation has been considered as an attractive therapeutic tool for the liver dysfunction [1]. However, this approach has certain boundaries such as serious shortage in organ donor and possibilities of rejection by the host immune system. In observation of these deficits, cell based hepatocytes transplantation has been suggested as the best alternative to cure liver dysfunction since it is simpler, non-invasive and could be cryopreserve for future use [2] Nevertheless, several studies have reported that the direct transplanted hepatocytes cells have lower survival rate and thus could not accomplish enough liver population successfully [1]. Consequently, researches are in search for other effective treatments and cell based therapies have

* Corresponding author at: cGMP Compliance Stem Cells Laboratory, Hygieia Innovation Sdn. Bhd, Lot 1G-2G, Lanai Complex, No. 2, Persiaran Seri Perdana, Persint 10, 62250 Federal Territory of Putrajaya, Malaysia. Tel.: +60 388902968; fax: +60 388902969.
** Corresponding author at: Department of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel.: +60 379674816; fax: +60 379674530.
E-mail addresses: vijay07001@gmail.com, vijay@hygieiainnovation.com (V. Govindasamy), sabrim@um.edu.my (S. Musa).

http://dx.doi.org/10.1016/j.bej.2014.04.007
1369-703X/© 2014 Elsevier B.V. All rights reserved.

University of Malaya

appeared to offer a promising alternative for treating liver dysfunction.

In this regards, mesenchymal stem cells (MSCs) which are known as a versatile and a 'universal' cell can be used for the treatment of several human diseases such as liver related diseases. Likewise, dental pulp stem cells from extracted deciduous teeth (SCDs) were shown to putatively form post natal stem cells that are capable of self-renewal, highly proliferative and also multipotent [3]. Previously, we as well as other researchers have shown there are tremendous differentiation potentials of SCDs such as neuronal differentiation [3], beta cell lines [4], and cardio differentiation [5]. Additionally, recent studies have found that SCDs may undergo hepatocyte differentiation if given veracious microenvironment for induction [6,7].

All the above mentioned research portrayal of SCDs were customarily conveyed in ex vivo conditions that contain basal medium supplemented with FBS which is a crucial nutrient for cells. However, one serious concern on the usage of FBS is the risk of animal orientated viral and pathogens. Moreover, the variation of composition in lot-to-lot collection of FBS has resulted in phenotypical differences [8]. From the point of view of animal ethics, the collection of FBS has caused great grief among the animal lovers as it involved the killing of prenatal cows as well as calf fetus [9]. Since numerous concerns have been put forth pertaining to the usage of FBS, consequently, a paradigm shift to animal serum-free alternatives is promoted by regulatory authorities and research communities in general. A chemically defined xeno-free media could be the preferred solution. However, such a formulation that allows for both isolation and expansion has not been fully achieved thus far. Further, making a chemically defined media as well as FBS itself ultimately could be an obstacle if the cells go for a large scale clinical stage [10].

Thus, initiation of human-based supplement, explicitly HPL, has been introduced increasingly in stem cell therapy as a potential surrogate to FBS. The components that reside within the HPL are; platelet derived growth factors, basic fibroblast growth factors, vascular endothelial growth factors, insulin growth factors and transforming growth factors-β are known to serve as cell culture growth factors [11]. Among the persuasive mediator released from platelet are adhesive protein, coagulant factors, mitogen, protein inhibitors and proteoglycan [12]. Hence, HPL may replace FBS in many cell culture systems. Despite this, the current body of literature on the usage of HPL in stem cells are heavily focused on its proliferation capacity and little is known on the differentiation capacity. We have previously reported that HPL permits extensive proliferation of SCDs for clinical application [10]. Here, we investigated the trans-differentiation potential of SCDs exposed to HPL media toward hepatic lineage cells. The hepatic lineage cells generated from SCDs which had been exposed to HPL media offer an alternative source of hepatocytes which will provide great advantages in liver disease treatments especially in autologous settings whereby both cell source and HPL can be derived from the same donor thus reducing the risk of disease transmission.

2. Materials and methods

2.1. Isolation of cells

This study was conducted under ethical approval from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [DF CD 0907/0042 (L)]. SCDs cultures were obtained from four young donors ($n = 4$; age 3–9 years old) as previously described [3,4]. Briefly, root surfaces were cleaned with Povidone-iodine (Sigma Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com) and the pulp were extirpated within 2 h post-extraction and processed. The pulp tissue was minced into small fragments prior to digestion in a solution of three mg/mL collagenase type I (Gibco, Grand Island, NY, http://www.invitrogen.com) for 40 min at 37 °C. After neutralization with 10% FBS, the cells were centrifuged and seeded in culture flasks.

It should be noted that human hepatocellular carcinoma cell line (HepG2, ABM, Biorev) was used as a positive control cell lines in the present study. These cells are highly differentiated and display many of the genotypic features of normal liver cells [13].

2.2. Human platelet lysate (HPL)

HPL was prepared as described by our group previously [10] in current good manufacturing practice (cGMP) condition. We also reported that HPL is free from animal origin, has been characterized, and passed the quality control test in terms of endotoxin level, mycoplasma, pH and sterility prior to the SCDs expansion and differentiation usage.

2.3. In vitro expansion of SCDs culture

For expansion of the SCDs culture, culture media was prepared using basal media of Knockout DMEM (Invitrogen) supplemented with 10% Australian characterized FBS (Hyclone, MA, USA, http://www.thermofisher.com), 0.01× Glutamax (Invitrogen) and 0.5% penicillin/streptomycin (Invitrogen). Since the amount of seeding materials is so small, taking the chance to lose the materials by adapting to new ingredients is risky. Therefore, we initially used the FBS to set the ball rolling since that is the commonly used nutrient in any media composition for culturing. At passage 1 (P1), the cells were divided into 2 groups, we retained the FBS in one group and introduced HPL supplemented with 0.001 mL of heparin (Heparinol) to the second group. The cells were cultured for 5 passages (P1–P5). We allowed the cells to acclimatize to HPL condition at least for one passage before we started with the differentiation process at P3 whereas other basic experiments except for growth kinetics were conducted at P5.

2.4. Growth kinetics

In this procedure the proliferation rate was determined by plating 5000 cells/cm^2 of each SCDs into T25 cm^2 culture flasks (BD Pharmingen). Three replicates were performed for each passage. Cells were detached by trypsinization, after reaching confluency of 90%. Cells were then counted and assessed for viability by means of trypan blue dye exclusion before splitting the cells into the next passage. Cells were re-plated for subsequent passages and a total of 5 passages were studied in this experiment. Growth kinetics was analyzed by calculating population doubling (PD) time. The PD time was obtained by the formula:

$$\mathrm{PDT} = \frac{\log_2(\mathrm{time})}{\log(\text{initial no. of cell} - \text{final no. of cell})}$$

2.5. Colony forming unit (CFU) assay

The colony forming unit (CFU) assay was determined by replating 100 cells in 35 mm dish (BD Bioscience) followed by 14 days of culture at 37 °C with 5% CO_2. Then, the cells were rinsed twice from growth media using Dulbecco's Phosphate Buffered Saline (DPBS, -Ca^{2+}, -Mg^{2+}; Invitrogen) and fixed with 100% methanol (Mallindkrodt, Hazelwood, USA, http://pharmaceuticals.covidien.com) for 20 min at room temperature (RT), followed by 3% crystal violet (Sigma Aldrich) staining. Next, the blue stain was

rinsed four times using tap water until the dishes became colorless. The dishes were then inverted downward on a clean cloth and allowed to air-dry for several minutes. Stained colonies with sizes larger than 2 mm were counted. The CFU of SCDs was calculated using the formula:

$$\text{CFU} = \frac{\text{the total number of colonies stained}}{\text{the initial number of cells}} \times 100\%$$

2.6. Senescence associated β-galactosidase (SA-β-gal) assay

The senescence associated β-galactosidase (SA-β-gal) assay was carried out using senescence β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA, http://www.cellsignal.com) and was done according to the manufacturer's procedures. The SA-β-gal assay of SCDs was calculated using the following formula:

$$\text{SA-}\beta\text{-gal} = \frac{\text{number of senescence cells}}{\text{total number of cell}} \times 100\%$$

2.7. Multi-lineages differentiation of SCDs

For the multi-lineages differentiation process a total of 1000 cell/cm^2 SCDs were cultured in a six-well plate (BD Bioscience) until confluent for both FBS and HPL culture media. Then, the culture media were removed and differentiation media was added: for osteogenic induction media the components were Knockout DMEM (Invitrogen), 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 0.1 μM dexamethasone sodium phosphate (Sigma Aldrich), 0.2 mM L-ascorbic 2 phosphate (Sigma Aldrich) and 10 mM β-glycerol-2-phosphate (Sigma Aldrich), and later evaluated by Von Kossa staining. As for the adipogenic media, the contents were Knockout DMEM (Invitrogen), 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 1 μM dexamethasone sodium phosphate (Sigma Aldrich), 10 μg/mL insulin (Sigma Aldrich), 200 μM Indomethacin (Sigma Aldrich) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich), and stained using Oil Red O (Sigma Aldrich) staining procedure. For chondrogenic media, we used Knockout DMEM (Invitrogen) with 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 5.35 μg/mL linoleic acid (Sigma Aldrich), 10 μg/mL L-ascorbic 2-phosphate (Sigma Aldrich), 10 μg/mL insulin (Sigma Aldrich), 1 μg/mL dexamethasone sodium phosphate (Sigma Aldrich) and 1.25 μg/mL bovine serum albumin (BSA; Sigma Aldrich), and was stained using Alcian blue staining (Sigma Aldrich).

2.8. Flow cytometric analysis

To determine whether culturing in different media supplement affects SCDs phenotype, cell surface markers were characterized using flow-cytometry at P5. On reaching 90% confluency, the cells were harvested with 0.05% trypsin (Invitrogen) and re-suspended in DPBS at a cell density of 1.5×10^6 cells/mL. An amount of 200 μL of cell suspension (1×10^5 cells) was incubated with labeled antibodies in the dark for 1 h at 37 °C. The following antibodies were used to mark the cell surface epitopes-CD44-phycoerythrin (PE), CD34-PE, CD45-PE, HLA-DR, CD73-fluoro isothyocyanate (FITC), CD90-FITC and CD166-PE, and CD105-FITC (all from BD Pharmingen). All analyses were standardized against negative control cells incubated with isotype-specific IgG1-PE and IgG1-FITC (BD Pharmingen). At least 10,000 events were acquired on Guava Technologies flow cytometer and the results were analyzed using Cytosoft, Version 5.2, Guava Technologies.

2.9. Hepatic differentiation

SCDs differentiation toward hepatic lineage cells under different media supplement was determined as described previously by Ishkitiev et al. [6] with some modifications. In short, initial hepatic induction media comprising Knockout DMEM (Invitrogen), 2% Australian characterized FBS (Hyclone) and 20 ng/mL recombinant human hepatocyte growth factor (HGF, R&D Systems, Minneapolis, MN, USA, http://www.rndsystems.com) were added for five consecutive days to the SCDs which were about 70% confluence. As for the HPL exposed cells, 2% FBS was replaced by 2% HPL at the initial hepatic induction. The maturation process were carried out on day 6 with the following components; Knockout DMEM (Invitrogen), 10 ng/mL Oncostatin M (R&D Systems), 10 nmol/L dexamethasone (Sigma Aldrich) and 1% Insulin-Transferrin-Selenium-X (ITS, Invitrogen). Fresh media were added every 3 days for a total of 16 days of maturation process. In addition, the morphological changes of SCDs were captured every day for 21 days using phase-contrast microscope and HepG2 morphology was presented as the positive control.

2.10. Real time PCR (qPCR)

Total RNA was extracted from HepG2 cells (used as positive control), undifferentiated SCDs, differentiating SCDs and differentiated SCDs using Trizol reagent following the manufacturer's instructions. Its purity was assessed by the absorbance ratio 260/280 nm. RNA integrity was examined by agarose gel electrophoresis. Reverse transcription of the above mentioned RNA along with purchased total liver RNA (adult, ClonTech) to cDNA was performed using Superscript II according to the manufacturer's instructions (Invitrogen). The forward and reverse primers of the chosen genes were designed and listed in Additional file 1: Table 1. The PCR started at 94 °C for 5 min and was performed as follows: denaturation at 94 °C for 30 s, annealing at a temperature as indicated in Table 1 for 45 s, and elongation at 72 °C for 45 s. After 30 cycles, the samples were incubated for an additional 10 min at 72 °C. Internal calibration curves were generated by the real time software. A melting curve analysis was carried out between 60 °C and 95 °C with a plate read every 0.5 °C after holding the temperature for 20 s. The threshold cycle number (C_T) at which the signals crossed a threshold set within the logarithmic phase and the peaks of melting curves were recorded. The relative quantitation of gene expression in terms of fold change was calculated using the $\Delta\Delta C_T$. All calculations were normalized using 18sRNA gene as a reference control.

2.11. Immunofluorescence

To analyze the presence of hepatic markers, HepG2 cells (positive control), undifferentiated (negative control) and differentiated SCDs were fixed for 20 min in 4% ice cold paraformaldehyde, treated with 0.1% Triton-X for optimal penetration of cell membranes, and incubated at room temperature (RT) in a blocking solution (0.5% BSA; Sigma Aldrich) for 30 min. Primary antibodies alpha-fetoprotein (AFP) (mouse, Abcam), hepatocyte nuclear factor 4 (HNF4α) (mouse, Abcam), Albumin (ALB) (mouse, Sigma), Ctyokeratin 18 (CK18) (mouse, Millipore), Cytokeratin 19 (CK19) (rabbit, Abcam), Ki-67 (Abcam) with dilution ratio of 1:400 for all samples, except for CK19 with 1:500 ratio, were incubated overnight at 4 °C, washed with DPBS (Invitrogen), and then incubated with secondary antibody (either fluoroisothiocyanate [FITC]-conjugated IgG or rhodamine-conjugated IgG) at 1:700 ratio at RT for 90 min. Slides were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, Chemicon, Temecula, CA, USA) for 5 min. Fluorescent images were captured by means of

Table 1
List of primers used to characterize DPSCs differentiated into hepatocyte.

Gene symbol	Primer sequence (5′–3′)	Base pair
18 s	F: CGGCTACCATCCAAGGAA R: GCTGGAATTACCGCGGCT	186
AFP	F: CATCCAGGAGAGCCAAGCAT R: CGCCACAGGCCAATAGTTTG	209
GATA 4	F: CAGAAAACGGAAGCCCAA R: TTGCTGGAGTTGCTGGAAG	256
SOX17	F:CTGTAGACCAGACCGCGACA R: CTGGTCGTCACTGGCGTATC	207
HNF3β	F: ACTACCCCGGCTACGGTTC R: AGGCCCGTTTTGTTCGTGA	296
CK19	F: CCATGCGCCAGTCTGTGGAG R: GTGGTGCTCTCCTCAATCTGCT	321
AAT	F: AGACCCTTTGAAGTCAAGCGACC R: CCATTGCTGAAGACCTTAGTGATGC	358
TDO	F: GGTTTAGAGCCACATGGATT R: ACAGTTGATCGCAGGTAGTG	424
TO	F: GGCAGCGAAGAAGTACAAATC R: TCGAACAGAATCCAACTCCC	217
CYP3A4	F: TCACCCTGATGTCCAGCAGAAACT R: TACTTTGGGTCACGGTGAAGAGCA	251
CK 18	F: CCATGCGCCAGTCTGTGGAG R: GTGGTGCTCTCCTCA ATCTGCT	321
GATA 6	F: GCCTCACTCCACTCGTGTCT R: TCAGATCAGCCACACAATATGA	540
HNF4α	F: GCTTGGTTCTCGTTGAGTGG R: CAGGAGCTTATAGGGCTCAGAC	730
TAT	F: GCTAAGGACGTCATTCTGACAAG R: GTCTCCATAGATCTCATCAGCTAAG	353
CYP7a1	F: GAGAAGGCAAACGGGTGAAC R: ATCGGGTCAATGCTTCTGTG	275
ALB	F: AAGGCACCCCGATTACTCCG R: TGCGAAGTCACCCATCACCG	213

a Olympus-BX63F-FL-CCD microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com).

2.12. In vitro functional assay

For the above process periodic acid-Schiff (PAS) (Sigma Aldrich) was used to detect the glycogen storage in the undifferentiated SCDs (control), differentiating SCDs and differentiated SCDs cells at 7, 14 and 21 days of hepatic differentiation protocol in the 35 mm dishes (BD Bioscience) according to the manufacturer's protocol.

Further, a study on urea secretion was carried out. Here, the cell culture media were collected from the undifferentiated SCDs (control) and differentiating SCDs at day 7, 14 and 21 after 24 h of exposure to 1 mmol/L NH_4Cl (Sigma Aldrich). At the same time a fresh culture medium supplemented with 1 mmol/L NH_4Cl (Sigma Aldrich) was used as a control. All these media were tested for urea production by using the glutathione kinetic method [14] and the optical densities were measured at 492 nm.

The undifferentiated SCDs and differentiated cells along with HepG2 cells were washed with DPBS and incubated in a medium containing 10 μg/mL 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine labeled acetylated low-density lipoprotein (Dil-Ac-LDL; Invitrogen, USA) for 24 h at 37 °C to examine the low density lipoprotein (LDL) uptake. Cells were fixed with 4% formaldehyde and, after washing, counterstained with Hoechst 33342 (Sigma–Aldrich, USA). Incorporation of fluorochrome-labeled LDL and Hoechst 33342 into cells was observed under a fluorescent microscope (Olympus, Japan). Albumin production was determined by using the albumin blue fluorescent (ABF) kit from Active Motif adhering to the manufacturer's specification.

2.13. Statistical analysis

All values are given as mean and standard deviation. Data were analyzed using the SPSS statistical software, version 19.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed using the two way analysis of variance (2-ANOVA). The significance level was set at $p = 0.05$. At the same time, Tukey post hoc multiple comparisons were carried out to determine the differences between the groups.

3. Results

3.1. Basic characterization of SCDs expanded in FBS and HPL

As to extensively characterize the SCDs expanded using two altered culture conditions, a number of parameters were evaluated. In terms of morphology, SCDs expanded in FBS formed thicker, flattened cells compared to smaller and long-spindle shaped cells in HPL. Moreover, the SCDs in the presence of HPL had a tendency to grow overlapping each other, forming highly condensed colonies and eventually SCDs were loosely segregated in FBS (Fig. 1A).

This was reflected in their proliferation rate. Both accumulated growth curve and PDT were higher in SCDs cultured under HPL (P1: 1.71 M ± 0.10; 19.70 ± 0.29 h, P5: 4.42 M ± 0.20; 28.94 ± 0.26 h respectively; $p < 0.05$; Fig. 1B and C) than FBS (P1: 1.11 M ± 0.07; 21.93 ± 0.35 h, P5: 3.17 M ± 0.18; 37.81 ± 0.44 h respectively; Fig. 1B and C). The CFUs at P5 also demonstrated lower rate of cellular proliferation in FBS (67.50% ± 6.45) as compared to HPL (83.75% ± 4.79). Likewise, senescence phenomenon was monitored by beta-galactosidase in P5. It was also observed that percent senescence activity cultured in HPL media (20 ± 3.21) was significantly lower in prolonged passages as compared with the cells cultured in FBS (28 ± 5.92; $p < 0.05$; Fig. 1E).

However, there is no significant visual trilineage differentiation between both the growths media. Both cells cultured under HPL and FBS demonstrated clumping of neutral lipid vacuoles, deposition of silver stained mineralized matrix, and formation of proteoglycan in adipogenesis, osteogenesis and chondrogenesis differentiation media respectively (Fig. 1F).

We have compared the immunophenotype of the final population obtained from P5, and have found that the cells cultured in HPL or FBS did not deviate from each other. The cells steadily shared the same MSC markers which is more than 90% positivity for CD44, CD73, CD90, CD105, and CD166, and lower than 2% for CD45, CD34 as well as HLA-DR (Supplementary Fig. 1).

3.2. Differentiation of SCDs toward hepatic lineage cells

Microscopic examination has revealed that SCDs cultured under HPL and FBS have started to show remarkable transition, from elongated fibroblast-like cells, to a round epithelial-shape by day 7 of the initiation step of hepatic differentiation. The contraction of the cytoplasm progressed further during the maturation stage that took place throughout the 16 days of the hepatocyte maturation media. At Day 21, we noticed that most of the treated cells under HPL and FBS became quite dense and round with clear nuclei similar to HepG2 cell lines. We also noticed oval-shaped cells with polygonal structures and visible bile canaliculi structures (Fig. 2 and Supplementary Fig. 2).

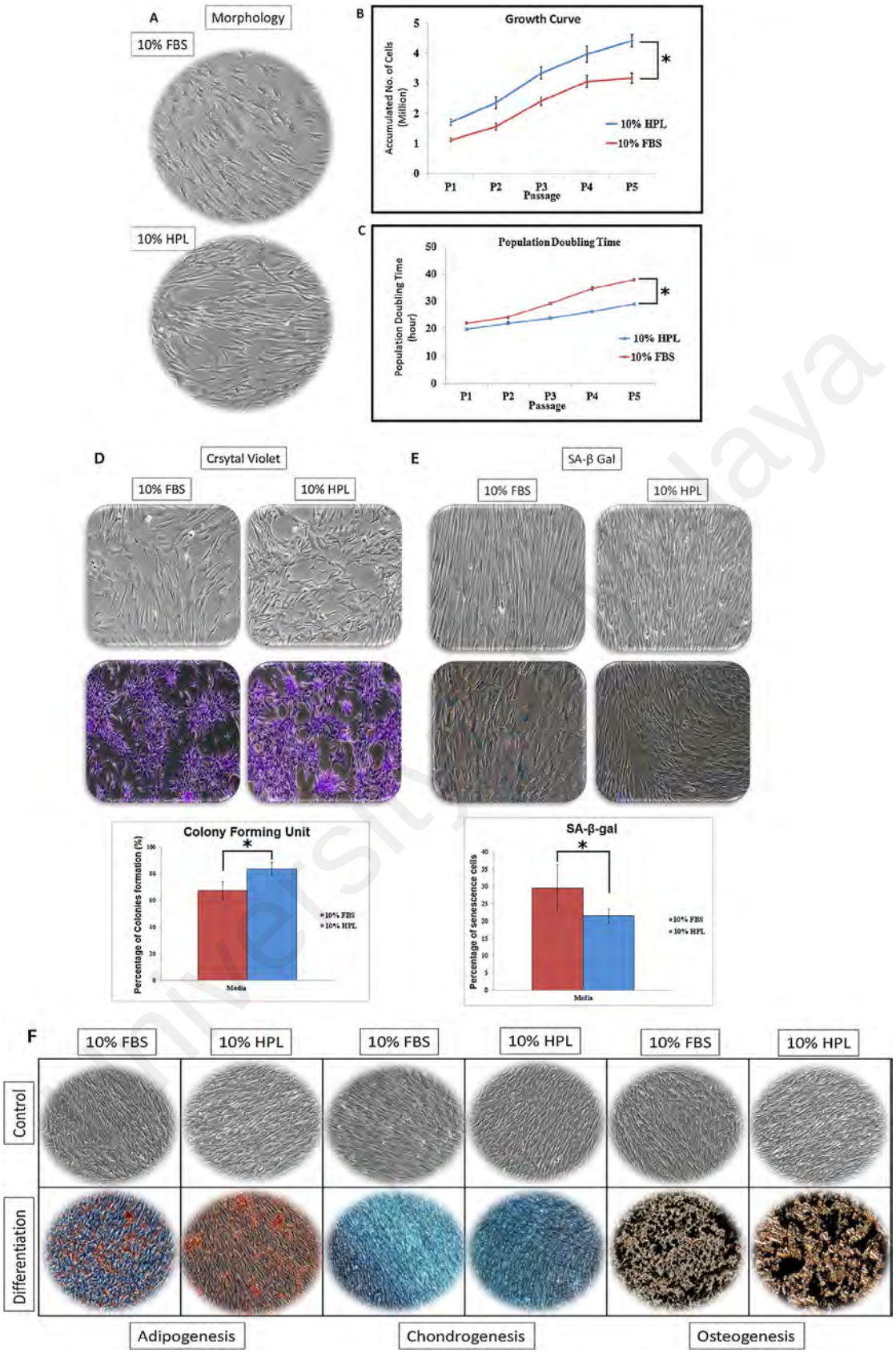


Fig. 1. Mesenchymal characteristics of SCDs cultured under HPL and FBS. (A) Morphological both SCDs culture under HPL and FBS appeared spindle shaped (scale bar = 100 μm). (B) and (C) Growth curve and population doubling time from P1 to P5 of respective cell culture. (D) Higher formation of colonies shown for SCDs cultured under HPL as compared to FBS. (E) Appearance of more senescent cells in SCDs cultured under FBS as to HPL condition. * indicates significance with p-value to be <0.05. (F) The panels display representative of visual observation of adipocytes detected by Oil Red O staining, chondrocytes detected by Alcian Blue staining and osteocytes detected by Von Kossa staining. * indicates significance with p-value to be <0.05. Results represent 4 culture replicates.

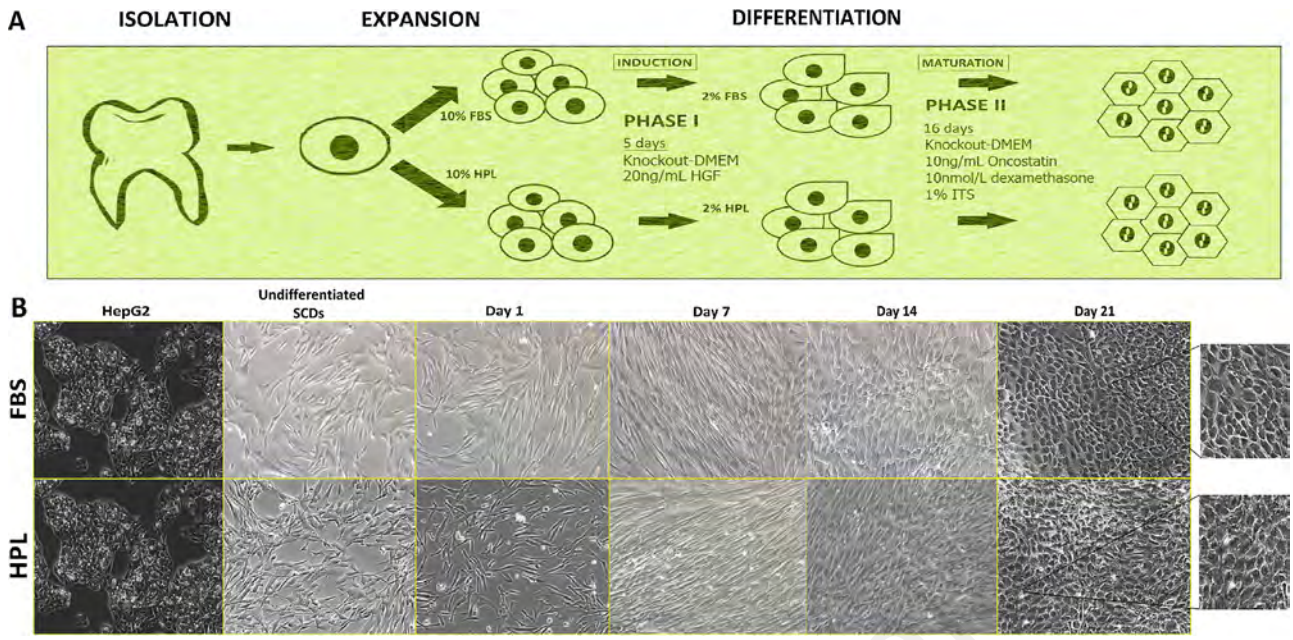


Fig. 2. Differentiation of SCDs into hepatic lineage (A) Schematic representation of the multistep differentiation protocol to generate hepatic lineage cells from SCDs under HPL and FBS condition. The time interval indicates change of media. SCDs upon exposure to both HPL and FBS, supplemented with growth factors to induce into hepatic lineage. The induction period last for the first 5 days followed by maturation period that last for another 16 days, making 21 days in total of the experiment period. (B) Photomicrographs (scale bar = 100 μm) of SCDs morphological changes during differentiation into hepatic lineage cells under HPL and FBS culture in comparison to positive control; HepG2. Attentively, magnification 20× images of Day 21 for both HPL and FBS present formation of polygonal shaped cells.

3.3. *Gene expression profile of hepatic lineage cells derived from SCDs*

To facilitate better understanding toward gene expression profile, we have categorized the expression patterns in a chronological structure, in which we found that a number of hepatic lineage genes during differentiation process of SCDs reiterated that of a liver development process in vivo (Figs. 3–5).

i) Expression of transcription factors, early and definitive endoderm markers – In the first priming stage of differentiation, SCDs were detoured through a meso-ectodermal origin to a definitive endodermal. Interestingly though GATA4, GATA6, sex-determining region Y box 17 (SOX17), hepatocyte nuclear factor 4 (HNF4α), hepatocyte nuclear factor 3 (HNF3β), and alpha-fetoprotein (AFP) are primitive endoderm as well as liver enrichment transcriptional factors, and we found that they were expressed in undifferentiated cells, exhibited more than 2-fold increase at day 7 as compared to undifferentiated cells and gradually decrease toward the end of the differentiation period. Further, the expression of all transcripts was greater than observed in HepG2 and adult liver tissue at undifferentiated cells at day 7, except for GATA6, HNF3β, and HNF4α.

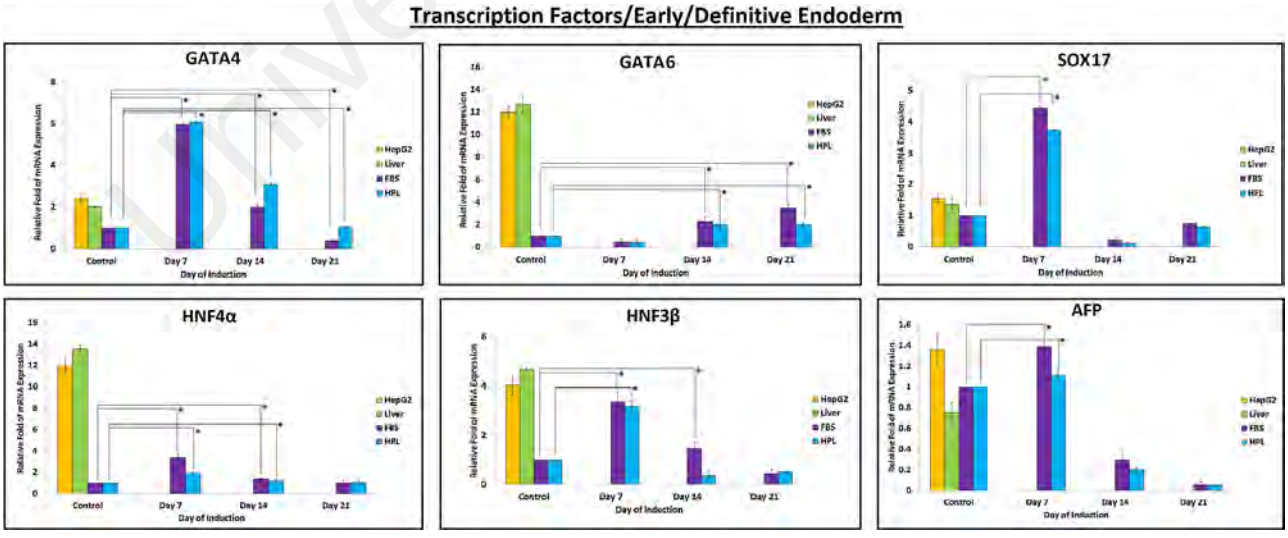


Fig. 3. Gene expression profile of SCDs during differentiation; transcription factors, early/definitive endoderm markers. qRT-PCR analysis showing expression levels of transcription markers; GATA4, GATA6 along with early and definitive endoderm markers;SOX17, HNF4α, HNF3β and AFP detected at durations (7, 14 and 21). For each marker, the expression level in undifferentiated SCDs cultured under HPL and FBS is used as standardization respectively (i.e., relative expression = 1) and normalized against 18sRNA. The expression levels are also compared with total RNA from HepG2 and adult liver tissue. The expressions that are significantly different from that of undifferentiated SCDs are marked with asterisk ($p < 0.05$). Mean ± SD values are shown from four independent experiments.

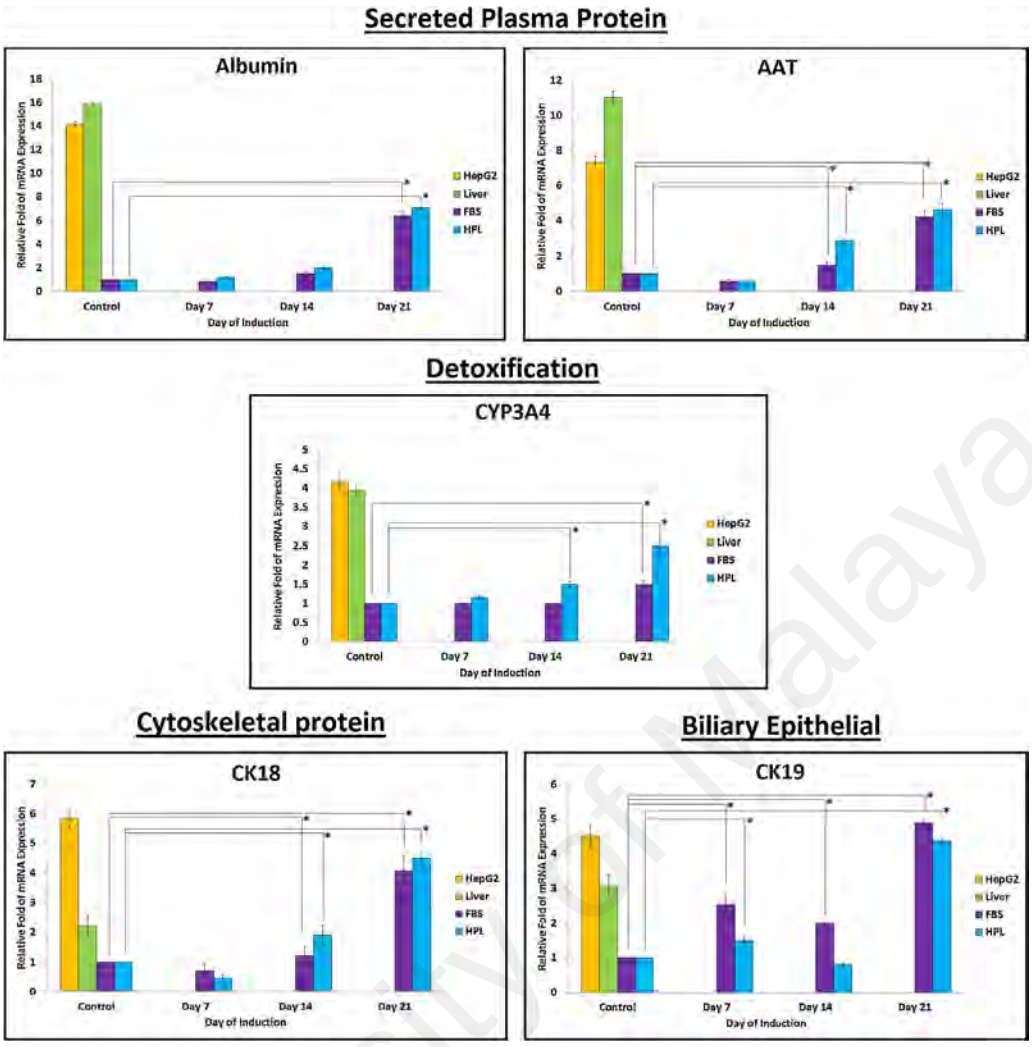


Fig. 4. Hepatic lineage-specific metabolism related markers expressed in differentiated SCDs. qRT-PCR analysis of metabolism related hepatic markers; TDO, TO, TAT and CYP7A1 during SCDs differentiation from day 7 to day 21 in comparison to undifferentiated SCDs, HepG2 and adult liver tissue. mRNA levels displayed have been normalized with respect to corresponding levels in undifferentiated SCDs culture under HPL and FBS. The expressions that are significantly different from that of undifferentiated SCDs are marked with asterisk ($p < 0.05$). Mean ± SD values are shown from four independent experiments.

ii) Expression of metabolism-related markers – Hepatocytes play important roles in amino acid metabolism tyrosine aminotransferase (TAT), tryptophan 2,3-dioxygenase (TDO) [15] and bile acid synthesis cytochrome P450 7A1 (CYP7A1) [16]. As shown in Fig. 4, none of these genes were expressed at undifferentiated cells. Except for CYP7A1, all other transcripts showed a significant up-regulation of 2–5-fold, consistently at every 7 day intervals in HPL compared to FBS. CYP7A1 remained at a very low level throughout the culture period. Despite having a noticeably increased metabolism, related markers were found in SCDs which differentiated into hepatic lineage cells, and the levels were admittedly lower than HepG2 as well as adult liver tissue.

iii) Secreted plasma proteins – One of the functions of the liver is the production and export of plasma proteins which are important in maintaining homeostasis of the human body [17]. Accordingly, SCDs cultured under HPL showed a significant up-regulation of ALB gene at day 7 (1.5-fold; $p < 0.05$), day 14 (2-fold; $p < 0.05$) and day 21 (7-fold; $p < 0.05$), whereas no significant change was detected in ALB expression under FBS at day 7, and less than 7-fold increment was detected at day 21 as compared to undifferentiated SCDs. In addition, the expression of alpha-1-antitrypsin (AAT) gene was only significant at day 21 with a more or less similar expression between SCDs cultured under HPL and FBS. In terms of detoxification-related gene, cytochrome P450 3A4 (CYP3A4) significant expression was only found in SCDs cultured under HPL (day 7:1.5-fold, $p < 0.05$; day 14: 2-fold, $p < 0.05$; day 21: 2.5-fold, $p < 0.05$) as compared to undifferentiated SCDs.

To discern any signs of heterogeneous population in our differentiated SCDs toward hepatic lineage cells, we checked the mRNAs for CK18 and CK19. These markers are commonly expressed in biliary epithelial cells and hepatoblast [18]. Surprisingly, both mRNAs were detected throughout in both culture conditions coupled with significant higher level than those from HepG2 and adult liver tissue. This indicates the presence of heterogeneous population in the SCDs had differentiated toward hepatic lineage.

