POTENTIAL OF HUMAN DECIDUOUS PERIODONTAL LIGAMENT STEM CELLS FOR USE IN HEPATIC LINEAGE AND THEIR EXOSOME APPLICATION ON HEPATIC INJURIES

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **PUNITHA R. VASANTHAN**

Registration/Matric No: **DHA120019**

Name of Degree: **DOCTOR OF PHILOSOPHY**

Title of Thesis : **POTENTIAL OF HUMAN DECIDUOUS PERIODONTAL LIGAMENT STEM CELLS FOR USE IN HEPATIC LINEAGE AND THEIR EXOSOMES APPLICATION ON HEPATIC INJURIES**

Field of Study: **REGENERATIVE DENTISTRY**

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ABSTRACT

Introduction: Human deciduous periodontal ligament stem cells represent as another source of stem cells from the dental origin. These cells were shown to have multi-potential attributes and also self renewing ability. These easily accessible stem cells candidate could also resolve issues concerning ethical and compatability for the use in regenerative purposes. However, their efficiency towards hepatic lineage differentiation has not been addressed so far. Meanwhile, the main contributor for regenerative properties were supposedly mediated by soluble factors and extracellular vesicles such as exosomes secreted by cells. **Objectives:** The aims of this study are to isolate, characterize and also to investigate the propensity of stem cells derived from periodontal ligament towards hepatic lineage. Besides that, the emergence of cell free based treatment have prompted the study on exosomes derived from periodontal ligament stem cells and their relevance in hepatic injury. **Methods:** Periodontal stem cells derived from human deciduous tooth were isolated and characterized using the gold standard characterization method. Differentiation of periodontal ligament stem cells into hepatocyte-like cells were conducted by the exposure of two phases media induction. First phase was performed in the presence of Hepatocyte growth factors and Nicotinamide to induce a definitive endoderm formation. In the subsequent phase, the cells were treated with Oncostatin M, Dexamethosone and Insulin Transferrin to generate hepatocyte-like cells. Hepatic-related characters of the generated hepatocyte-like cells were determined at both mRNA and protein level. Furthermore, functional assay was conducted to examine the functionality of the hepatocyte-like cells. On the other hand, the extracellular vesicles components were characterized prior to the application into assisting in *in vitro* hepatic injury induced by hydrogen peroxide on HepG2 cells. Polymer based precipitation were employed to isolate

exosomes and subjected to assessments on surface markers and protein quantification. **Results:** Foremost changes observed in the generation of hepatocyte-like cells were the morphological features in which these cells were transformed from fibroblastic shape to polygonal shape. Temporal expression of hepatic markers ranging from early endodermal up to late markers were detected in the hepatocyte-like cells. Differentiated periodontal ligament stem cells exhibited functional features of a hepatocyte during and at the end of the experiment duration. Crucial hepatic markers such as glycogen storage, albumin and urea secretion were also shown. In addition, exosomes isolated from periodontal ligament stem cells displayed positive exosome markers with correlation between cell confluence and exosome release. As for the *in vitro* hepatic injury, exosome from periodontal ligament stem cells conditioned media showed reduction in terms of HepG2 cells injuries after being exposed to oxidative stress. **Conclusions:** These findings exhibited the ability of periodontal ligament stem cells to be directed into hepatic lineage fate contributing towards usage beyond tooth regeneration. Along with that, the exosomes from periodontal ligament stem cells displayed hepatoprotective attributes in terms of reduction of oxidative stress. Ultimately, these cells can be regarded as an alternative autologous source potential not only for stem cell based treatment but also as a compelling alternative for non viable or cell free treatment in liver diseases.

Keywords: periodontal ligament, stem cells, hepatocytes, exosomes

ABSTRAK

Pengenalan: Sel stem dari sumber ligamen periodontal gigi susu merupakan salah satu daripada pelbagai sel stem yang boleh diperolehi secara mudah. Sel-sel ini telah pun diiktirafkan untuk mempunyai pelbagai potensi dalam kegunaan terapi penjanaan semula berasaskan sel. Kegunaan sel in bukan sekadar dalam sektor pergigian tetapi juga dalam sektor-sektor lain. Namun, pengunaan sel stem ini ke arah sel hepatik belum diterokai sehingga kini. Tambahan pula, kebanyakan terapi penjanaan semula dikatakan berdasarkan faktor-faktor yang dikeluarkan dari sel stem seperti bahan boleh larut dan '*extracellular vesicles' exosome*. **Objektif:** Pertama, sel stem telah diperolehi daripada sumber ligamen periodontal sebelum disiasat kompetensi sel stem tersebut ke arah sel hepatik iaitu sel hati. Kedua, untuk mengekstrakkan dan menilai *exosome* dari sel stem ligamen periodontal serta mengunakan *exosome* tersebut untuk aplikasi kecederaan hepatic secara *in vitro*. **Kaedah:** Sel stem dari ligamen periodontal gigi susu digunakan sebagai sumber sel stem dalam penyelidikan ini. Keupayaan sel stem dari ligamen periodontal gigi susu untuk menjadi sel hepatik dinilai setelah dibiarkan dalam dua media induksi berasingan. Media pertama yang digunakan mengandungi faktor *Hepatocyte Growth* dan *Nicotinamide* untuk membolehkan pembentukan sel *definitive endoderm.* Selepas itu, sel-sel tersebut dibiarkan dalam media yang kedua mengandungi *Oncostatin M, Dexamethasone* dan *Insulin Transferrin* bagi membentukkan sel hepatik yang seakan. Seterusnya sel-sel yang terbentuk akan dinilai dari segi *mRNA* dan *Protein* untuk memastikan sel tersebut mempunyai unsur-unsur seperti sel hepatik benar. Selain itu, keberkesanan pembentukan sel seperti hepatik dinilai dari segi fungsinya juga. Tambahan pula, salah satu komponen *extracellular vesicle* iaitu *exosome* juga dinilai dari segi fizikal sebelum digunakan untuk aplikasi kecederaan hepatik dari sel HepG2. *Polymer based*

precipitation merupakan kaedah yang digunakan untuk isolasikan *exosome* dari sel stem ligamen periodontal. **Keputusan:** Perkara yang pertama dilihat dalam pembentukan sel hepatik adalah perubahan morfologi dari bentuk *fibroblast* kepada bentuk *polygonal*. Expresi gen yang berkaitan dalam sel hepatik juga diperhatikan dari permulaan sehingga pembentukan sel hepatik. Selain itu, fungsi biologi penting sel hepatik seperti pemprosesan *albumin*, pemyimpanan *glycogen* dan juga sekresi urea juga diperhatikan. Dalam faktor penyelidikan *exosome* pula, diperhatikan ada marker yang positif dan juga hubungan antara extraksi *exosome* dan kepadatan sel. Tambahan pula, *exosome* dari sel stem menunjukan kebolehan untuk melindungi HepG2 dalam respons terhadap toksik daripada hidrogen peroksida. **Kesimpulan:** Segala penemuan dalam penyelidikan ini menunjukan keupayaan sel stem dari ligamen periodontal untuk bertukar untuk membentuk sel seakan hepatic. Ini secara langsung membolehkan sel-sel ini digunakan untuk terapi penjanaan semula dalam sektor rawatan penyakit berkaitan degeneratif hati. Namun, penyelidikan yang lebih lanjut diperlukan dalam *exosome* dari sel stem ligamen periodontal untuk membolehkan pengunaannya dalam terapi penjanaan semula.

Kata kunci: ligament periodontal, sel stem, hepatocyte, exosomes

ACKNOWLEDGEMENTS

Completion of this dissertation marks another important journey in my life. First and foremost, I would like to thank my supervisors **Prof. Dr. Sabri Musa**, **Prof Dr. Noor Hayaty Abu Kassim** and **Dr. Gokula Mohan** for their continuous support and advice. Thank you for generously providing me the opportunity to be part of your research team, freedom to shape my research works and unlimited courage throughout my candidature. The enthusiasm, passion and energy they have for research was contagious and motivational to me throughout my PhD pursuit. In addition, I would also thank Hygieia Innovation Sdn Bhd, for the contribution in terms of cGMP certified laboratory services. My biggest gratitude goes to my fellow lab mates, especially to **Pukana, Ashley, Loo Zhang Xin, Nazmul** and **Wijenthiran** for the help and encouragement given to me to overcome obstacles, for being there all the time with me, through ups and downs. Thank you for the friendship and joy that we have shared together. My appreciation also goes to all the RedReg (Regenerative Dentistry Group) members, supporting staffs in Paediatric Dentistry and Orthodontics department and Postgraduate administrative department for their kind assistance. Special thanks to **Faculty of Dentistry** for enrolling me as a postgraduate student and Bright Sparks Unit (**Bright Sparks Scholarship Program**) for the financial support. This research was funded by University of Malaya, **High Impact Research-Ministry of Higher Education**, Malaysia (UM.C/HIR/MOHE/DENT/02). Last but not least, I would like to express my deepest gratitude and dedicate this thesis to my husband **R. Sritheran** and daughter **Sri Meishaa** who stood by as the main pillar and also beloved immediate and in law family members for their love and encouragement which gave me the strength to surmount all challenges.

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LIST OF ABBREVIATIONS

MSC	: mesenchymal stem cell
SHED	: dental pulp stem cell from deciduous tooth
PDLSC	: periodontal ligament stem cell
DPSC	: dental pulp stem cell from permanent tooth
ESC	: embryonic stem cell
iPSC	: induced pluripotent stem cell
SCAP	: stem cell from apical papilla
ASC	: adult stem cell
HSC	: hematopoetic cell
DSC	: dental stem cell
FBS	: fetal bovine serum
HPL	: human platelet lysate
EV	: extracellular vesicles
ICM	: inner cell mass
OCT4	: octamer binding transcription factor 4
bFGF	: basic fibroblast growth factor
EMT	: epithelial to mesenchymal transition
CCl_4	: carbon tetrachloride
H_2O_2	: hydrogen peroxide

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Plentiful research has been conducted on the promising potential of mesenchymal stem cells (MSCs) in tissue repair and regeneration due to their inherent ability to self-renew and the possibilities they present in the area of multi-lineage differentiation (Wei et al., 2013; Han et al., 2019). In the context of cellular therapy for liver diseases, MSCs hold a promising future in producing hepatocyte-like cells and are being regarded worldwide as an alternative to orthotropic liver and/or hepatocytes transplantation (Peleman et al. 1987; Muraca et al. 2002; Dhawan et al. 2004; Stephenne et al. 2006; Kholodenko and Yarygin, 2017). Major issues such as the shortage of donor, lack of availability and the need for invasive methods have encouraged researchers to exploit the uses of MSCs. These synergize with snowballing evidence in the literature that shows the production of hepatocyte-like cells are morphologically as well as functionally comparable to primary hepatocytes from numerous sources of MSCs including 'off the shelf' bone marrow (Pournasr et al. 2011), adipose tissue (Yin et al. 2015), placental (Tahan and Tahan., 2014), and umbilical cord (Campard et al. 2008; Zhou et al., 2014; Yin et al., 2019).