3.4. Immunofluorescence staining of HNF4α, AFP, CK19, CK18 and ALB

The expression of a series of some genes in Figs. 3–5 was further analyzed at protein level (Fig. 6). The intensity of HNF4α expression

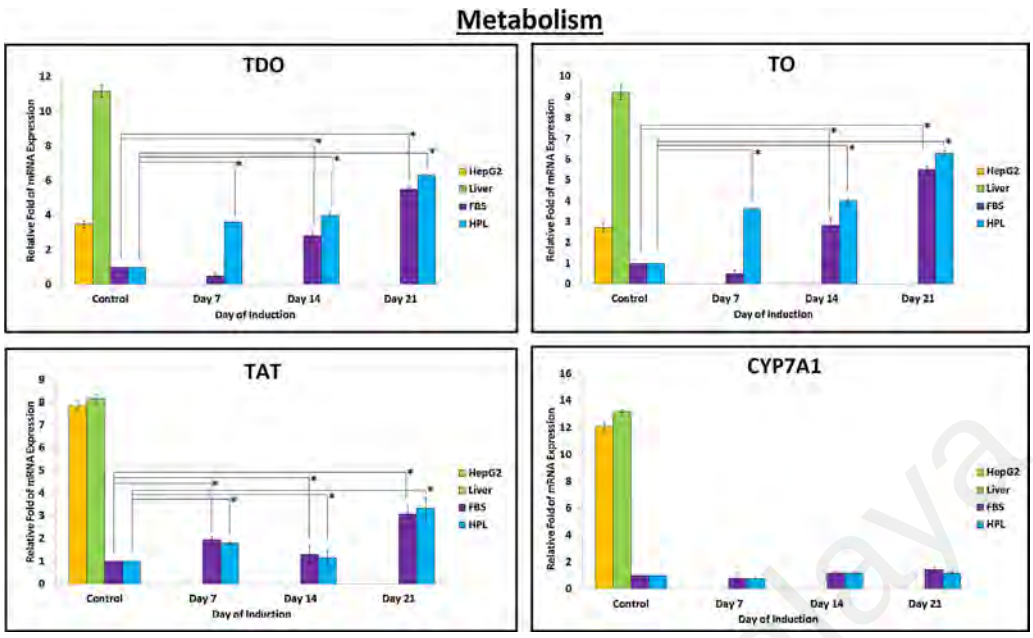


Fig. 5. Differentiation leads to expression of other hepatic functional transcriptomes. qRT-PCR analysis of secreted protein plasma related markers; ALB, AAT, detoxification related marker; CYP3A4, cytoskeletal related marker; CK18 and unforeseen biliary epithelial related marker; CK19 during SCDs differentiation in comparison to undifferentiated SCDs, HepG2 and adult liver tissue. mRNA levels displayed have been normalized with respect to corresponding levels in undifferentiated SCDs culture under HPL and FBS. The expressions that are significantly different from that of undifferentiated SCDs are marked with asterisk ($p < 0.05$). Mean ± SD values are shown from four independent experiments.

was similar for SCDs cultured under HPL and FBS throughout the differentiation period whereas intensity of AFP expression showed a gradual decrement during the same period. Further, CK18 and CK19 displayed a similar intensity level parallel to their mRNA expressions. As evidenced, ALB was not detected in the undifferentiated SCDs whereas there was a significant level of ALB expression at the end of the differentiation process under both culture conditions.

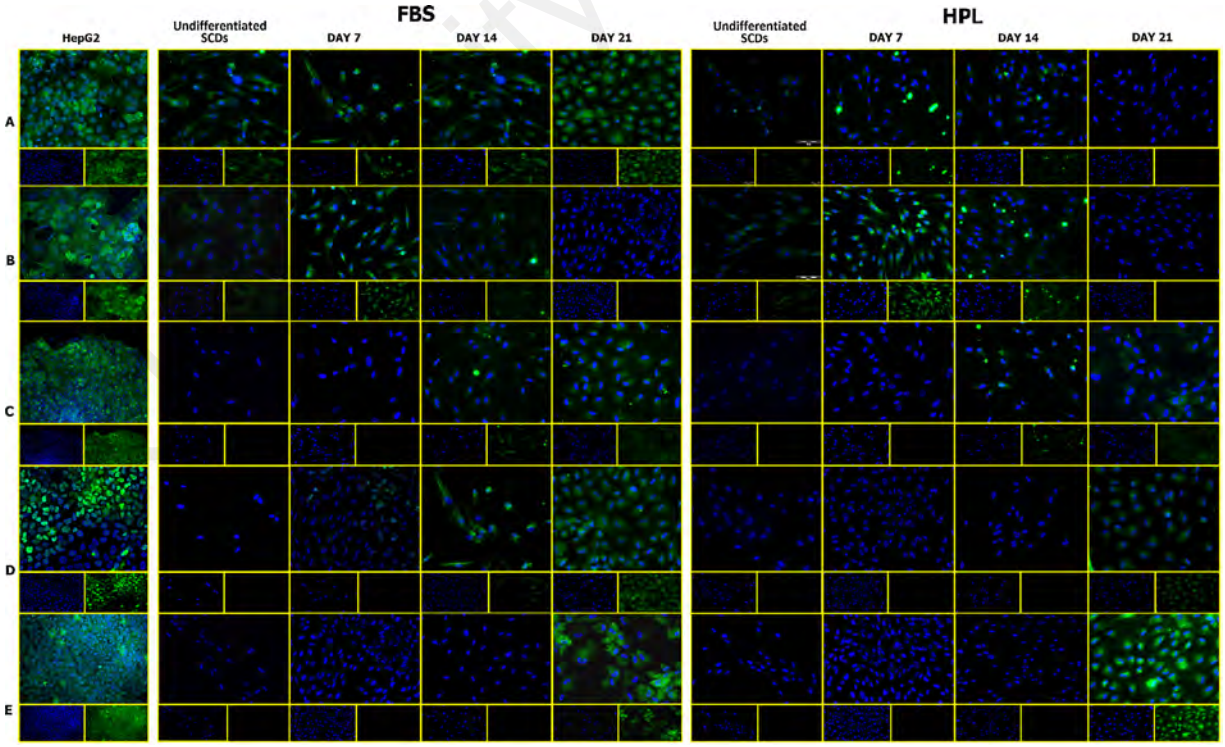


Fig. 6. Progressive alteration of selected protein level patterns in differentiating SCDs recapitulating developmental potency to hepatic lineage cells. Immunocyto chemistry analysis shows expression of various hepatic lineage markers; (A) HNF4α, (B) AFP, (C) CK18 and (E) ALB during differentiation process. The presence of (D) CK19 marker indicates heterogeneity. Scale bar = 50 μm. Each image denoted with right lower corner representing DAPI images and left lower corner representing FITC images and overlay stained images. HepG2 has been used as the positive control whereas undifferentiated SCDs are used as a negative control. Representative of 4 independent images are shown.

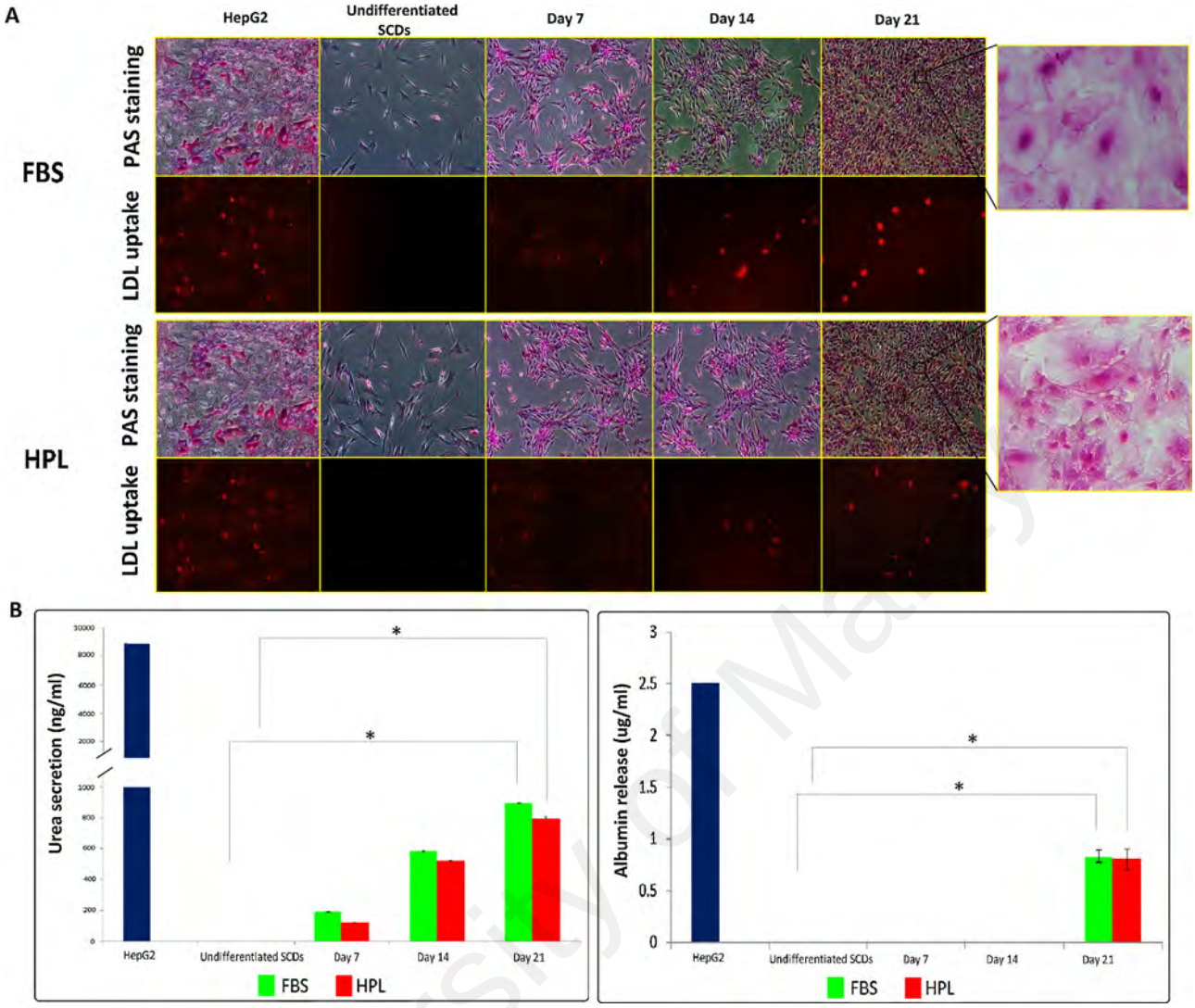


Fig. 7. Differentiation of SCDs into hepatic lineage imprints liver functions. (A) Panels display glycogen uptake by PAS staining and LDL uptake by the differentiated SCDs in both HPL and FBS condition. (B) They also exhibit secreted plasma protein (ALB), and urea production which are significantly higher in comparison to its original state (undifferentiated SCDs).* indicates significance with p-value to be <0.05. Mean ± SD values are shown from four independent experiments.

3.5. Functional characterization of SCDs derived hepatic lineage cells

To elucidate whether SCDs-derived hepatic lineage cells contains any functional mature hepatocytes population, we detoured the cells to several functional assays. Based on the findings of PAS staining, SCDs-derived hepatic lineage cells cultured in FBS and HPL were able to store glycogen and were detected as early as on day 7. However the intensity of PAS staining were not much varied between them throughout the experiment. Similar elevated level of LDL was observed in both culture conditions. Our analysis of ALB secretion indicated that undifferentiated SCDs did not secrete ALB, however after hepatic induction the secretion level of ALB was detected but only at day 21. Nevertheless, the value was far below from the HepG2 level. As for the urea secretion, a time dependent increase was observed (day 7: 123.337 ± 2.51, $p < 0.05$; day 14: 522.33 ± 1.15, $p < 0.05$; day 21: 893.54 ± 3.51, $p < 0.05$) under HPL culture condition as compared to FBS culture condition (day 7: 191.67 ± 2.08, $p < 0.05$; day 14: 583.75 ± 3.06, $p < 0.05$; day 21: 893.54 ± 3.12, $p < 0.05$). Undifferentiated SCDs were unable to perform these functions (Fig. 7).

3.6. The SCDs derived hepatic lineage cells are immature

The presence of CK19 mRNA transcripts in our study indicates that these cells contain heterogeneous population or perhaps immature cells as well. To ascertain our notion, we investigated the status of transcriptomes responsible for stem cell renewal in pre and post exposure stages of SCDs to hepatocyte specific differentiation program. Astonishingly, the RNA levels of stemness maintaining transcriptomes factors such as OCT4, Nanog, SOX2, and REX1 were not only maintained but slightly increased in differentiated SCDs compared to undifferentiated SCDs ($p > 0.0.5$). Further observation revealed that the protein level of Ki67, a marker of S/G1 cell cycle arrest, remain high in SCDs derived hepatic lineage cells compared to undifferentiated SCDs. These findings suggested that our differentiation protocol, although supporting the phenotype and functions of hepatocytes, does not fully subdue the stemness of SCDs (Fig. 8).

4. Discussion

The characterization of SCDs cultured in FBS and HPL were examined in several ways including proliferation rate, multilineage

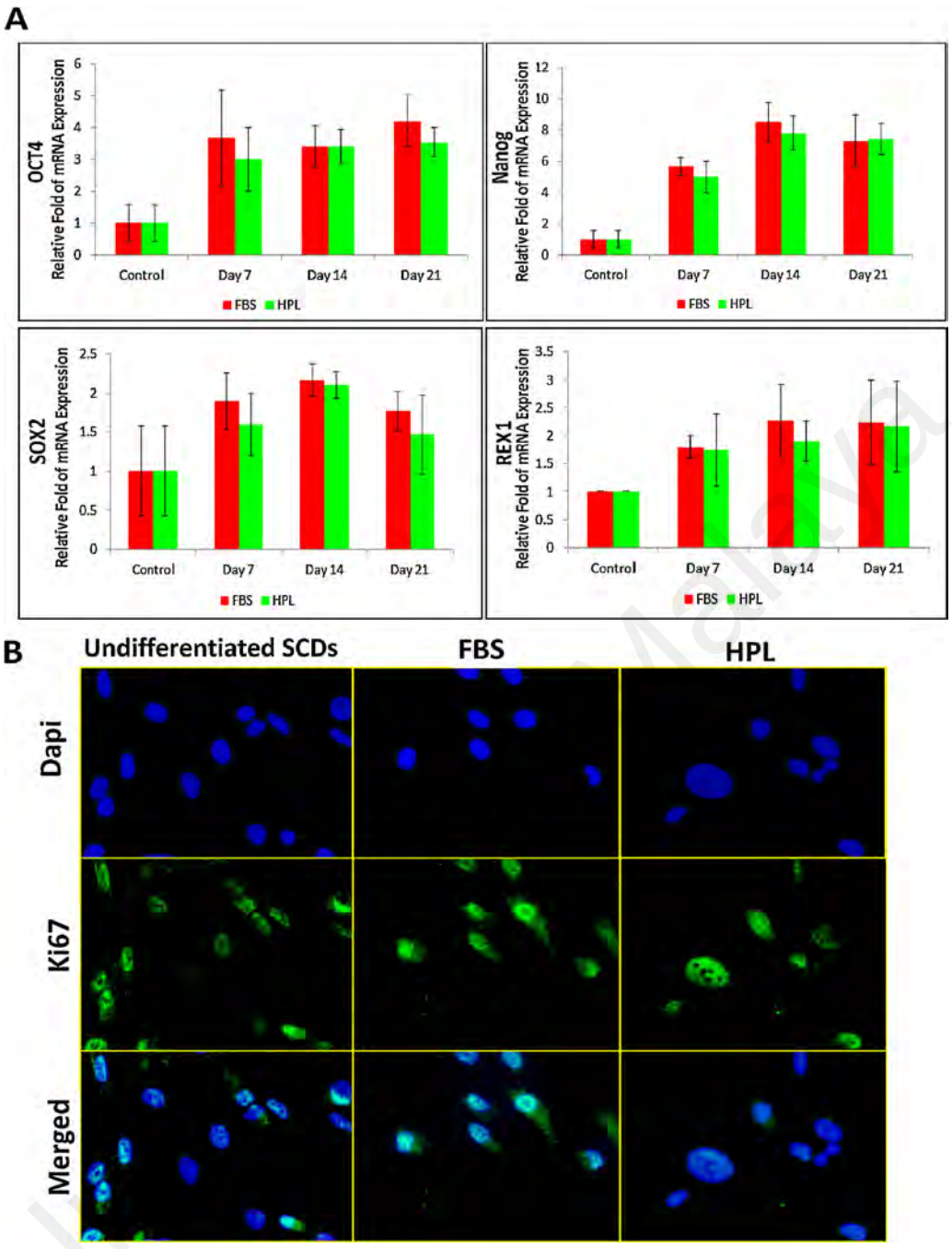


Fig. 8. Temporal gene level of some pluripotent markers in differentiated SCDs into hepatic lineage cells. (A) qRT-PCR analysis showing expression levels of OCT4, Nanog, SOX2, REX1. For each marker, the expression level in undifferentiated SCDs cultured under HPL and FBS is used as standardization respectively (i.e., relative expression = 1) and normalized against 18sRNA. No significance changes in term of gene expression between the control and differentiated days ($p > 0.05$). Cell, undifferentiated SCDs or SCDs derived hepatic lineage cells at day 21 were stained with anti-ki67 antibody (middle row image) and the nuclei were counterstained with DAPI (upper row image); an overlay of the two stains is depicted in the bottom row.

differentiation and cell phenotype analysis. Overall, the results displayed a significantly higher proliferation rate of SCDs expanded in HPL compared to FBS, coupled with the ability of cells to differentiate into mesoderm lineage and showed a similar percentage of cell surface markers. Similar observations were reported in many studies using different types of cell lines [10,19]. One possible reason is the abundance of growth factors found in HPL [10] in which HPL showed higher mitogenic effect as compared to FBS. In terms of their safety, cells cultured under HPL are found to be free from tumorigenesis with stable genomic contents [20].

The study was further continued with the differentiation of SCDs into hepatocyte lineage. SCDs cultured under FBS and HPL showed morphological transformation from a fibroblastic-like shape, to a polygonal shape, which conforms to one of the hepatocyte's characteristics. Binucleation was also observed in both media. The appearance of polygonal shaped cells with binuclei is indicative of hepatocyte cells and this could be the result of the cell's exit from mitosis due to the absence or abortion of cytokinesis [21–23].

Hepatocyte lineage cell are primarily regulated by a group of liver enrichment transcriptional factors as well as hepatic genes [22]. Therefore analyzing these genes is mandatory. In the present

study, a higher expression of SOX17, GATA4, GATA6, HNF3β, and HNF4α at both mRNA and protein levels were found in differentiated SCDs at the early stage of hepatic lineage development and gradually decreased toward the end of the experiment period. SOX17 and GATA4 are recognized as early markers for endoderm lineage [24] and GATA4 is localized in both early developing stage of the liver as well as the gastrointestinal tract [24], thus high expression of this marker gradually disappears in areas concentrated with hepatocytes. Conversely, HNF genes being the fundamental transcription factor for liver development [25] and is normally expressed in the hepatic diverticulum [26]. AFP gene was also gradually decreased toward the end of the experiment period. This observation is in agreement with previous reported studies [27,28]. AFP is the earliest hepatocyte specific marker that can be detected at the early phase of hepatocyte differentiation [28]. According to Ochiya et al. [27], although AFP is expressed in the early stage of hepatocyte differentiation, it decreases immediately after birth, and the total amount of mRNA expressed is identified to be less compared to during the fetal period.

Surprisingly, we found that undifferentiated SCDs expressed most of these markers. There is still a huge lacuna in our understanding behind this phenomenon. Nevertheless, there are reports on the role of endodermal in the development and evolution of pharyngeal arches in which neural crest cells are influenced by endodermal cues for jaw development [29]. Since SCDs are neural crest cells, we reckoned that they inherently possess some of these primitive or definitive endodermal markers. Another reason could be the epithelial-mesenchymal (EM) interactions which have been demonstrated to be essential for the process of organogenesis [30]. Studies have shown that endodermal generation depends on the successful completion of the EM transition via Wnt/β-catenin signaling functions. Remarkably, the same EM transition pathways are involved in the development of the tooth [31]. Therefore, we postulated that a fully developed dental tissue may carry some of the endodermal memories.

Next, we checked for specific hepatocytes makers in our differentiated SCDs into hepatic lineage cells. The expression of albumin as indicated by mRNA, protein as well as secretion level in the present study indicates the presence of mature hepatocytes in our differentiated SCDs. Albumin is a specific hepatocyte marker and can be categorized as a major protein in plasma that is involved in maintaining homeostasis within the body [32]. We also found that SCDs derived heaptic lineage cells in both culture conditions were able to express some of the specific late hepatocyte markers such as TO, TAT, TDO and specific cytochrome (CYP) family [23]. TAT mRNA is expressed in rat liver after birth [33], but is again detected earlier (mid-gestation) in human liver [34]. TAT represents an excellent enzymatic marker for pre or post natal hepatocyte specific differentiation. Since hormone-regulated TAT activity is strictly limited to the parenchymal cells of the adult liver, it has been used extensively for monitoring cellular differentiation in experimental models for liver development/maturation in vitro [35]. Mature hepatocytes also have a role in amino acid metabolism and plasma protein secretion. The presence of TO and TAT expression in the hepatic lineage cells derived from SCDs cultured in FBS and HPL showed that they have the potential to undergo amino acid metabolism, while expression of AAT represents the ability to secrete plasma protein. We also showed that the SCDs derived hepatic lineage cells differentiated under both culture conditions have the potential to store glycogen and accumulate fat (exemplified by expression of LDL expression) as well as the ability to synthesize urea though the levels are far below from HepG2 cell lines.

As has been noted, CYP family has mixed monooxygenases function and has been categorized as a major Phase-I enzyme. While, CYP3a4 is a marker involved in the metabolism of xenobiotics, CYP7a1, on the other hand, is considered as a hepatocyte maturation marker [32] as it is not expressed in fetal liver. Unfortunately, a lower expression of these genes were found in the present study indicating a fairly amount of immature characteristics in our differentiated SCDs. To add in, we also found the expression of CK19 which is a cell marker for bile duct epithelial cells and hepatic progenitor cell [24]. Generally, any hepatocytes cells from adults as well as embryonic stem cells (hESCs) contains 3 types of populations, namely, hepatocytes committed cells expressing AFP and ALB, cholangiocytes committed cells expressing CK19 and bipotential hepatoblast population expressing all these markers. The latter population develops into both biliary and hepatic cell lines and therefore are considered fetal source of hepatic progenitor cells [36,37]. To further establish the immature characteristics of SCDs derived hepatic lineage cells, we check for the sign of pluripotency in our cells. Startlingly, the expression pluripotent markers such as Nanog or OCT4 remained unchanged during the course of differentiation. Reports have suggested that Nanog is an essential marker for the conservation of definitive endoderm stage in hESCs, because the knockdown of Nanog expression by RNAi conciliated the capability of hESCs to become definitive endoderm [38]. Similarly, repression of OCT4 in human and mouse ESCs results in differentiation to trophectoderm [39] while over expression of OCT4 causes expression of definitive endoderm genes including SOX17 and Hex [40]. Correlating these reports to our present study, we believe that a number of hepatic progenitor cells remains in our SCD derived hepatic lineage cells which are orchestrated by pluripotent markers.

A progenitor cell of a particular cell tissue is perhaps the right source to treat tissue-orientated diseases. These cells will then perhaps differentiate into the target cells upon transplantation or at least release molecule signals that are similar and needed by the host tissue. Another benefit is the usage of these cells in transplantation due to the lack of HLA markers [41]. Commonly, ESCs is the supreme source of generating progenitor cells. However, our findings indicated that SCDs can be a potential substitute to ESC as it can be primed toward progenitor cells under appropriate cue and safely used in transplantation.

Despite the much hype of SCDs, the main caveat of this alternative cell source in regenerative medicine is the inability to generate a large number of cells that can cater for clinical use. This is due to the fact that the starting material (tissue) in isolating dental pulp stem cell is small. To overcome this, we introduced HPL into our culture system wherein we have reported that 5×10^8 of SCDs in a 5 cell stack system (approximately 3180 cm^2 total area of flask surface, Corning) can be generated within 14 days of culture period which can be produced at a much lower cost without compromising the quality [10]. The production of HPL is carried out routinely in our cGMP-compliant facility that requires a simple freeze-thaw method and can be prepared within 72 h. Further, a 300 mL of platelet can derive into approximately 200 mL of HPL. This can provide up to two times usage in a 10 cell stacks system (approximately 6360 cm^2 total area of flask surface, Corning) resulting to approximately 2×10^9 cells. Assuming only 50% of these population are able to differentiate into hepatic lineage cells, SCDs are able to provide 10 times transplantation, taking into account that patients with acute liver failure are given a minimum of 10^7 hepatocytes [42]. Remarkably, this procedure can be done in autologous settings whereby both cell source and HPL can be derived from the same donor thus reducing the risk of disease transmission.

5. Conclusion

In conclusion, the results of this study showed that the HPL is a potentially effective supplement to increase growth rate of SCDs.

Interestingly, HPL stimulates high proliferation of MSCs, and at the same time, is able to differentiate into hepatic lineage cells in HPL. Taken together, despite of the expression of some hepatic markers and functions it is necessary to optimize the concentration and exposure time for achieving liver-specific functions equal to normal mature hepatocytes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work is part of an on-going project between Hygieia Innovation and Faculty of Dentistry, University Malaya and supported by University Malaya, High Impact Research Chancellery grant (UM.C/625/HIR/032).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2014.04.007.

References

[1] S. Kakinuma, H. Nakauchi, M. Watanabe, Hepatic stem/progenitor cells and stem-cell transplantation for the treatment of liver disease, J. Gastroenterol. 44 (3) (2009) 167–172.
[2] Y. Kawashita, C. Guha, K. Yamanouchi, Y. Ito, Y. Kamohara, T. Kanematsu, Liver repopulation: a new concept of hepatocyte transplantation, Surg. Today 35 (9) (2005) 705–710.
[3] V. Govindasamy, A.N. Abdullah, V.S. Ronald, S. Musa, Z.A. Ab Aziz, R.B. Zain, S. Totey, R.R. Bhonde, N.H. Abu Kasim, Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth, J. Endod. 36 (9) (2010).
[4] V. Govindasamy, V.S. Ronald, A.N. Abdullah, K.R. Nathan, Z.A. Ab Aziz, M. Abdullah, S. Musa, N.H. Kasim, R.R. Bhonde, Differentiation of dental pulp stem cells into islet-like aggregates, J. Dent. Res. 90 (5) (2011) 646–652.
[5] C. Gandia, A. Arminan, J.M. Garcia-Verdugo, E. Lledo, A. Ruiz, M.D. Minana, J. Sanchez-Torrijos, R. Paya, V. Mirabet, F. Carbonell-Uberos, M. Llop, J.A. Montero, P. Sepulveda, Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction, Stem Cells 26 (3) (2008) 638–645.
[6] N. Ishkitiev, K. Yaegaki, B. Calenic, et al., Deciduous and permanent dental pulp mesenchymal cells acquire hepatic morphologic and functional features in vitro, J. Endod. 36 (2010) 911–920.
[7] N. Ishkitiev, K. Yaegaki, T. Imai, T. Tanaka, T. Nakahara, H. Ishikawa, V. Mitev, M. Haapasalo, High-purity hepatic lineage differentiated from dental pulp stem cells in serum-free medium, J. Endod. 38 (4) (2012) 475–480.
[8] J. van der Valk, D. Brunner, K. De Smet, A. FexSvenningsen, P. Honegger, L.E. Knudsen, T. Lindl, J. Noraberg, A. Price, M.L. Scarino, G. Gstraunthaler, Optimization of chemically defined cell culture media – replacing fetal bovine serum in mammalian in vitro methods, Toxicol. Vitro 24 (4) (2010) 1053–1063.
[9] J. van der Valk, D. Mellor, R. Brands, R. Fischer, F. Gruber, G. Gstraunthaler, L. Hellebrekers, J. Hyllner, F.H. Jonker, P. Prieto, M. Thalen, V. Baumans, The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture, Toxicol. Vitro 18 (1) (2004) 1–12.
[10] V. Govindasamy, V.S. Ronald, A.N. Abdullah, et al., Human platelet lysate permits scale-up of dental pulp stromal cells for clinical applications, Cytotherapy 13 (2011) 1221–1233.
[11] C. Doucet, I. Ernou, Y. Zhang, et al., Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications, J. Cell. Physiol. 205 (2005) 228–236.
[12] K. Bieback, Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures, Transfus. Med. Hemother. 40 (5) (2013) 326–335.
[13] S. Sassa, O. Sugita, R.A. Galbraith, A. Kappas, Drug metabolism by the human hepatoma cell, Hep G2, Biochem. Biophys. Res. Commun. 143 (1) (1987) 52–57.
[14] J.K. Fawcett, J.E. Scott, A rapid and precise method for the determination of urea, J. Clin. Pathol. 13 (March (2)) (1960) 156–159.
[15] P.J. Klover, R.A. Mooney, Hepatocytes: critical for glucose homeostasis, Int. J. Biochem. Cell Biol. 36 (5) (2004) 753–758.
[16] M. Massimi, S.R. Lear, S.L. Huling, A.L. Jones, S.K. Erickson, Cholesterol 7alpha-hydroxylase (CYP7A): patterns of messenger RNA expression during rat liver development, Hepatology 28 (4) (1998) 1064–1072.
[17] J.Y. Chiang, Bile acids: regulation of synthesis, J. Lipid Res. 50 (10) (2009) 1955–1966.
[18] S. Ishizaka, A. Shiroi, S. Kanda, et al, Development of hepatocytes from ES cells after transfection with the HNF-3β gene, FASEB J. 16 (2002) 1444–1446.
[19] A. Kocaoemer, S. Kern, H. Kluter, et al., Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue, Stem Cells 25 (2007) 1270–1278.
[20] Y. Wang, D.L. Huso, J. Harrington, et al., Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture, Cytotherapy 7 (2005) 509–519.
[21] I.S. Behbahan, Y. Duan, A. Lam, et al., New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes, Stem Cell Rev. 7 (2011) 748–759.
[22] J.E. Guidotti, O. Bregerie, A. Robert, et al., Liver cell polyploidization: a pivotal role for binuclear hepatocytes, J. Biol. Chem. 278 (2003) 19095–19101.
[23] M.R. Alison, R. Poulsom, W.R. Otto, et al., Recipes for adult stem cell plasticity: fusion cuisine or readymade? J. Clin. Pathol. 57 (2004) 113–120.
[24] N. Funakoshi, C. Duret, J.M. Pascussi, et al., Comparison of hepatic-like cell production from human embryonic stem cells and adult liver progenitor cells: CAR transduction activates a battery of detoxification genes, Stem Cell Rev. 7 (2011) 518–531.
[25] A.J. Watt, W.D. Garrison, S.A. Duncan, HNF4: a central regulator of hepatocyte differentiation and function, Hepatology 37 (2003) 1249–1253.
[26] S.A. Duncan, K. Manova, W.S. Chen, et al., Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 7598–7602.
[27] T. Ochiya, Y. Yamamoto, A. Banas, Commitment of stem cells into functional hepatocytes, Differentiation 79 (2010) 65–73.
[28] S. Kazemnejad, A. Allameh, A. Gharehbaghian, et al., Efficient replacing of fetal bovine serum with human platelet releasate during propagation and differentiation of human bone marrow-derived mesenchymal stem cells to functional hepatocytes-like cells, Vox Sanguinis 95 (2008) 149–158.
[29] A. Graham, J. Richardson, Developmental and evolutionary origins of the pharyngeal apparatus, Evodevo 3 (1) (2012) 24.
[30] J.B. Gurdon, The generation of diversity and pattern in animal development, Cell 68 (2) (1992) 185–199.
[31] I. Thesleff, M. Tummers, Tooth organogenesis and regeneration, in: StemBook, 2009, Retrieved March, 10, 2011 from http://www.ncbi.nlm.nih.gov/books/NBK27044/
[32] C.H. Yin, W. Chen, C.C. Hsiao, C.L. Chen, W.T. Wu, Improving albumin production of hepatic lineage cells from mouse embryonic stem cells in vitro, Biochem. Eng. J. 39 (3) (2008) 435–442.
[33] O. Greengard, Enzymic differentiation in mammalian liver injection of fetal rats with hormones causes the premature formation of liver enzymes, Science 163 (870) (1969) 891–895.
[34] S.M. Andersson, N.C. Raiha, J.J. Ohisalo, Tyrosine aminotransferase activity in human fetal liver, J. Dev. Physiol. 2 (1–2) (1980) 17–27.
[35] X.L. Shi, Y.D. Qiu, X.Y. Wu, T. Xie, Z.H. Zhu, L.L. Chen, L. Li, Y.T. Ding, In vitro differentiation of mouse bone marrow mononuclear cells into hepatocyte-like cells, Hepatol. Res. 31 (4) (2005) 223–231.
[36] D.A. Shafritz, M. Oertel, A. Menthena, Liver stem cells and prospects for liver reconstitution by transplanted cells, Hepatology 431 (2006) S89–S98.
[37] R. Zhao, S.A. Duncan, Embryonic development of the liver, Hepatology 41 (2005) 956–967.
[38] K.L. Jaremko, Y. Marikawa, Regulation of development competence and commitment towards the definitive endoderm lineage in human embryonic stem cells, Stem Cell Res. 10 (3) (2012) 489–502.
[39] Y. Babaie, R. Herwig, B. Greber, T.C. Brink, W. Wruck, D. Groth, H. Lehrach, T. Burdon, J. Adjaye, Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells, Stem Cells 25 (2) (2007) 500–510.
[40] S. Stefanovic, N. Abboud, S. Desilets, D. Nury, C. Cowan, M. Puceat, Interplay of Oct4 with Sox2 and Sox17: a molecular switch from stem cell pluripotency to specifying a cardiac fate, J. Cell Biol. 186 (5) (2009) 665–673.
[41] S. Musa, L.Z. Xin, V. Govindasamy, F.W. Fuen, N.H. Kasim, Global search for right cell type as a treatment modality for cardiovascular disease, Expert Opin. Biol. Ther. 14 (1) (2014) 63–73.
[42] I.J. Fox, J.R. Chowdhury, Hepatocyte transplantation, Am. J. Transplant. 4 (Suppl. 6) (2004) 7–13.

3.3 Publication 3 (Original research)

Jayaraman P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Vasanthan P, **Musa S**, Kasim NH. Expression patterns of immune genes in long-term cultured dental stem cells. Clin Oral Investig. 2016, 20(1):109-116

3.3.1 Contributions of co-authors:

Design and concept of study	SM, GV
Acquisition of data	JP, SM, GN, KW
Analysis of data	JP, SM, GV
Drafting of manuscript	JP, GV, SM
Revising manuscript for intellectual content	All authors

University of Malaya

Clin Oral Invest
DOI 10.1007/s00784-015-1497-1

ORIGINAL ARTICLE

Expression patterns of immune genes in long-term cultured dental stem cells

Pukana Jayaraman[1,2] · **Vijayendran Govindasamy**[1] · **Nareshwaran Gnanasegaran**[1,3] · **Wijenthiran Kunasekaran**[1,2] · **Punitha Vasanthan**[1,2] · **Sabri Musa**[2] · **Noor Hayaty Abu Kasim**[3]

Received: 11 February 2015 / Accepted: 20 May 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract
Background and objectives Long-term culture system is used to prevent the impediment of insufficient cells and is good for low starting materials such as dental pulp or periodontal ligament. In general, although cell viability and functionality are the most common aspects taken into consideration in culturing cells for a long term, they may not truly represent the biological state of the cells. Hence, we explored the behaviour of another important aspect which is the immune properties in long-term cultured cells.
Methods Dental pulp stem cells from deciduous (SHED; $n = 3$) and permanent (DPSCs; $n = 3$) teeth as well as periodontal ligament stem cells (PDLSCs; $n = 3$) were cultured under identical culture condition. The immune properties of each cell lines were profiled at passage 2 [P2] and passage 9 [P9] as early and late passages, respectively. This was further validated at the protein level using the Luminex platform.
Results A major shift of genes was noticed at P9 with SHED being the highest. SHED cultured at P9 displayed many genes representing pathogen recognition ($P < 0.001$), immune signalling ($P < 0.001$, pro-inflammatory ($P < 0.001$), anti-inflammatory ($P < 0.001$) and immune-related growth and stimulation factor ($P < 0.001$) as compared to DPSCs and PDLSCs. Surprisingly, SHED also expressed many cytotoxicity genes ($P < 0.001$).
Conclusions Communally, instabilities of immune genes from our findings suggest that long-term cultured cells may not be feasible for transplantation purposes.
Clinical relevance A complete biological characterization covering all major aspects including immune properties should be made as prerequisite criteria prior to the use of long-term cultured stem cells in clinical settings.

Keywords Immune properties · Gene expression profile · Regenerative medicine · Growth factors · Cytokines · Chemokine

Introduction

Stem cell (SC) therapy has entered a new dimension following the establishment of many clinical trials. Among the commonly used cells in clinical trials are mesenchymal stem cells derived from the bone marrow (BM-MSCs). Recently, stem cells of dental origin such as the permanent teeth (DPSCs), exfoliated deciduous teeth (SHED), periodontal ligament (PDLSCs), dental follicle (DF) and dental apical papilla (SCAP) have emerged as another attractive source of cells and have been geared to clinical trials especially in treating neuro-ectoderm-related diseases [1–4]. Despite this, a continuous uninterrupted supply of stem cells is needed for successful completion of any clinical trials. This is carried out by culturing the cells in multiple subcultures to attain the required cell number for transplantation purpose. While the process is easier by using BM-MSCs or umbilical cord stem cells, the

✉ Vijayendran Govindasamy
vijay@hygieiainnovation.com; vijay07001@gmail.com

✉ Sabri Musa
sabrim@um.edu.my

[1] GMP compliant Stem Cells Laboratory, Hygieia Innovation Sdn. Bhd, Federal Territory of Putrajaya, Lot 1G-2G, Lanai Complex, No.2, Persiaran Seri Perdana, Precinct 10, 62250 Putrajaya, Malaysia

[2] Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

[3] Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

Published online: 06 June 2015

University of Malaya

main caveat surrounding the usage of dental stem cells in a clinical set up is the low cell yield.

This is because, compared to other tissues, the starting material is extremely low and requires additional subcultures to achieve an adequate cell number. For example, a single umbilical cord is able to generate more than 1×10^8 cells within a subculture [5], while the use of dental tissues to generate a similar cell number requires an additional of four to five subcultures and that too in a pooled sample situation [6]. Despite this, using stem cells for transplantation has proven to be safe upon long-term expansion [7, 8]. Recently, it was reported that DPSCs cultured at long-term expansion are able to maintain cell function, proliferation and viability thus making them suitable for therapeutic usage [9]. While these results look promising, most of them focused in the plasticity, stemness and immunophenotyping However, another vital aspect worth considering in long-term cell culture systems is the immune properties. In general, stem cells are regarded as immunoprivileged cells with convincing evidence showing that they affect innate and adaptive immune cells in two possible steps: they decrease T- and B-cell proliferation by cell-cell contact and release a wide range of paracrine factors [10]. Similar mechanisms were also reported in stem cells of dental origins [11–13].

There are several methods to evaluate the immune properties of stem cells, and one of them is by looking at their gene level. In this context, gene expression profiling is a reliable method and has been commonly used in understanding the biology and the ontogeny of the cells [14, 15]. Here, we have investigated for the first time the immune properties of dental-derived stem cells in long-term expansion. It is believed that by understanding the pattern of immune properties of the dental stem cells especially at late culture will eventually add value to the existing knowledge on the safety of cell transplantation.

Materials and methods

Sample collection

Our experiments were conducted with institutional ethic approval (Medical Ethics Clearance Number DF CO1107/0066[L]), and the study subjects provided informed consent prior to the commencement of the study.

Isolation and expansion of cells

We have established dental pulp stem cells from deciduous teeth (SHED) [n = 3; ages 7–11 years] and permanent teeth (DPSCs) [n = 3; ages 24–35] as previously described [16] whereas periodontal ligament stem cells (PDLSCs) [n = 3; ages 7–11 years] were isolated and cultured up to passage 8 (P8) according to previously reported protocols with slight modification [17]. In this study, we have regarded cells at P2 and P9 as early and late passages, respectively.

TaqMan® Array Human Immune Gene Array

We have isolated total cellular RNA from all cell lines at P2 and P9 with TRIzol® RNA isolation reagent (Invitrogen). The complementary DNAs (cDNAs) were synthesized by using a 1-μg RNA sample through a reverse transcriptase enzyme kit (Invitrogen). We profiled the immune-related genes using TaqMan® Human Immune Gene Array. Briefly, we loaded the cDNAs on the microfluidic cards for thermal cycling on an ABI PRISM 7900HT Sequence Detection System (Fisher Scientific), and expression values for target genes were normalized to the expression of 18srRNA. For estimation of the fold change by TLDA when the initial transcript levels were undetectable, the initial cycle threshold (C_T) value was assigned to be 35, which would lead to a possible underestimation of the actual fold change.

Validation of immune genes by using Procarta® cytokine protein array

This study validated the secretion of immune genes randomly selected by using a customized human cytokine protein array (Panomics, Redwood City, CA). Briefly, we have collected conditioned mediums from all cell lines at P2 and P9, and an assay was performed according to the manufacturer's instruction. The results were analysed using Luminex platform.

Statistical analysis

We have conducted an experiment independently to ensure the reproducibility of the result. The descriptive statistical analyses were performed using the software SPSS for Windows (Version 11.0; SPSS Predictive Analytics, Chicago, IL) and the data analysed by using two-way analysis of variance (ANOVA) with the significance level set at $P = 0.05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between the groups.

Results

Gene expression variability between SHED, DPSCs and PDLSCs at early and late passages

To understand the immunological behaviour between these cell lines during their transition period from P2 (early passage) to P9 (late passage), we looked at the expression patterns of immuno-regulated genes. The genes were defined as being 2-fold up- or downregulated in each sample at P2 and P9 when

University of Malaya

comparing to SHED at P2 which acted as a control with an accompanying P value of 0.05. Since huge fold variations were noticed among all the samples, they were tentatively classified into five distinct categories based on their fold expression value as follows: upregulated genes consisting of three categories, namely highly upregulated (≥50-fold), medium upregulated (≥10- to 49.99-fold) and low upregulated (≥2- to 9.99-fold) followed by unchanged genes (≥0.5- to 1.99-fold) and downregulated (≤0.499-fold) categories. In this regard, in P2, it was noticed that in the highly upregulated category, there was no gene percentage expressed in SHED whereas there were no significant differences of gene expression percentage between DPSCs and PDLSCs. On the other hand, in the medium upregulated category, no significant differences of gene expression percentage were found among all the cell lines. PDLSCs showed a significantly lower percentage ($P < 0.05$) of genes in the low upregulated category as compared to SHED and DPSCs. Surprisingly, in P9, a tremendous augmentation of gene percentage was seen in all upregulated categories within all cell lines ($P < 0.05$). In terms of unchanged category, a significant swift ($P < 0.05$) was observed in SHED, DPSCs and PDLSCs from P2 to P9 where the genes were decreased from 78 to 6 %, 57 to 9 % and 60 to 9 % respectively. In contrast, a significant increment ($P < 0.05$) of gene percentages ranging from 8 to 17 % was observed in all the cell lines between the same passages (Fig. 1). To better understand the role played by each immuno-related gene within the cell lines and passages, we further categorized the genes according to their similar biological functions.

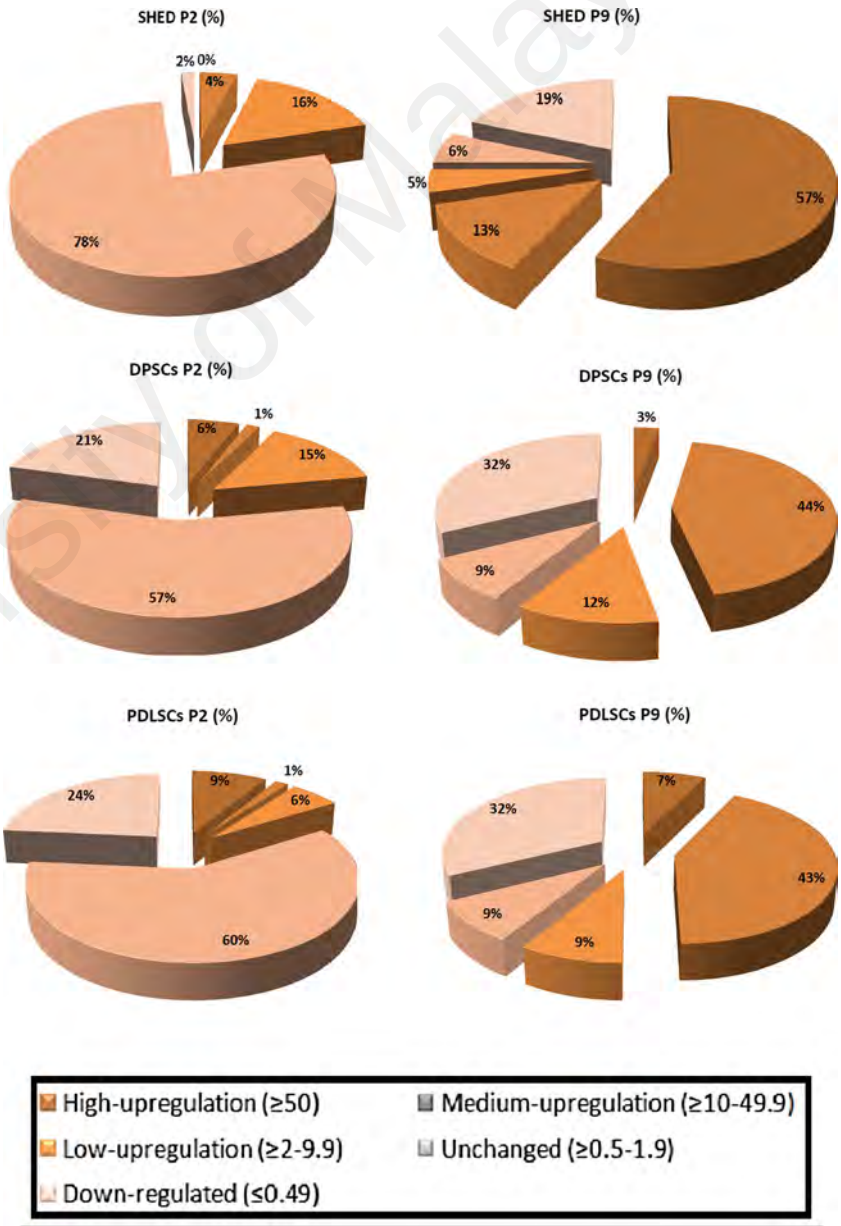


Fig. 1 Distribution of genes of SHED, DPSCs and PDLSCs at passage 2 (P2) and passage 9 (P9), respectively. A total of five categories, namely highly upregulated (fold change ≥50), medium upregulated (fold change of 10–49.9), low upregulated (fold change of 2–9.9), unchanged (fold change of 0.5–1.9) and downregulated (fold change of ≤0.49), were employed in this study to describe their gene expressions

University of Malaya

SHED cultured at P9 displayed many genes representing pathogen recognition as well as immune signalling and transduction

Among the 15 genes grouped under the category of pathogen recognition as well as signalling and transcription, 7 were highly significant (HLA-DRB1, CD80, CD34, TNFRSF18, PTPRC, SELE and SELP; $P < 0.001$) in SHED cultured at P9 as compared to SHED cultured at P2. A similar observation with lesser significant values ($P < 0.01$; $P < 0.05$) was observed for DPSCs and PDLSCs cultured in P9 as compared to P2 (Table 1).

Pro- and anti-inflammatory gene profiling of SHED, DPSCs and PDLSCs at P2 and P9

The transition of SHED from P2 to P9 resulted in the increment of seven pro-inflammatory genes namely TNF, ILIB, IFN- , LTA, IL-12B, IL-1A and IL-15 ($P < 0.001$). However, except for IFN- , not much significance was noticed for the same group of genes in DPSCs and PDLSCs during the transition period. On the other hand, tremendous increments ($P < 0.001$) of anti-inflammatory genes such as IL-4, IL-5, IL-7, IL-9, IL-10 and IL-13 were observed in SHED cultured at P9 as compared to P2. Surprisingly, we did not observe a similar expression in DPSCs and PDLSCs indicating SHED experiencing immune-related gene turbulence when cultured for a prolonged period of time (Table 2).

SHED cultured at P9 displayed many genes representing many growth and stimulation factors as well as chemokines

It is worth noting that growth and other stimulation factors also play an integral part in immune systems. Accordingly, we noticed that TGF-β1 and other stimulation factors such as CSF3 and IL2RA were highly expressed in SHED cultured at P9 as compared to P2 ($P < 0.001$), and again these genes were stagnant in DPSCs and PDLSCs in similar culture conditions. In terms of chemokine gene expression, only those genes related to the CXC group such as CCL5, CXCL11, CCR2, CCR4, CCR5 and CCR7 were noticeable in all cell lines at P9 (Table 3).

DPSCs and PDLSCs cultured at P9 expressed lesser cellular toxicity and apoptosis-related genes as compared to SHED cultured at P9

We postulated that the agitations of most of the immune genes in SHED cultured at P9 were most likely contributed by the elevated expression ($P < 0.001$) of cellular toxicity and

Table 1 Fold changes of genes related to pathogen recognition and signalling and transcription

PATHOGEN RECOGNITION & SIGNALING AND TRANSCRIPTION						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Phagocytes and Antigen Presenting Cells						
HLA-DRA	1.52 ± 0.04	38.77 ± 0.57**	2.50 ± 0.21	2.18 ± 0.12	0.92 ± 0.02	2.40 ± 0.10*
HLA-DRB1	1.48 ± 0.15	171.53 ± 0.50***	0.32 ± 0.03	9.25 ± 0.22**	0.35 ± 0.05	10.53 ± 0.50*
CD40	8.83 ± 0.28	0.78 ± 0.05	2.10 ± 0.10	0.04 ± 0.01	0.69 ± 0.06	0.06 ± 0.01
CD80	1.61 ± 0.04	403.19 ± 0.02***	0.74 ± 0.05	22.31 ± 0.10*	0.71 ± 0.01	25.47 ± 0.50**
CD86	1.68 ± 0.06	0	0	0	0	0
Other						
FAS	1.48 ± 0.05	4.75 ± 0.13*	0.66 ± 0.06	0	0.57 ± 0.06	0
CD34	1.54 ± 0.06	402.73 ± 0.64***	0.73 ± 0.03	22.24 ± 0.21**	0.70 ± 0.01	25.47 ± 1.29***
TNFRSF18	2.90 ± 0.10	42.66 ± 0.57***	0.46 ± 0.01	2.29 ± 0.09*	1.36 ± 0.05	2.70 ± 0.01
Scavenger Receptors						
CD68	16.56 ± 0.48*	0.06 ± 0.01	0.56 ± 0.05	0	0.54 ± 0.01	0
Nuclear transcription factor						
NFKB2	1.45 ± 0.05	0.03 ± 0.01	0.88 ± 0.02	0	0.46 ± 0.05	0
TBX21	2.79 ± 0.09*	0.42 ± 0.02	0	0.02 ± 0.01	0	0
PTPRC	1.45 ± 0.05	1700.49 ±	0.09 ± 0.01	907.17 ± 1.89***	7.49±0.43	561.03 ± 1.05***
Integrin ligands						
ICAM1	11.40 ± 0.36	8.28±0.28	5.57 ± 0.52**	0.46 ± 0.05	1.14 ± 0.15	0.55±0.01
SELE	1.19 ± 0.08	78.23 ± 1.08***	4.35 ± 0.05	42.50 ± 0.50*	69.30 ± 1.13	200.43 ± 0.52
SELP	1.57 ± 0.06	402.06 ± 1.10***	21.90 ± 0.78	238.30 ± 1.54***	25.13 ± 1.03	389.40 ± 0.53***

Highly up-regulated | Medium up-regulated | Low up-regulated | Unchanged | Down-regulated

*P value < 0.05, **P value < 0.005, ***P value < 0.001

Table 2 Fold change of genes related to cytokines

CYTOKINES						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Proinflammatory cytokines						
TNF	1.11±0.10	5850.72±0.87***	0.41±0.03	3.84±0.42*	0.80±0.04	4.37±0.01*
IL6	1.18±0.16	0.94±0.04	7.25±0.25	25.05±0.01	0.44±0.04	0.08±0.02
IL1B	1.95±0.05	191.74±1.56***	0.36±0.05	10.40±0.53**	2.65±0.13	12.43±0.51*
IFNG	1.76±0.05	34.63±0.55***	5799.67±0.58***	25.10±1.01	425.84±0.77	3842.23±0.68***
LTA	2.62±0.13	606.67±1.53***	0.77±0.06	21.59±0.52*	1.87±0.06	24.88±0.82*
IL17	1.18±0.03	0	0	0	0	0
IL12A	22.01±1.00*	1.32±0.09	1.75±0.05	0.08±0.01	0.54±0.04	0.08±0.01
IL12B	1.64±0.06	402.58±0.52***	1.17±0.29	20.92±0.89*	0.74±0.01	27.93±0.90*
IL18	0.85±0.05	14.52±0.50	0.50±0.10	0.80±0.01	0.12±0.01	0.93±0.01
IL1A	1.55±0.05	532.15±1.88***	0.72±0.02	39.37±0.64*	0.74±0.06	27.90±0.85*
IL15	1.63±0.06	429.45±0.51***	0.71±0.02	31.10±0.85*	1.22±0.10	20.80±0.72*
Th-2 type cytokines						
IL10	1.45±0.05	412.78±0.70***	0.75±0.05	27.87±0.61*	0.72±0.03	21.80±1.71*
IL4	1.93±0.06	532.82±0.74***	0.85±0.05	21.27±1.21*	0.76±0.05	25.43±0.51*
IL5	1.41±0.08	402.89±0.84***	1.17±0.15	22.63±0.71*	0.74±0.04	35.53±0.50*
IL13	1.24±0.05	302.74±0.65***	0.84±0.65	21.24±1.16*	5433.67±2.08**	44.80±0.72*
IL7	0.71±0.04	1232.71±1.12***	63.13±1.03*	2.25±0.22	0.28±0.11	588.73±0.64***
IL9	1.63±0.05	450.63±0.55***	1.61±0.08	34.13±0.15**	0.89±0.01	26.60±0.57*
Growth and stimulation factors						
TGFB1	1.42± 0.02	1278.06 ± 0.10***	0.91±0.02	0	1.03±0.03	0
CSF1	1.16 ± 0.14	0.26 ± 0.02	2.69±0.08	0.02±0.01	1.05±0.05	0.02±0.01
CSF2	1.28 ± 0.08	13.70 ± 1.12	1.09±0.10	0.77±0.03	0.23±0.06	0.81±0.02
CSF3	1.65 ± 0.05	411.87 ± 0.81**	0.82±0.02	21.37±0.55*	0.76±0.04	24.48±0.50*
VEGF	4.58 ± 0.08*	0.32 ± 0.02	0.83±0.03	0.03±0.01	1.21±0.36	0.02±0.02
IL2	1.64 ± 0.06	425.07 ± 0.12***	0.73±0.03	22.20±0.18*	0.71±0.03	25.45±0.51*
IL3	1.59 ± 0.09	412.08 ± 1.81***	0.84±0.04	23.42±0.52*	0.88±0.02	27.07±1.68*
IL2RA	2.52 ± 0.02	452.55 ± 0.51***	0	0	0	0
Chemokines						
IL8	3.24 ± 0.22*	0.16± 0.01	2.26±0.22	0	0.20±0.10	0
CC chemokines						
CCL2	0.46±0.06	0.36±0.01	10.62±0.54*	0.03±0.01	1357.47±0.50***	0.03±0.01
CCL3	1.36±0.05	28.76±0.67*	1.66±0.66	1.66±0.04	0.05±0.01	1.85±0.05
CCL5	1.65±0.05	402.73±0.64***	4.32±0.07	21.77±0.68*	10.53±0.50	26.13±1.63*
CCL19	1.3±0.10	0	0	0	0	0
CXC chemokines						
CXCL10	1.47±0.21	44.31±0.60*	3.72±0.25	2.44±0.05	1.32±0.07	2.52±0.45
CXCL11	1.78±0.08	423.73±1.10***	2.05±0.05	22.10±1.02*	0.62±0.08	26.13±0.81*
CCR2	1.63±0.03	412.84±0.78***	0.75±0.05	24.90±0.80*	0.81±0.03	25.77±1.08*
CCR4	0.65±0.06	123.46±0.50*	0.06±0.01	125.59±1.51**	1.62±0.07	110.47±1.35**
CCR5	1.55±0.05	440.78±0.70***	0.77±0.03	25.59±0.53*	4.21±0.26	25.13±1.03*
CCR7	1.73±0.10	450.37±0.55***	0.87±0.63	21.00±1.00*	5.84±0.14	26.40±0.53*
CXCR3	0.37±0.03	0	0	0	0	0

Highly up-regulated | Medium up-regulated | Low up-regulated | Unchanged | Down-regulated

*P value < 0.05, **P value < 0.005, ***P value < 0.001

University of Malaya

Table 3 Fold change of genes related to cellular cytotoxicity, surface molecules and apoptosis

CELLULAR CYTOTOXICITY, SURFACE MOLECULES & APOPTOSIS						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Cellular cytotoxicity						
GNLY	1.42 ± 0.08	359.40 ± 0.53***	0.68 ± 0.07	22.56 ± 0.51*	1.32 ± 0.16	21.50 ± 0.50***
GZMB	1.9 ± 0.06	50.40 ± 0.69**	1.24 ± 0.06	2.48 ± 0.42	0.14 ± 0.01	3.29 ± 0.26
PRF1	1.89 ± 0.08	305.17 ± 0.29***	1.80 ± 0.10	23.56 ± 1.26*	1.60 ± 0.10	30.63 ± 0.33*
Lymphocytes						
CD3E	1.20 ± 0.10	323.03 ± 0.06***	1.69 ± 0.11	24.00 ± 1.00*	1.62 ± 0.07	24.87 ± 0.81*
CD8A	1.23 ± 0.15	511.73 ± 0.64***	0.24 ± 0.06	21.33 ± 0.31*	0.88 ± 0.16	1993.00 ± 1.73***
CD4	1.72 ± 0.21	11.57 ± 1.37*	0.29 ± 0.09	0.66 ± 0.05	6.43 ± 0.51	0.76 ± 0.05
CD19	1.76 ± 0.18	512.57 ± 0.40***	0.60 ± 0.01	22.44 ± 0.51*	0.85 ± 0.05	31.47± 0.5**
CD28	7.32 ± 0.42	313.00 ± 0.50***	0.63 ± 0.04	16.77 ± 0.69*	0.54 ± 0.03	20.23 ± 0.68**
ICOS	1.75 ± 0.19	0.33 ± 0.11	0	0.04± 0.02	0	0.04± 0.03
CD38	1.49 ± 0.18	239.30 ± 0.21***	0.60 ± 0.05	13.42 ± 0.52*	0.85 ± 0.05	15.36 ± 0.56*
CD40LG	1.77 ± 0.17	256.27 ± 0.25***	0.77 ± 0.03	19.42 ± 0.52*	0.82 ± 0.08	29.17 ± 1.04*
CTLA4	8.30 ± 1.13	844.17 ± 0.29***	0.04 ± 0.01	0.09 ± 0.01	0.05 ± 0.02	0.27 ± 0.15
FASLG	1.62 ± 0.08	0	0	0	0	0
Apoptosis						
BAX	2.59 ± 0.52	0.64 ± 0.04	1.81 ± 0.08	0.10 ± 0.01	1.08 ± 0.07	0.32 ± 0.08
BCL2	8.63 ± 0.55	24.55 ± 0.51*	2.25 ± 0.25	1.42 ± 0.07	1.13 ± 0.03	1.68 ± 0.13
BCL2L1	1.82 ± 0.13	4.52 ± 0.50	0.61 ± 0.08	0.32 ± 0.16	1.07 ± 0.11	0.30 ± 0.20

Highly up-regulated | Medium up-regulated | Low up-regulated | Unchanged | Down-regulated

*P value < 0.05, **P value < 0.005, ***P value < 0.001

apoptosis such as GNLY, GZMB, PRF1, CD3E, CD8A, CD19, CD28, CD38, CD40LG and CTLA4.

Validation of immune genes using cytokine array

We randomly selected few genes that showed expression patterns in good agreement with the PCR array data (Fig. 2).

Discussion

The findings of this study have prompted a revisit of some of the key aspects of stem cells for clinical usage. One of it is the suitability of using pro-long cultured cells. Generally, cells cultured for a long term are considered to be safe [18] although a few studies reported that they undergo genomic instabilities [19, 20]. More recently, studies have shown that DPSCs cultured up to P14 too have maintained high mitogenic and functionality thus making them a safe source for therapeutic usage [9]. Nevertheless, the latter study as well as others in the past [21, 22] mainly focused on proliferation and functionality and may not truly represent the state of the cells. This is true in our situation that despite maintaining proliferation and functionality (data not shown), a significant shift was observed in the immunomodulatory aspect especially at long-term cultured cells. Interestingly, this shift happened across all tested cell lines with SHED being the highest.

There is a sharp increment of immune gene expression related to T-lymphocyte activation such as HLA-DR and CD80 across all cell lines at P9. We postulated that the presence of these molecules indicates that the cells are losing their stemness and transforming into terminally differentiated cells [23] and potential of creating a graft-versus-host disease scenario if transplanted in allogeneic settings. Further, we noticed a significant increase in the number of pro-inflammatory genes during the transition of the cell lines from P2 to P9. In vivo condition, these genes are expressed at large during the early inflammation phase to eradicate infection, and the overexpression of these genes especially TNF, IL1B, IFN- , LTA, IL12B, IL1A and IL2 could potentially harm the host tissues [24–26]. Likewise, we also noticed an increment of several of anti-inflammatory genes at P9. In an ideal situation, the expressions of pro-inflammatory and anti-inflammatory genes complement to each other, for example, the pattern of TNF is inversely proportional to IL-10 [27].In contrast, we found that both groups of genes were highly expressed in P9 indicating a disturbance in the genetic makeup though this needs further investigations. It was not surprising to see the elevation of TGF-β expression at P9. The pivotal function of TGF-β in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival [28] by suppressing pro-inflammatory cytokines. CCL5, CXCL11, CCR2, CCR4, CCR5 and CCR7 were also

University of Malaya

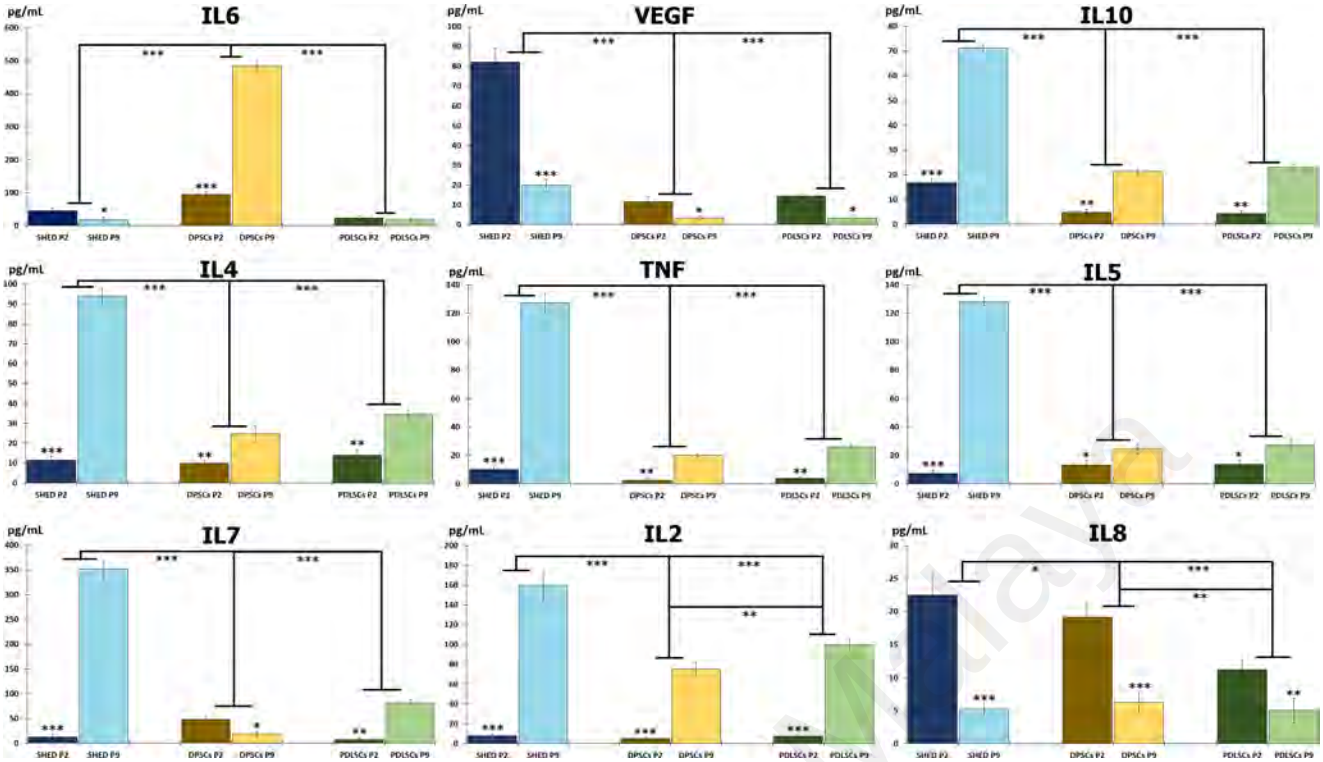


Fig. 2 Detection of cytokines released by SHED, DPSCs and PDLSCs at passage 2 (P2) and passage 9 (P9) via Luminex platform. The cytokines selected include interleukin 6 (IL6), vascular endothelial growth factor (VEGF), interleukin 10 (IL10), interleukin 4 (IL4), tumour necrosis factor (TNF), interleukin 5 (IL5), interleukin 7 (IL7), interleukin 2 (IL2) and interleukin 8 (IL8). (*P value <0.05, **P value <0.005 and ***P value <0.001)

upregulated in all cell lines at P9. All these molecules are members of the CXC chemokine family and, apart from playing a role in immunogenicity, they are potent promoters of angiogenesis and mediate their angiogenic properties [29]. Perhaps this might contribute to the continuous growth of the cells. It was surprising to notice that despite the cells being cultured in the same culture conditions, SHED surpassed other cell lines in expressing unwanted immune genes at a late passage. This perhaps is attributed to the high expression of cellular toxicity markers such as GNLY and GZMB in SHED as compared to DPSCs and PDLSCs. These genes are known to be involved as intracellular effectors of target cell death, with recent data suggesting that GNLY may have a role in the propagation of immune signals [30].

Collectively, occurrences of immune gene turbulence from our findings suggest that long-term culture conditions of stem cells may not be feasible for use in transplantation. Having said that, what is the remedy for using cells having a low starting material such as dental pulp or periodontal ligament tissue? To overcome this issue, large expansions of cells using a 3D bioreactor [31] are possibly made to dental stem cells at the early passage without triggering any side effects [32]. Apart from that, pooled allogeneic samples are another choice, but further characterization needs to be done to maintain their functionality. In conclusion, we proposed a comprehensive biological characterization which includes the immune properties before the stem cells cultured at a later passage are brought into clinical application.

Acknowledgments The work is part of an ongoing project between Hygieia Innovation and the Faculty of Dentistry, University Malaya, and supported by a University of Malaya-Ministry of Education high impact research grant (UM.C/HIR/MOHE/DENT/02).

Conflict of interest The authors declare that they have no competing interests.

References

1. Ponnaiyan D (2014) Do dental stem cells depict distinct characteristics?—Establishing their “phenotypic fingerprint”. Dent Res J (Isfahan) 11:163–172
2. Rodriguez-Lozano FJ, Insausti CL, Iniesta F, Blanquer M, Ramírez MC, Meseguer L, Meseguer-Henarejos AB, Marín N, Martínez S, Moraleda JM (2012) Mesenchymal dental stem cells in regenerative dentistry. Med Oral Patol Oral Cir Bucal 17:e1062–e1067
3. Sedgley CM, Botero TM (2012) Dental stem cells and their sources. Dent Clin N Am 56:549–561
4. Tan J, Xu X, Lin J, Fan L, Zheng Y, Kuang W (2014). Dental stem cell in tooth development and advances of adult dental stem cell in regenerative therapies. Curr Stem Cell Res Ther 9:1-9

5. Nekanti U, Rao VB, Bahirvani AG, Jan M, Totey S, Ta M (2010) Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. Stem Cells Dev 19:117–130
6. Govindasamy V, Ronald VS, Abdullah AN, Nathan KRG, Aziz ZACA, Abdullah M, Zain RB, Kasim NHA, Musa S, Bhonde RR (2011) Human platelet lysate permits scale-up of dental pulp stromal cells for clinical applications. Cytotherapy 13:1221–1233
7. Huang J, Sha H, Wang G, Bao G, Lu S, Luo Q, Tan Q (2015) Isolation and characterization of ex vivo expanded mesenchymal stem cells obtained from a surgical patient. Mol Med Rep 11:1777–1783
8. Zhuang Y, Li D, Fu J, Shi Q, Lu Y, Ju X (2015) Comparison of biological properties of umbilical cord-derived mesenchymal stem cells from early and late passages: immunomodulatory ability is enhanced in aged cells. Mol Med Rep 11:166–174
9. Martin-Piedra MA, Garzon I, Oliveira AC, Alfonso-Rodriguez CA, Carriel V, Scionti G, Alaminos M (2014) Cell viability and proliferation capability of long-term human dental pulp stem cell cultures. Cytotherapy 16:266–277
10. Meisel R, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D (2004) Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 103:4619–4621
11. Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S (2009) Immunomodulatory properties of human periodontal ligament stem cells. J Cell Physiol 219:667–676
12. Machado Cde V, Telles PD, Nascimento IL (2013) Immunological characteristics of mesenchymal stem cells. Rev Bras Hematol Hemoter 35:62–67
13. De Miguel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, Arnalich-Montiel F (2012) Immunosuppressive properties of mesenchymal stem cells: advances and applications. Curr Mol Med 12:574–591
14. Vasandan AB, Shankar SR, Prasad P, Jahnavi VS, Bhonde RR, Prasanna SJ (2014) Functional differences in mesenchymal stromal cells from human dental pulp and periodontal ligament. J Cell Mol Med 18:344–354
15. Winkler B, Taschik J, Haubitz I, Eyrich M, Schlegel PG, Wiegering V (2015) TGFbeta and IL10 have an impact on risk group and prognosis in childhood ALL. Pediatr Blood Cancer 62:72–79
16. Karaoz E, Dogan BN, Aksoy A, Gacar G, Akyüz S, Ayhan S, Genç ZS (2010) Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. Histochem Cell Biol 133:95–112
17. Jung IH, Kwon BS, Kim SH, Shim HE, Jun CM, Yun JH (2013) Optimal medium formulation for the long-term expansion and maintenance of human periodontal ligament stem cells. J Periodontol 84:1434–1444
18. Bogdanova A, Berzins U, Nikulshin S, Skrastina D, Ezerta A, Legzdina D, Kozlovska T (2014) Characterization of human adipose-derived stem cells cultured in autologous serum after subsequent passaging and long term cryopreservation. J Stem Cells 9: 135–148
19. Wang Y, Zhang Z, Chi Y, Zhang Q, Xu F, Yang Z, Meng L (2013). Long-term cultured mesenchymal stem cells frequently develop genomic mutations but do not undergo malignant transformation. Cell Death Dis 4:e950
20. Roselli EA, Lazzati S, Iseppon F, Manganini M, Marcato L, Gariboldi MB, Maggi F, Grati FR, Simoni G (2013) Fetal mesenchymal stromal cells from cryopreserved human chorionic villi: cytogenetic and molecular analysis of genome stability in long-term cultures. Cytotherapy 15:1340–1351
21. Scheers I, Lombard C, Paganelli M, Campard D, Najimi M, Gala JL, Decottignies A, Sokal E (2013). Human umbilical cord matrix stem cells maintain multilineage differentiation abilities and do not transform during long-term culture. PLoS One 8:e71374
22. Frazier TP, Gimble JM, Devay JW, Tucker HA, Chiu ES, Rowan BG (2013) Body mass index affects proliferation and osteogenic differentiation of human subcutaneous adipose tissue-derived stem cells. BMC Cell Biol 14:1–12
23. Laurin D, Kanitakis J, Bienvenu J, Bardin C, Bernaud J, Lebecque S, Gebuhrer L, Rigal D, Eljaafari A (2004) Allogeneic reaction induces dendritic cell maturation through proinflammatory cytokine secretion. Transplantation 77:267–275
24. Xu G, Liu G, Xiong S, Liu H, Chen X, Zheng X (2015) Histone methyltransferase Smyd2 is a negative regulator of macrophage activation by suppressing IL-6 and TNF-alpha production. J Biol Chem. doi:10.1074/jbc.M114.610345
25. Katz LH, Kopylov U, Fudim E, Yavzori M, Picard O, Ungar B, Eliakim R, Ben-Horin S, Chowers Y (2014) Expression of IL-2, IL-17 and TNF-alpha in patients with Crohn's disease treated with anti-TNF antibodies. Clin Res Hepatol Gastroenterol 38:491–498
26. Morales-Garcia G, Falfan-Valencia R, Garcia-Ramirez RA, Camarena A, Ramirez-Venegas A, Castillejos-López M, Pérez-Rodríguez M (2012) Pandemic influenza A/H1N1 virus infection and TNF, LTA, IL1B, IL6, IL8, and CCL polymorphisms in Mexican population: a case-control study. BMC Infect Dis 12:299
27. Strassmann G, Patil-Koota V, Finkelman F, Fong M, Kambayashi T (1994) Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. J Exp Med 180:2365–2370
28. Yoshimura A, Muto G (2011) TGF-beta function in immune suppression. Curr Top Microbiol Immunol 350:127–147
29. Balasubramanian S, Venugopal P, Sundarraj S, Zakaria Z, Majumdar AS, Ta M (2012) Comparison of chemokine and receptor gene expression between Wharton's jelly and bone marrow-derived mesenchymal stromal cells. Cytotherapy 14:26–33
30. Cullen SP, Brunet M, Martin SJ (2010) Granzymes in cancer and immunity. Cell Death Differ 17:616–623
31. Vecchiatini R, Penolazzi L, Lambertini E, Angelozzi M, Morganti C, Mazzitelli S, Trombelli L, Nastruzzi C, Piva R (2014) Effect of dynamic three-dimensional culture on osteogenic potential of human periodontal ligament-derived mesenchymal stem cells entrapped in alginate microbeads. J Periodontal Res. doi:10.1111/jre.12225
32. Elseberg CL, Salzig D, Czermak P (2014) Bioreactor expansion of human mesenchymal stem cells according to GMP requirements. Methods Mol Biol. doi:10.1007/7651_2014_117

University of Bern

3.4 Publication 4 (Original research)

Yazid FB, Gnanasegaran N, Kunasekaran W, Govindasamy V, **Musa S**: Comparison of immunomodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. Clinical Oral Investig. 2014, 18:2103-2112

3.4.1 Contributions of co-authors:

Design and concept of study	SM, GV
Acquisition of data	YFB, SM, KW, GN
Analysis of data	YFB, SM, GV, KW
Drafting of manuscript	YFB, GV
Revising manuscript for intellectual content	All authors

University of Malaya

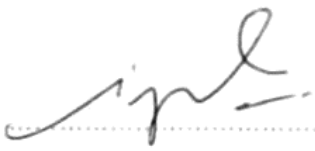
Cryocord Sdn Bhd

21st March 2019

Yazid FB, Gnanasegaran N, Kunasekaran W, Govindasamy V, Musa S: Comparison of immunomodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. Clinical Oral Investig. 2014, 18:2103-2112

As the corresponding author of the article named above, I consent to Sabri bin Musa including the published article above as part of his collection of published work to be submitted for his "PhD by prior publication" at University of Malaya, Kuala Lumpur.

Signed,



Full Name: Dr. Vijayendran Govindasamy

Designation: Senior Manager and Head of R&D Department

Full address: Suite 1-1, 1st Floor, Bio-X Centre, Persiaran Cyberpoint Selatan, Cyber 8, 63000, Cyberjaya, Selangor Malaysia

Email address: vijayendran@cryocord.com.my

Tel no: +603 8689 8888

Fax no:+603 8689 8866

University of Malaya

Clin Oral Invest
DOI 10.1007/s00784-014-1207-4

ORIGINAL ARTICLE

Comparison of immunodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth

Farinawati Binti Yazid · Nareshwaran Gnanasegaran · Wijenthiran Kunasekaran · Vijayendran Govindasamy · Sabri Musa

Received: 8 August 2013 / Accepted: 4 February 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract
Objectives The aim of this study was to investigate the immunodulatory properties of dental pulp stem cells derived from healthy (SCD) and inflamed pulp deciduous (SCDIP) tissues. The overall hypothesis is that SCDIP possess equal immune properties with SCD and could be used as an alternative tissue source in regenerative medicine.
Materials and methods An intra-oral examination was carried out to assess the status of the pulp tissues and group them according to healthy or inflamed. Primary cells were established from these groups, and basic mesenchymal stem cells (MSC) characterizations were conducted. The expression of human leukocyte antigen (HLA), namely HLA-G, HLA-DR, and HLA-ABC were examined in both cell lines using flow cytometry. We further compared the immunosuppressive effects of SCD and SCDIP on phytohemagglutinin-induced T cell proliferation. Supernatants were tested for cytokine profiling using multiplex array.
Results While SCD exhibited typical MSC characteristics, SCDIP on the other hand, did not. Compared with SCDIP, SCD effectively suppresses mitogen-induced T cells proliferation in a dose-dependent manner, as well as express a higher percentage of HLA-ABC and HLA-G. In addition, levels of several cytokines, such as TNF-α, TNF-β, and IL-2, were drastically suppressed in SCD than SCDIP. Furthermore, a high level of IL-10, an important anti-inflammatory cytokine, was present in SCD compared with SCDIP.
Conclusions These findings suggest that SCDIP is highly dysfunctional in terms of their stemness and immunomodulatory properties.
Clinical relevance SCDIP is not a viable therapeutic cell source especially when used in graft versus host disease (GvHD) and organ rejection.

Keywords Regenerative medicine · Immunogenicity · Cytokines · Cells · Deciduous teeth

F. B. Yazid · W. Kunasekaran · S. Musa
Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

S. Musa
e-mail: sabrim@um.edu.my

N. Gnanasegaran
Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

N. Gnanasegaran · W. Kunasekaran
Regenerative Dentistry Research Group (ReDReG), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

V. Govindasamy (✉)
Stem Cells Laboratory, Hygieia Innovation Sdn. Bhd, Lot 1G-2G, Komplex Lanai, No. 2, Persiaran Seri Perdana, Persint 10, 62250 Federal Territory of Putrajaya, Malaysia
e-mail: vijay@hygieiagroup.com

Introduction

Inflammation of the pulp, known as pulpitis, is one of the more common childhood diseases. Pulpitis occurs when dentin-pulp complex are triggered by noxious stimuli such as caries, dental trauma, exposure to irritants, as well as direct or indirect exposure of the pulp to oral environment contaminants [1]. Bacteria and their by-products are the primary aetiological agents in pulpitis. As the invading bacteria or their products reach the pulp, the first cell to encounter them is the odontoblast [2]. They provide a barrier function to the underlying tissue by orchestrating several inflammatory responses [3], which include the secretion of cytokines such as interleukin (IL)-4 [4], IL-6 [5], and IL-10 [4].

In addition, cells of the immune system such as T and B lymphocytes, macrophages, plasma cells, and neutrophils also

Published online: 19 February 2014

University of Malaya

infiltrate the pulp during the inflammation process and ultimately determine the inflammatory progression as well as clinical prognosis [6]. Current treatments in pulpitis include direct and indirect pulp capping and pulpotomy. However, treating pulpitis often ends up with complete removal of pulp (pulpectomy), which is regarded as a major pitfall in regenerative dentistry.