Following closely behind the next generation of MSCs therapy are those of dental origin (Huang et al., 2009; Estrela et al., 2011; Sharpe, 2016). Since the discovery of the first population of dental derived stem cells termed dental pulp stem cells (DPSCs) by Gronthos et al (2000), extensive knowledge on their dynamic features and functions have been obtained. Subsequently, various dental derived stem cells have been isolated such as dental pulp from exfoliated deciduous teeth (Muira et al., 2003), periodontal ligament (Seo et al., 2004), apical papilla, dental follicle (Morsczeck et al., 2005) and many others.

Although many source dependent differences in MSCs properties are being exploited for applications across the breadth of regenerative medicine and in particular for liver diseases, this innovative source have gained much attention because of their convenient harvesting procedures, excellent proliferation and differentiation capacity with the foremost advantage of no ethical restriction. These include Ishkitiev et al. (2012) who were able to obtain high purity hepatocyte-like cells expressing hepatic markers from dental pulp stem cells and most recently, Vasanthan et al. (2014) who demonstrate that dental pulp stem cells from deciduous teeth (SHEDs) were able to differentiate into hepatic lineage cells in either fetal bovine serum (FBS) or human platelet lysate (HPL) culture conditions.

Moreover, Seo et al. (2004) revealed the existence of stem cells in geographic proximity to dental pulp stem cells known as periodontal ligament stem cells (PDLSCs). These tissues are relatively obtained during the same course of harvesting dental pulp either via exfoliated or extracted tooth except for the area of isolation that differs, thus providing the usage of PDLSCs. Concomitantly, these cells were found to have MSCs properties for example, their multi-potential aptitude to differentiate into various cell lineages including osteoblast-like cells, neural cells, adipocytes (Tomokiyo et al., 2008) and even retinal cells (Huang et al., 2013). Similarly, recent investigations revealed the capability of human permanent PDLSCs to differentiate into hepatic cells and insulin producing cells signifying their ability to differentiate into endodermal lineages (Kawanabe et al., 2010; Lee et al., 2014). In addition, evidence of deciduous cells with superior qualities may provide better ability as stem cells source.

In the effort to regenerate liver or hepatocytes during injury, one of the multiple functional roles of participating MSCs is their potential to differentiate into hepatocyte which then

allow the replacement of damaged cells (Liu et al., 2015). As much, these human deciduous cells could be considered as potential candidates for the stem cell-based treatment of liver diseases. In brief, this study investigates the appropriate merits of human deciduous periodontal ligament stem cells (PDLSCs-D) with respect to their propensity to differentiate into hepatocyte-like cells.

Nevertheless, other possible mechanisms that take place during MSCs mediated- liver regeneration are by promoting residual hepatocyte regeneration and the inhibition of hepatic stellate cells activation or apoptosis via paracrine activity (Berardis et al., 2015). As much, the MSCs-mediated regeneration paradigm has been shifting toward a secretome based paracrine activity rather than its cellular differentiation and engraftment. Almost all the secretome released by MSCs contain factors with immunoregulatory, anti-apoptotic, and trophic activities (Ranganath et al., 2012). Of particular interest are extracellular vesicles (EV), which could be classified as exosomes and microvesicles depending on their origin and size. Exosomes are membrane bound nano-sized vesicle with diameters ranging from 30–100 nm and originate from specialized intracellular compartments, known as multivesicular bodies, while microvesicles are larger particles (100–1000 nm) originating from plasma membranes (Raposo et al., 2013). Numerous studies have demonstrated the importance of exosomes and microvesicles in intercellular communication. These vesicles can transfer membrane receptors, proteins, RNA, microRNA, lipids, and even organelles (mitochondria) between cells (Yu et al., 2014). Accumulating evidences demonstrate that the cargo composition of these vesicles depends on the cell type and their physiological state; accordingly, EVs derived from MSCs may have comparable therapeutic potential as the MSCs themselves.

Bearing this in mind and under the scope of the present review, we will also address the potential of PDLSC-D derived exosomes on hepatic injuries. This may provide insights on and perspectives for the future development of MSCs secretome with respect to the release of exosomes and which would have certain advantages over injections or the administration of viable MSCs thus creating stem cell free therapy for liver regeneration.

1.2 Research questions

The main goals of this research are to study the possibilities of periodontal ligament stem cells as good candidates for the treatment of hepatic injuries. The following research questions are addressed:

i. Are stem cells derived from deciduous periodontal ligament a potential MSCs source?

ii. Are stem cells derived from deciduous periodontal ligament able to differentiate into hepatic-like cells?

iii. Do exosomes derived from periodontal ligament stem cells provide the potential for the cell-free therapy treatment for hepatic injuries?

1.3 Aims and objectives of the study

This study has four interrelated aims as outlined below:

Aim I: To obtain PDLSCs-D from primary source. The objectives were:

1. To isolate stem cells from human deciduous periodontal ligament
2. To characterize and verify the MSCs status of PDLSCs-D
3. To determine growth kinetics of PDLSCs-D

Aim II: To study the potential of PDLSCs-D differentiation into hepatic lineage. The objectives were:

1. To induce differentiation towards hepatic lineage using chemically defined media
2. To assess the morphology of the differentiated cells from PDLSCs-D
3. To ascertain gene expressions for hepatocytes of differentiated PDLSCs-D using real time polymerase chain reaction (qPCR) and immunocytochemistry techniques
4. To assess the functional properties of generated hepatocytes from differentiated PDLSCs-D

Aim III: To obtain exosomes derived from PDLSCs-D culture medium. The objectives were:

1. To isolate exosomes derived from PDLSCs-D
2. To quantify and characterize exosomes derived from PDLSCs-D
3. To determine the correlation between cell confluence and exosome release

Aim IV: To determine the efficacy of exosomes derived from PDLSCs-D in the treatment of *in vitro* hepatic injuries. The objectives were:

1. To create an *in vitro* model of hydrogen peroxide (H_2O_2) induced- hepatic injury using HepG2 cells
2. To optimize and evaluate the cytotoxicity of exosomes derived from PDLSCs-D
3. To investigate the potential of exosomes derived from PDLSCs-D in treating *in vitro* hepatic injuries

1.4 Organization of the thesis

The manuscript will elucidate the background of the current study, our aims, research questions and significance of the study prior to reviewing the available literary research context in the following chapter two. Chapter three briefly elaborates the properties of periodontal ligament stem cells using mesenchymal stem cells criteria. Chapter four explores the propensity of the mentioned cells towards hepatic lineage. Chapter five focuses on the exosomes derived from periodontal ligament stem cells followed by application of the exosomes in Chapter six. Finally, Chapter seven conclude the thesis with recommendation for future works.

CHAPTER 2: LITERATURE REVIEW

2.1 Stem cells

Since the first discovery of stem cells from mouse embryos in 1981 (Evans and Kaufman, 1981), more accurate studies were able to discover methods for separation or isolation of stem cells from human embryo (Thomson et al., 1998). Consequently, over the past decades, regenerative medicine field has benefited from the abundant findings in stem cell biology that laid to the provision of cell-based therapies. Emergence of stem cell-based therapy/ treatment have led to elaboration of novel ideas and concepts for various treatment for deliberating diseases and have been very promising since they aim at the full restoration of lost or damaged tissues simultaneously ensuring their functionality (Mahla, 2016). Stem cells have been brought into the regenerative limelight due to for the current scenario whereby patients with severe injuries or chronic diseases have to deal with undisputable transplantation demands from the donated tissues or organ. The above statements are directly attributed to the two backbone features of stem cells which are; potential to self-renew and to give rise to diverse form of cell types for tissue repair and regeneration (Weissman, 2000).

Stem cells which are well defined as undifferentiated form of cells in human can be categorised to that of their developmental potential or to the extent into which they can give rise to various cell type or better known as differentiation potential. A hierarchy of potential are categorised as totipotent, pluripotent, multipotent and unipotent by virtue of how primitive they are (Alison et al., 2002). A totipotent stem cell can differentiate into all cell types in the body including embryo and placenta in which a fertilised egg (zygote) is considered to be totipotent. As for pluripotent stem cell, it is derived from a totipotent stem cells that can make up the tissues from all three germ layers (ectoderm, endoderm, mesoderm) except embryo that give rise to placenta or other supporting tissues (Zhao et al., 2003). A multipotent stem cell can develop into a restricted subsets of cell type of a cell lineage. For an example, liver stem cells can form all cellular

components of a liver organ (Fausto, 2004). However, the use of the term 'multipotential' may be to some extent redundant if it is true, as it appears now to be, that certain adult stem cells, can differentiate into cells that arise from the three germ layers. Meanwhile unipotent stem cell exhibit contribution to one particular cell type, usually the same type of cell in which it was isolated from and considered to be terminally differentiated (Wagers and Weissman, 2004).

Nevertheless, terminology used to classify stem cells is rather perplexing therefore, most commonly stem cells are regarded based on their origin or location (Fortier, 2005). Conservatively, there have been four primary tissue sources to obtain human stem cells; embryo, fetus, neonatal and postnatal (adult stem cells) (Pera and Dottori, 2005). Hence, stem cells are broadly classified as embryonic stem cells (ESCs), adult stem cells (ASCs) and induced pluripotent stem cells (iPSCs) in which the first two groups occur naturally while iPSCs are generated through reprogramming of cells.

2.1.1 Embryonic stem cells

Embryonic stem cells (ESCs) are stated as the top of the hierarchical stem cell tree and are derived from inner cell masses (ICM) of mammalian blastocyst (Pera et al., 2000). Embryonic stem cells are obtained from zygote cell which is fertilized *in vitro* and usually is 4-5 days embryo that is in the form of a hollow ball called blastocyst. Blastocyst is composed of three parts: the trophoblast layer that is surrounding blastocyst, a hollow cavity inside the blastocyst and inner cell mass that changes to embryo. These cells are pluripotent cells in nature and can give rise up to 200 types of cells (Thomson et al., 1998). The stemness and trans-differentiation fate of these cells are governed by functional status of transcriptional factors such as Octamer binding transcription factor 4 (OCT4), Proto-oncogene (cMYC), Krupple-like factor family (KLF4), teratoma protein coding gene (NANOG), SRY-box transcription factor 2 (SOX2) and so forth (Thomson et al., 2011). During embryonic development, ICM develops into two distinct cell layers, the epiblast

and hypoblast. The hypoblast forms the yolk sac which becomes redundant in human while the latter differentiates into three primordial germ layers; ectoderm, mesoderm and endoderm. To date, tremendous studies have demonstrated the ability of ESCs to differentiate into a number of pathologically relevant cells, including neural cells (Dhara and Stice, 2008; Chuang et al., 2015), insulin-producing cells (Segev et al., 2004; Schiesser et al., 2014), hepatocyte-like cells (Cai et al., 2007; Palakkan et al., 2015) Although these cells represent potential inexhaustible source of somatic cells under given culture condition to differentiate, unfortunately, there are a lot of resistances to ESCs research. Most of it relies on the ethical issues either from the personal moral views or from religious stand points. Many people see the donating of the embryos to science as terminating a possible human life (Johnson, 2008; Einsedel et al., 2009, Hyun, 2010). Besides that, the propensity of developing teratomas has created a major drawback to their clinical use (Rohwedel et al., 2001).

2.1.2 Induced Pluripotent stem cells

The alternative candidate that might circumvent the ethical concerns and risk of rejection related to ESCs are the induced pluripotent stem cells (iPSCs). Indeed, iPSCs are embryonic – like cells derived from multipotent cells by forced expression of the functional pluripotent transcriptional factors (Takayashi and Yamanaka, 2006). Three main research streams involving nuclear transfer or better known as cloning (Gurdon, 1962), discovery of the 'master' transcriptional factors that regulates the fate of a given lineage (Schneuwly et al., 1987) and also the optimization of culture condition using specific key factors such as basic fibroblast growth factor (bFGF) to prolong the pluripotency state (Smith et al., 1988) gave an impetus for the discovery of iPSCs. Thus, numerous research work was explored in generating iPSCs by means of genetic reprogramming either via retroviruses or lentiviruses from terminally differentiated cells that aid in treating diseases. iPSCs revolutionized the regenerative medicine due to the fact its patient-specific nature and can be obtained from autologous tissues. Likewise, in the

endodermal lineage, great emphasis was given to hepatocyte differentiation (Si-Tayeb et al., 2010; Ma et al., 2013; Calabrese et al., 2019). iPSCs were also shown to convert into insulin-producing cells (Zhang et al., 2009; Pellegrini et al., 2015). On the other hand, ectodermal differentiation of iPSCs mainly targets neural lineage (Chambers et al., 2009; Denham and Dottori, 2011; Yuan et al., 2013). Studies have also demonstrated the ability of iPSCs to differentiate into cardiac cells (Ieda et al., 2010; Carpenter et al., 2012).

However, the shortcomings of iPSCs were shown in terms of high tumorigenic potential resulting from usage of cells containing viruses that causes genome integration and activation of oncogenic cMYC which is part of the pluripotent transcriptional factors (Okita et al., 2007; Unzu et al., 2016). Moreover, donor and cell type contribute to the development potential and differentiation capacity of iPSCs as most of the background mutation of parental cells account for genetic heterogeneity (Young et al., 2012). Regardless of the iPSCs technology, continuous menace of these cells in inducing genetic damage and the ever-present malignant cell growth have tapered the potential and the fate of these cells.

2.1.3 Adult stem cells

Development of multicellular organism has traditionally seen as an organized hierarchy system that culminates the formation of billions of cells with well-defined responsibilities. Generally, multipotent adult stem cells (ASCs) are seen to be at the second top of the hierarchic system followed by committed progenitor cells in the middle and lineage –restricted precursor cells that give rise to terminally differentiated cells being at the bottom (Young and Black, 2004). However, recent studies on ASCs plasticity contradict the dogma that the differentiation and commitment of adult stem cells are restricted to their own tissues. They are able to differentiate across boundaries of lineage, tissues and germ layer (Anderson et al., 2001). One of the most common ASCs are the hematopoietic stem cells (HSCs) (Metcalf, 2007), a well characterized

multipotent stem cell population that reside predominantly in bone marrow and differentiate into all lineages of mature blood cells (Kondo et al., 2003). These cells are responsible for the production of blood in a process called hematopoiesis. Another subpopulation found in bone marrow are the stromal cells which were designated as mesenchymal stem cells (MSCs) (Caplan, 1991) have been shown to differentiate into multiple mesoderm type cells (e.g. osteoblast, chondrocytes, adipocytes) (Pittenger et al., 1999; Jiang et al., 2002; Dominici et al., 2006; Bianco, 2008). Opportunely, MSCs with similar biological features to those derived from bone marrow have been isolated not only from other mesodermal origin as in adipose (Zuk et al., 2001; Romanov et al., 2005; Wystrychowski et al., 2016), muscles (Meregalli et al., 2011), heart (Beltrami et al., 2003; Chong et al., 2011) but also from non-mesodermal origin including brain (Paul et al., 2012; Appaix et al., 2014; Lojewski et al., 2015), liver (Herrera et al., 2006; Najimi et al., 2007; Wang et al., 2016), skin (Riekstina et al., 2008; Orciani and Primio, 2013), umbilical cord (Lee et al., 2004a; Choudhery et al., 2013) and many more. Hence, virtually all postnatal tissues with repair and/or regenerative capabilities is likely to harbour MSCs in each tissues specific are called ‘stem-cell niche’ (da Silva Meirelles, 2006). Moreover, they are able to differentiate into numerous cell types including myocyte (Narita et al., 2008; Ostrovidov et al., 2015), cardiomyocyte (Zhang et al., 2002; Jiang et al., 2016), neuronal (Anghileri et al., 2008; Pavlova et al., 2012), hepatic (Lee et al., 2004b; Snykers et al., 2011) , pancreatic (Timper et al., 2006; Wang et al., 2017).

In contrast with ESCs, these cells can be harvested from autologous tissues, thus overcoming the immunologic disparities associated with the expression of major histocompatibility complex (MHC) proteins in ESCs (Drukker et al., 2002; Grompe, 2002). In summary, the multilineage potential, ability to allude host’s immune rejection and their relative ease of isolation and expansion have taken MSCs to great strides forward in regenerative medicine field.

2.2 Dental derived mesenchymal stem cells (DSCs)

Most recent source of MSCs that have gained much attention are from the field of dentistry termed dental stem cells (DSCs). They are regarded as excellent source as they can be obtained non-invasively from the teeth that are indicated for extraction and discarded as medical waste without any ethical restriction (d'Aquino et al, 2009). To date, bountiful DSCs have been isolated and characterised (Liu et al., 2015) from various part of the tooth ever since the discovery of dental pulp stem cells by Gronthos et al., (2000). Theoretically, all the DSCs were originated from the neural crest-mesenchymal cells during tooth development (Zhang et al., 2006a; Takashima et al., 2007; Wang et al., 2012).

There are several types of dental mesenchymal stem cells that have been studied over the past few years and named after according to its tissues origin within a tooth including the dental follicle stem cells (DFSCs) that are derived from human third molar tooth (Handa et al., 2002; Morsczeck et al., 2005), dental pulp stem cells (DPSCs) from human permanent teeth (Gronthos et al., 2002), stem cells from human exfoliated deciduous teeth (SHED) (Miura et al., 2003) or human extracted deciduous teeth (SCD) (Govindasamy et al., 2010), periodontal ligament stem cells (PDLSCs-D) (Seo et al., 2004; Chen et al., 2006), stem cells from apical papilla (SCAP) (Sonoyama et al., 2008) and stem cells from gingival connective tissues (Mitrano et al., 2010). Although stem cells populations of dental origin are collectively referred as DSCs, not all dental MSCs are equal in terms of their phenotypes and functional properties (Volpani and Sharpe, 2013). In support of this notion, several attempts have prompted the usage of stem cells from periodontal ligament that might differs according to the isolation location in this study.

2.2.1 Periodontal Ligament stem cells (PDLSCs-D)

Periodontal ligament (PDL) are specialised connective tissues that connect tooth to the alveolar bone found embedded between cementum and alveolar bone socket (Beertsen et al., 1997) in the area surrounding root surfaces. These tissues have multitude role not only restricted to supporting teeth, contributing to tooth nutrition and homeostasis but also repair damaged tissue (Bartold et al., 2000; Shimono et al., 2003). These tissues are known to possess heterogeneous cell populations such as fibroblast, osteoclast, osteoblast, cementoblast, and epithelial cells (McCulloch and Bordin, 1991; Seo et al., 2004). Further investigation on the phenotypes in a PDL cell population (Nagatomo et al., 2006; Kim et al., 2012; Wang et al., 2012) found that the heterogeneous is due to the existence of small sub population of MSCs.

Periodontal ligament stem cells (PDLSCs-D) were first discovered by Seo et al., (2004) providing unique reservoir of mesenchymal stem cells (MSCs) from an easily accessible tissue source. PDLSCs-D were shown to have similar characteristics as dental pulp stem cells namely in terms of the plastic adherent features and colony forming units (Shi et al., 2005). Numerous research work has demonstrated the ability of PDLSCs-D differentiate into cells and tissues that are most likely related to periodontium (Liu et al., 2008; Ji et al., 2013). However, studies have proven that differentiation potential of PDLSCs-D are not restricted to the cells or tissue origin but were able to commit to other cells types such as adipocytes, chondrocytes and osteocytes (Gay et al., 2007; Zhu and Liang, 2015). PDLSCs-D have also shown to possess immunomodulatory properties comparable to BM-MSCs in which the low immunogenicity and immunosuppressive effects on T cells and B cells allow use of allogeneic PDLSCs-D (Wada et al., 2009).