Mesencyhmal stem cells (MSC) are unspecialized cells, which divide to replenish dying cells and regenerate damaged tissues [7]. These cells were originally discovered in bone marrow and are also found in a variety of tissues, such as adipose, umbilical cord, liver, skin, pancreas, and synovial fluid [8]. In 2000, Gronthos et al. discovered a subpopulation of MSC in the pulp (DPSC) that is highly proliferative and is able to differentiate into various cell lineages [9]. These cells have also shown remarkable outcomes in preclinical studies, including for dentin regeneration [10].

Another striking feature of MSC is their low immunogenicity and immunoregulatory effects. MSC can modulate immunological responses by preserving dendritic cell immaturity [11], angling the CD4+ T-helper population phenotypes, and modulating CD8+ cytotoxic T lymphocyte and natural killer functions [12]. MSC acts through a multitude of pathways, including the prostaglandin E2, nitric oxide, and indoleamine 2,3-dioxygenase [13, 14]. Furthermore, MSC is also involved in the modulation of Treg cells [15]. Previous studies reported that DPSC displays an increased level of immunosuppressive activities when compared with bone marrow MSC. Huang et al. also showed that the implantation of DPSC derived from rhesus monkeys into the hippocampus of mice did not cause any immune rejection [16]. However, stem cells from inflamed tissue have been shown to lose osteogenic potential [17]. Furthermore, there are also reports on impaired immune functions, for example, in a study conducted by Liu et al. where stem cells isolated from inflamed periodontal ligament tissue showed a lower expression of many cytokines, including IL-17 and IL10, as well as lower production of Treg cells [18]. They further concluded that these imbalances may trigger an unsteady immune response, leading to alveolar bone loss in periodontitis. While this study focused on inflamed periodontal ligament tissue, we extended a similar approach to pulp tissue, which had not been reported yet. Therefore, this study was carried out to investigate the immunogenic properties of stem cells derived from clinical specimen of healthy (SCD) and inflamed deciduous teeth (SCDIP). It was believed that when a pulp is inflamed, there is no value in it and it will be discarded, but with the discovery of DPSC, the compromised pulp tissue can perhaps be salvaged into something beneficial such as a potential source in regenerative medicine.

Methodology

Sample population

The study was conducted using samples from pediatric donors. Prior to the commencement of subject recruitment, approval for the study was obtained from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Ethics approval number, DF CD1201/0013[P]).

Tooth selection

An intra-oral examination was done to assess the status of subjects' pulp tissue based on several criteria (Table 1), where they were grouped into healthy or inflamed. The study was conducted with three samples for each group, and all experiments were repeated independently to ensure the reproducibility of the results.

Pulp collection and isolation of cells

Stem cell cultures derived from SCD and SCDIP were established as previously described by our group [19]. Briefly, root surfaces were cleaned with povidone–iodine (Sigma Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com), and the pulp were extirpated within 2 h post-extraction and processed. The pulp tissue were minced into smaller fragments prior to digestion in a solution of 3 mg/mL collagenase type I (Gibco, Grand Island, NY, http://www.invitrogen.com) for 40 min at 37 °C. After neutralization with 10 % fetal bovine serum (FBS), the cells were centrifuged and seeded in culture flasks.

Both cells were cultured in identical culture condition, namely in T 75 cm^2 culture flasks (BD Pharmingen, San Diego CA, USA; http://www.bdbiosciences.com) with culture medium containing 1× KO-DMEM, 200 units/mL and 200 μg/mL of penicillin/streptomycin (Invitrogen); 0.01× Glutamax (Invitrogen) and 10 % FBS with humidified atmosphere of 95 % of air and 5 % of CO_2 at 37 °C, as well as cell seeding of 1,000 cell/cm^2. Nonadherent cells were removed 48 h after initial plating. The medium was replaced every 3 days until the cells reached 80–90 % confluency.

Growth kinetics

The proliferation rate for both groups was determined by plating 5000 cells per cm^2 into separate T25 cm^2 culture flask (BD Pharmingen). There were three replicates for each passage. Both SCD and SCDIP cells were detached by trypsinization after reaching 90 % confluency. Cells were counted and assessed for viability using trypan blue dye exclusion before the next subculture. Cells were re-plated for subsequent subculture, and a total of five subcultures were

Table 1 Inclusion and exclusion criteria for both healthy and inflamed samples

Healthy sample			
Donor details			
Donor	Age	No. of teeth	Type of teeth
I	5	2	Molars
II	6	2	Molars
III	5	2	Incisor and molars
Inclusion criteria			
Healthy children with no medical problems.			
Sound tooth			
Tooth that was indicated for extraction, i.e., for serial extractions, balancing extractions, or for orthodontic purpose			
Root resorption was less than half based on radiography examination			
Exclusion criteria			
Children with medical problems			
Aged below 3 years old or above 12 years old			
Dental pulp sample was obtained from the permanent teeth			
Tooth with caries			
Tooth was not indicated for extraction			
Root resorption was more than half based on radiograph examination			
Inflamed sample			
Donor details			
Donor	Age	No. of teeth	Type of teeth
I	5	3	molars
II	4	3	molars
III	4	4	Incisors and molars
Inclusion criteria			
Children with no medical problems			
Clinically diagnosed as deep caries with dentinal involvement or reversible pulpitis that was indicated for pulpotomy			
Tooth that was indicated for extraction, i.e., serial extractions, balancing extractions, or for orthodontic purpose			
Root resorption was less than one half based on the radiograph examination			
Exclusion criteria			
Children with medical problems			
Aged below 3 years old or above 12 years old			
Dental pulp sample was obtained from the permanent teeth			
Clinically diagnosed as irreversible pulpitis, history of abscess, and swelling			
Tooth was not indicated for extraction			
Root resorption was more than half based on the radiograph examination			

studied in this experiment. Growth kinetics was analyzed by calculating population doubling (PD) time. The PD time was obtained using:

$$\mathrm{PDT} = t\mathrm{plg2}/(1\mathrm{gNH}-1\mathrm{gNI}).$$

NI is the inoculums cell number; NH is the cell harvest number; and t is the time of the culture (in hours).

Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) was carried out as previously described by our group [20]. The following antibodies were used to mark the cell surface epitopes—CD90-phycoerythrin (PE), CD73-PE, CD166-PE, CD34-PE, CD45-fluorescein-isothyocyanate (FITC), HLA-DR-FITC, HLA-ABC-PE, HLA-G-FITC, and HLA-DR-FITC (all from BD Pharmingen). All analyses were standardized against negative control cells incubated with isotype-specific IgG1-PE and IgG1-FITC (BD Pharmingen). Cells were acquired on FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA) and were identified by light scatter for 10,000 gated events and analyzed using BD CellQuest™ Pro software (BD Biosciences).

Multilineage differentiation of stem cell deciduous

In vitro multilineage differentiation studies were performed to determine the multilineage capacity of mesenchymal cells. All samples were cultivated and differentiated into osteoblast, adipocyte, and chondrocyte lineages in the appropriate induction media as previously described [21]. In brief, adipogenic differentiation was initiated by inducing the cells with 200 mM indomethacin, 0.5 mM 3-isobutyl-1-methyxanthine (IBMX), 10 mg/mL insulin, and 1 mM dexamethasone (all reagents from Sigma-Aldrich). Lipid droplets in the adipocytes generated were visualized by staining with Red Oil staining (Sigma-Aldrich). For chondrogenic differentiation, the cells were supplemented with ITS_1 (Sigma-Aldrich), 50 mM L-ascorbic acid-2 phosphate, 55 mM sodium pyruvate (Invitrogen), 25 mM L-proline (Sigma-Aldrich), and 10 ng/mL transforming growth factor-beta (TGF-β; Sigma-Aldrich). Assessment of proteoglycan accumulation was visualized by Alzarin Blue staining (Sigma-Aldrich). The osteogenic differentiation was stimulated in a 3-week culture with 10^{-7} M dexamethasone, 10 mM β-glycerol phosphate (Fluka, Buchs, Switzerland) and 100 mM L-ascorbic acid-2 phosphate. Assessment of calcium accumulation was visualized by Von Kossa staining (Sigma-Aldrich). Important genes related to bone, fat, and cartilage development were also checked, and the primers are listed in Table 2.

Semiquantitative and quantitative gene expression via polymerase chain reaction

Polymerase chain reaction (PCR) was performed in 0.2 mL Eppendorf tubes (Axygen) with a final volume of 12.5 μL. cDNA amplification was performed in a thermocycler using Taq polymerase supplied with KCl buffer and 1.5 mM $MgCl_2$ (Invitrogen) at 94 °C for 1 min, 58 °C for 30 s, and 72 °C for 1 min. PCR products were resolved on 1.5 % agarose (Invitrogen)

University of Malaya

Table 2 List of genes with primer sequence and the product size

Gene name	Forward (5′-3′)	Reverse (5′-3′)	Base pair
RUNX2	GTCACTGTGCTGAAGAGGCT	GTCACTGTGCTGAAGAGGCT	119
OSTEOCALCIN	CAGAGGTGCAGCCTTTGTGTC	TCACAGTCCGGATTGAGCTCA	150
PPARγ2	ACAGCAAACCCCTATTCCATGCTGT	TCCCAAAGTTGGTGGGCCAGAA	159
LPL	TGGACTGGCTGTCACGGGCT	GCCAGCAGCATGGGCTCCAA	167
AGGRECAN	AGGGCGAGTGGAATGATGTT	GGTGGCTGTGCCCTTTTTAC	68
COLLAGEN 2A1	CTGCAAAATAAAATCTCGGTGTTCT	GGGCATTTGACTCACACCAGT	101
18s rRNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT	186

gel in 1× Tris borate–EDTA buffer. The expression levels of the genes were quantified in duplicates, using SYBR Green Master Mix (Applied Biosystems). PCR reactions were carried out on an ABI 7900HT RT-PCR system (Applied Biosystems), and the results were analyzed using a software called SDS v 2.1. Gene expressions were analyzed via comparative CT Method (ΔΔCT) and were normalized to 18s rRNA. The primer sequences are listed in Table 2.

Immunosuppression assay

Isolation and activation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors for culturing, after written consent were taken using a protocol approved by the Faculty of Dentistry, University Malaya. PBMCs were isolated by density gradient centrifugation. The cells were cultured in RPMI-1640 media supplemented with 2 mM glutamine, 10 % FBS, and 2× antibiotic antimycotic (GIBCO, Invitrogen). For mitogenic activation experiments, PBMCs were stimulated with 10 μg/mL phytohemagglutinin (PHA; Sigma Aldrich).

Mitotic inactivation of cell by mitomycin C

SCD and SCDIP cells were seeded at 2,500 cells/T25 cm^2 flask. After 24 h, 10 μg/mL of mitomycin C (Sigma Aldrich) was added to inhibit stem cells' proliferation, and incubation for 2.5 h at 37 °C were followed by six extensive washes with Knockout DMEM media. The cells were then plated into 96-well plates (BD FALCON, NJ, USA) at ratios of 1:12, 1:24, and 1:48 (SCD/PBMC) per well in culture media consisting of RPMI-1640 (GIBCO-BLR, USA) supplemented with 2 mM glutamine, 10 % FBS, and antibiotic antimycotic.

PBMC-stem cells co-cultures

The activated PBMC were added into the same 96-well plates (BD FALCON, NJ, USA), in which both SCD healthy and inflamed had been plated earlier, at different wells following the different concentration ratios of 1:12, 1:24, and 1:48 (SCD/PBMC). The positive control consisted of only PHA-stimulated PBMC. The co-cultures were incubated at 37 °C, 5 % CO_2, and proliferation rates were measured at 12, 24, 48, 72, and 96 h. Cell proliferation was analyzed using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma Aldrich) assay, and data were obtained with the following formula:

$$\%\ \text{changes in PBMC proliferation} = 100 \times\ [(\text{cells} + \text{PBMC} + \text{PHA})]/(\text{PBMC} + \text{PHA})$$

Cytokine analysis with Procarta® Immunoassay from Affymetrix

Collected media samples were centrifuged at 3,000 rpm for 10 min to remove cell debris and filtered through a 0.2-mm filter. For cytokine arrays, media were concentrated by a factor of 50 using Amicon Ultra-15 centrifugal filter devices with 3 kDa molecular weight cutoff (Millipore, Billerica, MA). Human cytokine antibody array (Panomics, Redwood City, CA) was used to identify the cytokines present in conditioned media. The assay was performed according to the manufacturer's instructions. Briefly, after blocking of nonspecific binding sites, membranes were incubated in nonconditioned or AF-MSC-CM media overnight at 4 °C. After the membranes were washed, biotin-conjugated antibody was added and the whole mixture was incubated for 16 h at 4 °C. The membranes were then washed again and incubated with horseradish peroxidase-conjugated streptavidin at RT for 45 min.

University of Malaya

Finally, the membranes were incubated with detection buffer (ECL solution) and exposed to Hyperfilm. Plate seal was removed, and the filter plate was placed in the Luminex machine for analysis.

Statistical analysis

Results were presented as comparison of average±standard deviation of a three biological replicates (n=3) or samples. The data were analyzed using SPSS, version 19.0 (SPSS Inc, Chicago, IL, USA). Statistical calculations using two-way ANOVA test were carried out, and a p value of less than 0.05 was considered to be significant.

Results

Characterization of stem cells derived SCD and SCDIP

The isolated SCD and SCDIP formed a homogenous monolayer of adherent, spindle-shaped, fibroblast-like cells. SCD began to proliferate faster, and reached confluency after 10–12 days, approximately 3–5 days earlier than SCDIP (Fig. 1a). The SCD and SCDIP displayed similar proliferation rate throughout the five passages (p<0.05). The average numbers of cells obtained for SCD and SCDIP at subculture 1 were $8.75\pm2.17\times10^5$ and $5.41\pm1.91\times10^5$, respectively. At subculture 5, the accumulated cell count for SCD and SCDIP were $39.75\pm1.66\times10^5$ and $35.00\pm1.44\times10^5$, respectively

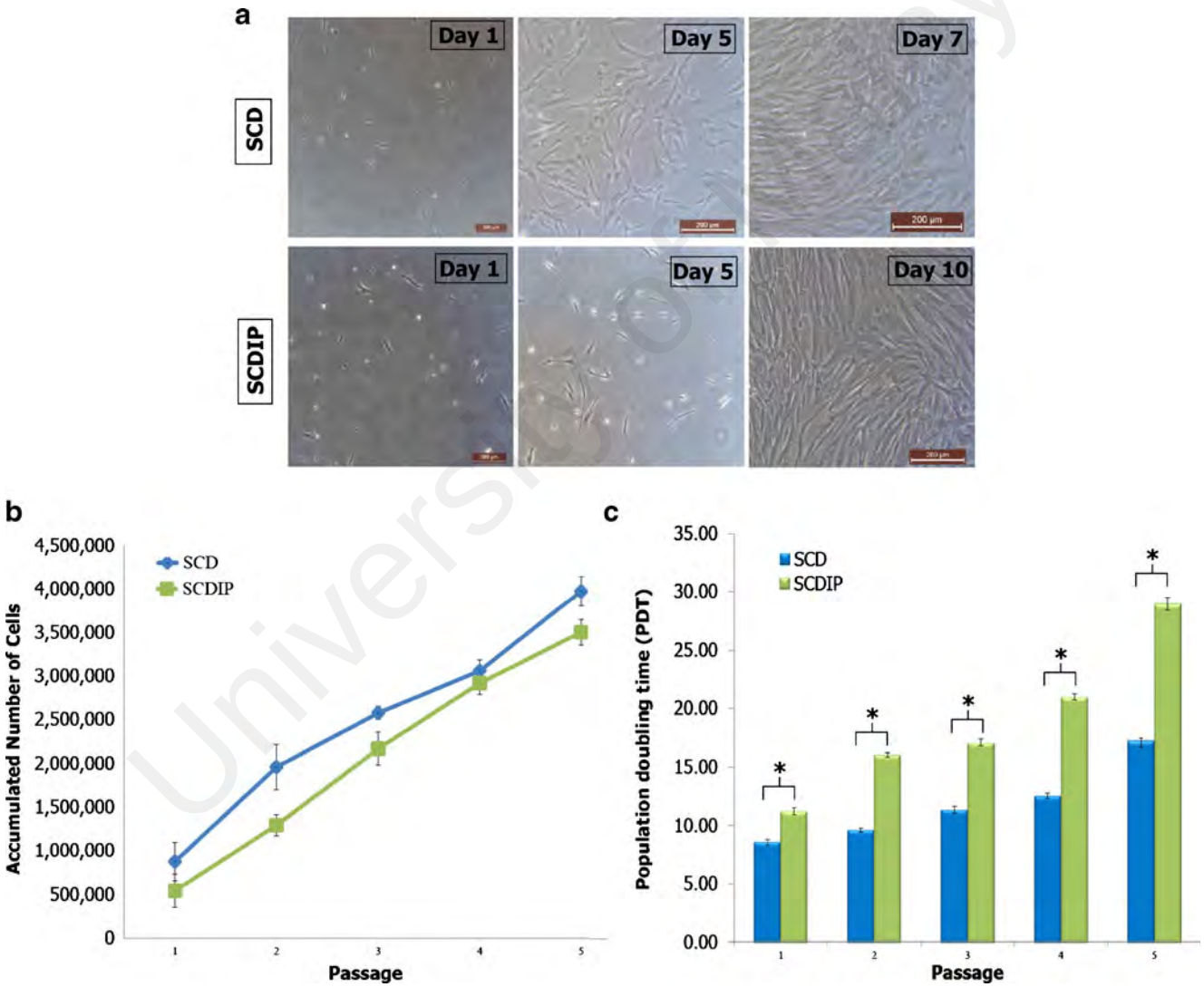


Fig. 1 Characterization of deciduous stem cell (*SCDs*) and inflamed deciduous stem cell (*SCDIP*). **a** Cell culture morphology pictures up to their confluency. Pictures were taken at ×10 magnification. **b** Accumulated cell count of SCD and SCDIP from subcultures 1 to 5. **c** Population doubling time (*PDT*) of both SCD and SCDIP from subcultures 1 to 5. In all experiments, the results represent average of three culture replicates with standard deviation, and a representative photomicrograph was given for each experiment

(Fig. 1b). We further characterized the PDT of SCD and SCDIP throughout the five subcultures. Based on our observation, the time required for PD varied, between approximately 8 h (SC1) and 18 h (SC5) for SCD, whereas in SCDIP, approximately 12 h (SC1) and 30 h (SC5) (Fig. 1c).

Adipogenic, chondrogenic, and osteogenic differentiation were confirmed in both cell types by the accumulation of neutral lipid vacuoles, secretion of cartilage-specific proteoglycans staining, and the deposition of a silver-stained mineralized matrix, respectively (Fig. 2a). Despite being able to differentiate, gene expression profile of the SCDIP into the aforesaid lineages exhibited impaired formation as compared with SCD (Fig. 2b, c). Furthermore, the expression of cell surface markers namely CD73, CD90, and CD166 were slightly higher (95.22, 93.10, and 91.19 %) than SCDIP (93.22, 91.03, and 91.04 %). Conversely, hematopoietic markers such as CD34 and CD45 were not expressed in both cell lines.

HLA expression

The expression of HLA-DR was negative (SCD, 0.5 %±0.2; SCDIP, 1.2 %±0.5) in both cell lines. Conversely, the expression of HLA-ABC was detected at a varying degree, in which the expression was higher in SCD (96.7 %±0.35) as compared with SCDIP (64.24 %±0.52). Notably, the expression of HLA-G, which plays a role in immune tolerance, was also highly expressed in SCD (84.2 %±0.61) as compared with SCDIP (1.2 %±0.5). These results indicate that SCDIP has an inferior immune suppressive function relative to SCD.

Suppression of mitogen-induced T cell proliferation by the SCD and SCDIP

Figure 3 shows PHA-induced proliferation of T cells in the PBMC co-cultured either in the absence or presence of SCD or SCDIP in various ratios. At five different time intervals,

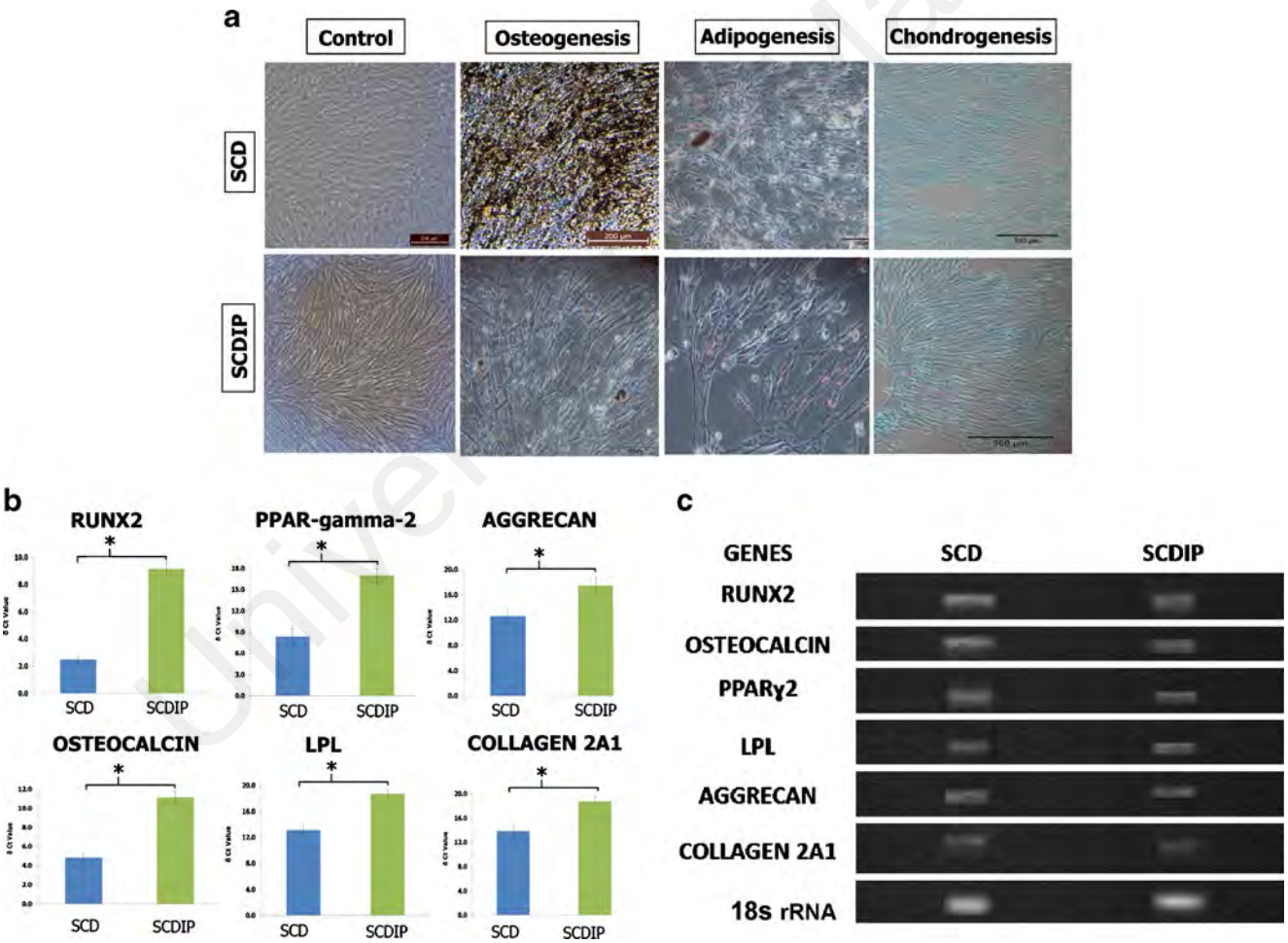


Fig. 2 MSC characterization of deciduous stem cell (*SCDs*) and inflamed deciduous stem cell (*SCDIP*). **a** In vitro multi-differentiation of SCD and SCDIP. **b**, **c** Gene expression of trilineage differentiation markers for SCD and SCDIP by means of quantitative and semiquantitative analyses, respectively. The lower a cycle threshold (C_T) value is, the more copies are present in the specific sample. Values are presented after normalized to 18s mRNA levels. In all experiments, the results represent average of three culture replicates with standard deviation and a representative photomicrograph was given for each experiment

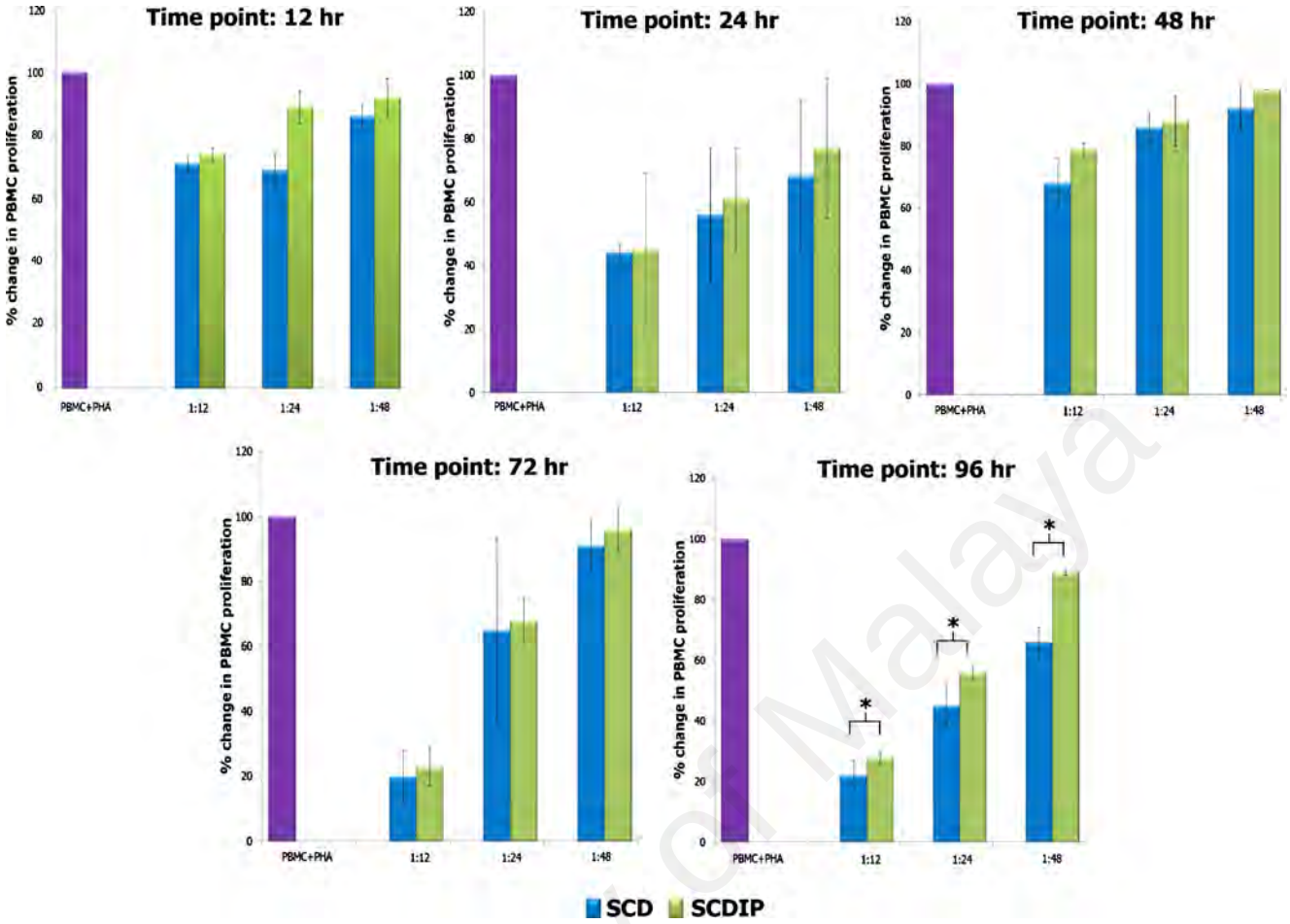


Fig. 3 Proliferation activity of peripheral blood mononuclear cells (*PBMC*) after co-culture at different ratio with either deciduous stem cell (*SCDs*) or inflamed deciduous stem cell (*SCDIP*) via MTT assay. Significant differences with p value <0.05 were marked with an *asterisk*

both cell lines displayed a similar pattern of dose dependent decrement of T cells proliferation. This indicates the immunosuppresion capacity of SCD and SCDIP. However, SCD suppresses the T cell proliferation better than SCDIP ($p<0.05$), demonstrating that SCD possesses lower immunogenicity compared with SCDIP.

Cytokine secretion profile of activated PBMC in co-cultures with SCD and SCDIP

To identify the contribution of soluble factors in immunosuppression, the supernatants of mitogen-activated T cells cultured in the absence or presence of each cell line were examined in multifaceted immunomodulatory mediators, namely IL-2, IL-10, tumor necrosis factor (TNF)-α and TNF-β (Fig. 4). The levels of IL-2, TNF-α, and TNF-β were higher in SCDIP as compared with SCD. Meanwhile, the expression of IL-2 was higher in SCD. Despite the ability of SCDIP to suppress some amount of pro-inflammatory cytokines, SCD appears to be more effective at enhancing immune suppression capacity.

Discussion

Dental pulp stem cells from deciduous teeth (SCD) are another rising candidate for stem cell therapy because of their similar characteristics and differentiation potential with BMMSC [9]. Previously, we reported that SCD has the capacity to differentiate into osteocyte, chondrocyte, adipocyte, neuronal-like cells, and cells like aggregates [19–21]. SCD is also shown to alleviate neurodegenerative diseases like Alzheimer's and Parkinson's in preclinical models. Additionally, SCD were shown to have the ability to form bone when transplanted in vivo [22]. Nevertheless, it is difficult to obtain SCD from a healthy pulp unless the tooth is being indicated for extraction due to balancing, compensating, or serial extraction. Generally, pulp therapy treatment often requires the removal of the inflamed pulp, and recent studies show that these tissues still contain viable cells that have stem cell properties [23, 24]. As common stem cells characteristics between SCD and SCDIP, e.g., cell surface markers, PD and mesoderm differentiation were reported in previous papers [23–25]; here, we examined whether the same population of cells possess immunological characteristics.

University of Malaya

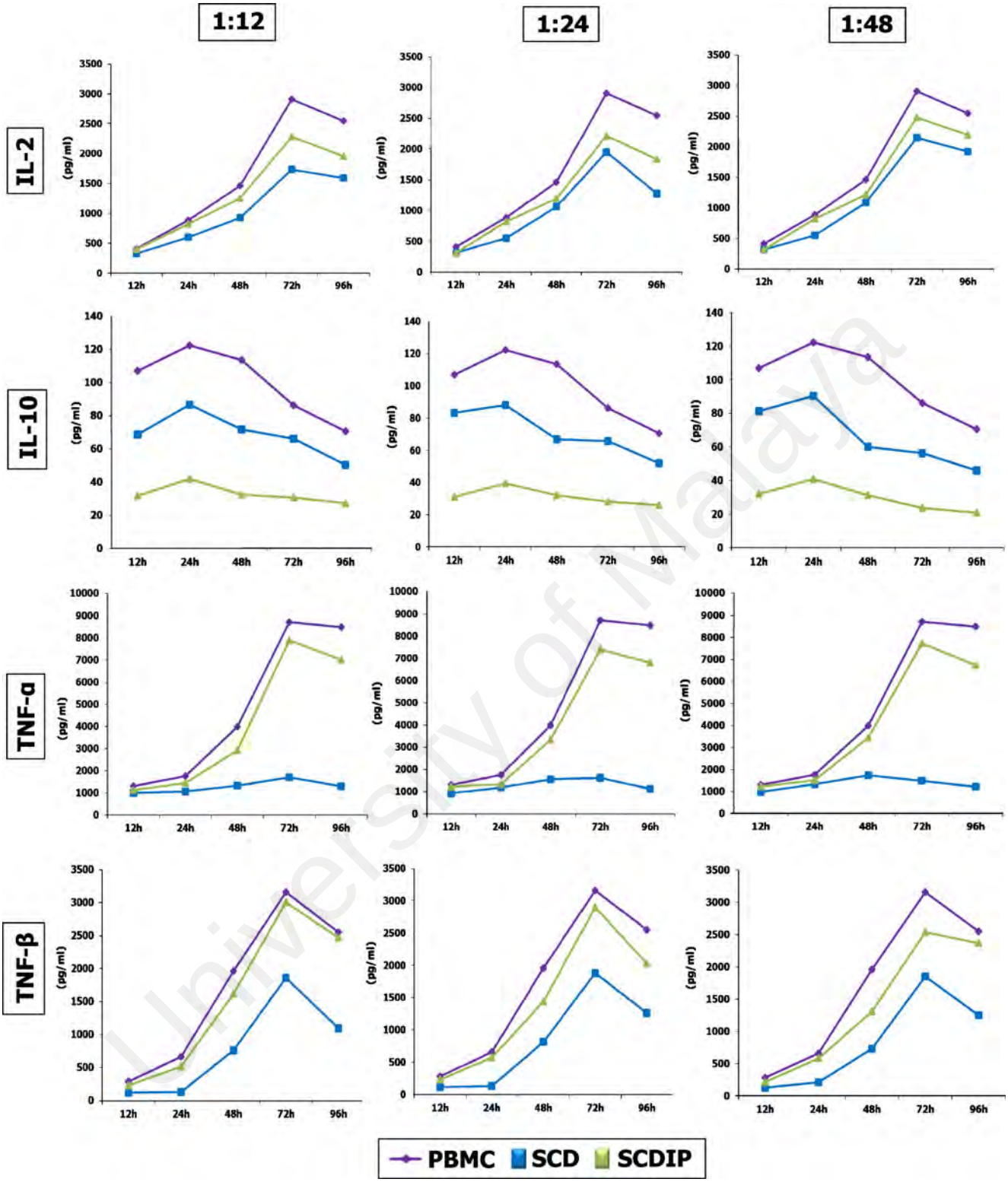


Fig. 4 Amount of selected cytokines namely interleukin-2 (*IL-2*), interleukin-10 (*IL-10*), tumor necrosis factor-α (*TNF-α*), and tumor necrosis factor-β (*TNF-β*) secreted at different co-culture ratio between peripheral blood mononuclear cells (*PBMC*) and either deciduous stem cell (*SCD*) or inflamed deciduous stem cell (*SCDIP*)

The expression of HLA is important in regards to immune regulation. In the present study, both SCD and SCDIP were positive for HLA-ABC and HLA-G and negative for HLA-DR, which is similar in other MSC [26, 27]. Interestingly, in

contrast to SCDIP, higher expressions of HLA-G were seen in SCD. HLA-G is a specific MHC-I antigen that is critical to maintain immune tolerance during pregnancy and also indicates a stronger immunosuppression [28]. Moreover, a study has also documented the role of HLA-G as a prognostic indicator of graft tolerance [29]. For the mixed lymphocyte reaction (MLR), we found that the immunogenicity of SCD was low and remained for 96 h. By contrast, the degree of immunosuppression in SCDIP was less. A similar scenario was reported by Liu et al. [18], whereby inflamed periodontal ligament stem cells showed significantly diminished inhibition of T cell proliferation compared with the healthy cells. Moreover, Plumas et al. [30] demonstrated that MSC inhibits PHA-induced T cell proliferation by inducing early apoptosis but had no effects on resting T cells. They confirmed that activated T cells die through apoptotic mechanism in the presence of MSC in MLR. Furthermore, in line with a previous study by LeBlanc et al. [31], it was also revealed that PBMC suppression took place in dose dependent manner. Past studies have shown that MSC inherently has the ability to migrate to injured tissue and participate in restoring the functions. MSC repairs injury site by either differentiating to replace damaged cell or secreting appropriate cytokines or growth factors [32]. Thus, it can be speculated that the lower degree of immunosuppression found in SCDIP is mainly due to their inability to release soluble factors to decelerate the proliferation of the T cells.

In this study, levels of IL-2, IL-10, TNF-α, and TNF-β secretion from activated T cells increased over time but were drastically reduced when T- cells were co-cultured with SCD. However, SCDIP only minimally inhibits the secretion of these cytokines. IL-2 is a lympho-cytotrophic hormone that is responsible in signaling T lymphocyte proliferation which plays a role in the immune response [33]. IL-2 is an important expansion factor for most or all types, of activated T cells, and it also plays a central role in downregulating immune responses. As IL-2 is an important growth and expansion factor for T helper cells, it can indirectly influence the production of all T cell-derived cytokines [34].

The TNF cytokine family has been shown to be essential in the mediation of both innate inflammatory processes [35] in response to tissue injury, as well as being a crucial regulatory component in bone remodeling [36]. The pattern of TNF-α and TNF-β inhibition were inversely proportional to IL-10 whereby SCD had a higher inhibition level compared to SCDIP. Thus, this study demonstrates that both SCD and SCDIP inhibits the secretion of pro-inflammatory cytokines (IL-2 and TNF-α) and increases the secretion of anti-inflammatory cytokine (IL-10). Concurrent findings were also observed by Liu et al. [37], who reported that the MSC were capable of inducing IL-10 expression and inhibiting TNF-α expression. This indicated a possible link between the two cytokines and MSC. Aggarwal and Pittenger [38] also reported that MSC interacts with each of the isolated cells of the immune system and are capable of altering the outcome of the immune cell response by inhibiting two of the most important pro-inflammatory cytokines (i.e., TNF-α and IFN-γ) and by increasing expression of suppressive cytokines, including IL-10.

In conclusion, SCDIP has minimal ability to suppress pro-inflammatory and stimulate anti-inflammatory responses. Our findings suggest allogeneic/autologous transplantation using SCDIP is not a viable therapeutic method especially when used in graft-versus-host disease (GVHD) and organ rejection. However, further studies would be required to assess the functionability, immunomodulatory effects, and immune responses in animal models to ascertain the suppression capacity of SCDIP.

Acknowledgments This work is part of an ongoing project between Hygieia Innovation and Faculty of Dentistry, University Malaya and supported by University Malaya, High Impact Research Chancellery grant (UM.C/625/HIR/032).

Conflict of interest The authors declare that no competing financial interests exist.

References

1. Izumi T, Kobayashi I, Okamura K, Sakai H (1995) Immunohistochemical study on the immunocompetent cells of the pulp in human non-carious and carious teeth. Arch Oral Biol 40(7): 609–614
2. Leprince JG, Zeitlin BD, Tolar M, Peters OA (2012) Interactions between immune system and mesenchymal stem cells in dental pulp and periapical tissues. Int Endod J 45(8):689–701
3. Veerayutthwilai O, Byers MR, Pham TT, Darveau RP, Dale BA (2007) Differential regulation of immune responses by odontoblasts. Oral Microbiol Immunol 22:5–13
4. Hahn CL, Best AM, Tew JG (2000) Cytokine induction by Streptococcus mutans and pulpal pathogenesis. Infect Immun 68(12):6785–6789
5. Barkhordar RA, Hayashi C, Hussain MZ (1999) Detection of interleukin-6 in human pulp and periapical lesions. Endod Dent Traumatol 15:26–27
6. Hahn CL, Liewehr FR (2007) Update on the adaptive immune responses of the dental pulp. J Endod 33(7):773–781
7. Anderson DJ, Gage FH, Weissman IL (2001) Can stem cells cross lineage boundaries? Nat Med 7(4):393–395
8. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276(5309):71–74
9. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 7:13625–13630
10. Liu H, Gronthos S, Shi S (2006) Dental pulp stem cells. Methods Enzymol 419:99–113
11. English K, Barry FP, Mahon BP (2008) Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. Immunol Lett 115:50–58
12. Uccelli A, Moretta L, Pistoia V (2006) Immunoregulatory function of mesenchymal stem cells. Eur J Immunol 36:2566–2573

University of Malaya

13. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2:141–150
14. Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML (2010) Mesenchymal stem cells: mechanisms of immunomodulation and homing. Cell Transplant 19:667–679
15. English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP (2009) Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4 + CD25 (high) forkhead box P3+ regulatory T cells. Clin Exp Immunol 156:149–160
16. Huang AHC, Snyder BR, Cheng PH, Chan AW (2008) Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. Stem Cells 26(10):2654–2663
17. Li X, Makarov SS (2006) An essential role of NF-κB in the "tumor-like" phenotype of arthritic synoviocytes. Proc Natl Acad Sci U S A 103:17432–17437
18. Liu D, Xu J, Liu O, Fan Z, Liu Y, Wang F, Ding G, Wei F, Zhang C, Wang S (2012) Mesenchymal stem cells derived from inflamed periodontal ligaments exhibit impaired immunomodulation. J Clin Periodontol 39(12):1174–1182. doi:10.1111/jcpe.12009
19. Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH (2010) Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. J Endod 36(9):1504–1515
20. Abu Kasim NH, Govindasamy V, Gnanasegaran N, Musa S, Pradeep PJ, Srijaya TC, Aziz ZA (2012) Unique molecular signatures influencing the biological function and fate of post-natal stem cells isolated from different sources. J Tissue Eng Regen Med. doi:10.1002/term.1663
21. Govindasamy V, Ronald VS, Abdullah AN, Ganesan Nathan KR, Aziz ZA, Abdullah M, Zain RB, Kasim NH, Musa S, Bhonde RR (2011) Human platelet lysate permits scale-up of dental pulp stromal cells for clinical applications. Cytotherapy 13(10):1221–1233
22. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100(10):5807–5812
23. Alongi DJ, Yamaza T, Song Y, Fouad AF, Romberg EE, Shi S, Tuan RS, Huang GT (2010) Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. Regen Med 5(4):617–631
24. Kim J, Park JC, Kim SH, Im GI, Kim BS, Lee JB, Choi EY, Song JS, Cho KS, Kim CS (2013) Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth. Oral Dis. doi:10.1111/odi.12089
25. Wang Z, Pan J, Wright JT, Bencharit S, Zhang S, Everett ET, Teixeira FB, Preisser JS (2010) Putative Stem cells in human dental pulp with irreversible pulpitis: an exploratory study. J Endod 36(5):820–825
26. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99(10):3838–3843
27. Yoo KH, Jang IK, Lee MW, Kim HE, Yang MS, Eom Y, Lee JE, Kim YJ, Yang SK, Jung HL, Sung KW, Kim CW, Koo HH (2009) Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol 259(2):150–156. doi:10.1016/j.cellimm.2009.06.010
28. Hunt JS, Petroff MG, McIntire RH, Ober C (2005) HLA-G and immune tolerance in pregnancy. FASEB J 19(7):681–693
29. Menier C, Rouas-Freiss N, Favier B, LeMaoult J, Moreau P, Carosella ED (2010) Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule. Tissue Antigens 75(3):201–206
30. Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC (2005) Mesenchymal stem cells induce apoptosis of activated T cells. Leukemia 19(9):1597–1604
31. Le Blanc K, Rasmusson I, Götherström C, Seidel C, Sundberg B, Sundin M, Rosendahl K, Tammik C, Ringdén O (2004) Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. Scand J Immunol 60(3):307–315
32. Prockop DJ, Gregory CA, Spees JL (2003) One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc Natl Acad Sci U S A 100(Suppl 1):11917–11923
33. Smith KA (1988) Interleukin-2: inception, impact, and implications. Science 240(4856):1169–1176
34. Gaffen SL, Liu KD (2004) Overview of interleukin-2 function, production and clinical applications. Cytokine 28(3):109–123
35. Feghali CA, Wright TM (1997) Cytokines in acute and chronic inflammation. Front Biosci 2(1):d12–d26
36. Kimble RB, Bain S, Pacifici R (1997) The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. J Bone Miner Res 12(6):935–941
37. Liu N, Chen R, Du H, Wang J, Zhang Y, Wen J (2009) Expression of IL-10 and TNF-alpha in rats with cerebral infarction after transplantation with mesenchymal stem cells. Cell Mol Immunol 6(3):207–213. doi:10.1038/cmi.2009.28
38. Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105(4):1815–1822

University of Malaya

3.5 Publication 5 (Original research)

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, **Musa S**, Abu Kasim NH: Differential expression of basal microRNAs' patterns in human dental pulp stem cells. J Cell Mol Med. 2015, 19(3):566-580

3.5.1 Contributions of co-authors:

Design and concept of study	SM, GV
Acquisition of data	VP, SM, GN, KW, GV
Analysis of data	VP, SM, GV,
Drafting of manuscript	VP, GV
Revising manuscript for intellectual content	All authors

University of Malaya

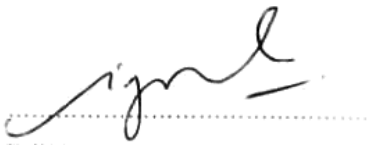
CryoCord Sdn Bhd

21st March 2019

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Musa S, Abu Kasim NH: Differential expression of basal microRNAs' patterns in human dental pulp stem cells. J Cell Mol Med. 2015, 19(3):566-580

As the corresponding author of the article named above, I consent to Sabri bin Musa including the published article above as part of his collection of published work to be submitted for his "PhD by prior publication" at University of Malaya, Kuala Lumpur.

Signed,



Full Name: Dr. Vijayendran Govindasamy

Designation: Senior Manager and Head of R&D Department

Full address: Suite 1-1, 1st Floor, Bio-X Centre, Persiaran Cyberpoint Selatan, Cyber 8, 63000, Cyberjaya, Selangor Malaysia

Email address: vijayendran@cryocord.com.my

Tel no: +603 8689 8888

Fax no:+603 8689 8866

University of Malaya

J. Cell. Mol. Med. Vol 19, No 3, 2015 pp. 566-580

Differential expression of basal microRNAs' patterns in human dental pulp stem cells

Punitha Vasanthan [a], Vijayendran Govindasamy [b, *], Nareshwaran Gnanasegaran [c], Wijenthiran Kunasekaran [a], Sabri Musa [a], Noor Hayaty Abu Kasim [c]

[a] *Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia*
[b] *Komplex Lanai, Hygieia Innovation Sdn. Bhd, Federal Territory of Putrajaya, Malaysia*
[c] *Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia*

Received: February 27, 2014; Accepted: July 2, 2014

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate translation of mRNA into protein and play a crucial role for almost all biological activities. However, the identification of miRNAs from mesenchymal stem cells (MSCs), especially from dental pulp, is poorly understood. In this study, dental pulp stem cells (DPSCs) were characterized in terms of their proliferation and differentiation capacity. Furthermore, 104 known mature miRNAs were profiled by using real-time PCR. Notably, we observed 19 up-regulated miRNAs and 29 significantly down-regulated miRNAs in DPSCs in comparison with bone marrow MSCs (BM-MSCs). The 19 up-regulated miRNAs were subjected to ingenuity analysis, which were composed into 25 functional networks. We have chosen top 2 functional networks, which comprised 10 miRNA (hsa-miR-516a-3p, hsa-miR-125b-1-3p, hsa-miR-221-5p, hsa-miR-7, hsa-miR-584-5p, hsa-miR-190a, hsa-miR-106a-5p, hsa-mir-376a-5p, hsa-mir-377-5p and hsa-let-7f-2-3p). Prediction of target mRNAs and associated biological pathways regulated by each of this miRNA was carried out. We paid special attention to hsa-miR-516a-3p and hsa-miR-7-5p as these miRNAs were highly expressed upon validation with qRT-PCR analysis. We further proceeded with loss-of-function analysis with these miRNAs and we observed that hsa-miR-516a-3p knockdown induced a significant increase in the expression of WNT5A. Likewise, the knockdown of hsa-miR-7-5p increased the expression of EGFR. Nevertheless, further validation revealed the role of WNT5A as an indirect target of hsa-miR-516a-3p. These results provide new insights into the dynamic role of miRNA expression in DPSCs. In conclusion, using miRNA signatures in human as a prediction tool will enable us to elucidate the biological processes occurring in DPSCs.

Keywords: medical biotechnology • gene expression • signalling network • mesenchymal stem cells

Introduction

Dental pulp stem cells (DPSCs) have emerged as a promising source of cells for numerous applications in regenerative medicine. Once thought to be seed cells only for tooth tissue regeneration, currently, these cells are being investigated for repair of tissues outside the tooth. We have shown that DPSCs are able to differentiate into myriad types of cells [1]. Likewise, others have a successful outcome of using these cells in pre-clinical animal disease models [2]. DPSCs are present in 'cell-rich zones' within the dental pulp region and are considered to have similar characteristics as BM-MSCs, *e.g.*, self-renewal capability and multi-lineage differentiation [3, 4]. Previously, we conducted a gene profiling study between DPSCs and other types of MSCs and found that although these cells shared basic MSCs criteria, they retained unique gene characteristics which make them different from one another. For instance, DPSCs are primed towards neuro-ectoderm lineages as compared with other cell lines [5]. We reckoned that these phenomena are because of molecular networks and regulatory pathways. However, knowledge of these fundamental cues in DPSCs is still insufficient. Hence, optimal conditions and signals, especially involving gene expression regulation governing the fate of DPSCs, need to be identified.

One of the molecular regulatory factors that have received increasing attention is miRNAs, which have the ability to regulate

*Correspondence to: Vijayendran GOVINDASAMY, Ph.D.,
Hygieia Innovation Sdn. Bhd, Lot 1G-2G, Komplex Lanai, No.2,
Persiaran Seri Perdana, Persint 10, 62250,
Federal Territory of Putrajaya, Malaysia.
Tel.: +60388902968
Fax: +60388902969
E-mail: vijay@hygieiainnovation.com (or) vijay07001@gmail.com

doi: 10.1111/jcmm.12381

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

many target genes and control gene expression through translational repression and degradation [6]. MicroRNA (miRNAs) are ~20–22 nucleotides in length and well known to govern a broad array of cellular functions by influencing the abundance and translation efficiency of cognate mRNA. One single miRNA can target multiple sites of genes on mRNA transcripts, and conversely, a single mRNA can be targeted by multiple miRNAs [7, 8]. This regulation is performed on two bases: cis regulation in which miRNA directs target mRNA and either represses their translation or regulates degradation at post-transcriptional level. On the other hand, miRNA also appears to provide a subsequent effect that may exert the level of other mRNA as well as protein interactions through trans-regulatory mechanisms [9].

They are also essential regulators that can contribute to intrinsic stem-cell (SC) properties such as self-renewal, SC pluripotency and differentiation [10]. For instance, differentiation was found to be directly associated with cell cycle exit in which miRNA tends to cause negative regulation of oncogenes, which otherwise would promote proliferation [11]. Moreover, they were shown to be involved in differentiation [12], controlling developmental time-point and homoeostasis through diverse cellular processes by focusing on specific pathways within cells.

Thus, in this study, expression profiling of miRNAs found in DPSCs as compared with the BM-MSCs, which is always regarded as a golden cell source in regenerative medicine, was carried out for the first time to uncover molecular signatures and regulatory pathways that could broaden our understanding of the roles of miRNAs for future experimental and clinical applications. Ultimately, this can be used as a primordial approach to comprehend the DPSCs' biological progressions to ensure success when applied in SC therapy.

Materials and methods

Tissue collection and isolation of cells

This study was conducted with written consents from all donors after being reviewed and approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Medical Ethics Clearance Number: DF CO1107/0066(L)].

A volume of 60 ml of BM-MSCs aspirates was obtained from the iliac crest of three healthy donors ($n = 3$) under deep sedation (age: 24–35) as previously described by us [5]. Briefly, the BM-MSCs were diluted (1:1) in knockout (KO) DMEM (Invitrogen, Carlsbad, CA, USA; www.invitrogen.com), and centrifuged at 706.3524 × g for 10 min. to remove anti-coagulants. After centrifugation, the mononuclear cells (MNC) were isolated by layering onto a lymphoprep density-gradient media (1:2; Axis-Shield PoC AS). The MNC present in the buffy coat were then washed with culture medium (consisting of basal media of KO-DMEM, 10% Australian characterized foetal bovine serum (FBS, Hyclone, Thermo Scientific Inc, Waltham, MA, USA, http://www.thermofisher.com), 1% Glutamax (Invitrogen) and 0.5% Penicillin/Streptomycin (Invitrogen). The mononuclear fractions that also contained SCs were plated onto culture flasks. Separately, DPSCs cultures were isolated from sound and intact third molars of adults (age: 24–35, $n = 3$) as previously described by us [13]. Prior to isolation, root surfaces were cleaned with povidone-iodine (Sigma-Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com) and the pulp was removed within 2 hrs post-extraction. The pulp tissues were minced into smaller fragments, and treated with a solution of 3 mg/ml of collagenase type I (Gibco, Grand Island, NY, USA; http://www.invitrogen.com) for 40 min. at 37°C. After inactivation with 10% FBS, the cells were then centrifuged and seeded in a conventional tissue culture flask. Similar culture conditions were provided for both cells, namely in T75 cm^2 culture flasks (BD Pharmingen, San Diego CA, USA; http://www.bdbiosciences.com) with culture medium containing KO-DMEM, 0.5% and 10,000 μg/ml of Penicillin/Streptomycin (Invitrogen); 0.01× Glutamax (Invitrogen) and 10% FBS with humidified atmosphere of 95% air and 5% CO_2 at 37°C. Non-adherent cells were removed after 48 hrs of initial plating by intensely washing the flask. The medium was replaced every 3 days until the cells reached 80–90% confluence.

Growth Kinetics

Analysis of proliferation capacity was determined by plating 25,000 cells/cm^2 of each SC into separate T75 cm^2 culture flasks (BD Pharmingen). When the cells reach 90% confluence, they were then trypsinized. Cells were counted and evaluated for viability by means of Tryphan Blue dye exclusion before sub-culturing. Cells were re-plated for a total of 5 subsequent passages (P1–P5), with three replicates for each passage. To compare the expansion rate for both cells, the population doubling time (PDT) values were determined. The PDT was obtained by using the formula:

$$\mathrm{PDT} = t \times \log 2/(\log N_h - \log N_i)$$

N_i: the inoculums cell number; N_h is the cell harvest number and t is the duration of the culture (in hours).

Cell cycle analysis

The cells were pre-seeded on a 35-mm tissue culture dish (BD Pharmigen) at a density of 5000 cells/cm^2. Upon reaching 90% confluence, the cells were detached, fixed and permeabilized in 70% ethanol and left overnight at 4°C. Thereafter, 500 μl was extracted (containing 1×10^6 cells), and DNA was stained with Propidium iodide/RNAse staining buffer (BD Pharmigen) for 15 min. at room temperature and subsequently washed in Dulbecco's PBS (DPBS; Invitrogen). DNA content was analysed on Guava Technologies (Millipore, Billerica, MA, USA) flow cytometer by using Cytosoft, Version 5.2, Guava Technologies software.

Flow cytometric analysis

At P3, cells were harvested by trypsinization with 0.05% trypsin (Invitrogen) upon reaching 90% confluence, and re-suspended in DPBS to reach a final cell density of 1.5×10^6 cells/ml. An amount of 200 μl of cell suspension (1×10^5 cells) was incubated in the dark for 1 hr at 37°C with Phycoerythrin-conjugated antibodies against CD44, CD73, CD166, CD105 and CD34, and fluoro isothycyanate-conjugated antibodies against CD45 and HLA-DR (all from BD Pharmingen) for specific surface antigens analysis by using flow cytometer. Excess antibodies were removed by washing with DPBS. All analyses were standardized against negative control cells incubated with Isotype-specific IgG1-PE

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

and IgG1-FITC (BD Pharmingen). At least 10,000 events were acquired on Guava Technologies flow cytometer, and the results were analysed by using Cytosoft, Version 5.2, Guava Technologies.

In vitro tri-lineage differentiation assay

A total of 1000 of P3 cells/cm^2 in 6-well plates were grown until confluence, and induced to multi-lineage differentiation as defined by Govindasamy *et al.* [13] with the following formulae: adipogenic differentiation medium: media supplemented with 10% FBS, 200 μM indomethacin, 0.5 mM 3-Isobutyl-L-methyxanthine, 10 μg/ml insulin and 1 μM dexamethasone (all reagents from Sigma-Aldrich); chondrogenesis differentiation medium: media supplemented with ITS+1 (Sigma-Aldrich), 50 μM of L-ascorbic acid-2 phosphates, 55 μM of sodium pyruvate (Invitrogen), 25 μM of L-proline (Sigma-Aldrich) and 10 ng/ml of a transformation growth factor-beta (TGF-β) (Sigma-Aldrich); osteogenic differentiation medium: media supplemented with 10% FBS, 10^{-7} M dexamethasone, 10 mM-glycerol phosphate (Fluka, Buchs, Switzerland) and 100 μM of L-ascorbic acid-2 phosphate.

Evaluation of tri-lineage differentiation

After ~21 days of differentiation, the cells were fixed for cytochemical staining. Lipid droplets were visualized by using Oil Red O staining (Sigma-Aldrich), proteoglycans accumulation was visualized by Alcian Blue staining (Sigma-Aldrich) and calcium accumulation was visualized by using Von Kossa staining (Sigma-Aldrich) for adipogenic, chondrogenic and osteogenic differentiation respectively. The cells were also analysed by using quantitative RT-PCR (qRT-PCR). Total RNA was extracted by using Trizol (Invitrogen) and reverse-transcribed into cDNA by using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The qRT-PCR mixture contained cDNA, forward and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reactions were conducted by using AbiPrism 7000 Sequence Detection System (Applied Biosystems) with initial enzyme activation at 95°C for 10 min., followed by 45 cycles of denaturation at 95°C for 15 sec. and annealing and extension at 60°C for 60 sec. The expression level of genes of interest was normalized against housekeeping gene GAPDH. The fold change was calculated by using the equation $2^{-\Delta\Delta CT}$. Primer sequences are presented in Table S1.

miRNAs isolation

Culture medium was aspirated, discarded and rinsed with DPBS. Cells at P3 ($n = 3$) were then trypsinized to detach them from the flask and counted. Immediately, culture medium was added to inactivate the trypsin, and centrifuged to pellet the cells. An estimated 10^2 to 10^3 million cells were collected for the miRNA isolation. The foremost step carried out for the mirVana miRNA Isolation Kit procedure was to disrupt samples in a denaturing lysis buffer. Next, samples were subjected to Acid-Phenol:Chloroform extraction, which provides a robust front-end purification that also removes most DNA [14]. The procedure to obtain miRNAs was according to the manufacturer's protocol (mirVana miRNA isolation kit, Ambion, Life Technologies, Austin, TX, USA).

Profiling of miRNAs

Profile analysis of human encoded miRNAs was performed by using the TaqMan MicroRNA Assay (Applied Biosystems). Briefly, TaqMan MicroRNA Assays included two steps: stem loop reverse transcription (RT) followed by real-time quantitative PCR (90 ng/Rx with 24-multiplex primers). Each of the 10 μl RT reaction tube which included 90 ng total RNA, 50 nM stem-loop RT primers, 1× RT buffer, 1.25 mM each of dNTPs, 0.25 U/μl RNase inhibitor and 10 U/μl MultiScribe Reverse Transcriptase was incubated in a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) for 30 min. at 16°C and at 42°C, followed by 5 min. at 85°C and then maintained at 4°C. RT products were diluted 20 times with dH_2O prior to the setting up of the PCR reaction. Real-time PCR for each miRNA was carried out in triplicates, and each 10 μl reaction mixture included 2 μl of diluted RT product, 5 μl of 2× TaqMan Universal PCR Master Mix and 0.2 μM TaqMan probe. The reaction tube was incubated in an Applied Biosystems 7900HT Sequence Detection System at 95°C for 10 min., followed by 40 cycles at 95°C for 15 sec. and 60°C for 1 min. The threshold cycle (C_t) is defined as the fraction of cycle number at which the fluorescence exceeds the fixed threshold of 0.2. As an endogenous control, total RNA input was normalized based on the Ct values of the TaqMan U6 snRNA assay. The fold change was calculated as $2 - Ct \times K$, where Ct = [Ct miRNA − Ct U6snRNA] and K is a constant [15].

Quantitative validation of miRNA using qRT-PCR

Quantitative reverse transcription-PCR (RT-PCR) was carried out by using 25 ng of total RNA by using the mirVana quantitative RT-PCR miRNA Detection Kit (Ambion, Life Technologies) with mirVana quantitative RT-PCR primer sets (Ambion, Life Technologies) for the 10 miRNAs of interest that are listed in Table S2. Detection of amplification was performed with SYBR green nucleic acid stain (Invitrogen) by using an Applied Biosystems-Real time Detection System. The miRNAs expression levels were calculated by using comparative cycle threshold (C_t) method. C_t values of target miRNAs were normalized in relation to U6 snRNA, which is an internal control gene. The fold change was calculated by using the equation $2^{-\Delta\Delta CT}$.

Pathway analysis and prediction

Predicted miRNA targets were determined by using the miRanda algorithm (http://microrna.sanger.ac.uk/targets/v5/) and TargetScan v4.2 (http://www.targetscan.org/). Common predicted targets as well as targets from each database were subjected to pathway exploration by using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). An IPA (Core) Analysis is the process of mapping uploaded data to the IPA Knowledge Base (KB), and creating molecular networks by generating pathways algorithmically. This pathway was developed by dividing data into diseases and biological functions that are overrepresented in our data. To avoid exceeding the maximum gene list size allowed by the IPA program, we limited targets based on assigned score by each program. Therefore, scores of at least 17 and 20.31 were set for miRanda and TargetScan respectively. Using this software and its accompanying interactive database,

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

the top-ranked pathways were determined based on the incidence of predicted miRNA targets in a list of canonical pathways provided by the software. IPA also produced the top-ranked networks where the predicted miRNA targets were found according to gene ontology. Additionally, the biological functions associated with these networks are also provided.

Transient transfection of miRNA mimics and inhibitors

The miRNA mimics, inhibitors and negative controls for hsa-miR-516a-3p and hsa-miR-7-5p were purchased from (*mir*Vana®, Life Technologies™). DPSCs were transfected with the mimic, inhibitor and negative control at final concentrations of 20 nM. The siPORT NeoFX transfection agent (Ambion, Austin, TX, USA) was used according to the manufacturer's instructions. Briefly, cells were digested with 0.25% trypsin when they reached 80% confluence. The transfection agent was mixed, and incubated for 10 min. at room temperature. Cell suspension was overlaid onto the transfection complexes, and incubated at 37°C for 24 and 48 hrs for further miRNA and mRNA analysis. Transfection efficiency was determined by qRT-PCR.

Real-time RT-PCR of mRNA expression

The transfected cells were analysed for selected target mRNA expression by using quantitative RT-PCR. Total RNA was extracted by using Trizol (Invitrogen), and was then reverse-transcribed into cDNA by using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The qRT-PCR mixture contained cDNA, forward and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems). The reactions were conducted by using AbiPrism 7000 Sequence Detection System (Applied Biosystems) with initial enzyme activation at 95°C for 10 min., followed by 45 cycles of denaturation at 95°C for 15 sec., and annealing and extension at 60°C for 60 sec. The expression levels of wingless-type MMTV integration site family, member 5A (WNT5A) and epidermal growth factor receptor (EGFR) were normalized against the housekeeping gene alpha-tubulin. The relative expression levels were normalized against Human cDNAs (Positive control), which

Table 1 Sorted Log_2 (fold change) of 104 miRNA between DPSCs and BMSCs using ΔΔCts. 53.85% of ΔΔCts (56 of 104 determined assays), were between +1 and −1

Up-regulated	Down-regulated		Between +1/−1		
hsa-miR-516a-3p	hsa-miR-20a*	hsa-miR-154*	hsa-miR-15b*	hsa-miR-509-3p	hsa-miR-188-5p
hsa-miR-7-5p	hsa-miR-659	hsa-miR-630	hsa-miR-138-1*	hsa-miR-601	hsa-miR-214*
RNU43	hsa-miR-126*	hsa-miR-379*	hsa-miR-149*	hsa-miR-543	hsa-miR-432*
hsa-miR-526b*	hsa-miR-181a-2*	hsa-miR-335*	hsa-miR-151-3p	hsa-miR-589*	hsa-miR-130b*
hsa-miR-376a*	hsa-miR-801	hsa-miR-923	hsa-miR-19b-1*	hsa-miR-625*	RNU48
hsa-let-7f-2-3p	hsa-miR-34b*	hsa-miR-550	hsa-miR-27b*	hsa-miR-638	hsa-miR-93*
hsa-miR-106a	hsa-miR-27a*	hsa-miR-10b*	hsa-miR-22*	hsa-miR-643	hsa-miR-7-1*
hsa-miR-190a	hsa-miR-454*	hsa-miR-18a*	hsa-miR-26a-1*	hsa-miR-656	hsa-miR-505*
hsa-miR-378	hsa-miR-513-3p	hsa-miR-15a*	hsa-miR-26b*	hsa-miR-769-5p	hsa-miR-181a*
hsa-miR-125b-1*	hsa-miR-29c*	hsa-miR-500*	hsa-miR-30e*	hsa-miR-877	hsa-miR-222*
hsa-miR-629*	hsa-miR-16-1*		hsa-miR-30a*	hsa-miR-942	hsa-miR-135a*
hsa-miR-939	hsa-miR-941		hsa-miR-30d	RNU24	hsa-miR-493*
hsa-miR-377*	hsa-miR-432		hsa-miR-30e	RNU44	hsa-miR-145*
hsa-miR-565	hsa-miR-136*		hsa-miR-34a*	RNU6B	hsa-miR-875-5p
hsa-miR-766	hsa-miR-661		hsa-miR-411*	hsa-miR-768-3p	hsa-miR-30d*
hsa-miR-148b*	hsa-miR-99a*		hsa-miR-409-3p	hsa-miR-373*	hsa-miR-550*
hsa-miR-221*	hsa-miR-520c-3p		hsa-miR-424*	hsa-let-7i*	hsa-miR-21*
hsa-miR-584	hsa-miR-99b*		hsa-miR-425*	hsa-miR-100*	hsa-miR-760
hsa-miR-564	hsa-miR-206		hsa-miR-770-5p	hsa-miR-30a	

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

University of Malaya

had also been normalized to 1. The fold change was calculated by using the equation $2^{-\Delta\Delta CT}$. Primer sequences are presented in Table S1.

Western blot analysis

Western blot analysis was performed after the whole transfected cell lysate was extracted by using Cytobuster (Novagen, Milipore, Billerica, MA, USA) lysis buffer. The cell lysate was then treated with protease inhibitor cocktail (Milipore). Prior to loading on to gel, protein quantification was carried out against bovine serum albumin (ThermoScientific, Wilmington, DE, USA) by using Bradford method. The proteins were loaded on 10% sodium dodecyl sulphate-polyacrylamide gels, and then transferred to polyvinylidene fluoride membranes. Blocking and washing were performed according to the manufacturer's instructions (Western blot kit, Pierce ECL, ThermoScientific). The membranes were left overnight with the following primary antibodies: rabbit anti-human WNT5A;

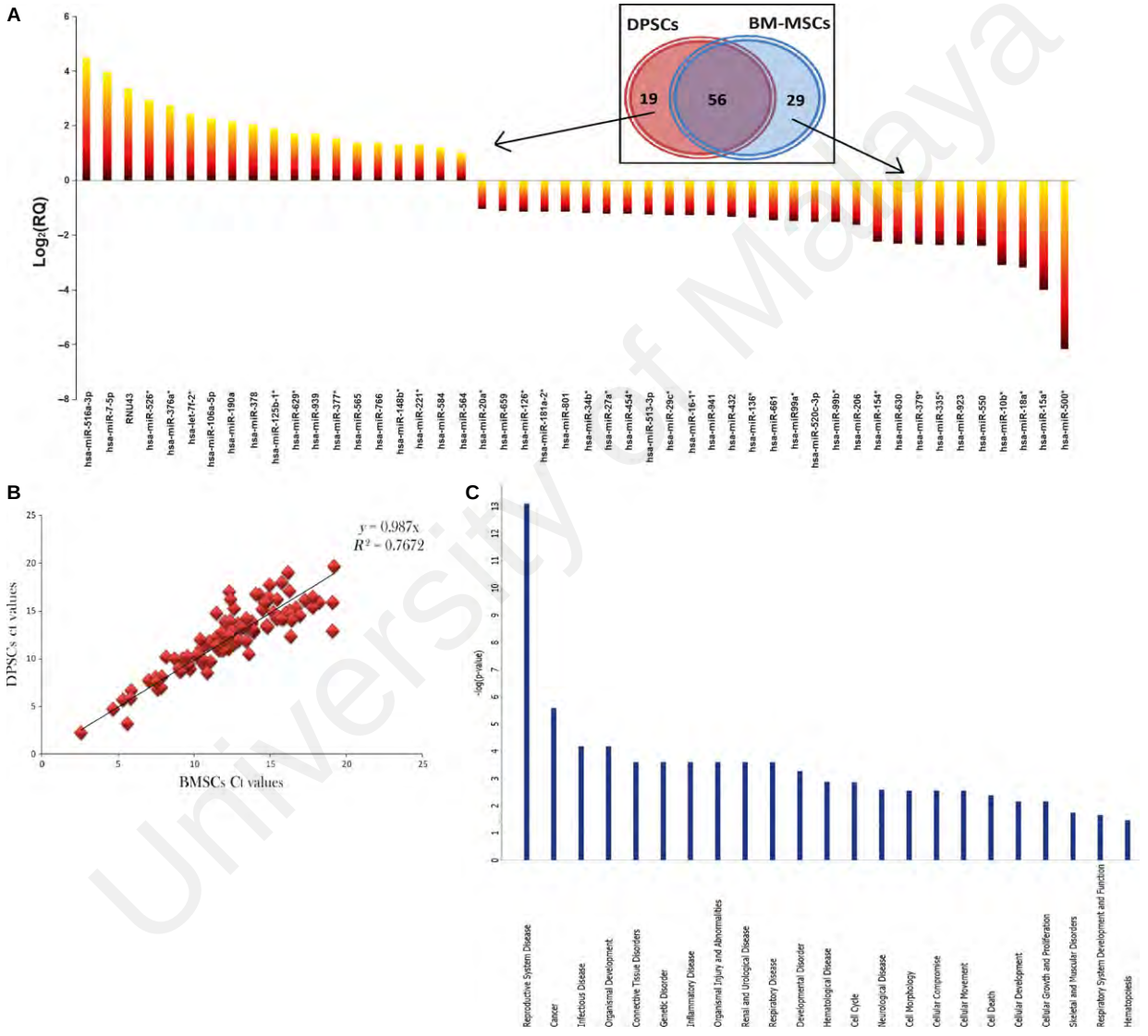


Fig. 1 miRNAs manifestation in DPSCs compared with BM-MSCs. (**A**) The $\log_2$ of RQ value was used to plot the relative fold change. *Y*-axis: $\log_2$ RQ, *X*-axis: miRNA. Sorted $\log_2$ RQ shows 29 miRNAs with decreased expression and 19 with increased expression in DPSCs. The most significant difference was seen in hsa-miR-500* with decreased expression, and hsa-miR-516a-3p with increased expression [RQ = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct$ (DPSCs) − ΔCt (BM-MSCs), ΔCt = Ct (target miRNA) − Ct (endogenous control)]. Venn diagram showing the number of shared and specific miRNAs for DPSCs and BM-MSCs. (**B**) Scatter plot and correlation analysis between DPSCs and BM-MSCs with standard correlation found to be R2 is over 76%. (**C**) Major functions inclined by miRNA action on putative target genes using IPA of MSCs of BM *versus* DP. Height of bar is determined by projected involvement of the particular pathways.

University of Malaya

© 2014 The Authors.

rabbit anti-human EGFR; and alpha tubulin as control (Abcam, Cambridge, UK). Thereafter, the membranes were incubated with peroxidase-conjugated secondary antibody (Abcam). The blots were visualized by using a chemiluminescence detection system.

Reporter vectors and luciferase assay

The oligonucleotides of the putative hsa-miR-516a-3p recognition element, at the nucleotides of 1650-1656 of the 3′-untranslated region (3′-UTR) of the human WNT5A gene wild-type, were designed by using human genomic DNA with flanking Pst1 and EcoRV sites (forward: 5′-*CTGCAG*TCCAGTTGGGATTATTC-3′ and 5′-*GATATC*TTCAACCCAACA CGC-3′). Meanwhile, the mutant type was constructed by deleting 3 nucleotides of the seed region with the flanking EcoRV and HindIII sites (forward: 5′-*GATATC*TCAAAGTATTTTGTAC-3′ and 5′-*AAGCTT*CCTCA GAAACAAGG-3′). After annealing the sense and anti-sense oligonucleotides, the DNA fragment products were double digested by using the above-indicated restriction sites and cloned into pSV40-CLuc (New England Biolabs, Ipswich, MA, USA) vector. The resulting vector wild-type indicated as pSV40-WNT5A-WT or mutant type as pSV40-WNT5A-MT was then transfected by using Lipofectamine 2000 (Invitrogen) into hsa-miR-516a-3p mimics (miR-516) DPSCs (*mir*Vana®, Life Technologies™) or miR-negative control (miR-NC) DPSCs (2×10^4 cells) seeded in a 24-well plates. After 48 hrs of incubation, the cell extracts were prepared for luciferase assay. A thymidine kinase promoter-driven secreted Gaussian luciferase (pTK-GLuc, New England Biolabs) was used as an internal control. The relative luciferase activity was calculated by normalizing transfection efficiency to the internal control. All experiments were carried out in 3 technical replicates.

Statistical analysis

All values are given as mean and SD. Data were analysed by using the SPSS statistical software, version 19.0 (SPSS Inc, Chicago, IL, USA). The

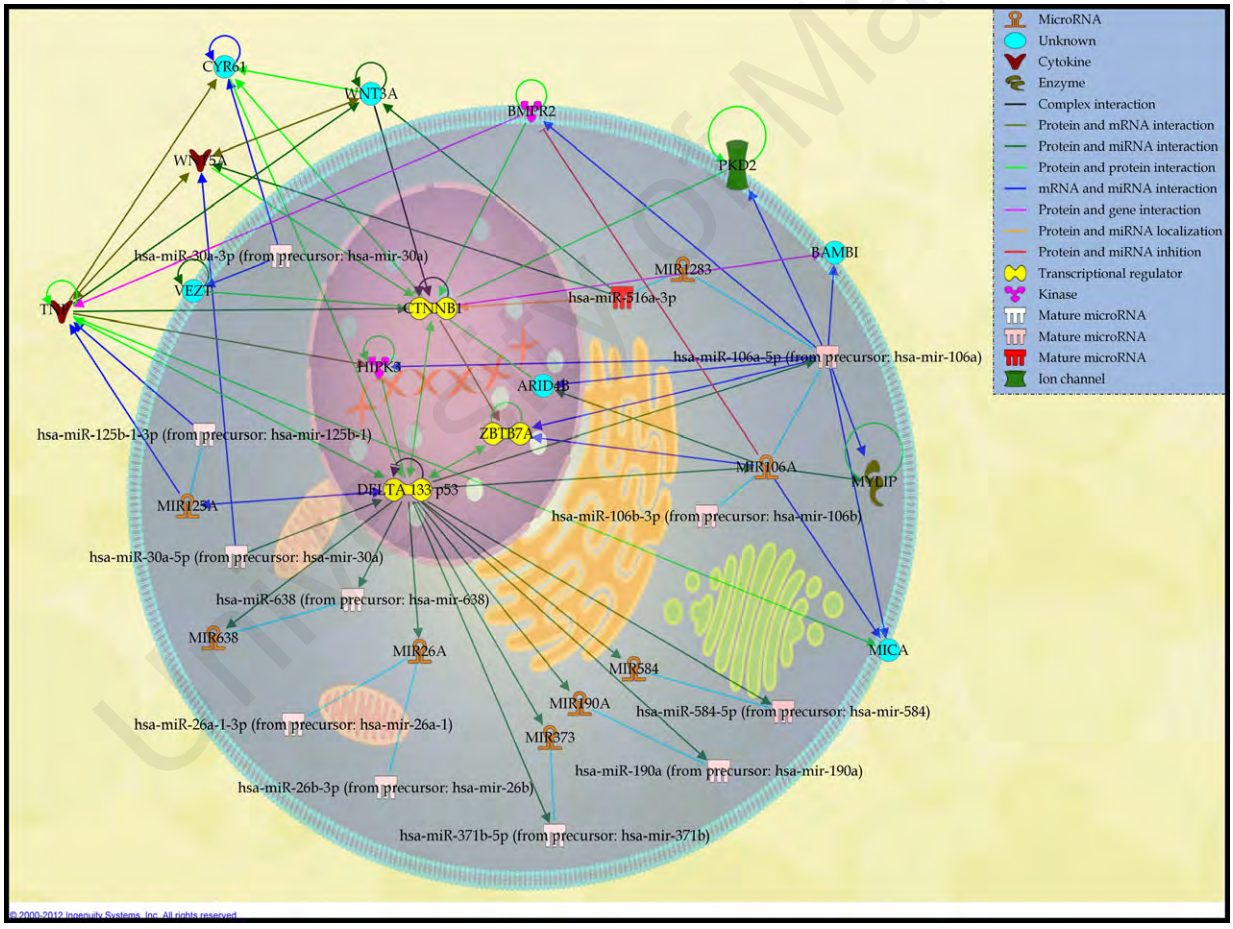


Fig. 2 Network 1: Schematic representation describing the interaction between 5 highly expressed miRNAs found in DPSCs with their associated target mRNAs and cellular proteins related to cancer, reproductive system disease and genetic disorder. The miRNAs are namely hsa-miR-516a-3p, hsa-miR-125b, hsa-miR-190a, hsa-miR-106a and hsa-miR-584-5p. Network was constructed by using Ingenuity software based on expression relationships described in the literature. For miRNA analysis, the colour intensities (from pink to red) were correlated with fold change intensities, in which miRNAs overexpressed in functional analysis, are indicated in red.

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

data were analysed by using two-way ANOVA. The significance level was set at $P = 0.05$. Tukey post-hoc multiple comparisons were carried out to determine the differences between the groups. Mean ± SD values are shown from either three independent biological or technical experiments.