2.3 Dental stem cells (DSCs); Beyond tooth regeneration

Majority of research on dental stem cells (DSCs) have been employing them into tooth regeneration mainly in the loss or damage dental and periodontal structures (Sloan and Smith, 2007; Feng, et al., 2011; Nakashima and Iohara, 2014; Sharpe, 2016). However substantial number of findings have garnered interest in application of these cells as a potential source for regeneration purpose beyond tooth structure (Park et al., 2016). Likewise, DSCs has been shown to differentiate into neuronal-like cells (Li et al., 2013; Chang et al., 2014; Gnanasegaran et al., 2017), retinal-like cells (Huang et al., 2013; Syed-Picard et al., 2014), pancreatic-like cells (Govindasamy et al., 2011), , hepatocytes-like cells (Iskhitiev et al., 2010; Vasanthan et al., 2014), cardiomyocytes-like cells (Loo et al., 2014). Ultimately, yielding impressive outcomes such as promote wound regeneration (Jayaraman et al., 2013) promote neuroregenerative, promote angiogenesis (Hilkens, 2014) and many more. The fact that these cells are readily available and fascinating benefit of granting autologous settings have made the headline in the arena of stem cell-based therapeutics. As in this research, liver injury related regeneration was kept as the main focus.

2.4 Liver regeneration

Liver being a major organ for metabolism and detoxification in the human body, have the ability to regenerate on its own through endogenous tissue-repairing activities (Taub, 2004; Ishibashi et al., 2009; Fausto, 2012). In the case of liver injury, the prominent mode of treatment is by the activation of liver progenitor cells residing within liver in which proliferation and trans-differentiation from epithelial to mesenchymal of the injured adult liver cells to hepatic mesenchyme (Choi and Diehl, 2009). As a final attempt, stem cells from bone marrow would actually move in to the liver injury site via portal and periportal as an intermediate cell population and eventually matures into hepatocytes (Grompe, 2003). Nevertheless, as an effect

of overwhelmed degree of injury inflicted due to various factors such as viruses, alcohol, metabolism and autoimmune diseases, liver loses its ability and proceed to chronic stage injury resulting in hepatocytes death. Cirrhosis is known as the common chronic condition wherein scar tissues replaces the healthy tissues. Ultimately, creating options like liver transplantation as the only definite solution to treat such conditions. Inopportunely, the latter is not applicable due to the scarcity of donor livers and complication due to long term immunosuppression (Zhang and Wang, 2013; Jadlowiec and Taner, 2016). In addition, hepatocytes transplantation has been proposed as alternative treatment however due to the similar reason as stated above, the applicability remains limited (Gramignoli et al., 2015). The emergence of other source cells besides intrahepatic cells, such as MSCs that can be differentiated into hepatocytes (Snyker et al., 2009; Kim et al., 2017; Kwak et al., 2018) have been assisting along the way.

2.5 Applications of MSCs in regenerative medicine associated with liver diseases

Tremendous amount of studies has been carried out in engaging various sources of MSCs in treating either liver fibrosis or liver cirrhosis (Wang et al., 2018). Likewise, MSCs derived from bone marrow (Pournasr et al., 2011), adipose (Zhang et al., 2014; Yin et al., 2015), umbilical cord (Tsai et al., 2009; Zhou et al., 2014) have been reported to develop hepatocytes and improve liver injury. Although, previously hepatocytes were obtained from endodermal cell differentiation, but existing studies have also proven the ability of non-endodermal cells to differentiate into hepatocytes as well. The above information corresponds well with the pioneering report by Iskhitiev et al., (2010) in generating hepatocytes from DPSCs as well as in SHEDs. Vasanthan et al., (2014) have also reported the ability of DPSCs differentiation using serum and human platelet lysate. This approach will prevent xenogeneic contamination during transplantation. Hence, DSCs as the most suitable candidate for cell-based therapy have provided an impetus to consider for the treatment of liver disease related regeneration due to the

fact of easily discarded for personal growth without any ethical issue protruding. Over the years, remarkable studies have been portraying the potential of DSCs in treating liver related injuries in *in vitro* and *in vivo* conditions (Ikeda et al., 2008; Yamaza et al., 2015; Ishkitiev et al., 2015; Hirata et al., 2016) by preventing liver fibrosis and increase level of albumin and bilirubin. However, source of DSCs is mainly focused on pulp attained stem cells and even though stem cells from other location such as PDLSCs-D considered to have similar properties still yet to be elucidated.

2.6 Challenges and drawbacks of MSCs applications as therapeutic agent

Current data and ongoing research suggest that MSCs based therapy may provide a fascinating approach in repairing defected tissues by means of transplantation of autologous or allogeneic cells. However, several key parameters need to be addressed in order to optimize through clinical research, mainly in terms of culture conditions, optimal transplantation route, coaxing cells to the right cell type, survival and engraftment of transplanted cells (Volarevic et al., 2014; Zhang et al., 2018). Initially, MSCs were often-cited on the ground wherein the cells augment the structure and function of the damaged and disease tissue via direct cell replacement (Yang et al., 2015). The regenerative potential of MSCs has previously been shown in animal studies, however, most studies have remained vague in identifying the cell populations post-transplantation and their direct distribution to tissue regeneration (Chen et al., 2015). However, it soon became apparent that MSCs engraftment and differentiation at the injury sites are very low and transient. The precise mechanisms underlying MSCs therapy for regeneration is still yet to be elucidated. This prompted to the paradigm shift from cell-based-regeneration via trans-differentiation mechanism to cell-free-based regeneration via paracrine mechanism (van Poll et al., 2008; Zhang et al., 2012; Xagorari et al., 2013; Liu et al., 2015).

2.7 MSCs secretome: Approach to enhance regenerative benefits of MSCs

In support to the cell free based regeneration via paracrine mechanism hypothesis, many studies have observed the use of secretome derived MSCs conditioned media for regenerative medicine. This secretome consists of special set of factors such as soluble proteins, free nucleic acid, lipid in extracellular vesicles (EV) that are secreted into the extracellular space and changes in response to fluctuations in various conditions (Yu et al., 2014; Vizoso et al., 2017). EV were first described by Pan and Johnstone in (1983) classified into exosomes, microvesicles and apoptotic body depending on their origin and size (Raposo et al., 2013). Of note, exosome once thought to be a 'trash bag' for cells to discard unwanted proteins became more prevalent for studies due to the fact of exerting similar functions in cell to cell communication as their derived cells. Eventually leading to research hypothesis, in which exosome is said to be the paracrine mediator for MSCs in regenerative medicine.

2.8 Exosomes: Biogenesis

Exosome which tend be homogenous can be distinguished from other subsets of EVs by the size, morphology and functions (Yu et al., 2014). Typically, exosome have diameter between 40 nm to 100 nm with density of 1.13 -1.19 g/ml in a sucrose solution and can be isolated by centrifugation at 100,000 x g (Petersen et al., 2015). Identification of exosomes are more easily conducted by looking into the cell surface markers such as CD9, CD81 and CD63. Generally, exosomes membrane is enriched with lipids such as cholesterol, ceramide and sphingolipids along with that are essential for the biogenesis of exosomes. Besides that, exosome also contains biological active molecules such as proteins, mRNAs and miRNAs. Their biogenesis are generally derived from endosomes that forms intraluminal vesicles (ILV) by inward budding of the plasma membrane into the lumen of late multivesicular bodies / endosomes. As a result of fusion of the multivesicular bodies with the delimiting plasma membrane, these ILVs are release by exocytosis and eventually enters the extracellular space containing matrix and body fluids

such as blood plasma, urine and saliva. These secreted ILVs containing cytoplasmic molecules such as proteins, mRNAs and miRNAs are referred as exosomes. Subsequently, exosomes may integrate with other cells by direct membrane fusion, endocytosis or cell-type specific phagocytosis (Raposo et al., 2013; Rani et al., 2015).

2.8.1 Properties of exosome derived from MSCs

Exosomes are known to release either constitutively or in a regulated manner with the most unique function which enables cell to cell communication in a widely separated location in the body (Vlassov et al., 2012). Exosomes roles are basically in a component-dependent manner implicated in physiological and pathological process. MSCs derived exosomes have been extensively explored due to the fact that they able to carry out functions similar to MSCs such as repairing damaged tissues, suppressing inflammatory responses and regulating immune systems.

One of the most crucial components of exosomes are the presence of clustering proteins mainly surface markers which are altered under various pathological conditions, thus being regarded as diagnostic biomarker (Raimando et al., 2011; Muller, 2012). Moreover, presences of proteasome from MSCs derived exosomes have been shown to regulate degradation of intracellular oxidatively damaged proteins that eventually contribute to cardioprotective activity (Lai et al., 2012). Although MSCs are known to express cytokines and growth factors, the proteins present in MSCs are lacking in the signalling peptides. However, the packaging of protein together with mRNA and miRNA in a membrane bound vesicles explains the method on how the MSCs exerts multiple role in biological process (Teixera et al., 2013; Phinney and Pittenger, 2017). As a consequence, components found in exosomes can modify the phenotype and / or the physiological state of the target / recipient cell by modulating cellular processes such as proliferation, differentiation and survival (Cocucci and Meldolesi, 2015). Moreover, the low

immunogenicity, long-half life in circulation and ability of cross the brain-blood barrier (Sun et al., 2010; Kalani et al., 2014) have brought exosomes derived from MSCs into the limelight for regenerative therapy in comparison to the MSCs themselves. Nevertheless, these mentioned roles of exosomes may vary in accordance to the origin of the cells, physiological or pathological state of the target / recipient cells and also the extracellular microenvironment all together.

2.8.2 Application of exosomes derived from MSCs in liver diseases

At present, few studies have elaborated the role of exosomes derived from MSCs in liver diseases. As reported by Li et al., (2013) umbilical cord MSCs derived exosomes were able to significantly ameliorate carbon tetrachloride (CCl_4) induced liver fibrosis by inhibiting epithelial to mesenchymal transition (EMT) and protecting hepatocytes. During drug-induced liver injury by acetaminophen or Hydrogen peroxide (H_2O_2), exosomes elicit hepatoprotective effects by suppressing hepatocyte apoptosis (Tan et al., 2014) *in vitro* and in vivo studies. Similarly, exosomes from human menstrual blood derived MSCs were able to promote anti-apoptotic activity in acute liver failure (Chen et al., 2017). Recently, Damania et al., (2018) demonstrated antiapoptotic and prosurvival effects along with antioxidant effects on *in vitro* and *in vivo* liver injury models. Results from the previous proof-of-concepts studies are very promising with revealing benefits of exosomes such as smaller and less complex, lower possibilities of immune rejection while devoid of viable cells (Ren et al., 2019).

On the basis of the above literature review, we proposed an ideal approach of using DSCs of particular interest the PDLSCs-D and as well as their exosomes for the relevance in regenerative medicine as in this study focusing on liver injury.