Results

Elementary depiction of dental pulp and bone marrow SCs

For physiognomies interpretation, both BM-MSCs and DPSCs showed appearance of fibroblastoid cells in spindle-shaped morphology (Fig. S1A), which was retained in all subcultures. DPSCs cultures consistently displayed a higher incidence (12.7% ± 2.1%) of cells in the S + G2 + M phases of the cell cycle with ~85% of cells in phase G1/GO when compared with BM-MSCs (5.7% ± 1.2%) of cells in S + G2 + M with ~90% of the cells in phase G1/GO (Fig. S1B). The accumulation cell number was also compared for BM-MSCs and DPSCs throughout 5 passages (Fig. S1C). The graph for DPSCs showed a lag phase for 2 passages, and then multiplying at a rapid rate before reaching a plateau stage earlier than BM-MSCs. Furthermore, the PDT for DPSCs in P1 was 20.50 ± 1.39 hrs, whereas 27.01 ± 0.73 hrs was recorded for BM-MSCs. At P5, the PDT was 23.12 ± 0.65 hrs for DPSCs and 33.16 ± 0.97 hrs for BM-MSCs respectively (Fig. S1C). Collectively, these results show DPSCs having a higher proliferation rate as compared with BM-MSCs, conforming to our previous [5] as well as other independent studies [16]. Moreover, antigenic phenotypes for both cells were examined by using flow cytometric analyses as shown in Figure S2. The results revealed that DPSCs were positive (>85%) for many markers similar to BM-MSCs: CD44, CD 73, CD90, CD105 and CD166. At the same

Table 2 Top two associated network functions generated by using Ingenuity Pathway Analysis

Network	miRNA	Abbr.	Entrez gene name	Function
Reproductive system disease, Cancer, Genetic disorder	miR-638	ARID4B	AT-rich interactive domain 4B (RBP1-like)	Other
	miR-26a-1-3p	BAMBI	BMP and activin membrane-bound	Other
	miR-294-5p	BMPR2	inhibitor homologue	Kinase
	miR-30c-5p/miR-30c/miR-30b-5p	CTNNB1	bone morphogenetic protein receptor, type II	Transcription
	miR-30a-3p/miR-30d-3p/miR-30e	CYR61	catenin (cadherin-associated protein),	Regulator
	miR-26b-3p/miR-26b*/miR-26a-2-3p	HIPK3	beta 1, 88 kD	Other
	miR-125b-1-3p/miR-125b-3p	MICA	cysteine-rich, angiogenic inducer, 61	Kinase
	miR-190a	MYLIP	homeodomain interacting protein kinase 3	Other
	miR-106a	PKD2	MHC class I polypeptide-related sequence A	Enzyme
	miR-584-5p	TNF	myosin regulatory light-chain interacting	Ion channel
	miR-17-5p/miR-20b-5p/miR-93-5p	VEZT	protein polycystic kidney disease 2	Cytokine
	miR-516a-3p/miR-516b-3p	WNT3A	(autosomal dominant)	Other
		WNT5A	tumour necrosis factor	Cytokine
		ZBTB7A	vezatin, adherens junctions transmembrane protein	Cytokine
			wingless-type MMTV integration site family, member 3A	Transcription regulator
			wingless-type MMTV integration site family, member 5A	
			zinc finger and BTB domain containing 7A	
Genetic disorder, Skeletal and muscular disorder, Developmental disorder	miR-543-3p/miR-543*/miR-543	DICER1	dicer 1, ribonuclease type III	Enzyme
	miR-409-3p (human, mouse)	EGFR	epidermal growth factor receptor	Kinase
	miR-409-5p	EIF2C2	eukaryotic translation initiation factor 2C, 2	Translation regulator
	miR-4712-5p/miR-770-5p	FOS	FBJ murine osteosarcoma viral oncogene homologue	Transcription factor
	miR-425-3p/miR-425*	IRS1	insulin receptor substrate 1	Enzyme
	miR-656	NR0B2	nuclear receptor subfamily 0, group B, member 2	Ligand dependent nuclear receptor
	miR-539			
	miR-431			
	miR-495			
	miR-494			
	miR-487			
	miR-382			
	miR-7-5p/miR-7a-5p/miR-7a			
	miR-221-5p/miR-221*			
	miR-377-5p/miR-672-5p/miR-672			
	let-7f-2-3p			
	miR-376a-5p			

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

time, DPSCs were negative (<2%) for haematopoietic surface markers, such as CD34, CD45, HLA-DR.

Cultivation of confluent DPSCs and BM-MSCs was then introduced to multipotent differentiation (Fig. S3A). Accumulation of neutral lipid vacuoles indicated by the Oil Red O stain revealed adipogenic differentiation in both cell lines. However, the observation showed larger and dense lipid vacuoles (red colour) in BM-MSCs compared with DPSCs, which were smaller and scattered remotely throughout the flask. Similar effects were seen when there was a higher exposure of dark-stained mineralized matrix in BM-MSCs in comparison with DPSCs, which indicates efficient osteogenic differentiation. Chondrogenic differentiation was confirmed with the presence of proteoglycan by using Alcian Blue in both cell lines. No staining was seen in undifferentiated cells; however the data are not shown here. Besides that, the cells also showed mRNA expression of runt-related transcription 2, osteocalcin, peroxisome proliferation activated receptor 2 lipoprotein lipase, aggrecan and collagen 2A1 (COL2A1). These findings are typical for osteoblast cells, adipocytes and chondrocytes (Fig. S3B).

Differential expression of miRNAs between dental pulp and bone marrow SCs

Based on the analysis of the 104 miRNAs, it is clearly shown in Table 1, that 48 miRNAs were differentially expressed between BM-MSCs and DPSCs. Among the differentially expressed miRNAs, 19 of them were up-regulated in DPSCs, while 29 were down-regulated. In addition, a total of 56 miRNAs (53.8%) with $\Delta\Delta$Cts value between +1 and −1 were shown to be commonly expressed between the two subsets of cells (Fig. 1A). Furthermore, there was a high correlation of miRNA expression pattern between DPSCs and BM-MSCs, with R^2 76% (Fig. 1B). The fold change value of each miRNA is presented in Table S3.

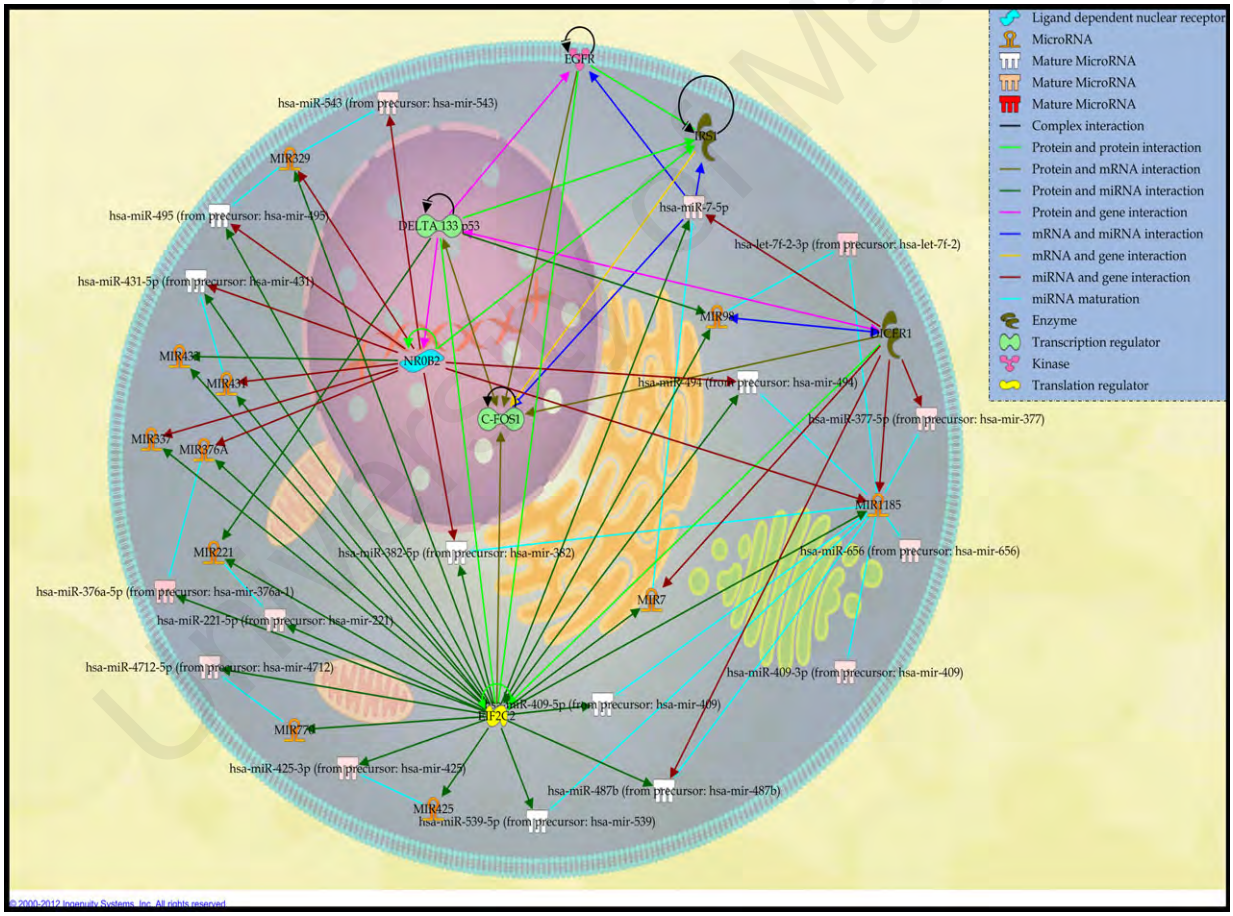


Fig. 3 Network 2: Schematic representation describing the interaction between 5 highly expressed miRNAs found in DPSCs with their associated target mRNAs as well as cellular proteins related to genetic, developmental, skeletal and muscular disorder. The miRNAs are namely hsa-miR-7-5p, hsa-miR-221-5p, hsa-miR-377-5p, hsa-miR-376a-5p, and let-7f-2-3p. Network was constructed by using Ingenuity software based on expression relationships described in the literature. For miRNA analysis, the colour intensities (from pink to red) were correlated with fold change intensities, in which miRNAs overexpressed in functional analysis, are indicated in red.

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

Analysis of up-regulated miRNAs expressed in DPSCs

The biological functions generated from the up-regulated miRNA demonstrated its involvement in various pathways with the top 5 in the reproductive system disease, cancer, infectious disease, organismal development, connective tissue disorders and genetic disorders, whereas the lowest 5 pathways are in cellular development, cellular growth and proliferation, skeletal and muscular disorders, respiratory system development and function as well as haematopoiesis (Fig. 1C). Within this, the software analysis identified 25 associated network functions whereby a few pathways may involve in creating a network function. Amongst this, top 2 networks were further analysed based on the statistical significance ($P < 0.01$) and biological relevance. In the first network that comprises of 12 miRNA, only 5 miRNAs were up-regulated (Fig. 2, Table 2). Among this, hsa-miR-516a-3p was noted with highest expression level and this miRNA is reported to regulate WNT and CTNNB1 mRNAs, which play an important role in WNT pathways. Next is has-miR-106a (developed from has-miR-106b), which regulates a diverse range of mRNA related to transcription regulators such as ZBTB7A, enzyme such as MYLIP and kinase such as BMPR2, MICA and ARID4B. The next up-regulated miRNA is hsa-miR-125b-1-3p, which regulates inflammatory-related mRNA, TNF. Surprisingly, we found that 2 up-regulated miRNAs, hsa-miR-584-5p and miR-190a, were co-regulated by DELTA 133 p53 mRNA, which is known to be involved in tumourigenesis.

A total of 5 up-regulated miRNAs of 16 miRNAs were noticed in 2nd network (Fig. 3, Table 2). Among these, hsa-miR-376a-5p and hsa-miR-221-5p were regulated by translation regulator-related mRNA, EIF2C2, while hsa-miR-377-5p directly regulated by DICER1. The most highly up-regulated miRNA is the hsa-miR-7-5p, which controls and acts on kinase-related gene EGFR, enzyme-related gene IRS1 and also transcriptional regulator C-FOS1. Apart from that, it is also shown that EIF2C2 and DICER1 modulate hsa-miR-7-5p. However, there were no possible targets for hsa-let-7f-2-3p.

Validation of the differentially expressed miRNAs by using qRT-PCR

The distinct expressions of the 10 miRNAs found in DPSCs in both networks were further validated by using qRT-PCR analysis. The results for up-regulated miRNAs in DPSCs are shown in Figure 4, which are significant relative to BM-MSCs. Consistent with the array results, the hsa-miR-516a-3p and hsa-miR-7-5p exhibited substantial increase in expression in DPSCs and further carried out in the downstream work.

hsa-miR-516a-3p, the highly expressed microRNA, indirectly targets WNT5A gene

To examine the role of miR-516a-3p in DPSCs, we focused our attention on elucidating the role of these microRNA on its target mRNA.

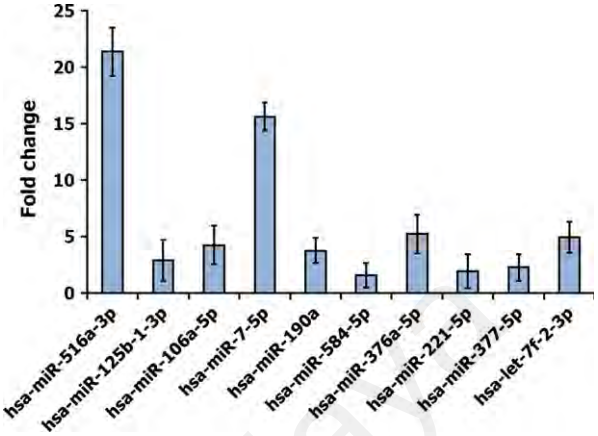


Fig. 4 Validation of 10 highly expressed miRNAs in DPSCs using qRT-PCR. Generally, the higher a fold change value, the more copies are present in the specific sample. The miRNAs expression levels were calculated by using comparative cycle threshold (Ct) method. Ct values of target miRNAs were normalized in relation to U6 snRNA, which is an internal control gene. The fold change was calculated by using the equation $2^{-\Delta\Delta CT}$.

Computational analysis indicated that WNT5A is a potential hsa-miR-516a-3p target because its 3′-UTR is matched to the hsa-miR-516a-3p seed region (Fig. 5A). To investigate whether WNT5A is regulated post-transcriptionally, we examined the expression on mRNA and protein level by performing gain- or loss-function assay. In DPSCs, dramatic reduction of WNT5A mRNA was detected in overexpressed hsa-miR-516a-3p by qRT-PCR analysis as well as in the western blot result, while knockdown of has-miR-516a-3p enhanced their expression (Fig. 5B).

However, based on literature, Takei *et al.*, [17] have debated that expression of WNT5A and WNT3A is indirectly regulated by hsa-miR-516a-3p because of other target, specifically SULF1 (extracellular sulfatase). Therefore, we proceeded to find the relative interaction between the hsa-miR-516a-3p and its predicted WNT5A mRNA 3′-UTR target sites by generating reporter vectors containing seed region complementarity to the miRNA upstream of the open reading frame (Fig. 6A). These were constructed by using wild-type of WNT5A 3′-UTR sequences and the same sequence with four point mutation (deletion; Fig. 6B). In DPSCs transfected with mimic miR-516, no effect on the expression of reporter was observed comparable to the DPSCs transfected with miR-NC (Fig. 6C) impeding the specificity of the binding sequences. This finding is in agreement with the reported result by Takei *et al.*,[17].

EGFR gene as a direct target of microRNA hsa-miR-7-5p

Likewise, we found that EGFR is a potential hsa-miR-7-5p target as its 3'UTR is matched with hsa-miR-7-5p seed region (Fig. 7A). Consistently, our results displayed that overexpression of the miRNA

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

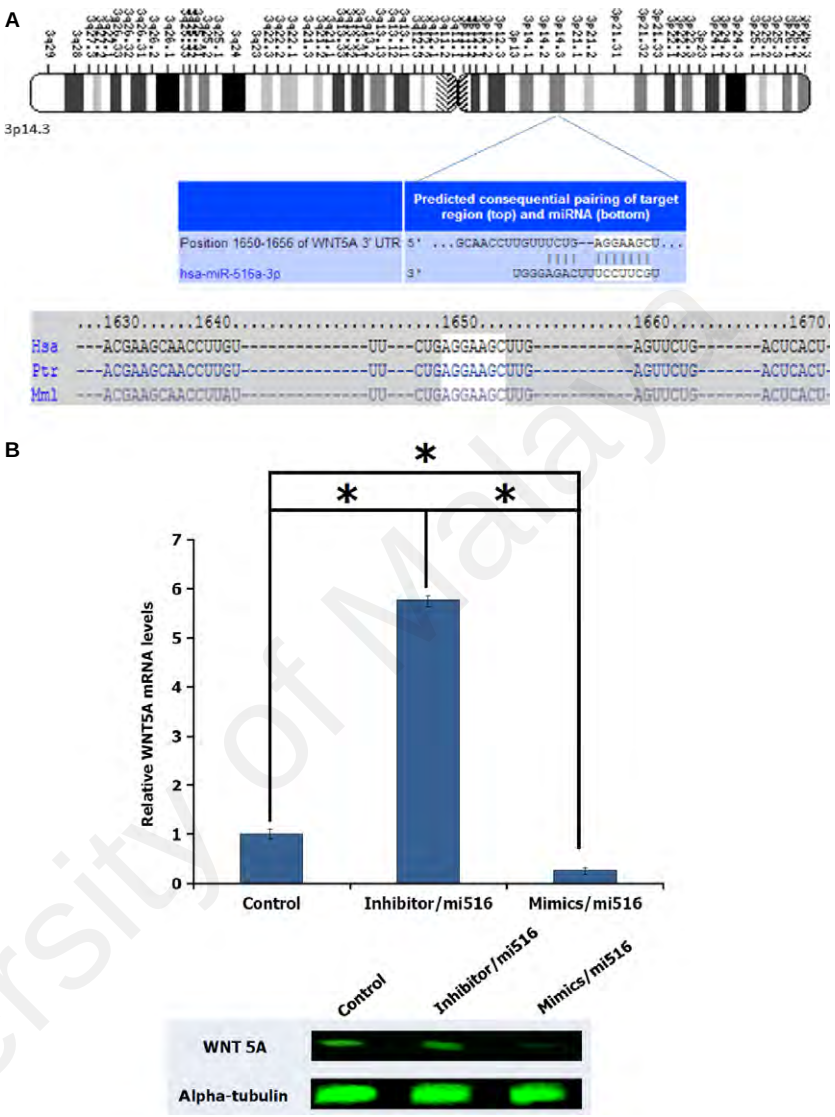


Fig. 5 WNT5A is a potential hsa-miR-516a-3p target. (**A**) Sequence alignment of hsa-miR-516a-3p and predicted binding sites in the 3′-UTR of WNT5A (http://www.targetscan.org). (**B**) Quantification of WNT5A mRNA expression levels in response to the mimic and inhibitory effect of hsa-miR-516a-3p. (**C**) Protein level expression of the results shown in (**B**). Data are shown as the mean of SD values ($n = 3$).

reduced mRNA and protein level of EGFR, while knockdown of the miRNA increased the mRNA as well as the protein level of EGFR (Fig. 7B). Our results correspond well with previous works [18] conducted to validate the interaction between hsa-miR-7-5p and EGFR 3′-UTR target sites by using reporter assay. Hence, no further validation was carried out for hsa-miR-7-5p and EGFR target relation.

Discussion

In the present study, DPSCs exhibited typical MSCs characteristics; fibroblastoid morphology, proliferation, multipotent differentiation capability and the expression of a typical set of surface protein. Nevertheless, variations were still noted between DPSCs and BM-MSCs and a key factor that attributes to this phenomenon probably because of its intrinsic molecular propensity that governs the fate of the cells. Various gene expressions are being controlled by miRNAs, and they also partly act in mutual negative feedback loops with protein factors to control cell fate decisions that are elicited by signal transduction activity. These findings implicate miRNAs as important mediators of gene regulation in response to cell–cell signalling [19]. Dysregulation of these molecules often ends with an uncontrolled growth stage in the cell population [20]. Hence, there is a need to identify miRNA activity in DPSCs to enable understanding of expression patterns that might be applicable prior to its usage in cell therapy.

Based on 2 network systems, 2 miRNAs were highly up-regulated and the remaining was moderate. Here, we briefly discuss the functions of each up-regulated miRNAs. An augmented level of hsa-miR-125b-1-3p expression was observed in the present study. This miRNA was involved in the regulation of TNF, a pro-inflammatory

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
University of Malaya

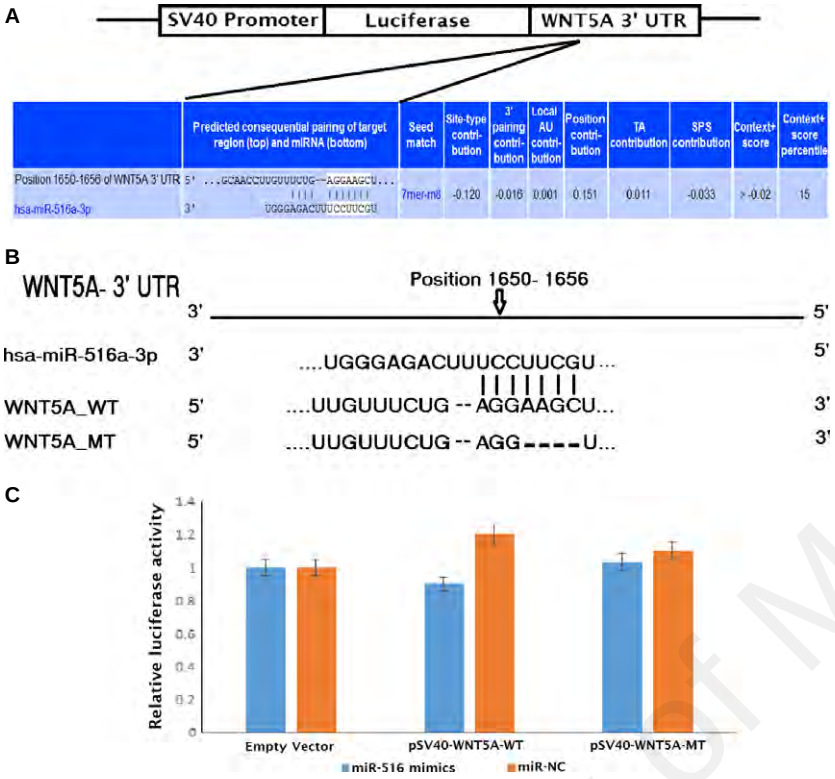


Fig. 6 Validation of WNT5A gene as a target gene of hsa-miR-516a-3p. (**A**) Schematic diagram of luciferase reporter constructs for consensus hsa-miR-516a-3p target sites at the 3′-UTR region. (**B**) The sequence alignment of the predicted hsa-miR-516a-3p binding site in the 3′-UTR region of human WNT5A is shown with the seed target sequence (UCCUUCG). (**C**) Luciferase reporter vector containing hsa-miR-516a-3p target seed region of WNT5A (wild-type) or same vector without target seed region (empty vector) or same vector with deletion of the target seed region (mutant) were cotransfected with miR-516 mimics or negative control respectively. Data are representative of at least three technical experiments.

cytokine. This gene is said to function as critical feedback in signal molecules between immune cells and MSCs for MSCs-mediated immunosuppressive activities [21]. Interestingly, there are also reports indicating that hsa-miR-125b-1-3p plays a vital role in suppressing osteogenic differentiation in MSCs [22]. Furthermore, hsa-miR-125b-1-3p has been indicated to be involved in MSCs ageing, with a study reporting down-regulation of expression in MSCs in old primates compared with young primates [23]. Similarly, the higher expression of hsa-miR-125-1-3p in DPSCs could be as a result of the fact that the cells were isolated at an early stage of adult tooth development [24]. Overexpression of hsa-miR-125b-1-3p, which was done elsewhere, showed a significant increase in SC population, while depletion of the miRNA increased the non-SC population through WNT signalling [25]. This was also reported by Lee *et al.* [26], who described that the depletion of human hsa-miR-125b-1-3p leads to the critical role of proliferation of differentiated cells.

Hsa-miR-106a-5p has been reported to target BMP groups, thus inhibiting the cells from osteogenesis [27]. We believe that this miRNA down-regulates BMPR2, which is a kinase-related gene acting as potent inducer for osteogenesis differentiation and cell growth through Smad signalling [28]. The computational data also predicted that hsa-miR-106a-5p is connected to various mRNAs such as ARID4B, ZBTB7A, BAMB1, PKD2, BMPR2, MYLIP and MICA. Among these mRNAs, ARID4B are cell cycle inhibitors [29], and the elevated expression of hsa-miR-106a-5p suggests that it enables cell proliferation in DPSCs. On the other hand, ZBTB7A, a transcription factor, regulates differentiation in multiple tissues and cell lineage, mainly oligodendrocyte lineage cells [30]. However, in DPSCs, the hsa-miR-106a-5p could suppress the response of this gene and allow proliferation rather than differentiation. Apart from this, according to Shangguan *et al.* [31], increase in BAMB1 expression could block the differentiation of MSCs into carcinoma cells through TGF-β/Smad signalling in BM-MSCs. Besides that, BAMB1 are also a negative regulator for adipogenesis [32], while positively modulating WNT signalling [33] to promote cell cycle progression. In DPSCs, hsa-miR-106a-5p probably suppresses the BAMB1 and could instead play the antagonistic role. Hsa-miR-106a-5p also reacts to PKD2, which allows calcium influx [34] into cells that would trigger maturing of cells into specialized functions. Sun *et al.* [35] reported that miR-17 directly targets PKD2, and post-transcriptionally represses PKD2 expression, which leads to cell proliferation. Hence, in DPSCs, hsa-miR-106a-5p is predicted to suppress PKD2 to enable cell proliferation. The other gene that is regulated by hsa-miR-106a-5p is MICA, which is known to activate natural killer receptor and induce immune surveillance in cancer cells [36]. One of the roles performed by MICA is to promote cytotoxic response during infection by binding with endothelial cells of the graft and induce cell destruction. The suppression of this immune-related gene in DPSCs by RNA interference may be used in transplantation, and also as a therapeutic target gene.

Hsa-mir-376a-5p has been known to suppress proliferation while inducing apoptosis in hepatocellular carcinoma cells [37]. This miRNA, along with hsa-mir-377-5p, also known as chondro-miRs (with

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

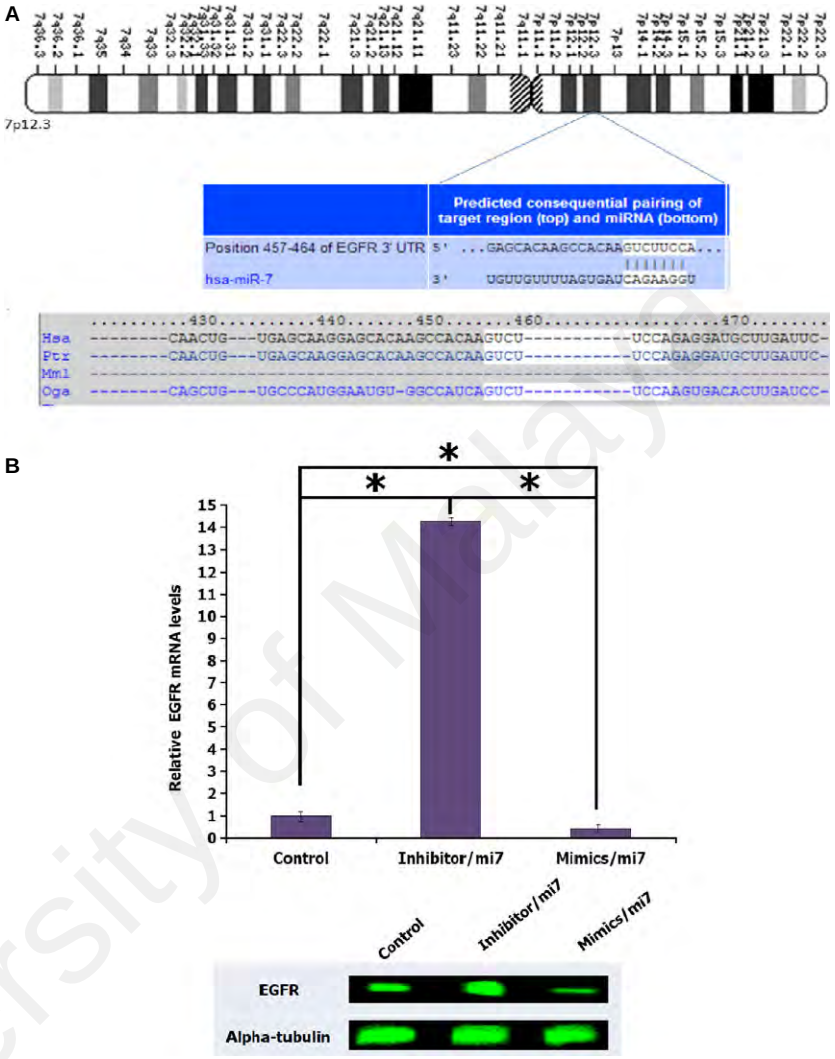


Fig. 7 EGFR is a potential hsa-miR-7-5p target. (**A**) Sequence alignment of hsa-miR-7-5p and predicted binding sites in the 3′-UTR of EGFR (http://www.targetscan.org). (**B**) Quantification of EGFR mRNA expression levels in response to the mimic and inhibitory effect of hsa-miR-7-5p. (**C**) Protein level expression of the results shown in (**B**). Data are shown as the mean of SD values ($n = 3$).

chondrocyte targets such as TGFbR, MAP3K, collagens, SMADs and cadherins), acts as a mediator of chondrogenic signalling pathways. This includes cell–cell interactions, TGF-β and MAPK signalling, which suggests a mechanism for genetic induction of chondrogenic differentiation [38]. Along with that, hsa-miR-221-5p in DPSCs tends to inhibit osteogenesis in MSCs [39]. Another miRNA, which is present in DPSCs, hsa-let-7f-2-3p, is known to be a pro-differentiation factor with 'anti stemness' properties [40]. Our results predicted that translation factor EIF2C2 regulates these miRNAs. EIF2C2 is a short-interfering RNA that mediates gene silencing, which suggests its involvement in controlling lineage-restricted pathway [41]. DICER-1 and EIF2C2 interact together to function as a translation initiation factor for short interfering RNA-mediated post-transcriptional gene silencing similar to the role played by miRNA. Bahubeshi *et al.*, [42] demonstrated that DICER1 could function as the sole member of the miRNA pathway in which germline mutations induced the carrier to develop a human disease. Therefore, the functions carried out by the above miRNAs are suppressed because of the effects of DICER-1 and EIF2C2, bringing about the loss of chondrogenic differentiation or perhaps contributing to other lineage development. Hsa-miR-190a is one of the poorly characterized miRNAs. Previous study focused on the involvement of this miRNA in the development of tolerant to μ-opioid receptor agonists with NEUROD1 (a neural differentiation marker) as the direct target for mir-190 [43]. Likewise, hsa-miR-584 is known to play an important role in tumourigenesis process by inhibiting them [44].

We paid special attention to hsa-miR-516a-3p and hsa-miR-7-5p as these miRNAs were highly expressed upon validation with qRT-PCR analysis. We further proceeded with loss-of-function analysis with these miRNAs and we observed that hsa-miR-516a-3p knock-down induced a significant increase in the expression of WNT5A. This gene is involved in controlling cell fate decision by integral involvement in maintenance and growth [45]. Furthermore, in tooth development, this gene is involved in regulating 4 cell signal pathways,

© 2014 The Authors.

Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
University of Malaya

namely JNK and AKT signal pathways as well as P42/44/MAPK and P38/MAPK pathways, which have close relation with cell proliferation and differentiation [46]. Apart from this, we also predicted that hsa-miR-516a-3p down-regulates CTNNB1 gene, also known as beta-catenin, which is associated with WNT signalling for SCs renewal [47]. WNT, together with Beta-catenin signalling pathway, represent a diverse group of molecules involved in controlling transcription of pluripotent genes, self-renewal and differentiation in most of the SCs found in adult tissues [48]. Meanwhile, Blauwkamp *et al.* [49] reported that different levels of WNT signalling lead to distinct lineage-specific differentiation properties in human embryonic SCs. As in haematopoietic SCs, WNT signalling together with beta-catenin form a complex pathway which are shown to be more essential for development rather than for maintenance [50]. Hence, we suggest that hsa-miR-516a-3p acts by suppressing the expression of WNT5A genes involved in WNT signalling pathway *via* altering/elevating SCs from undifferentiated to differentiated state. Nonetheless, it was puzzling to observe higher proliferation and less differentiation capacity in DPSCs when it was supposed to be the other way round. One of the possible reasons is the high expression of several pluripotency transcription factors such as Oct-4, Sox-2 and Nanog in DPSCs [16] with Wnt signalling pathway directly encompassing these genes. In addition, Oct 4 is involved in the maintenance of SCs fate *via* interaction with Wnt signalling pathway [51]. Nevertheless, our further work to investigate the predicted interaction between the hsa-miR-516a-3p and mRNA WNT5A does not satisfy the criteria of miRNA and target prediction. This outcome corresponded to the work conducted by Takei *et al.*, [17] that the WNT5A expression changes are most probably because of SULF1, which plays an important role in promoting WNT signalling pathway [52]. Consistent with the above findings, Hayano *et al.*,[53] have postulated that regulation of WNT signalling is modulated by SULF enzymes, which eventually control the differentiation of mouse pulp cells into odontoblasts. Our findings and notions warrant further investigation on the relationship between hsa-miR-516a-3p and WNT signalling pathway.

Another gene, EGFR, is known to suppress the osteoblast differentiation by inhibiting expression of transcription factors [54]. As in neural SCs, the EGFR is known to promote cell number and self-regeneration [55]. We found in this study that the positive interaction between hsa-miR-7-5p and its target EGFR through gain and loss assay. Validation of this interaction was found in a few studies that confirm that hsa-miR-7-5p directly targets EGFR [56, 57]. Therefore, we assume that in DPSCs, the role of EGFR is suppressed, which ultimately maintains SC numbers.

In addition to targeting EGFR, hsa-miR-7-5p targets upstream regulator, insulin receptor substrate (IRS-1) of the Akt pathway, which is essential for regulation of cell cycle progression, cell survival and cellular growth as noted by Kefas *et al.* [58]. Besides that, hsa-miR-7-5p also down-regulates C-FOS1 transcription regulator, which is known as a marker for neuron activity. C-FOS1 is a member of AP-1 transcription factors that activate many genes, including those involved in cell growth and proliferation [59]. Thus, hsa-miR-7-5p in DPSC is predicted to inhibit C-FOS1, eventually decreasing cell proliferation and growth. EGFR, IRS-1 and C-FOS-related genes are also connected to MAPK signalling pathway that regulates proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis using a diverse range of stimuli [60]. Therefore, we suggest that the role of hsa-miR-7-5p in gene regulation may suppress cell cycle progression and proliferation, either for differentiation or to maintain DPSCs in a quiescent state.

Conclusion

In conclusion, our data suggest that miRNAs expressed in DPSCs preferentially express and integrate appropriately as a group, rather than playing a solitary role to create a functional switch between self-renewal, stemness and lineage development. These findings, along with further studies, can introduce a new dimension of gene regulation in controlling SC fate and behaviour in DPSCs, and facilitate development of therapeutic approaches for various diseases.

Acknowledgements

This work is part of an on-going project between Hygieia Innovation Sdn. Bhd. And Faculty of Dentistry, University of Malaya and was supported by University of Malaya, High Impact Research- Ministry of Higher Education, Malaysia (UM.C/HIR/MOHE/DENT/02).

Conflicts of interest

The authors declare no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. DPSCs exhibit favourable phenotype of MSCs.

Figure S2. Characterization of DPSCs by mesenchymal stem cell-related surface markers by FCM analysis.

Figure S3. DPSCs are bona fide MSCs based on multipotency ability.

Figure S4. Quantitative assessment on two highly expressed miRNAs; hsa-miR-516a-3p and hsa-miR-7-5p in DPSCs.

Table S1. The primer sequences for tri-lineage RT-PCR evaluation.

Table S2. The product ID of the 10 miRNAs for validation by qRT-PCR.

Table S3. Fold change value of differentially expressed miRNAs in DPSCs.

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

References

1. **Govindasamy V, Abdullah AN, Ronald VS, *et al.*** Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. *J Endod*. 2010; 36: 1504–15.
2. **Kanafi MM, Rajeshwari YB, Gupta S, *et al.*** Transplantation of islet-like cell clusters derived from human dental pulp stem cells restores normoglycemia in diabetic mice. *Cytotherapy*. 2013; 15: 1228–36.
3. **Gronthos S, Mankani M, Brahim J, *et al.*** Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci USA*. 2000; 97: 13625–30.
4. **Gronthos S, Brahim J, Li W, *et al.*** Stem cell properties of human dental pulp stem cells. *J Dent Res*. 2002; 81: 531–5.
5. **Abu Kasim NH, Govindasamy V, Gnanasegaran N, *et al.*** Unique molecular signatures influencing the biological function and fate of post-natal stem cells isolated from different sources. *J Tissue Eng Regen Med*. 2012; DOI:10.1002/term.1663.
6. **Bartel DP.** MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116: 281–97.
7. **John B, Enright AJ, Aravin A, *et al.*** Human MicroRNA targets. *PLoS Biol*. 2004; 2: e363.
8. **Lim LP, Lau NC, Garrett-Engele P, *et al.*** Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005; 433: 769–73.
9. **Ivey KN, Muth A, Arnold J, *et al.*** MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell*. 2008; 2: 219–29.
10. **Ivey KN, Srivastava D.** MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell*. 2010; 7: 36–41.
11. **Shivdasani RA.** MicroRNAs: regulators of gene expression and cell differentiation. *Blood*. 2006; 108: 3646–53.
12. **Kloosterman WP, Plasterk RH.** The diverse functions of microRNAs in animal development and disease. *Dev Cell*. 2006; 11: 441–50.
13. **Govindasamy V, Ronald VS, Abdullah AN, *et al.*** Differentiation of dental pulp stem cells into islet-like aggregates. *J Dent Res*. 2011; 90: 646–52.
14. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987; 162: 156–9.
15. **Qureshi R, Sacan A.** A novel method for the normalization of microRNA RT-PCR data. *BMC Med Genomics*. 2013; 6: S14.
16. **Tamaki Y, Nakahara T, Ishikawa H, *et al.*** *In vitro* analysis of mesenchymal stem cells derived from human teeth and bone marrow. *Odontology*. 2013; 101: 121–32.
17. **Takei Y, Takigahira M, Mihara K, *et al.*** The metastasis-associated microRNA miR-516a-3p is a novel therapeutic target for inhibiting peritoneal dissemination of human scirrhous gastric cancer. *Cancer Res*. 2011; 71: 1442–53.
18. **Webster RJ, Giles KM, Price KJ, *et al.*** Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. *J Biol Chem*. 2009; 284: 5731–41.
19. **Lakshmipathy U, Hart RP.** Concise review: microRNA expression in multipotent mesenchymal stromal cells. *Stem cells*. 2008; 26: 356–63.
20. **Shenouda SK, Alahari SK.** MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metast Rev*. 2009; 28: 369–78.
21. **Aggarwal S, Pittenger MF.** Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005; 105: 1815–22.
22. **Mizuno Y, Yagi K, Tokuzawa Y, *et al.*** miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochem Biophys Res Commun*. 2008; 368: 267–72.
23. **Yu JM, Wu X, Gimble JM, *et al.*** Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. *Aging Cell*. 2011; 10: 66–79.
24. **Bakopoulou A, Leyhausen G, Volk J, *et al.*** Comparative analysis of *in vitro* osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch Oral Biol*. 2011; 56: 709–21.
25. **Liu Z, Liu H, Desai S, *et al.*** miR-125b functions as a key mediator for snail-induced stem cell propagation and chemoresistance. *J Biol Chem*. 2013; 288: 4334–45.
26. **Lee YS, Kim HK, Chung S, *et al.*** Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *J Biol Chem*. 2005; 280: 16635–41.
27. **Li Q, Gregory RI.** MicroRNA regulation of stem cell fate. *Cell Stem Cell*. 2008; 2: 195–6.
28. **Bonor J, Adams EL, Bragdon B, *et al.*** Initiation of BMP2 signaling in domains on the plasma membrane. *J Cell Physiol*. 2012; 227: 2880–8.
29. **Kim VN.** Cell cycle micromanagement in embryonic stem cells. *Nat Genet*. 2008; 40: 1391–2.
30. **Maeda T, Hobbs RM, Merghoub T, *et al.*** Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. *Nature*. 2005; 433: 278–85.
31. **Shangguan L, Ti X, Krause U, *et al.*** Inhibition of TGF-β/Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. *Stem cells*. 2012; 30: 2810–9.
32. **Luo X, Hutley LJ, Webster JA, *et al.*** Identification of BMP and activin membrane-bound inhibitor (BAMBI) as a potent negative regulator of adipogenesis and modulator of autocrine/paracrine adipogenic factors. *Diabetes*. 2012; 61: 124–36.
33. **Lin Z, Gao C, Ning Y, *et al.*** The pseudoreceptor BMP and activin membrane-bound inhibitor positively modulates Wnt/beta-catenin signaling. *J Biol Chem*. 2008; 283: 33053–8.
34. **Sutters M, Germino GG.** Autosomal dominant polycystic kidney disease: molecular genetics and pathophysiology. *J Lab Clin Med*. 2003; 141: 91–101.
35. **Sun H, Li QW, Lv XY, *et al.*** MicroRNA-17 post-transcriptionally regulates polycystic kidney disease-2 gene and promotes cell proliferation. *Mol Biol Rep*. 2010; 37: 2951–8.
36. **Zwirner NW, Fuertes MB, Girart MV, *et al.*** Immunobiology of the human MHC class I chain-related gene A (MICA): from transplantation immunology to tumor immune escape. *Immunologia*. 2006; 25: 25–38.
37. **Zheng Y, Yin L, Chen H, *et al.*** miR-376a suppresses proliferation and induces apoptosis in hepatocellular carcinoma. *FEBS Lett*. 2012; 586: 2396–403.
38. **Bakhshandeh B, Soleimani M, Hafizi M, *et al.*** MicroRNA signature associated with osteogenic lineage commitment. *Mol Biol Rep*. 2012; 39: 7569–81.
39. **Bakhshandeh B, Soleimani M, Paylakhi SH, *et al.*** A microRNA signature associated with chondrogenic lineage commitment. *J Genet*. 2012; 91: 171–82.
40. **Viswanathan SR, Daley GQ, Gregory RI.** Selective blockade of microRNA processing by Lin28. *Science*. 2008; 320: 97–100.
41. **Doi N, Zenno S, Ueda R, *et al.*** Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Curr Biol*. 2003; 13: 41–6.