CHAPTER 3: ISOLATION AND CHARACTERISATION OF STEM CELLS FROM HUMAN DECIDUOUS PERIODONTAL LIGAMENT

3.1 Introduction

Human dental stem cells (HSCs), a subtype of adult stem cells have been progressively acknowledged in the field of regenerative medicine, with the publications about the subject increasing every year due to their technical and practical superiorities. Postnatal dental pulp stem cells (DPSCs) were the first population of stem cells derived from dental origin (Gronthos et al., 2000) followed by discovery of stem cells from exfoliated deciduous teeth (SHEDs) by Miura et al., (2003). A year later, Seo et al., (2004) isolated periodontal ligament stem cells (PDLSCs-D) using protocol similar to the one used to ascertain both DPSCs and SHEDs. The latter source of stem cells from dental origin obtained from deciduous teeth have been gaining attention for future therapies (Song et al., 2012). As these cells were characterised, they were found to be expressing similar characteristics as the bone marrow stem cell (BMMSCs) in that they can self-renew as well as differentiate into mesenchymal cell lineages (osteoblasts, adipocytes, and chondrocytes). Although these cells are known to be originated from neural crest, PDLSCs-D exhibit common properties of MSCs in cell morphology, differentiation potential and immune-modulatory ability (Wada et al., 2009). Basically, their original function is in development and the homeostasis of tooth structures, many applications of these cells in dentistry have aimed at tooth structure regeneration (Bansal et al., 2015). Studies have demonstrated the ability of PDLSCs-D for regeneration of periodontal complex (Zhu and Liang, 2015). However, the application in other than tooth structures has been attempted extensively (Park et al., 2016). These dental stem cells (DSCs) may represent a good alternative due to the ease with which they can be obtained and lack of

morbidity at the donor site. In addition, these inexhaustible proliferative stem cells which is a critical goal for stem cells research and therapy may provide innate benefit as an autologous source. Although they are collectively known as DSCs, not all dental MSCs are equal in terms of their phenotypes and functional properties (Volpani and Sharpe, 2013). On the basis of the International Society of Cellular Therapy (ISCT), minimal criteria of MSCs are plastic adherence, expression of specific surface antigen (Ag) and multi-differentiation potential (Dominici et al., 2006). Therefore, the main aim of this study is to isolate and characterise PDLSCs-D from primary source.

Experimental works were carried out to meet the following objectives:

Aim I: To obtain PDLSCs-D from primary source. The objectives were:

1. To isolate stem cells from human deciduous periodontal ligament
2. To characterize and verification of the MSCs status of PDLSCs-D
3. To determine growth kinetics of PDLSCs-D

3.2. Materials and Methods

3.2.1 Ethics approval for collection of human extracted deciduous teeth

Sample collections were conducted with prior written informed consent according to the guidelines set by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number: DF CD 1411/0087 (P) (Appendix A).

3.2.2 Media preparation

Different types of media and buffers are required for stem cell isolation, expansion and cryopreservation. Composition and procedure to prepare those media are described as following.

3.2.2.1 Preparation of transportation medium

Transportation medium is required to transport the extirpated dental periodontal ligament. Transportation medium was prepared using the Dulbecco's modified Eagle's Medium Knock-out (DMEM-KO) (Invitrogen, Carlsbad, CA, USA) supplemented with 1% Penicillin/Streptomycin (Invitrogen). The complete transportation medium was sterilised by filtration through a 0.2 μm membrane filter (Thermo Fisher, Scientific Inc, Waltham, MA) and stored at 4˚C until further use.

3.2.2.2 Preparation of washing buffer

Washing buffer was prepared by adding Dulbecco's Phosphate Buffered Saline (DPBS; - Ca^{2+}, -Mg^{2+}) (Invitrogen) supplemented with 0.5% Penicillin/Streptomycin (Invitrogen) in a 1:1 ratio. Prepared washing buffer was sterilised by filtration through a 0.2µm membrane filter (Thermo Scientific) and transferred into 1.5 ml centrifuge tube (Axygen, Union City, CA, USA). Washing buffer was always prepared freshly.

3.2.2.3 Preparation of culture medium

Periodontal ligament stem cells (PDLSCs-D) culture medium was prepared by using DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen) with the composition as shown in Table 3.1.

Table 3.1: Composition of culture media

Component	Manufacturer	Volume
Australian characterised Fetal bovine serum (FBS)	Hyclone, Thermo Fisher, Scientific Inc, Waltham, MA	50.0 ml
Penicillin/Streptomycin	Invitrogen	2.5 ml
Glutamax	Invitrogen	5.0 ml
DMEM-KO	Invitrogen	442.5 ml

3.2.2.4 Preparation of solution for tissue digestion

Tissues digestion solution is required to digest the tissue to isolate stem cells. For PDLSCs-D isolation, 1% Collagenase Type I (Gibco, Grand Island, NY, USA) was used by dissolving 100 mg of Collagenase Type I (Gibco) into 99 ml of DPBS (Gibco; -Ca^{2+},

-Mg^{2+}) containing 100 units/ml Penicillin/Streptomycin (Invitrogen). Prepared solution was then sterilised by filtration through 0.2µm membrane filter (Thermo Scientific) and stored at 4˚C until further use.

3.2.2.5 Preparation of freezing medium

Freezing medium was used for cryopreserving the stem cells. Freezing medium was prepared by mixing 90% of FBS (Thermo Scientific) with 10% of Dimethyl Sulphoxide (DMSO) (Sigma Aldrich, Steinhem, Germany). Prepared freezing medium was sterilised by filtration through 0.2 µm membrane filter (Thermo Scientific). Freezing medium was always prepared freshly.

3.2.3 Isolation of stem cells from human deciduous periodontal ligament (PDLSCs-D)

Samples of periodontal ligament (PDL) from deciduous teeth were obtained from healthy donors (n=3, 4 to 8 years old) who undergone a routine extraction at Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya.

The PDLSCs-D were isolated according to the procedure described by Osman et al., (2013) in this study. The tooth surface was cleaned with Povidone-iodine (Sigma Aldrich, St. Louis, MO, USA) and the PDL tissues were gently scraped from the root surface using a sterile scalpel. The PDL tissues were then gently transferred using sterile tweezers into a fresh transportation medium. In brief, the steps for sample collection of PDL were illustrated in Figure 3.1.

The PDL tissues were washed in a washing buffer for three times. Mechanical destruction was performed by mincing tissues into small fragments using sterile scissors and followed by enzymatic destruction by immersion of the PDL tissues into 1% of Collagenase Type I (Gibco) and incubated for 40 minutes at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. After collagenase treatment, the mixture was neutralized with 5 ml of culture media and the entire mixture were transferred into 15 ml centrifuge tube (BD Bioscience, Franklin Lakes, NJ, USA) prior to centrifugation at 25 ºC for 6 minutes at 1250 rpm. The supernatant was discarded, and the pellet was gently re-suspended with 1 ml of culture media, seeded into a new T-25 cm^2 culture flask (BD Pharmingen, San Diego, CA, USA) pre-loaded with 5 ml of culture media. The culture flask was incubated at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Non-adherent cells were removed 48 hours after the initial plating. Culture medium was replenished every three days until cells reached 80 – 90% confluence. The morphology of the cells was captured using an inverted microscope (Olympus model XC-50, Tokyo, Japan).

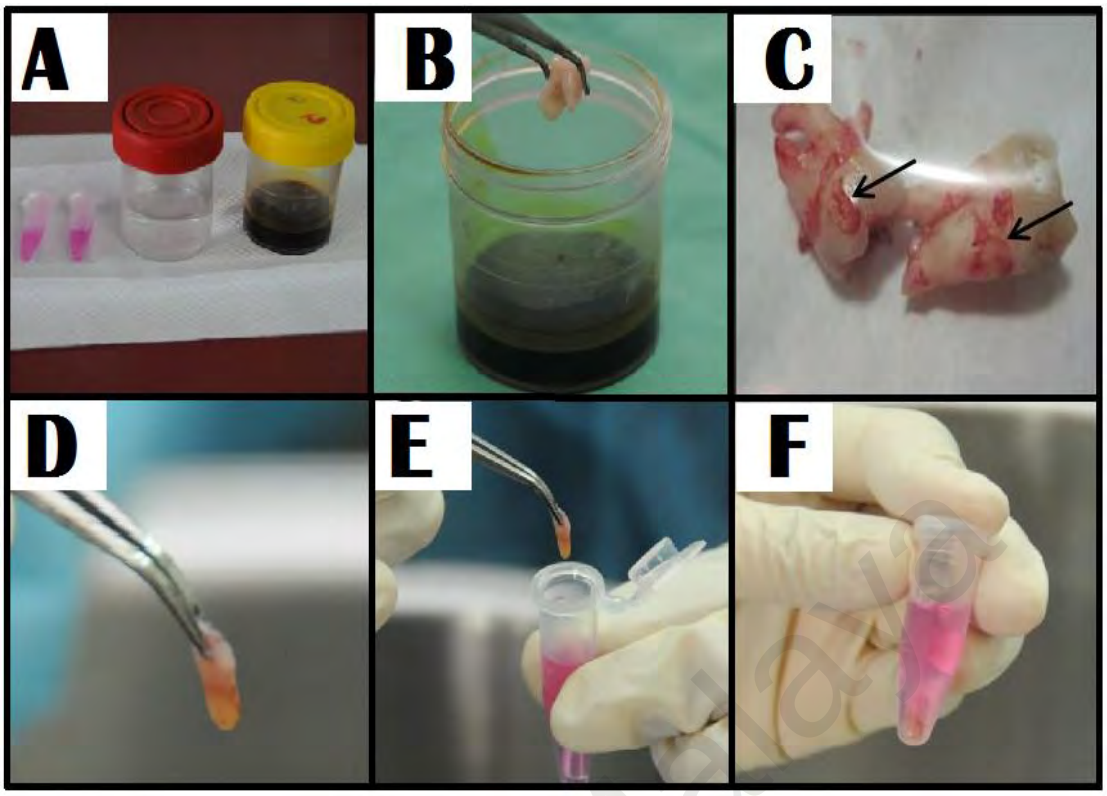


Figure 3.1: Isolation of periodontal ligament. A) from left to right; transportation medium, washing buffer and Povidone-iodine (PVP-I) solution. B) Cleaning of tooth surface with PVP-I solution. C) Periodontal ligament tissues (PDL). D) Scrapped PDL tissue with tweezers. E) PDL tissue transferred to transportation medium. F) Immersed PDL tissue in transportation medium.

3.2.4 Expansion and cryopreservation of PDLSCs-D

Once cells reached 80 – 90 % confluence, cells were rinsed twice using 5 ml DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove culture media and debris. Subsequently, 2 ml of 0.05% EDTA-Trypsin (Invitrogen) were added to into the culture flask and incubated at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2 to detach cells via enzymatic reaction. Immediately after complete detachment, 8 ml of culture media were added to neutralise as to avoid cell apoptosis. Next, the cells in the culture media were transferred into 15 ml centrifuge tube (Axygen) and were centrifuged at 1250 rpm for 6 minutes at 25 ºC in which supernatant was discarded and cells re-suspended into culture media. Aliquot of solution was used to calculate total cell count. A total of 10 µl from the solution was mixed with 90 µl of Trypan Blue stain (Sigma Aldrich) and 10 µl of the mixture was transferred to a haemocytometer (Sigma Aldrich) and total cell count was determined by viewing under microscope (Olympus). Cells not stained with Trypan Blue stain were counted based on the following formula: [Number of cells x dilution factor x 10^4 x volume of media used for re-suspending the cells] /4. After cell count was conducted, some of the newly harvested cells were re-suspended in 7 ml of culture media, seeded into a new T-25 cm^2 culture flask (BD Pharmingen) and incubated at 37 ºC in humidified atmosphere of 95% air and 5% CO_2 at a cell seeding density of 1000 cells/ cm^2. The culture media was replenished every three days until cells reached 80 – 90% confluence for expansion of subsequent study. Meanwhile the remaining cells were frozen with approximately 1 million cells for 1 ml of freezing media. The frozen cells with freezing solution were then transferred into cryovial, labelled and kept in vapour phase liquid nitrogen.

3.2.5 Identification of MSC-like properties of stem cells from human deciduous periodontal ligament (PDLSCs-D)

According to the International Society for Cellular Therapy (ISCT) MSC should have adherence to plastic, specific surface antigen (Ag) expression, and multipotent differentiation potential (Dominici et al., 2006). In addition, PDLSCs-D- were also assessed selected of MSCs markers using primary antibodies.

3.2.5.1 Plastic adherence of stem cells from PDLSCs-D

Plastic adherence of PDLSc was confirmed by viewing the culture flask under inverted microscope (Olympus).

3.2.5.2 Specific cell surface markers analysis on PDLSCs-D

Immunophenotyping of PDLSCs-D were examined by using flow cytometry at passage 3. On reaching 80% confluence, the PDLSCs-D were harvested using 0.05% EDTA- trypsin (Invitrogen) and re-suspended into 10 ml DPBS (-Ca^{2+}, -Mg^{2+}) (Invitrogen). The cell suspensions were centrifuged at 1250 rpm for 6 minutes at 25°C. Next, the supernatant was discarded, and the cell pellet was re-suspended again with DPBS (-Ca^{2+}, -Mg^{2+}) (Invitrogen) at a cell density of 1.5 x 10^6 cells / ml. A total of 200 µl of DPBS (-Ca^{2+}, -Mg^{2+}) (Invitrogen), approximately 1 x 10^5 cells were aliquot into several test tubes. The cells were then mixed with labelled antibodies and incubated for 1 hour at 37°C under dark condition. Then, the cells were washed with 2 ml of DPBS (-Ca^{2+}, -Mg^{2+}) (Invitrogen). The antibodies used to mark the cell surface epitopes are listed in Table 3.2. Excess antibodies were removed by washing with DPBS. 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.

Table 3.2: Antibodies used for cell surface markers analysis

Cell surface antigen	Conjugated dye	Volume used	Manufacturer
CD34	Phycoerythrin (PE)	20 µl	BD Pharmingen
CD44	Phycoerythrin (PE)	20 µl	BD Pharmingen
CD45	Phycoerythrin (PE)	20 µl	BD Pharmingen
CD105	Phycoerythrin (PE)	20 µl	BD Pharmingen
HLA-DR	Phycoerythrin (PE)	20 µl	BD Pharmingen
CD73	Fluoroisothycyanate (FITC)	20 µl	BD Pharmingen
CD90	Fluoroisothycyanate (FITC)	20 µl	BD Pharmingen
CD166	Fluoroisothycyanate (FITC)	20 µl	BD Pharmingen

3.2.5.3 Multi-lineage differentiation of PDLSCs-D

PDLSCs-D (Section 3.2) at passage 3 (P3) were culture at 1000 cells/cm^2 cell density in a 6- wells plate (BD Bioscience) and grown to a confluence in humidified atmosphere of 95% air and 5% CO_2 at 37°C. Once the cells attained 80% confluence, the culture medium were then changed to specific induction media for adipogenic, chondrogenic and osteogenic differentiation accordingly.

3.2.5.3.1 Adipogenic differentiation

PDLSCs-D were exposed to adipogenic induction medium that contains DMEM-KO (Invitrogen), 10% 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-isobuthyl-1-methyxanthine

(Sigma Aldrich) for 21 days, with medium changed three times per week for adipogenic differentiation.

Adipogenic differentiation of PDLSCs-D were visualised using Oil Red O (Sigma Aldrich) staining procedure. Cell fixation were done using 4% parafolmaldehyde (PFA) (Sigma Aldrich) for 30 minutes at (23 ± 1°C) and rinsed with 1 ml of DPBS (-Ca^{2+}, -Mg^{2+}) (Invitrogen) twice. Next, the differentiated cells were washed twice using diethylpyrocarbonate-treated water (DEPC-treated water, Merck, Whitehouse Station, NJ, USA). Oil Red O working solution was gently added and incubation was done for 50 minutes at (23 ± 1°C). The stained solution was carefully removed and rinsed with DEPC-treated water for three times. The nuclei were stained with hematoxylin (Sigma Aldrich) for 10 minutes. The formation of lipid droplet was observed as red droplet under the microscope. The preparation of 4% PFA and Oil Red O working solution is in Appendix

3.2.5.3.2 Chondrogenic differentiation

Chondrogenic differentiation of PDLSCs-D were stimulated using induction media prepared by adding DMEM-KO (Invitrogen) with 10% 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), 1.0 µg/ml Dexamethasone Sodium Phosphate (Sigma Aldrich) and 1.25 µg/ml Bovine Serum Albumin (BSA, Sigma Aldrich). PDLSCs-D with 80% confluence were subjected to chondrogenic induction media for 21 days.

Alcian blue staining (Sigma Aldrich) was used to ascertain the chondrogenic differentiation of PDLSCs-D. PDLSCs-D were washed twice using DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and fixed using 4% PFA (Sigma Aldrich) for 10 minutes at (23 ±

1°C). A total of 1 ml of Alcian Blue working solution was added into the well and PDLSCs-D were incubated for 30 minutes at room temperature (23 ± 1°C). The wells were washed three times using tap water followed by another 2 times wash using ddH_2O. The blue stained cells were observed under microscope for the presence of sulphate proteoglycan accumulation which will be stained in blue colour. The preparation for Alcian Blue working solution is described in Appendix

3.2.5.3.3 Osteogenic differentiation

For osteogenic differentiation, the culture media for PDLSCs-D was replaced with osteogenic induction media containing DMEM-KO (Invitrogen), 10% 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-glycero-2-phosphate (Sigma Aldrich) for 21 days. The induction media was changed twice a week. Osteogenesis of PDLSCs-D was evaluated using Von Kossa staining.

As to determine calcium mineralisation, PDLSCs-D were fixed using 4% PFA (Sigma Aldrich) for 15 minutes at (23 ± 1°C) and rinsed once with 5 ml of ddH_2O. Differentiated PDLSCs-D were then incubated with 1% silver nitrate (Sigma Aldrich) for 60 minutes under a bright light. Staining procedures was completed by rinsing once with ddH_2O and then observed under microscope (Olympus). The calcium mineralization will be observed as black coloured clump or precipitation. The preparation of 1% silver nitrate solution are shown in Appendix.

3.2.5.4 Immunocytochemistry on selected MSCs markers

To analyses presence of selected MSCs markers, PDLSCs-D 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 Octamer-binding transcription factor 4 (OCT4) (mouse, Abcam), SRY Box 2 (SOX2) (mouse, Abcam), and Stage specific embryonic antigen (SSE4) (mouse, Abcam) with dilution ratio of 1: 400 for all samples were incubated overnight at 4°C, washed with DPBS (Invitrogen), and then incubated with secondary antibody, fluoro isothiocyanate [FITC]-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 a Nikon-Eclipse-90i microscope (Nikon, Tokyo, Japan, http:// www.nikon.com).

3.2.6 Cell Proliferation and Doubling Time

The proliferation rate of PDLSCs-D cultured in media were evaluated in terms of population doubling time (PDT). Cells (n=3) at 70-80% confluence were trypsinised (Invitrogen) and live cells were counted with Trypan Blue (Sigma Aldrich) dye exclusion method. A total of 1000 cell/ cm^2 of PDLSCs-D were cultured into a new T-25 cm^2 flask and incubated at 37˚C humidified atmosphere of 95% air and 5% CO_2 until 90% confluence. Their confluence was determined under microscope (Olympus). Three independent replicates were performed for each passage and time pointed for a total of five passages. Data were analysed and plotted using Microsoft Excel. Growth kinetics was analysed by calculating population doubling (PD) and population doubling time

(PDT) which were calculated using the following equation respectively;

$$X = \log_{10}(N_H) - \log_{10}(N_I)] / \log_{10}(2)$$

Where X = population doublings, NI =Inoculum cell number, and NH = cell harvest number.

$$T2 = \frac{\Delta t}{\log_2(\frac{\Delta N}{N_0} + 1)}$$

Where N_0 is the number of cells at the beginning of observation, ΔN is the increase in the number of cells during the period of time of the length Δ t, and *T2* is the doubling time (Korzynska and Zychowicz, 2008). To obtain cumulative population doubling (CPD), the PD at the current passage was added to the PD of previous passages (Cristofalo, Allen, Pignolo, Martin and Beck, 1998; Li et al., 2015). Cumulative cell number was then calculated by multiplying the initial seeding cell number of 100,000 cells at passage 1 by the fold expansion at each passage up to 5 (Dolley-Sonnevillem Romeo and Melkoumian, 2013).

3.2.7 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, U.S.A.). The significance level was set at P=0.05. Tukey Post-hoc multiple comparisons were carried out to determine the differences between the groups.