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

42. **Bahubeshi A, Tischkowitz M, Foulkes WD.** miRNA processing and human cancer: DICER1 cuts the mustard. *Sci Transl Med*. 2011; 3: 111ps46.
43. **Zheng H, Zeng Y, Zhang X, *et al.*** mu-Opioid receptor agonists differentially regulate the expression of miR-190 and NeuroD. *Mol Pharmacol*. 2010; 77: 102–9.
44. **Ueno K, Hirata H, Shahryari V, *et al.*** Tumour suppressor microRNA-584 directly targets oncogene Rock-1 and decreases invasion ability in human clear cell renal cell carcinoma. *Br J Cancer*. 2011; 104: 308–15.
45. **Reya T, Clevers H.** Wnt signalling in stem cells and cancer. *Nature*. 2005; 434: 843–50.
46. **Peng L, Ren LB, Dong G, *et al.*** Wnt5a promotes differentiation of human dental papilla cells. *Int Endod J*. 2010; 43: 404–12.
47. **Davidson G, Niehrs C.** Emerging links between CDK cell cycle regulators and Wnt signaling. *Trends Cell Biol*. 2010; 20: 453–60.
48. **Holland JD, Klaus A, Garratt AN, *et al.*** Wnt signaling in stem and cancer stem cells. *Curr Opin Chem Biol*. 2013; 25: 254–64.
49. **Blauwkamp TA, Nigam S, Ardehali R, *et al.*** Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nat Commun*. 2012; 3: 1070.
50. **Reya T, Duncan AW, Ailles L, *et al.*** A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*. 2003; 423: 409–14.
51. **Davidson KC, Adams AM, Goodson JM, *et al.*** Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. *Proc Natl Acad Sci USA*. 2012; 109: 4485–90.
52. **Nawroth R, van Zante A, Cervantes S, *et al.*** Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells. *PLoS ONE*. 2007; 2: e392.
53. **Hayano S, Kurosaka H, Yanagita T, *et al.*** Roles of heparan sulfate sulfation in dentinogenesis. *J Biol Chem*. 2012; 287: 12217–29.
54. **Zhu J, Shimizu E, Zhang X, *et al.*** EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. *J Cell Biochem*. 2011; 112: 1749–60.
55. **Aguirre A, Rubio ME, Gallo V.** Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. *Nature*. 2010; 467: 323–7.
56. **Zhou X, Hu Y, Dai L, *et al.*** MicroRNA-7 inhibits tumor metastasis and reverses epithelial-mesenchymal transition through AKT/ERK1/2 inactivation by targeting EGFR in epithelial ovarian cancer. *PLoS ONE*. 2014; 9: e96718.
57. **Xie J, Chen M, Zhou J, *et al.*** miR-7 inhibits the invasion and metastasis of gastric cancer cells by suppressing epidermal growth factor receptor expression. *Oncol Rep*. 2014; 31: 1715–22.
58. **Kefas B, Godlewski J, Comeau L, *et al.*** microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res*. 2008; 68: 3566–72.
59. **Karin M, Liu Z, Zandi E.** AP-1 function and regulation. *Curr Opin Cell Biol*. 1997; 9: 240–6.
60. **Pearson G, Robinson F, Beers Gibson T, *et al.*** Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*. 2001; 22: 153–83.

University of Miami

© 2014 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

3.6 Publication 6 (Review article)

Xin LZ, Govindasamy V, **Musa S**, Abu Kasim NH: Dental stem cells as an alternative source for cardiac regeneration. Medical Hypotheses. 2013, 81(4):704-706

3.6.1 Contributions of co-authors:

Design and concept of study	SM, GV, AKNH
Acquisition of data	XLZ, SM
Analysis of data	XLZ, SM
Drafting of manuscript	XLZ, SM, GV
Revising manuscript for intellectual content	All authors

University of Malaya

Medical Hypotheses 81 (2013) 704–706

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Dental stem cells as an alternative source for cardiac regeneration

Loo Zhang Xin [a], Vijayendran Govindasamy [b,*], Sabri Musa [a,*], Noor Hayaty Abu Kasim [c]

[a] Department of Children Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Hygieia Innovation Sdn. Bhd (852106-M), Lot 1G-2G, Lanai Complex No. 2, Persiaran Seri Perdana, Precint 10, 62250 Federal Territory of Putrajaya, Malaysia
[c] Department of Conservative Dentistry, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 30 April 2013
Accepted 17 July 2013

ABSTRACT

Dental tissues contains stem cells or progenitors that have high proliferative capacity, are clonogenic *in vitro* and demonstrate the ability to differentiate to multiple type cells involving neurons, bone, cartilage, fat and smooth muscle. Numerous experiments have demonstrated that the multipotent stem cells are not rejected by immune system and therefore it may be possible to use these cells in allogeneic settings. In addition, these remarkable cells are easily abundantly available couple with less invasive procedure in isolating comparing to bone marrow aspiration. Here we proposed dental stem cells as candidate for cardiac regeneration based on its immature characteristic and propensity towards cardiac lineage via PI3-Kinase/Aktsignalling pathway.

© 2013 Elsevier Ltd. All rights reserved.

Introduction

Out of the 57 million global deaths in 2008, 17 millions are attributed to cardiovascular diseases (CD) [1]. Current treatments include the using drugs such as anti-hypertensive or anti-arrhythmias which mainly act to delay the progression of heart failure. Other advance treatments include surgical intervention such as stent and bypass that restore blood supplying to the ischemia myocardium to save the remaining cardiomyocytes [2]. But the limitation of these treatments is that it does not encourage tissue regeneration coupled with invasive in nature. Therefore, novel therapeutic approaches are urgently required to reduce the mobility and mortality of CD. In this scenario, cell replacement therapy represents a promising option for myocardial repair and several reports now suggest that cardiomyoctes are produced throughout the lifetime of the adult [3] and hence eliminating the old dogmatic view that defines myocardium as a tissue incapable of self-renewal. Nevertheless, the productions of cardiomyocytes are far lower as compared to epithelium and bone marrow (BM) cells. This prompted for the continuous search for an alternative cells source that capable in regenerating cells.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have always being considered as the leading candidates for cardiac regeneration albeit the usage of these cell lines in clinical application are caveated with genetic manipulation and ethical controversies [4]. In terms of adult/mesenchymal stem cells (MSCs), BM-MSCs have been the most frequently studied (www.clinicaltrial.gov). Recently, dental stem cells (DSCs) obtained from dental tissue present itself as an attractive candidate in regenerative medicine. DSCs were first discovered by Gronthos et al. [5] and subsequently various different types of dental stem cells been identified in dental origin namely exfoliated primary teeth (SHED), dental pulp from permanent teeth (DPSCs), buccal mucosa, apical papilla and periodontal ligament (PDLSCs) [6]. These cells are easier to obtain, using less invasive procedures and ethically free than isolating BM-MSCs. Apart from differentiating into odontoblast/osteoblast like cells [7], DSCs are also reported to differentiate into functional active neurons [8–10], mature melanocytes [11], smooth muscle cells [12], islet like aggregates [13] and hepatic cells [14] as well. A striking feature of DSCs was that is has the ability to differentiate into cardiomyocytes *in vitro* [15] as well as promoting angiogenesis in pre-clinical model [16] been reported recently (Fig. 1).

Hypothesis and evaluation of the hypothesis

Based on the available data, there is a coherent idea to hypothesize that DSCs an alternative source for cardiac regeneration. Herein we outlined the evidence to support the hypothesis.

Inherent pluripotent capacity of DSCs

One of the unique characteristics of ESCs or iPSCs is the ability of these cells to differentiate into any desired cell types. The main reason is the highly expression of several pluripotency transcription factors such as Oct-4, Sox-2 and Nanog [17]. Likewise, many researchers have reported that SHED is more primitive in nature and expressing almost all pluripotent genes [12,13]. Perhaps due

* Corresponding authors. Tel.: +60 3 88902968; fax: +60 3 88902969 (V. Govindasamy). Tel.: +60 3 79674816; fax: +60 3 79674530 (S. Musa).
E-mail addresses: vijay@hygieiagroup.com (V. Govindasamy), sabrim@um.edu.my (S. Musa).

0306-9877/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mehy.2013.07.032

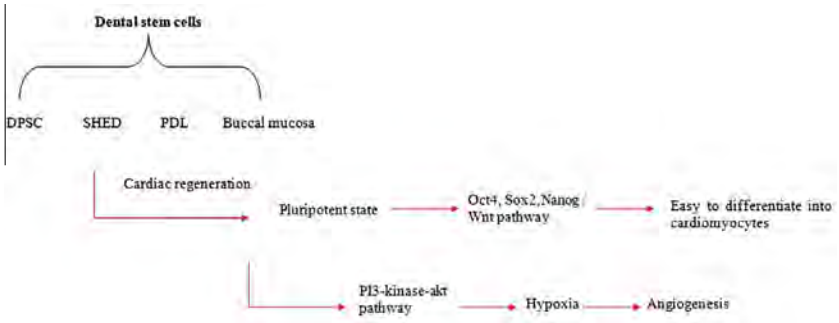


Fig. 1. Schematic illustration of the potential therapeutic effects of human dental derived stem cells on myocardial infarction.

to this, DSCs could be effectively reprogrammed into iPSCs [18]. Further, Wnt, TGFβ/Activin/Nodal and BMP signaling have important roles with pluripotent genes [19]. Sato et al. have shown a downstream effector of the Wnt pathway, an inhibitor of glycogen synthase kinase-3 (GSK3) [20]. In addition, Oct 4 involved in the maintenance of stem cells fate via interaction with Wnt/β-catenin signaling pathway [21]. Furthermore, dental pulp expresses abundantly TGFβ family members [22]. Surprisingly, tooth development includes all members of this pathway [23] which further prove the notion that these cells being primitive cells and hence are more easily to be directed into a specific lineage as compared to other stem cells sources. C-kit$^+$ cells also present in DPSCs which indicated that portion of the cells can be enriched to be used for downstream application similarly to a study using kinase insert domain protein receptor (KDR) from ESCs derived population were also used to develop human cardiovascular progenitor cells [24,25].

PI3-Kinase (K) and Akt pathway: The cross-link between DSCs, angiogenesis and cardiomyocytes

The PI3K-Akt signaling pathway is involved in fundamental cellular processes including protein synthesis, proliferation and survival of cardiovascular system development [26]. McDevitt et al. have shown this pathway as a significant mediator in the proliferation of cardiomyocytes derived from human ESCs and activation of PI3K and ERK related signaling cascades leads to increased survival of cardiomyocytes [27]. Studies also have shown that PI3K-Akt signaling play a critical role in the angiogenesis [28]. In the context of cardiovascular system development, an onset to cardiac arrest leads to infarct zone in hypoxia condition, acidosis and lack of substrates as well as accumulation of metabolites. This stimulate the production pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietin 1 (Ang1) and 2 (Ang2), placental growth factor (PGF), and platelet-derived growth factor-B (PDGFB) [29].

These cytokines encouraged angiogenesis and perhaps could explain the underlying improvement of cardiac function such as inhibiting apoptosis, increase survival of cells, and stimulate continuous angiogenesis by activating the PI3K-Akt pathway [30]. On the other hand, Aranha et al. have shown that hypoxia enhances the angiogenic potential in DPSCs [29]. Further, Gandia et al. have shown that improvement in cardiac function when DPSCs were intramyocardially delivered to the rats and that improved heart function may be attributed to angiogenesis [16]. Here we postulate that PI3K-Akt pathway might plays a vital role in directing DSCs into cardiomyoctes and further studies on this ought to be done. Some transcription factors and receptors such as HAND2, GATA6 and KDR [31], which link to the angiogenesis pathway, were also found to express in cardiomyocytes differentiation. This indicates that these genes play a multifaceted function. Surprisingly, these genes were also detected in DSCs [32,33] and thereby highlighting their cardiac regeneration potential.

Conclusion

The cumulative amounts of data suggest the potential DSCs in cardiac regeneration and if it does work, this approach will lofty the goal of making DSCs a new drug for myocardial repair.

Conflict of interest statement

None.

Acknowledgments

The work is part of an on-going project between Hygieia Innovation and Faculty of Dentistry, University Malaya and supported by University of Malaya, High Impact Research-Ministry of Higher Education, Malaysia (UM.C/HIR/MOHE/DENT/02).

References

[1] World health organization. Causes of death 2008. <http://apps.who.int/ghodata/?vid=10015>.
[2] Jessup M, Abraham WT, Casey DE, Feldman AM, Francis GS, Ganiats TG, et al. 2009 Focused update: ACCF/AHA guidelines for the diagnosis and management of heart failure in adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation 2009;119(14):1977–2016.
[3] Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001;344(23):1750–7.
[4] Wen Z, Liao Q, Hu Y, Liu S, You L, Zhao Y. Human adipose-derived stromal/stem cells: a novel approach to inhibiting acute pancreatitis. Med Hypotheses 2013;80(5):598–600.
[5] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 2000;97:13625–30.
[6] Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 2009;88(9):792–806.
[7] Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. J Dent Res 2002;81(8):531–5.
[8] Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 2008;26:1787–95.
[9] Kadar K, Kiraly M, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, et al. Differentiation potential of stem cells from human dental origin – promise for tissue engineering. J Physiol Pharmacol 2009;7:167–75.
[10] Osathanon T, Nowwarote N, Pavasant P. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCγ signaling pathway. J Cell Biochem 2011;112(7):1807–16.
[11] Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, et al. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 2008;17(6):1175–84.
[12] Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, et al. Isolation and characterization of a population of immature

dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells Tissues Organs 2006;184(3–4):105–16.
[13] Govindasamy V, Ronald VS, Abdullah AN, Nathan KR, Ab Aziz ZA, Abdullah M, et al. Differentiation of dental pulp stem cells into islet-like aggregates. J Dent Res 2011;90(5):646–52.
[14] Ishkitiev N, Yaegaki K, Imai T, Tanaka T, Nakahara T, Ishikawa H, et al. High-purity hepatic lineage differentiated from dental pulp stem cells in serum-free medium. J Endod 2012;38(4):475–80.
[15] Ferro F, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, Beltrami AP, et al. Dental pulp stem cells differentiation reveals new insights in Oct 4 a dynamics. PLoS One 2012;7(7):e41774. http://dx.doi.org/10.1371/journal.pone.0041774.
[16] Gandia C, Arminan A, Garcia-Verdugo JM, Lledo E, Ruiz A, Minana MD, et al. Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. Stem Cells 2008;26(3):638–45.
[17] Yamanaka S, Li J, Kania G, Elliot S, Wersto RP, Van Eyk J, et al. Pluripotency of embryonic stem cells. Cell Tissue Res 2008;331(1):5–22.
[18] Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. IPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev 2010;19(4):469–80.
[19] James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGF beta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 2005;132(6):1273–82.
[20] Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specificinhibitor. Nat Med 2004;10(1):55–63.
[21] Abu-Remaileh M, Gerson A, Farago M, Nathan G, Alkalay I, ZinsRousso S, et al. Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/β-catenin signalling. EMBO J 2010;29(19):3236–48.
[22] Yildirim S. Reprogramming of DPSC to induced pluripotent stem cells Springer briefs. Stem Cells 2013:65–71.
[23] Thesleff I, Vaahtokari A, Partanen AM. Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. Int J Dev Biol 1995;39(1):35–50.
[24] Mangano C, Paino F, d'Aquino R, De Rosa A, Iezzi G, Piattelli A, et al. Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. PLoS One 2011;6(4):e18721. http://dx.doi.org/10.1371/journal.pone.0018721.
[25] Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell derived population. Nature 2008;453(7194):524–8.
[26] McNamara CR, Degterev A. Small-molecule inhibitors of the PI3K signaling network. Fut Med Chem 2011;3(5):549–65.
[27] McDevitt TC, Laflamme MA, Murry CE. Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI3-kinase/Akt signaling pathway. J Mol Cell Cardiol 2005;39(6):865–73.
[28] Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407(6801):249–57.
[29] Aranha AM, Zhang Z, Neiva KG, Costa CA, Hebling J, Nor JE. Hypoxia enhances the angiogenic potential of human dental pulp cells. J Endod 2010;36:1633–7.
[30] Hung SC, Pochampally RR, Chen SC, Hsu SC, Prockop DJ. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells 2007;25(9):2363–70.
[31] Xu C. Differentiation and enrichment of cardiomyocytes from human pluripotent stem cells. J Mol Cell Cardiol 2012;52:1203–12.
[32] Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, et al. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. J Endod 2010;36(9):1504–15.
[33] Abe M, Tamamura Y, Yamagishi H, Maeda T, Kato J, Tabata MJ. Cell tissue res et al. tooth-type specific expression of dHAND/Hand 2: possible involvement in murine lower incisor morphogenesis. Cell Tissue Res 2002;310(2):201–12.

University of Melbourne

3.7 Publication 7 (Review article)

Pukana Jayaraman, Prakash Nathan, Punitha Vasanthan, **Sabri Musa** and Vijayendran Govindasamy. Stem cells conditioned medium: a new approach to skin wound healing management. Cell Biol Int 37 (2013) 1122–1128

3.7.1 Contribution of co-authors:

Design and concept of study	PJ, SM
Acquisition of data	PJ, SM, PV, PN
Analysis of data	PJ, SM, GV
Drafting of manuscript	PJ, SM
Revising manuscript for intellectual content	All authors

University of Malaya

Cell Biology International ISSN 1065-6995
doi: 10.1002/cbin.10138

SHORT COMMUNICATION

Stem cells conditioned medium: a new approach to skin wound healing management

Pukana Jayaraman[1], Prakash Nathan[2], Punitha Vasanthan[1], Sabri Musa[1]* and Vijayendran Govindasamy[2,3]*

1 Department of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya 50603, Kuala Lumpur, Malaysia
2 Hygieia Innovation Sdn Bhd (852106-M), Lot 1G-2G, Lanai Complex No. 2, Persiaran Seri Perdana, Precint 10, Federal Territory of Putrajaya Malaysia
3 KOZA Sdn Bhd (947767-T), 13.3A, Menara 1 Mont Kiara, Jalan Kiara, 50480 Mont Kiara, Kuala Lumpur, Malaysia

University of Malaya

Abstract

Stem cell biology has gained remarkable interest in recent years, driven by the hope of finding cures for numerous diseases including skin wound healing through transplantation medicine. Initially upon transplantation, these cells home to and differentiate within the injured tissue into specialised cells. Contrariwise, it now appears that only a small percentage of transplanted cells integrate and survive in host tissues. Thus, the foremost mechanism by which stem cells participate in tissue repair seems to be related to their trophic factors. Indeed, stem cells provide the microenvironment with a wide range of growth factors, cytokines and chemokines, which can broadly defined as the stem cells secretome. In in vitro condition, these molecules can be traced from the conditioned medium or spent media harvested from cultured cells. Conditioned medium now serves as a new treatment modality in regenerative medicine and has shown a successful outcome in some diseases. With the emergence of this approach, we described the possibility of using stem cells conditioned medium as a novel and promising alternative to skin wound healing treatment. Numerous pre-clinical data have shown the possibility and efficacy of this treatment. Despite this, significant challenges need to be addressed before translating this technology to the bedside.

Keywords: cytokines; growth factors; host tissues; paracrine activities; regenerative medicine; tissue repair

Introduction

Statistics reported by the World Health Organization (WHO) estimated that each year over 300,000 people die of skin injury, with the highest death documented in South-East Asian countries (Mock, 2007). In general, skin wound healing takes around 2 weeks depending on the wound severity (acute or chronic; Szpaderska et al., 2003; Figure 1). The slow recovery of natural wound healing has resulted in the entry of exogenous wound healing treatments. Since then, many treatments have proved to quicken the healing (Figure 2). Nevertheless, cost build-up and inconsistency in healing are the major pitfalls of these treatments. This resulted in the discovery of more advanced treatments, such as tissue engineering (Chen et al., 2009), gene therapy (Song et al., 2012), platelet-rich plasma (Park et al., 2011), growth factors (GF) (Penn et al., 2012) and stem cells (SC) therapy (Lee et al., 2012). Among these, SC has become the centre of attraction in wound healing by promoting microvascular remodelling (Dulmovits and Herman, 2012) and enhancement of neovascularisation (Choi et al., 2013). Many approaches can be envisioned for using SC in the support of wound healing. Obviously the first approach that comes to the mind is the injection of SC directly into the wound; reports have shown that SC plays a major role in strengthening wound healing by secreting a multitude of trophic and survival signals including GF, chemokines and cytokines (Chen and Tredget, 2008). They serve as a tool among cells to communicate and these molecules can be traced in the conditioned medium (CM) or spent medium harvested from cultured cells (Shohara et al., 2012). Most recently, CM has been used in pre-clinical studies as a substitute for numerous cellular based therapies including wound healing (Walter et al., 2010). This has encouraged the use of CM in wound healing by modulating wound repair without SC being present in the wound. Nonetheless, details of this method remain uncertain and must be proved before taken as fact.

Corresponding author: e-mail: sabrim@um.edu.my (S. Musa); vijay@hygieiagroup.com (V. Govindasamy)

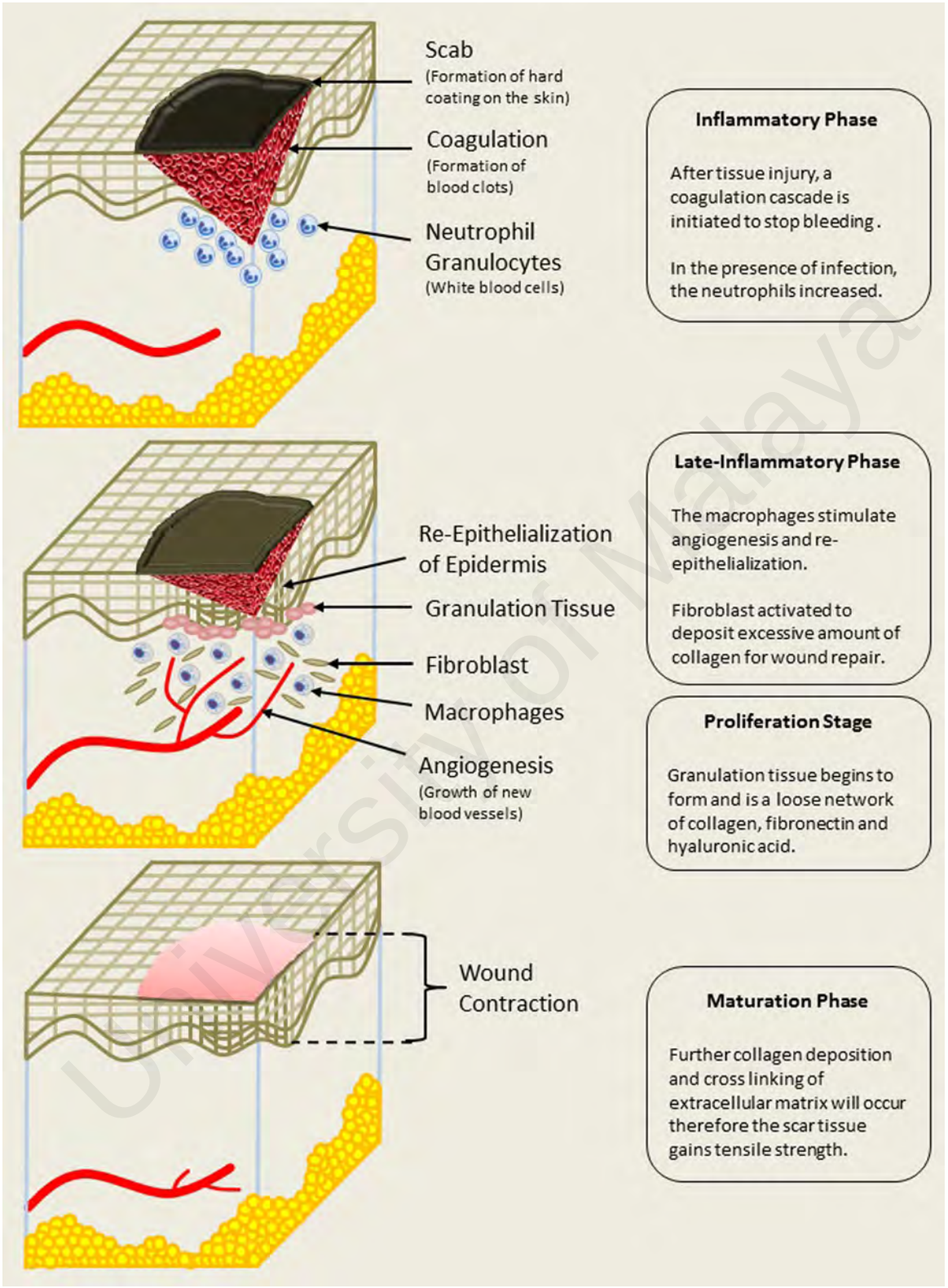


Figure 1 Wound healing stages.

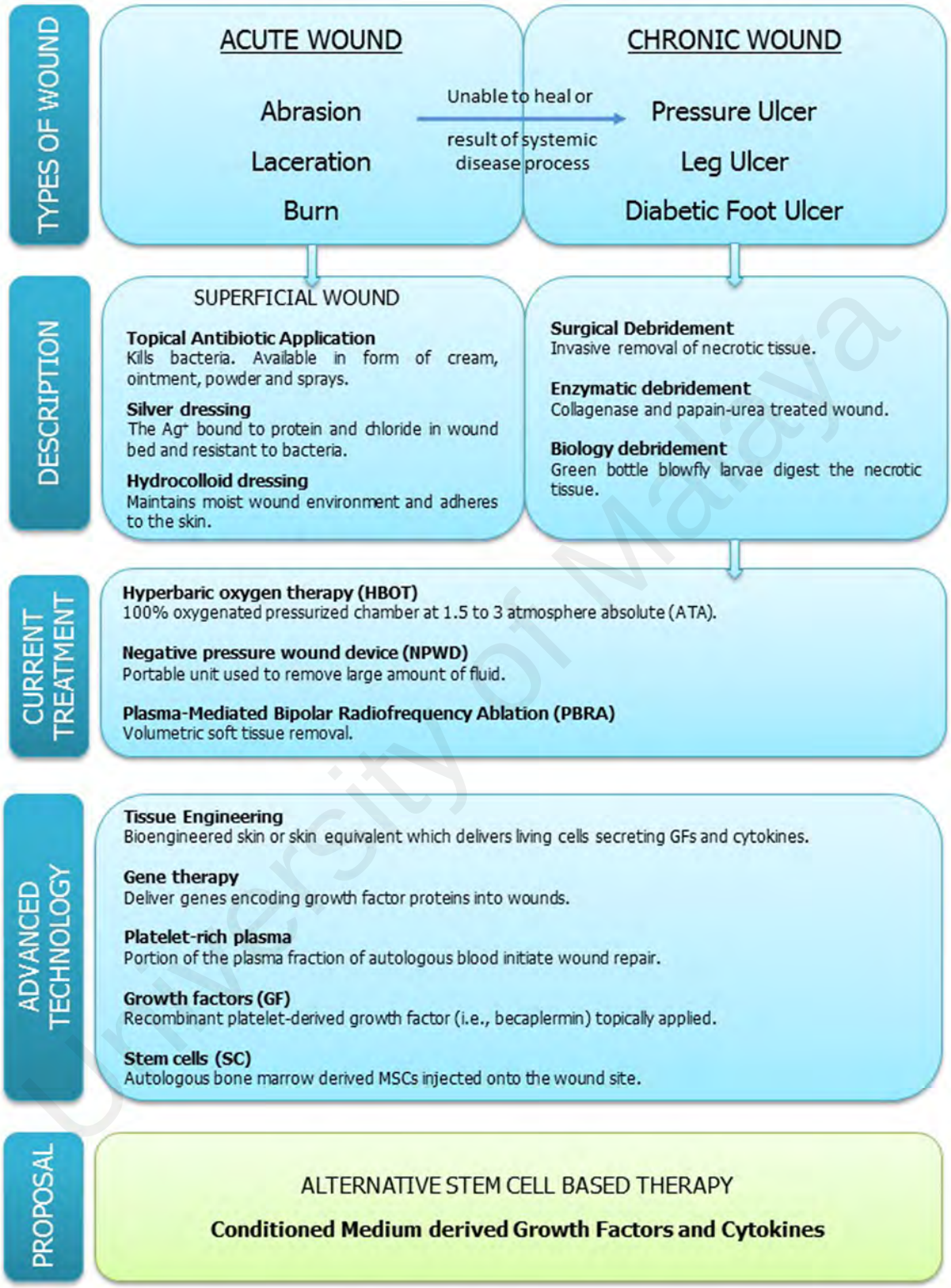


Figure 2 Treatment modalities for wound healing.

University of Malaya

Cell free therapy: an alternative in wound healing management?

One of the major limitations of SC based treatment is the low survivability of cells after being transplanted in the host (Modo et al., 2002). In addition, there are reports suggesting similar characteristics exist between mesenchymal stem cell (MSC) and cancer SC (Kucia et al., 2005). There is even evidence suggesting that SC within normal tissues are of cancerous origin (Sell, 2010). Therefore, to ensure the safety of SC based therapies, developing an alternative approach to direct transplantation of stem cells is necessary. The use of SC-CM instead of direct implantation of SC perhaps offers a better solution to overcome the limitation of cell based therapy.

Lee et al. (2011) reported that CM of human embryonic stem cell (hESC)-derived endothelial precursor cells (EPC) containing high level of GF and cytokines such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), fractalkine, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-6 were successfully used in the treatment of excisional wound healing in rats. Chen and Tredget (2008) suggested a significantly increased wound closure using bone marrow. Oesenchymal stem cell (BMMSC)-CM compared to fibroblast-CM which promises novel therapies for wound repair. They reported that BMMSC-CM secreted higher paracrine factors such as vascular endothelial growth factor (VEGF)-α, insulin like growth factor (IGF), EGF, keratinocyte growth factor (KGF), angioprotein-1 (Ang-1), stromal derived factor-1 and erythropoietin (EPO) compared to fibroblast-CM, indicating that the origin of cells are significantly contributing to the production of paracrine factors. Adipose derived stem cell (ADSC)-CM also has regenerative effects on skin wounds. It stimulates both collagen synthesis and migration of dermal fibroblasts hence promoting wound healing and improving wrinkling in animal models (Kim et al., 2009). ADSC-CMs upregulate the transcription of type I procollagen-alpha-1 chain gene of fibroblasts and involve Rho-associated kinase (RhoA-ROCK) signalling, which leads to the proliferation of keratinocytes and dermal fibroblasts. Dental pulp stem cell (DPSC)-CM has the ability to enhance wound healing by increasing collagen synthesis, and activating proliferation and migration activity of human dermal fibroblast (HDF) (Ueda and Nishino, 2010). Inoue et al. (2013) reported that DPSC-CM enhances vasculogenesis, migration and differentiation of endogenous neuronal progenitor cells in ischemic brain injury in a rat model.

How does SC-CM works in wound healing?

Skin injury causes blood vessel damage and leakage of blood constituents into the wound site. Hemostasis begins immediately after wounding, with vascular constriction and formation of fibrin clot (Szpaderska et al., 2003). As depictured in Figure 1 in the natural wound healing process, the migration of inflammatory cells into the wound by chemotaxis starts with the infiltration of neutrophils, macrophages and lymphocytes (Gosain and DiPietro, 2004). These cells are a major source of GF through phosphoinositide 3-kinase (PI3K) P13K/Akt, and Janus kinase and Signal Transducer and Activator of Transcription (Jak-STAT) pathways. For example, neutrophils initiate VEGF and transforming growth factor-β (TGF-β), whereas lymphocytes initiate tumour necrosis factor (TNF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-1 and macrophage secretes bFGF, EGF, platelet-derived growth factor (PDGF), GM-CSF, TGF-α, TGF-β, IL-1 and TNF. Depending on the individuals, the expression of these groups of cytokines and GF determine the duration of wound healing process. At this juncture, the introduction of SC-CM to the site of injury may accelerate the recovery process. This is because, apart from host tissues, SC-CM has a wide range of cytokines and GF related directly to the wound healing process (Table 1). The next phase of recovery involves angiogenesis, whereby molecules such as VEGF, bFGF, EGF and TGF-β promote new blood vessel, sustain the newly formed granulation tissues and help in the survival of endogenous keratinocytes. In the late phase of the wound healing process, GFs such as EGF, GM-CSF and hepatocyte GF (HGF) prompt keratinocytes to migrate from the basal population around the wound edge to cover the lesion and differentiate into squamous keratinizing epidermal cells (Metcalfe and Ferguson, 2007; Figure 3.

Challenges to SC-CM therapy

Numerous questions remain to be answered before SC-CM can be used as an efficient therapeutic tool, the key ones being addressed below.

Secretome factors

The level of paracrine factors secreted by different SC resources plays an important role on their influences on cell recruitment and wound repair (Friedenstein et al., 1966). Hence, the question is how to increase the paracrine factors in SC-CM enough for them to be used for the treatment Hypoxia treatment is perhaps one of the ways. Hypoxic stress is a condition that reduces oxygen, which will improve cellular functions depending on the cell type, position and microenvironment. When ADSC are cultured under hypoxic conditions in vitro, the proliferative and self-renewal capacities of the cells are significantly improved, enhancing the secretion of certain GFs (Efimenko et al., 2010). Kinnaird et al. (2004) and Lee et al. (2009) reported a wide variety of

University of Malaya

Table 1 List of cytokines secreted by SC-CM.

Paracrine factors	Function	Phase of wound
Transforming growth factor beta (TGF-β)	Stimulates migration of macrophages, dermal fibroblasts. Increases angiogenesis and granulation tissue for re-epithelialisation process	Inflammatory, proliferation
Transforming growth factor-alpha (TGF-α)	Stimulates epithelial cells and granulation tissue for re-epithelialization process	Inflammatory, proliferation
Basic fibroblast growth factor (bFGF)	Increases fibroblast proliferation, angiogenesis and matrix deposition	Proliferation, maturation
Interleukin-6 (IL-6)	Influencing inflammatory cells influx and promotes reepithelialisation	Inflammatory, proliferation
Interleukin-8 (IL-8)	Promotes skin re-epithelialisation by increasing keratinocyte migration and proliferation	Proliferation
Interleukin-1 (IL-1)	Increases pro-inflammatory cell and fibroblast proliferation	Inflammatory, proliferation
Epidermal growth factor (EGF)	Enhance migration of keratinocyte and fibroblast. Increased granulation tissue	Proliferation
Vascular endothelial growth factor (VEGF)	Endothelial survival and migration and proliferation. Regulates angiogenesis and granulation tissue formation	Inflammatory, proliferation
Platelet-derived growth factor (PDGF)	Increase macrophage activation, fibroblast proliferation, angiogenesis and collagen metabolism	Inflammatory, proliferation, maturation
Keratinocyte growth factor (KGF)	Stimulation of keratinocytes' proliferation and migration	Proliferation
Granulocyte-colony stimulating factor (G-CSF)	Initiate inflammatory cells and increases keratinocytes	Inflammatory, proliferation
Granulocyte macrophage-colony stimulating factor (GM-SCF)	Proliferation of epidermal cell	Proliferation
Tumour necrosis factor (TNF)	Increases fibroblast	Proliferation
Insulin like-growth factor (IGF-1)	Fibroblast and collagen synthesis	Proliferation, maturation
Hepatocyte growth factor (HGF)	Promotes reepithelialisation, vasculogenesis and granulation tissue formation	Proliferation
Macrophage chemotactic protein-1α (MCP-1) and RANTES	Promote dermal wound healing as a chemoattractant to cells of the immune system particularly macrophages	Proliferation
Collagen type 1 and fibronectin	Stimulates fibroblast and keratinocycte cell adhesion and migration	Maturation
SPARC	Cell-matrix interaction	Maturation
Insulin-like growth factor binding protein 7 (IGFBP-7)	Regulate proliferation and migration of keratinocytes	Inflammatory, proliferation
Connective tissue growth factor (CTGF)	Chemo attractant for fibroblast	Proliferation

cytokine genes expressed in MSC-CM collected in hypoxic condition; it has promoted in vitro proliferation and migration of endothelial cells as well as collagen synthesis. Another aspect is the timing of collection of the CM from the cells. Walter et al. (2010) showed that CM collected and filtered after 72 h incubation from a population of MSC at passage II used to replace CM in scratch wound assay medium successfully improved the healing ability in this assay.

Choice of cells

MSC derived from various tissue sources are different from each other, indicating their propensity towards a specific lineage (Pal et al., 2009; Nekanti et al., 2010). Similarly, we suggest that there will be variation in terms of cytokine and GF among various cell sources whereby the right source needs to be identified to provide maximum efficacy in wound healing treatments.