3.3 Results

3.3.1 Isolation of stem cells from periodontal ligament

Preliminary outgrowth of spindle shaped PDLSCs-D were observed four days after onset of culture and shown to form homogenous monolayer on day seven as shown in Fig. 3.2. Cells showed migration from the primary periodontal ligament tissues towards the media. The PDLSCs-D were shown to cover approximately 80% of the flasks at day 14.

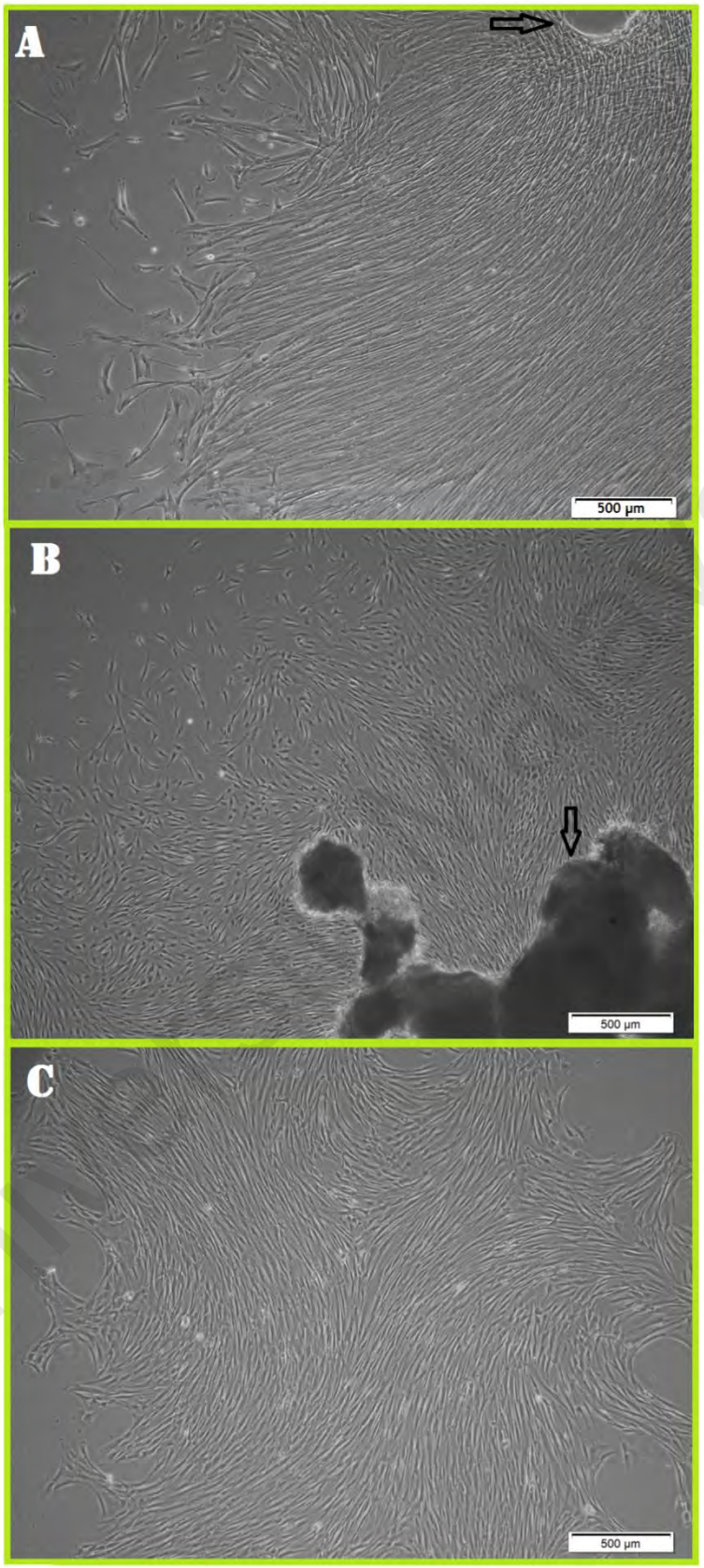


Figure 3.2: Micrograph showing outgrown of PDLSCs-D from primary periodontal ligament tissues as shown by arrows in A and B. Confluence PDLSCs-D shown in C. Scale bars: 500µm

3.3.2 MSCs-like properties of PDLSCs-D isolated from human deciduous periodontal ligament

3.3.2.1 Morphology PDLSCs-D

Based on the observation, tissues from periodontal ligament adhere to the culture flasks within 24 hrs of culture. Cells observed under the microscope (Olympus) (Fig. 3.3) were found to be in spindle-shaped and in homogenous monolayer thus confirming its plastic adhering capability.

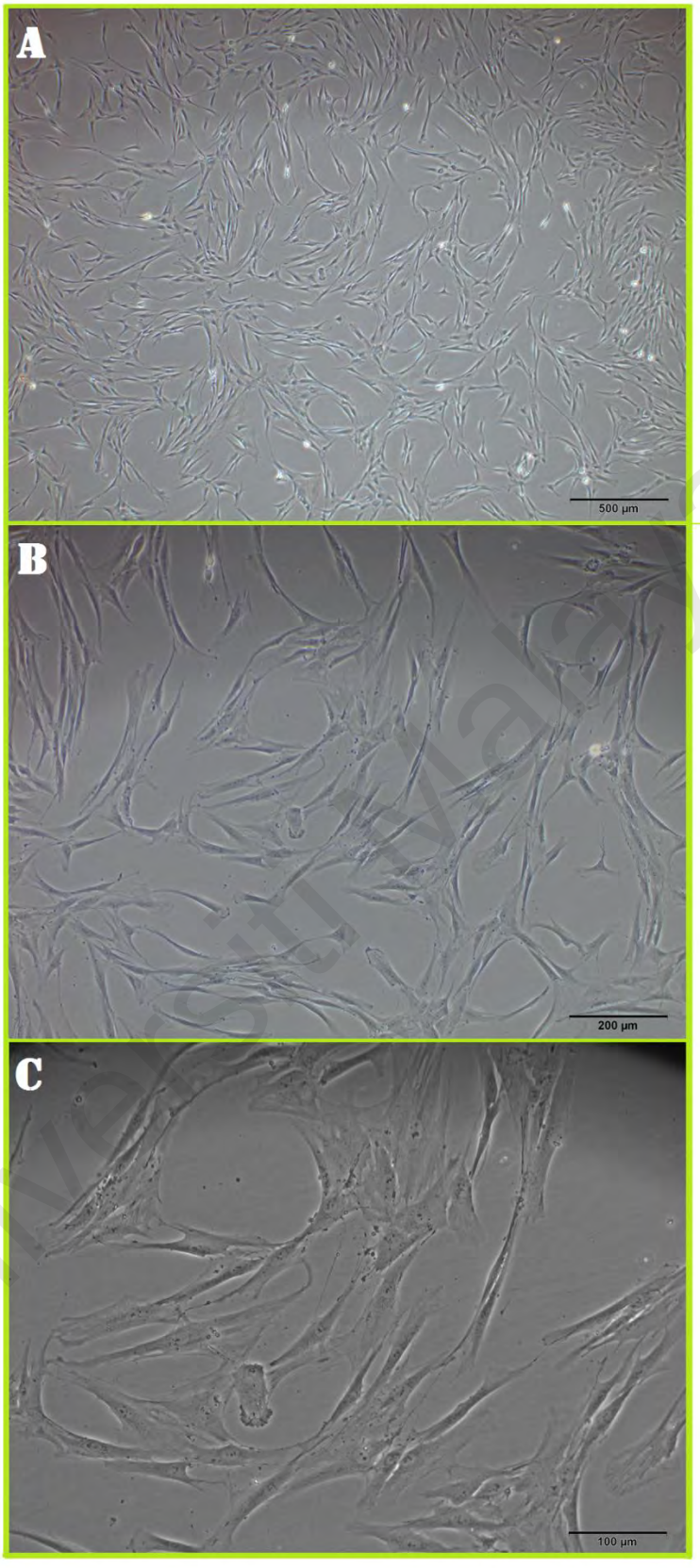


Figure 3.3: Micrograph showing PDLSCs-D isolated from human deciduous periodontal ligament. Plastic adherence ability shown in the diagram with cells in homogenous monolayer. A) Scale bars: 500µm B) Scale bars: 200µm and C) Scale bars: 100µm.

3.3.2.2 Expression of specific cell surface markers by PDLSCs-D

The immunophenotypic profile of PDLSCs-D was determined by testing a panel of surface markers using flow cytometer. Results as shown in Fig. 3.4 revealed that PDLSCs-D were positive (> 85%) for many commonly positive markers such as CD105, CD73, CD90, CD44 and CD166 meanwhile negative (<2%) for hematopoietic surface markers such as CD34, CD45, HLA-DR.