Safety issues

Stem cells culture is usually expanded in basic media with fetal bovine serum (FBS) or other serum supplements such as human platelet lysate (HPL) (Lohmann et al., 2012). The collection of SC-CM with serum supplemented condition method may not be adequate as it can introduce animal derived cytokines and GFs to the medium. For better therapeutic usage of SC-CM, the use of completely defined serum-free conditions is desirable, but

University of Malaya

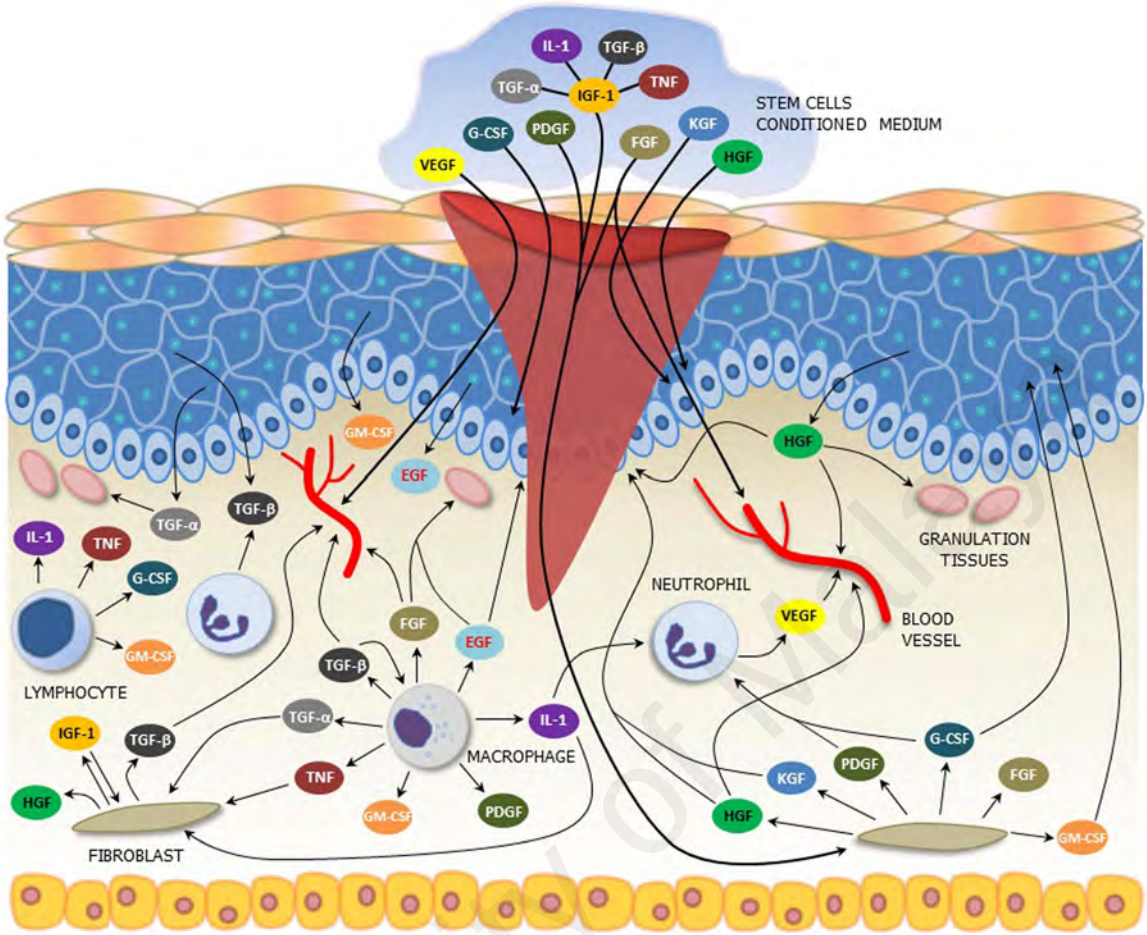


Figure 3 Mechanism of wound healing using SC-CM.

may escalate production cost. There is also the possibility of introduction of dead cells and extracellular matrix well as cell debris in SC-CM.

Delivery method

Multiple combinations of administration routes are perhaps the best in the case of SC-CM. In a wound healing model, Chen and Tredget (2008) gave each excision wound 80 μL MSC-CM by subcutaneous injection and 20 μL by topical application on the bed, and showed remarkable recovery (Friedenstein et al., 1966).

Conclusion

It is clear that SC-CM technology is a rapidly advancing field that promises to have a substantial impact on the treatment of skin wound healing. Therefore, gaining a more complete understanding of growth factors and cytokines in the SC-CM is crucial, together with finding better solutions to some of the key questions we have raised. In addition, knowledge of SC-CM could persuade academic, pharmaceutical and regulatory scientists to agree on a common path forward that will maximize the possibility of clinical realization of SC-CM therapies.

Acknowledgements and funding

The work is part of an on-going project between Hygieia Innovation and Faculty of Dentistry, University Malaya and supported by University of Malaya, High Impact Research-Ministry of Higher Education, Malaysia (UM.C/HIR/MOHE/DENT/02).

Conflict of interest

None.

References

Chen L, Tredget EE, Wu PY, Wu Y (2008) Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE 3(4): e1886.

Chen M, Przyborowski M, Berthiaume F (2009) Stem cells for skin tissue engineering and wound healing. Crit Rev Biomed Eng 37 (4–5): 399–421.

Choi M, Lee HS, Naidansaren P, Kim HK, Eunju O, Cha JH, Ahn HY, Yang PI, Shin JC, Joe YA (2013) Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. Int J Biochem Cell Biol 45(3): 560–70.

Dulmovits BM, Herman IM (2012) Microvascular remodeling and wound healing: a role for pericytes. Int J Biochem Cell Biol 44 (11): 1800–12.

Efimenko A, Starostina EE, Rubina KA, Kalinina NI, Parfenova EV (2010) Viability and angiogenic activity of mesenchymal stromal cells from adipose tissue and bone marrow in hypoxia and inflammation in vitro. Tsitologiia 52(2): 144–54.

Friedenstein AJ, Piatetzky S, II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16(3): 381–90.

Gosain A, DiPietro LA (2004) Aging and wound healing. World J Surg 28(3): 321–6.

Inoue T, Suqiyama M, Hattori H, Wakita H, Wakabayashi T, Ueda M (2013) Stem cells from human exfoliated deciduous tooth-derived conditioned medium enhance recovery of focal cerebral ischemia in rats. Tissue Eng Part A 19(1–2): 24–9.

Kim WS, Park BS, Sung JH (2009) Protective role of adipose-derived stem cells and their soluble factors in photoaging. Arch Dermatol Res 301(5): 329–36.

Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94(5): 678–85.

Kucia M, Reca R, Miekus K, Wanzeck J, Wojokowski W, Janowska-Wieczorek A, Ratajczak J, Ratajczak MZ (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells 23(7): 879–94.

Lee EY, Xia Y, Kim WS, Kim MH, Kim TH, Kim KJ, Park BS, Sung JH (2009) Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. Wound Repair Regen 17(4): 540–7.

Lee MJ, Kim J, Lee KI, Shin JM, Chae JI, Chung HM (2011) Enhancement of wound healing by secretary factors of endothelial precursor cells derived from human embryonic stem cells. Cytotherapy 13(2): 165–78.

Lee SH, Jin SY, Song JS, Seo KK, Cho KH (2012) Paracrine effects of adipose-derived stem cells on keratinocytes and dermal fibroblasts. Ann Dermatol 24(2): 136–43.

Lohmann M, Walenda G, Hemeda H, Joussen S, Drescher W, Jockenhoevel S, Hutschenreuter G, Zenke M, Wagner W (2012) Donor age of human platelet lysate affects proliferation and differentiation of mesenchymal stem cells. PLoS ONE 7(5): e37839.

Metcalfe AD, Ferguson MW (2007) Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. J R Soc Interface 4(14): 413–37.

Mock C (2007) WHO joins forces with International society for burn injuries to confront global burden of burns. Inj Prev 13(5): 303.

Modo M, Rezaie P, Heuschling P, Patel S, Male DK, Hodges H (2002) Transplantation of neural stemcells in a rat model of stroke: assessment of short-term graft survival and acute host immunological response. J Brain Res 958: 70–82.

Nekanti U, Rao VB, Bahirvani AG, Jan M, Totey S, Ta M (2010) Long-term expansion and pluripotent marker array analysis of Wharton's Jelly-derived mesenchymal stem cells. Stem Cells Dev 19: 117–30.

Pal R, Totey S, Mamidi MK, Bhat VS, Totey S (2009) Propensity of human embryonic stem cell lines during early stage of lineage specification controls their terminal differentiation into mature cell types. Exp Biol Med 234(10): 1230–43.

Park HB, Yang JH, Chung KH (2011) Characterization of the cytokine profile of platelet rich plasma (PRP) and PRP-induced cell proliferation and migration: upregulation of matrix metalloproteinase-1 and -9 in HaCaT cells. Korean J Hematol 46(4): 265–73.

Penn JW, Grobbelaar AO, Rolfe KJ (2012) The role of the TGF-β family in wound healing, burns and scarring: a review. Int J Burns Trauma 2(1): 18–28.

Sell S (2010) On the stem cell origin of cancer. Am J Pathol 176(6): 2584–494.

Shohara R, Yamamoto A, Takikawa S, Iwase A, Hibi H, Kikkawa F, Ueda M (2012) Mesenchymal stromal cells of human umbilical cord Wharton's jelly accelerate wound healing by paracrine mechanisms. Cytotherapy 14(10): 1171–81.

Song SH, Lee MO, Lee JS, Jeong HC, Kim HG, Kim WS, Hur M, Cha HJ (2012) Genetic modification of human adipose-derived stem cells for promoting wound healing. J Dermatol Sci 66(2): 98–107.

Szpaderska AM, Egozi EI, Gamelli RL, DiPietro LA (2003) The effect of thrombocytopenia on dermal wound healing. J Invest Dermatol 120(6): 1130–7.

Ueda M, Nishino Y (2010) Cell-based cytokine therapy for skin rejuvenation. J Craniofac Surg 21(6): 1861–6.

Walter MN, Wright KT, Fuller HR, MacNeil S, Johnson WEB / givenNames> (2010) Mesenchymal stem cell conditioned medium accelerates skin wound healing: an in vitro study of fibroblast and keratinocyte scratch assays. Exp Res Cell 316: 1271–81.

Received 26 March 2013; accepted 30 April 2013.
Final version published online 24 June 2013.

University of Malaya

CONCLUSION

4.1 Conclusion

Taking into account the limitations of the research works and review papers discussed in chapter 3, the conclusions of this research can be summarized as follows:

SCDs possess higher proliferation rates than DPSCs. Both SCDs and DPSCs are able to differentiate into odontoblast, adipocytes, and chondrocytes, thus qualifying for the minimum requirements of MSCs. However, the quantification results of osteogenic and adipogenic differentiation indicate that SCDs have better differentiation capabilities over DPSCs as they significantly expressed more pluripotent markers thereby supporting the idea that they comprise a more primitive or pluripotent population of cells compared to DPSCs. In conclusion, our work shows that gene variations occur within the different sources of the same stem cells and that these variations determine their lineage propensity towards a specific destination. We infer that SCDs retained their plasticity over the passages whereas DPSCs lost this attribute and were seen to be more inclined towards a neuronal lineage. Our work demonstrates that both SHED/SCD and DPSCs exhibit specific gene expression profiles, with a clear-cut inclination of DPSCs toward neuronal lineage. This conclusion was drawn on the basis of the first objective in which cellular, morphology, and culture characteristics were compared among three different stem cells of dental origin. This is with reference to the existence of subtle differences although cells from dental origin, collectively known as dental MSCs, do not equally possess similar phenotypes and functional properties.

In regard to the dynamics of miRNA expressions in DPSCs, they were found to be preferentially expressed and appropriately integrated as a group rather than playing a solitary role in creating a functional switch between self-renewal, stemness, and lineage development. The use of miRNA signatures in humans as a prediction tool allows us to

University of Malaya

explain the biological processes occurring in DPSCs. These findings can introduce a new dimension of gene regulation in controlling the fate of SCs and their behaviour in DPSCs and facilitate the development of therapeutic approaches for various diseases.

In terms of the differentiation potential of dental derived stem cells, SHED/SCD were shown to differentiate into hepatic lineages effectively even with HPL replacement. HPL is a potentially effective supplement that enhances the growth rate of SCDs and stimulates a high proliferation of MSCs. Finally, SHED/SCD differentiation undertaken using HPL provides an autologous setting or, to use another term, xeno-free contamination where both cell source and HPL are derived from the same donor thus reducing the risk of disease transmission. Based on the review of the related papers, we hypothesize and propose dental stem cells as likely candidates for cardiac regeneration based on their immature characteristics and propensity towards cardiac lineage via the PI3-Kinase/Akt signaling pathway. It is clear that the differentiation potential shown by stem cells derived from various dental sources offer sound prospects for their use in areas that go beyond tooth regeneration.

Various aspects were also explored in this study in regard to analyzing the immunological effects and therapeutic potential of dental derived stem cells. Firstly, this work prompted a revisit of some of the key aspects of stem cells for clinical usage such as the suitability of using prolonged cultured cells. This work shows that despite the cells being treated under similar culture conditions, SHED surpassed other cell lines (DPSCs and PDLSCs) in expressing unwanted immune genes at a late passage. In conclusion, long-term passaging may have an effect on immune-related genes of dental stem cells (DPSCs and PDLSCs) and suggest that a complete biological characterization covering all major aspects including immune properties should be made a prerequisite prior to the use of long-term cultured stem cells in clinical applications. Secondly, we

University of Malaya

examined whether the same population of cells (SCDs and SCDIPs) with common stem cell traits possess immunological characteristics. Both SCDs and SCDIPs were positive for HLA-ABC and HLA-G and negative for HLA-DR, which is similar in other MSCs. Interestingly, in contrast to SCDIP, higher expressions of HLA-G were seen in SCD. SCDIP has minimal ability to suppress pro-inflammatory and stimulate anti-inflammatory responses. It can be concluded that SCDIPs are highly dysfunctional in terms of their stemness and immunomodulatory properties and do not offer a viable therapeutic cell source.

4.2 Recommendations/Future Works

As most possibilities presented by these findings are mainly based on the characteristics of dental stem cells, further steps have to be considered in employing them as candidates in stem cell therapy. As such, future work should focus on the application of these cells in *in vitro* as well as *in vivo* diabetic-wound-healing models. This will ensure their safety and the efficacy in treating such conditions. It should be kept in mind however that while the various studies and research on regenerative therapy using exogenous stem cells transplantation are based on the premise that they would engraft, differentiate, and replace damaged cells, ambiguity remains in understanding the comprehensive effects of such a procedure. All these observations are physically and temporally incompatible with the differentiation hypothesis. As such they have prompted an alternative hypothesis where the transplanted stem cells mediate their therapeutic effects through the secretion of paracrine factors that promote survival and favour tissue regeneration. During such secretion, exosomes, which are one of the several groups of vesicles released, have a crucial role in intercellular communication and perform almost extensive MSCs functions. Therefore, a study on exosomes to

University of Malaya

determine the degree of reliability of these hypotheses in stem-cells-free treatments should be conducted.

University of Malaya

REFERENCES

Abatangelo, G., Ferroni, L., Bressan, E., Gardin, C., Pinton, P., Cortivo, R., Sivolella, S. & Zavan, B. (2011). Stemness Ability of Human Dental Pulp Stem Cells Related to the in Vivo and in Vitro Aging. *Italian Journal of Anatomy and Embryology.* 116(1), 8

About, I., Bottero, M. J., de Denato, P., Camps, J., Franquin, J. C. & Mitsiadis, T. A. (2000). Human dentine production in vitro. *Experimental Cell Research, 258*(1), 33-41

Aggarwal, S & Pittenger, M. F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood, 105,* 1815-1822.

Alge, D. L., Zhou, D., Adams, L. L., Wyss, B. K., Shadday, M. D., Woods, E. J., Chu, G. T. M & Goebel, W. S. (2010). Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. *Journal of Tissue Engineering and Regenerative Medicine, 4*(1), 73–81.

Alliot-Licht, B., Bluteau, G., Magne, D., Lopez-Cazaux, S., Lieubeau, B., Daculsi, G., et al (2005). Dexamethasone stimulates differentiation of odontoblast-like cells in human dental pulp cultures. *Cell and Tissue Research, 321*(3), 391-400

Barry, F. & Murphy, M. (2013). Mesenchymal stem cells in joint disease and repair. *Nature Reviews, Rheumatology, 910*, 584–594.

Bartel, D. P. (2004). MicroRNAs. *Cell*, 116(2), 281–297.

Béry, A. (2014). Extraction of healthy permanent teeth: An ethical and legal approach. *Journal of Dentofacial Anomalies and Orthodontics*, 17(4), 404.

Beyar, R. (2011). Challenges in Organ Transplantation. *Rambam Maimonides Medical Journal*, 2(2).

Bongso, A., & Richards, M. (2004). History and perspective of stem cell research. *Best Practice & Research Clinical Obstetrics and Gynaecology, 18*(6), 827-842

Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A. & Nikbin, B. (2006). Aging of mesenchymal stem cell in vitro. *BMC Cell Biology. 7*(14), doi: 10.1186/1471-2121-7-14

Bussolati, B. (2011). Stem cells for organ repair. *Organogenesis*, 7(2), 95–95.

University of Malaya

Caplan, A. I. (1991). Mesenchymal Stem Cells. *Journal of Orthopaedic Research, 9*, 641-650.

Casagrande, L., Cordeiro, M.M., Nör, S. A & Nör, J. E. (2011). Dental pulp stem cells in regenerative dentistry. *Odontology,* 99, 1–7.

Cawthorn, W. P., Scheller, E. L., & MacDougald, O. A. (2012). Adipose tissue stem cells: the great WAT hope. *Trends Endocrinol Metab., 23*(6), 270-277.

Chalisserry, E. P., Nam, S. Y., Park, S. H. & Anil, S. (2017). Therapeutic potential of dental stem cells. *Journal of Tissue Engineering, 8,* 1–17.

Chatterjee, S., & Davies, M. J. (2015). Current management of diabetes mellitus and future directions in care. *Postgraduate Medical Journal*, 91(1081), 612–621.

Corcione, A., Benvenuto, F., Ferretti, E., Giunti, D., Cappiello, V., Cazzanti, F, … Uccelli, A. (2006). Human mesenchymal stem cells modulate B-cell functions. *Blood, 107,* 367-372.

Cui, G. H., Wang, Y. Y., Li, C. J., Shi, C. H. & Wang, W. S. (2016). Efficacy of mesenchymal stem cells in treating patients with osteoarthritis of the knee: A meta-analysis. *Experimental and Therapeutic Medicine, 12*(5), 3390–3400.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S. … Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy, 8*(4), 315-317.

Egusa, H., Sonoyama, W., Nishimura, M., Atsuta, I., & Akiyama, K. (2012). Stem cells in dentistry - Part I : Stem cell sources. *Journal of Prosthodontic Research, 56*, 151-165.

Fan, L. X., Hu, C. X., Chen, J. J., Cen, P. P., Wang, J & Li, L. J. (2016). Interaction between Mesenchymal Stem Cells and B-Cells. *International Journal of Molecular Sciences, 17*(5), 650.

Fernandez, T. D. S & Fernandez, C. D. S. (2016). Mesenchymal Stem Cells: Biological Characteristics and Potential Clinical Applications for Haematopoietic Stem Cell Transplantation. *Pluripotent Stem Cells,* 495-519, doi: 10.5772/63772

Forbes, S., Vig, P., Poulsom, R., Thomas, H., & Alison, M. (2002). Hepatic stem cells. *J Pathol, 197*, 510-518.

University of Malaya

Freitag, J., Bates, D., Boyd, R., Shah, K., Barnard, A, Huguenin, L & Tenen, A. (2016). Mesenchymal stem cell therapy in the treatment of osteoarthritis: reparative pathways, safety and efficacy – a review. *BMC Musculoskeletal Disorders, 17,* 230.

Freund, C., Davis, R.P., Gkatzis, K., et al. (2010). The first reported generation of human induced pluripotent stem cells (iPS cells) and iPS cell-derived cardiomyocytes in the Netherlands. *Neth Heart J 18*(1):51-4

Gage, F. H. (2000). Mammalian Neural Stem Cells *SCIENCE, 287*, 1433-1438.

Gao, F., Chiu, S. M., Motan, D.A.L., Zhang, Z., Chen, L., Ji, H. L., Tse, H. F., Fu, Q. L. & Lian, Q. (2016). Mesenchymal stem cells and immunomodulation: current status and future prospects. *Cell Death and Disease 7*(1), doi:10.1038/cddis.2015.327

Gay, I. C., Chen, S. & MacDougall, M. (2007). Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthodontics & Craniofacial Research, 10*(3), 149-60.

Guleria, M., Dua, H., Rohila, S., & Sharma, A. K. (2014). Stem Cells In Dentistry. *Indian Journal of Dental Sciences, 6*(4), 107-111.

Govindasamy, V., Ronald, V. S., Abdullah, A. N., Ganesan Nathan, K. R., Ab. Aziz, Z.A.C., M. Abdullah , … & Bhonde, R. R. (2011). Differentiation of Dental Pulp Stem Cells into Islet-like Aggregates, *Journal of Dental Research, 90*(5), 646-652.

Govindasamy, V., Abdullah A. N., Ronald, V. S., Musa, S, Ab Aziz, Z. A. C, Zain, R. B,…. Abu Kasim, N. H. 2010 Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth. *Journal of Endodontics, 36*(9), 1504-1515.

Grinnemo, K. H., Månsson, A., Dellgren, G., Klingberg, D., Wardell, E., Drvota, V., Tammik, C., Holgersson, J., Ringdén, O., Sylvén, C. & Le Blanc, K. (2004). Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *The Journal of Thoracic and Cardiovascular Surgery 127*(5), 1293-300.

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., & Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America, 97* (25), 13625-13630.

University of Malaya

Halleux, C., Sottile, V., Gasser, J.A. & Seuwen, K. (2001). Multi-lineage potential of human mesenchymal stem cells following clonal expansion. *Journal of Musculoskeletal & Neuronal Interactions, 2*(1), 71-6.

Han, I., Kwon, B. S., Park, H. K., & Kim, K. S. (2017). Differentiation Potential of Mesenchymal Stem Cells Is Related to Their Intrinsic Mechanical Properties. *International Neurourology Journal*, 21(Suppl 1), S24–31.

Heins, N., Englund, M. C., Sjoblom, C., Dahl, U., Tonning, A., Bergh, C., et al. (2004). Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells, 22*(3), 367-376

Hilkens, P., Driesen, R. B., Wolfs, E., Gervois, P., Vangansewinkel, T., Ratajczak, J…. Lambrichts, I. (2016). Cryopreservation and Banking of Dental Stem Cells. *Advances in Experimental Medicine and Biology, 951*, 199-235.

Hosoya, A. & Nakamura, H. (2015). Ability of stem and progenitor cells in the dental pulp to form hard tissue. *Japanese Dental Science Review, 51*(3), 75-83.

Huang, G. T., Gronthos, S. & Shi, S. (2009). Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *Journal of Dental Research, 88*(9), 792-806.

Huang, G. T., Sonoyama, W., Liu, Y., Liu, H., Wang, S., & Shi, S. (2008). The hidden treasure in apical papilla, the potential role in pulp/dentine regeneration and bioroot engineering. *Journal of Endodontics*, 34(6), 645-651

Ieda, M., Fu, J. D., Delgado-Olguin, P., et al. (2010) Direct reprogramming of fibroblasts into Functional cardiomyocytes by defined factors. *Cell, 142*(3):375-86

Jeon, B. G., Jang, S. J., Park, J. S., Subbarao, R. B., Jeon, G. J., Park, B. W. & Rho, G. J. (2015). Differentiation potential of mesenchymal stem cells isolated from human dental tissues into non-mesodermal Lineage. *Animal Cells and Systems, 19*(5), 321–331.

Jiang, X. X., Zhang, Y., Liu, B., Zhang, S. X., Wu, Y., Yu, X. D. & Mao, N. (2005). Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood, 105*, 4120-4126.

Kabir, R., Gupta, M., Aggarwal, A., Sharma, D., Sarin, A. & Kola, M. Z. (2014). Imperative Role of Dental Pulp Stem Cells in Regenerative Therapies: A Systematic Review, *Nigerian Journal of Surgery, 20*(1), 1–8.

University of Malaya

Kadar, K., Kiraly, M., Porcsalmy, B., Molnar, B., Racz, G. Z., Blazsek, J., Kallo, K. … Varga, G. (2009). Differentiation potential of stem cells from human dental origin – promise for tissue engineering. *Journal of Physiology and Pharmacology, 60*, 167–175.

Kawanabe, N., Murata, S., Murakami, K., Ishihara, Y., Hayano, S., Kurosaka, H., Kamioka, H., Takano-Yamamoto, T. & Yamashiro, T. (2010). Isolation of multipotent stem cells in human periodontal ligament using stage-specific embryonic antigen-4. *Differentiation* 79(2):74–83. doi:10.1016/j.diff.2009.10.005

Kern, S., Eichler, H., Stoeve, J., Klüter, H. & Bieback, K. (2006). Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *Stem Cells.* 24(5):1294-301.

Kim, H. J & Park, J. S. (2017). Usage of Human Mesenchymal Stem Cells in Cell-based Therapy: Advantages and Disadvantages. *Developmental Reproduction, 21*(1), 1-10.

Kim, H. S., Kim, K. H., Kim, S. H., Kim, Y. S., Koo, K. T., Kim, T. I … Lee, Y. M. (2010). Immunomodulatory effect of canine periodontal ligament stem cells on allogenic and xenogenic peripheral blood mononuclear cells. *Journal of Periodontal and Implant Science, 40*(6), 265–270.

Kolar, M. K., Itte, V. N., Kingham, P. J. Lev N. Novikov, L. N., Wiberg, M. & Kelk, P. (2017). The neurotrophic effects of diferent human dental mesenchymal stem cells. *Scientific Reports, 7.* doi: 10.1038/s41598-017-12969-1

Krampera, M., Glennie, S., Dyson, J., Scott, D., Laylor, R., Simpson, E. & Dazzi, F. (2003). Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood, 101*(9), 3722-3729.

Kumar, G. S. (2011). *Orban's Oral Histology & Embryology*: Elsevier India

Lalu, M. M., McIntyre, L., Pugliese, P., Fergusson, D., Winston, B. W., Marshall, J. C., Granton, J. & Stewart, D. J. (2012). Safety of Cell Therapy with Mesenchymal Stromal Cells (SafeCell): A Systematic Review and Meta-Analysis of Clinical Trials. *Public Library of Science (PLoS ONE), 7*(10): e47559, doi:10.1371/journal.pone.0047559

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E. & Ringden, O. (2003). HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Experimental Hematology, 31,* 890–896.

University of Malaya

Le Blanc, K. (2003). Immunomodulatory effects of fetal and adult mesenchymal stem cells, *Cytotherapy, 5*(6), 485-489.

Lee, J. S., An, S.Y., Kwon, I.K., Heo, J.S. (2014). Transdifferentiation of human periodontal ligament stem cells into pancreatic cell lineage. *Cell Biochem Funct* 32:605–611. doi:10.1002/cbf.3057

Li, F. F., Guo, X. & Chen, S. Y. (2017). Function and Therapeutic Potential of Mesenchymal Stem Cells in Atherosclerosis. *Frontiers in Cardiovascular Medicine, 4*, doi:10.3389/fcvm.2017.00032

Li, N., Long, B., Han, W., Yuan, S., & Wang, K. (2017). microRNAs: important regulators of stem cells. *Stem Cell Research & Therapy*, 8(1).

Marei, M. K. & Backly, R. M. E. (2018). Dental Mesenchymal Stem Cell-Based Translational Regenerative Dentistry: From Artificial to Biological Replacement. *Frontiers in Bioengineering and Biotechnology, 6.*

Marti, L. C., Ribeiro, A. A. F. & Hamerschlak, N. (2011). Immunomodulatory effect of mesenchymal stem cells. *The Einstein Journal of Biology and Medicine, 9*(2 Pt.1), 224-8.

Mathieu, J., & Ruohola-Baker, H. (2013). Regulation of Stem Cell Populations by microRNAs. *Advances in Experimental Medicine and Biology*, 786: 329–351

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L. W., Robey, P. G. & Shi, S. (2003). SHED: Stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences of the United States of America, 100*(10), 5807-5812.

Mohr, A. & Zwacka, R. (2018). The future of mesenchymal stem cell-based therapeutic approaches for cancer – From cells to ghosts. *Cancer Letters, 414,* 239-249.

Monfrini, M., Donzelli, E., Rodriguez-Menendez, V, Ballarini, E., Carozzi, V. A., Chiorazzi, A., Scuteri, A. (2017). Therapeutic potential of Mesenchymal Stem Cells for the treatment of diabetic peripheral neuropathy. *Experimental Neurology, 288,* 75-84.

Moreira, A., Kahlenberg, S. & Hornsby, P. (2017). Therapeutic potential of mesenchymal stem cells for diabetes. *Journal of Molecular Endocrinology, 59*(3), R109-R120.

Morsczeck, C., Götz, W., Schierholz, J., F, F. Z., Kühn, U., Möhl, C., . . . Hoffmann, K. (2005). Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix biology, 24*(2), 155-165.

University of Malaya

Nakashima, M., Iohara, K., Murakami, M., Nakamura, H., Sato, Y., Ariji, Y. & Matsushita, M. (2017). Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: a pilot clinical study. *Stem Cell Research & Therapy, 8,* 61.

Osathanon, T., Sawangmake, C., Nowwarote, N. & Pavasant, P. (2014). Neurogenic differentiation of human dental pulp stem cells using different induction protocols. *Oral Diseases, 20,* 352–358.

Parekkadan, B & Milwid, J. M. (2010). Mesenchymal Stem Cells as Therapeutics. *Annual Review of Biomedical Engineering, 12,* 87–117.

Park, Y. J., Cha, S. H. & Park, Y. S. (2015). Regenerative Applications Using Tooth Derived Stem Cells in Other Than Tooth Regeneration: A Literature Review. *Stem Cells International, 2016,* doi: 10.1155/2016/9305986

Patel, D. M., Shah, J. & Srivastava, A. S. (2013). Therapeutic Potential of Mesenchymal Stem Cells in Regenerative Medicine. *Stem Cells International, 2013*, doi:10.1155/2013/496218

Peeters, C., Leijs, M.J., Reijman, M., van Osch, G. & Bos, P. (2013). Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review. *Osteoarthritis Cartilage, 21,* 1465–1473.

Pers, Y. M., Ruiz, M., Noel, D. & Jorgensen, C. (2015). Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives. *Osteoarthritis and Cartilage, 23*(11), 2027-2035.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R, Mosca, J.D., Moorman, M.A, Simonetti, D.W., Craig, S & Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science,* 284(5411), 143-7.

Potdar, P.D. & Jethmalani, Y.D. (2015). Human dental pulp stem cells: Applications in future regenerative medicine. *World Journal of Stem Cells,* 7(5): 839-851.

Potten, C. S. (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos Trans R Soc Lond B Biol Sci., 353*(1370), 821-830.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *SCIENCE*, 276(5309), 71-74

Rasmusson, I. (2006). Immune modulation by mesenchymal stem cells. *Experimental Cell Research, 312*(12), 2169-79.

Rimondini, L., & Mele, S. (2009). Stem cell technologies for tissue regeneration in dentistry. *Minerva Stomatologica, 58*(10), 483-500

University of Malaya

Rodrigues, M., Griffith, L. G. & Wells, A. (2010). Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Research & Therapy, 1*(4), 32.

Rosenwasser, R. F., Anderson, K., & Epstein, B. J. (2013). Long-term care and prevention. *Advances in Myocardial Infarction Management*, 98–115.

Saidi, R. F., & Hejazii Kenari, S. K. (2014). Challenges of organ shortage for transplantation: solutions and opportunities. *International journal of organ transplantation medicine*, *5*(3), 87-96.

Saito, M. T., Silvério, K. G., Casati, M. Z., Sallum, E. S. & Nociti Jr, F. H. (2015). Tooth-derived stem cells: Update and perspectives. *World Journal of Stem Cells, 7*(2), 399-407.

Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J… Shi, (2004). S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *The Lancet, 364*(9429), 149-55.

Sharpe, P. T. (2016). Dental mesenchymal stem cells. *Development, 143*, 2273-2280.

Shi, S. & Gronthos, S. (2003). Perivascular Niche of Postnatal Mesenchymal Stem Cells in Human Bone Marrow and Dental Pulp. *Journal of Bone and Mineral Research.* 18(4):696-704.

Slack, J. M. W. (2008). Origin of Stem Cells in Organogenesis. *SCIENCE, 322*(5907), 1498-1501.

Sonoyama, W., Liu, Y., Fang, D., Yamaza, T., Seo, B.-M., Zhang, C., . . . Wang, S. (2006). Mesenchymal Stem Cell-Mediated Functional Tooth Regeneration in Swine *PLoS ONE, 20*(1), e79.

Sonoyama, W., Liu, Y., Yamaza, T., Tuan, R., Wang, S., Shi, S., & Huang, G. (2008). Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod, 34*(2), 166-171.

Sotiropoulou, P. A., Perez, S.A., Gritzapis, A.D., Baxevanis, C.N. & Papamichail, M. (2006). Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells, 24*(1), 74-85.

Spaggiari, G. M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M. C. & Moretta, L. (2008). Mesenchymal stem cells inhibit natural killer–cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2, 3-dioxygenase and prostaglandin E2. *Blood, 111,* 1327-1333.

Spaggiari, G. M. & Moretta, M. (2013). Cellular and molecular interactions of mesenchymal stem cells in innate immunity. *Immunology and Cell Biology, 91,* 27–31.

University of Malaya

Stanko, P., Altanerova, U., Jakubechova, J., Repiska, V & Altaner, C. (2018). Dental Mesenchymal Stem/Stromal Cells and Their Exosomes. *Stem Cells International, 2018,* doi:10.1155/2018/8973613

Suchanek, J., Soukop, T., Ivancakova, R., Karbanova, J., Hubkova, V., Pytlik, R., et al (2007). Human dental pulp stem cells-isolation and long term cultivation. *Acta Medical (Hradec Kralove), 50*(3), 195-201

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*, 663-676.

Taşh, P. N., Aydin, S. & Şahin, F. (2016). Immunomodulatory Properties of Stem Cells Derived from Dental Tissues. In: Şahin F., Doğan A., Demirci S. (eds) Dental Stem Cells. *Stem Cell Biology and Regenerative Medicine*. *Springer, Cham.* 29-45, doi: 10.1007/978-3-319-28947-2_2

Telles, P. D., Machado, M. A. d. A. M., Sakai, V. T., & Nor, J. E. (2011). Pulp tissue from primary teeth: new source of stem cells. *J Appl Oral Sci, 19*(3), 189-194.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *SCIENCE, 282*, 1145-1147.

Tomasello, L., Mauceri, R., Coppola, A., Pitrone, M., Pizzo, G., Campisi, G., Pizzolanti, G. & Giordano, C. (2017). Mesenchymal stem cells derived from inflamed dental pulpal and gingival tissue: a potential application for bone formation. *Stem Cell Research & Therapy, 8,* doi: 10.1186/s13287-017-0633-z

Trubiani, O., Di Primio, R., Traini, T., Pizzicannella, J., Scarano, A., Piattelli, A & Caputi, S. (2005). Morphological and cytofluorimetric analysis of adult mesenchymal stem cells expanded ex vivo from periodontal ligament. *International Journal of Immunopathology and Pharmacology, 18*(2), 213-221.

Ullah, I., Subbarao, R. B., Kim, E. J., Bharti, D., Jang, S. J.,Park, J. S., … Rho, G. J. (2016). In vitro comparative analysis of human dental stem cells from a single donor and its neuronal differentiation potential evaluated by electrophysiology. *Life Sciences, 154,* 39-51.

Van der Sanden, B., Dhobb, M., Berger, F., & Wion, D. (2010). Optimizing Stem Cell Culture. *Journal of Cellular Biochemistry*, 111(4), 801–807.

Van der Valk, J., Brunner, D., De Smet, K., Fex Svenningsen, Å., Honegger, P., Knudsen, L. E., Lindl,T., Noraberg, J., Price, A., Scarino, M.L., Gstraunthaler, G. (2010). Optimization of Chemically Defined Cell Culture Media – Replacing Fetal Bovine Serum in Mammalian in Vitro Methods. *Toxicology in Vitro*, 24(4), 1053–1063.

University of Malaya

Wada, N., Gronthos, S. & Bartold, P. M. (2013). Immunomodulatory effects of stem cells. *Periodontology 2000, 63,* 198–216.

Wang, L., Zhao, Y & Shi, S. (2012). Interplay between Mesenchymal Stem Cells and Lymphocytes: Implications for Immunotherapy and Tissue Regeneration. *Journal of Dental Research, 91*(11), 1003–1010.

Watt, F. M. (1998). Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philos Trans R Soc Lond B Biol Sci., 353*(1370), 831-837.

Wei, X., Yang, X., Han, Z. P., Qu, F. F., Shao, L. & Shi, Y. F. (2013). Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacologica Sinica, 34*, 747–754.

Weissman, I. L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *SCIENCE,* 287(5457), 1442-1446.

Wise, G. E., Frazier-Bowers, S., & D'Souza, R. N. (2002). Cellular, Molecular, And Genetic Determinants Of Tooth Eruption. *Crit. Rev. Oral Biol. Med, 13*, 323-334.

Xiao, L. & Tsutsui, T. (2013). Human dental mesenchymal stem cells and neural regeneration. *Human Cell, 26(3),* 91–96.

Yamaza, T., Kentaro, A., Chen, C., Liu, Y., Shi, Y., Gronthos, S., Wang, S & Shi, S. (2010). Immunomodulatory properties of stem cells from human exfoliated deciduous teeth. *Stem Cell Research and Therapy, 1*(1), 5.

Yildirim, S., Zibandeh, N., Genc, D., Ozcan, E. M., Goker, K & Akkoc, T. (2016). The Comparison of the Immunologic Properties of Stem Cells Isolated from Human Exfoliated Deciduous Teeth, Dental Pulp, and Dental Follicles. *Stem Cells International, 2016,* doi: 10.1155/2016/4682875

Yoshida, Y. & Yamanaka, S. (2011). iPS cells: a source of cardiac regeneration. *J Mol Cell Cardiol 50*(2):327-32

Yu, S., Diao, S., Wang, J. S., Ding, G., Yang, D. M. & Fan, Z. P. (2014). Comparative Analysis of Proliferation and Differentiation Potentials of Stem Cells from Inflamed Pulp of Deciduous Teeth and Stem Cells from Exfoliated Deciduous Teeth. *BioMed Research International, (2014)*, doi: 10.1155/2014/930907

Zhao, Q. J., Ren, H. Y. & Han, Z. C. (2016). Mesenchymal stem cells: Immunomodulatory capability and clinical potential in immune diseases. *Journal of Cellular Immunotherapy 2*(1), 3-20.

University of Malaya

APPENDIX A : LIST OF PUBLICATIONS

1 Govindasamy V, Abdullah AN, Sainik Ronald V, **Musa S**, Che Ab. Aziz ZA, Zain RB, Totey S, Bhonde RR, Abu Kasim NH. Inherent Differential Propensity of Dental Pulp Stem Cells Derived from Human Deciduous and Permanent Teeth. Journal of Endodontics. 2010, 36(9):1504-1515

2 Vasanthan P, Gnanasegaran N, Govindasamy V, Abdullah AN, Jayaraman P, Ronald VS, **Musa S**, Kasim NHA: Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. Biochemical Engineering Journal. 2014, 88:142-153

3 Jayaraman P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Vasanthan P, **Musa S**, Kasim NH. Expression patterns of immune genes in long-term cultured dental stem cells. Clin Oral Investig. 2016, 20(1):109-116

4 Yazid FB, Gnanasegaran N, Kunasekaran W, Govindasamy V, **Musa S**: Comparison of immunomodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. Clinical Oral Investig. 2014, 18:2103-2112

5 Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, **Musa S**, Abu Kasim NH: Differential expression of basal microRNAs' patterns in human dental pulp stem cells. J Cell Mol Med. 2015, 19(3):566-580

6 Xin LZ, Govindasamy V, **Musa S**, Abu Kasim NH: Dental stem cells as an alternative source for cardiac regeneration. Medical Hypotheses. 2013, 81(4):704-706

7 Pukana Jayaraman, Prakash Nathan, Punitha Vasanthan, **Sabri Musa** and Vijayendran Govindasamy. Stem cells conditioned medium: a new approach to skin wound healing management. Cell Biol Int 37 (2013) 1122–1128

University of Malaya