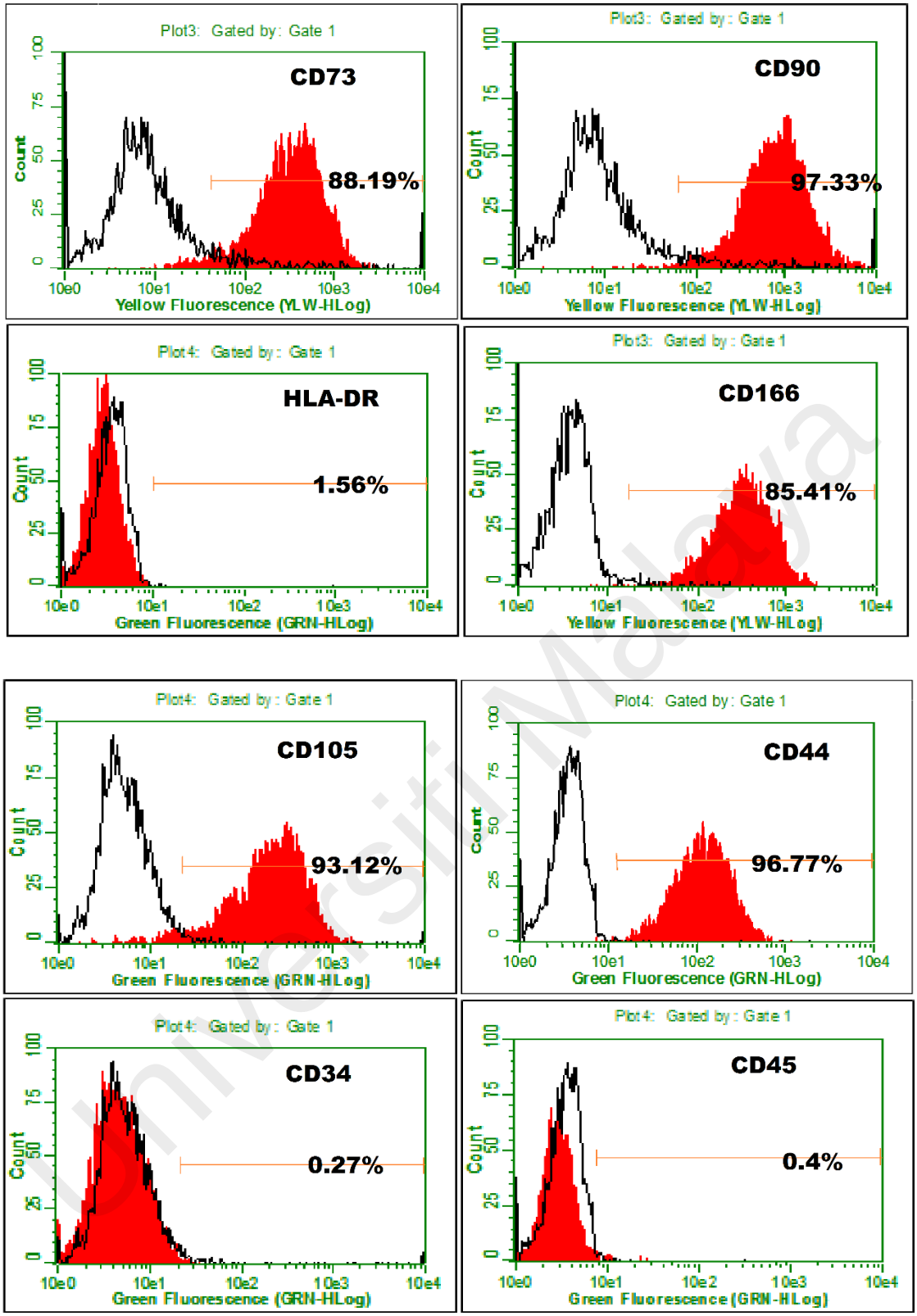


Figure 3.4: Expression of MSCs specific cell surface markers. Isolated PDLSCs-D shown to be positively expressing the gold standard of MSCs markers with the absence or indeterminably low for markers involved during hematopoiesis.

3.3.2.3 Multi-differentiation potential of PDLSCs-D

These cells ability to differentiate into multi-lineage were confirmed by standard induction protocols. Differentiation capacity of PDLSCs-D were as shown in Fig. 3.5, including formation of proteoglycan indicating chondrogenesis (Fig. 3.5A), deposition of silver stained mineralized matrix indicating osteogenesis or bone forming ability (Fig. 3.5B) and exhibiting clumping of neutral lipid vacuoles indicates the ability to produce adipocytes (Fig. 3.5C).

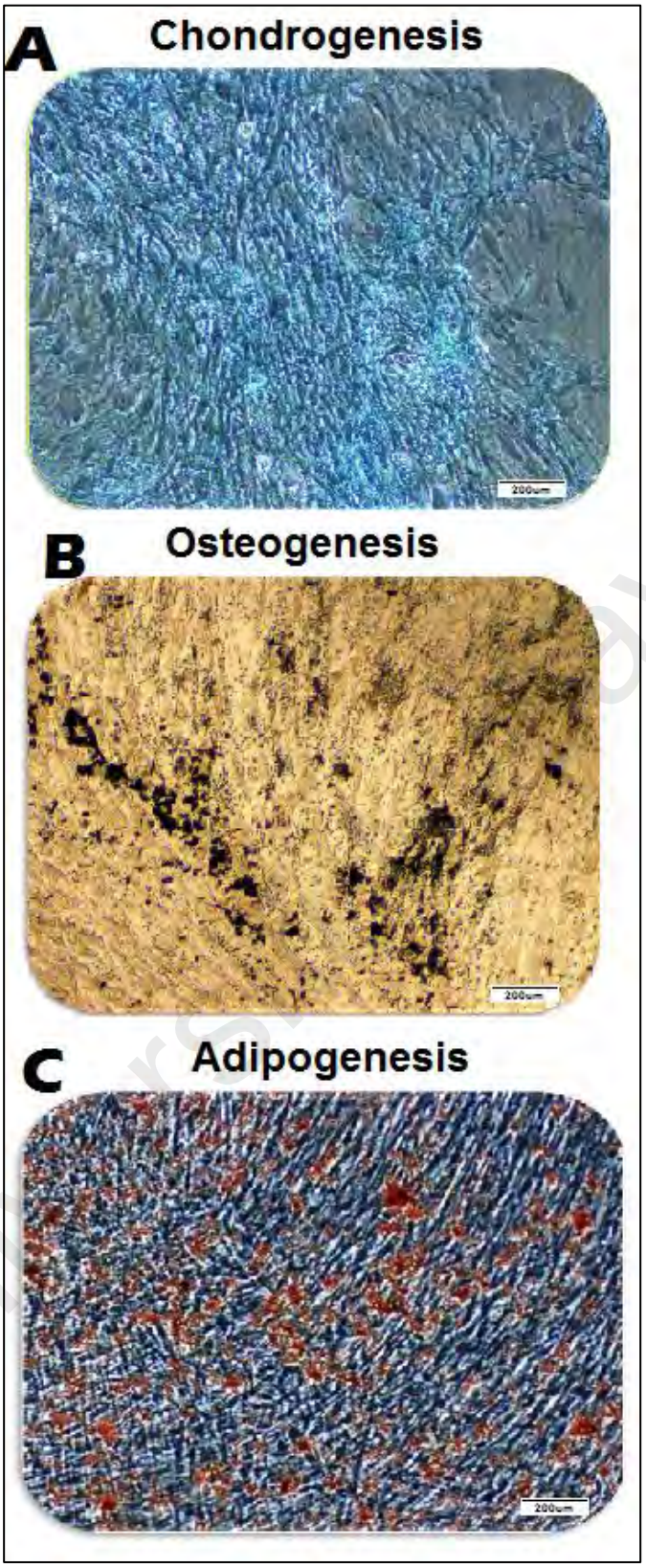


Figure 3.5: Multi-differentiation potential of PDLSCs-D. The panel display representative of visual observation (A-C) chondrocyte detected by Alcian Blue staining, osteocyte detected by Von Kossa staining and adipocyte detected by Oil Red O staining. Scale bars: 200µm

3.3.2.4 Immunocytochemistry of selected MSCs markers on PDLSCs-D

To determine pluripotency of PDLSCs-D, immunofluorescence test on selected markers compromising OCT4, SOX2 and SSEA4 were performed on PDLSCs-D. Primary human PDLSCs-D from deciduous tooth stably expressed OCT4 and SOX2 that indicates the pluritpotent stem cells state which is a major key for maintaining stem cells renewal capacity. In addition, SSEA4 was also present in PDLSCs-D, a marker of undifferentiated state in human embryonic stem cells.

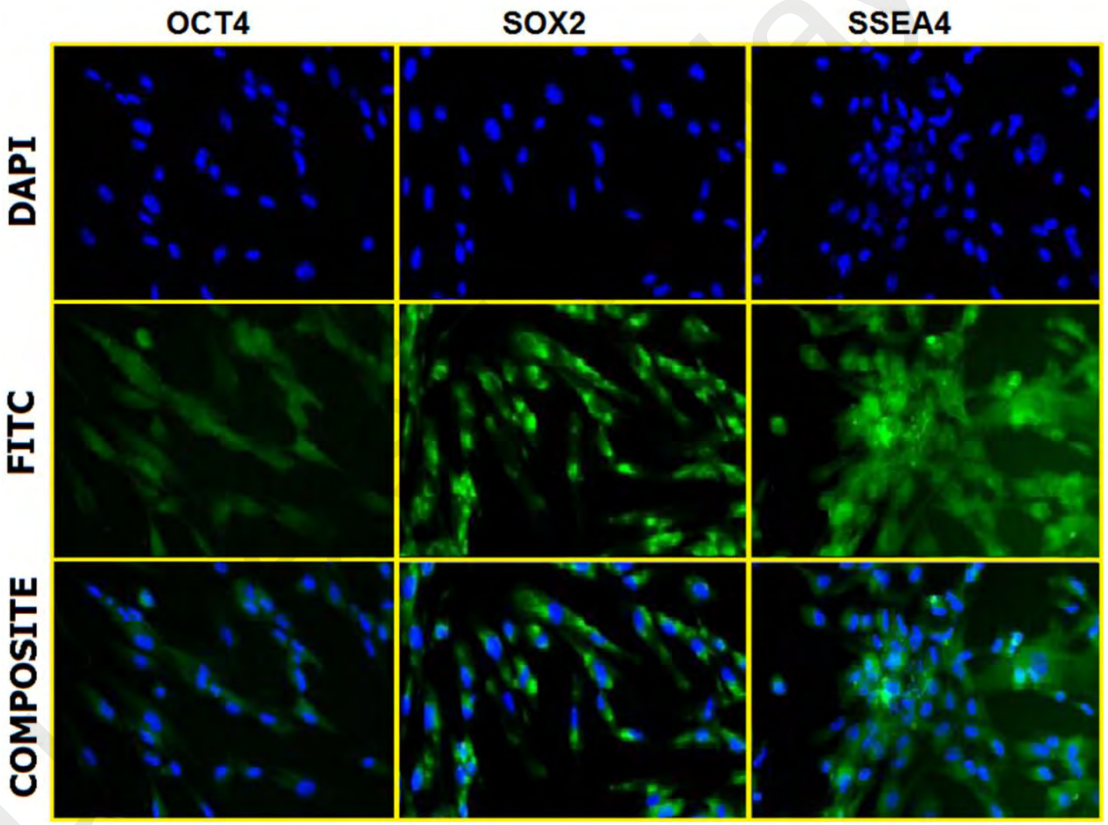


Figure 3.6: Immunocytochemistry of selected MSCs markers. The green fluorescence (FITC) stained cell cytoplasmic with targeted markers while DAPI stained the nucleus. Composite were the overlay stained image of DAPI and respective target (OCT4, SOX2 and SSEA4). Scale bar = 50µm

3.3.3 Growth kinetics of PDLSCs-D

Population doubling time of isolated samples (n=3) were calculated from passage 1 until passage 5 (P1-P5). PDLSCs-D showed a gradual increase of population doubling with increase of passages. Accumulation of cell number and cumulative population doubling of passage 1 until passage 5 were shown in Fig 3.7. Based on the results, PDLSCs-D tended to double their population at average of (23.81± 2.8 hr).

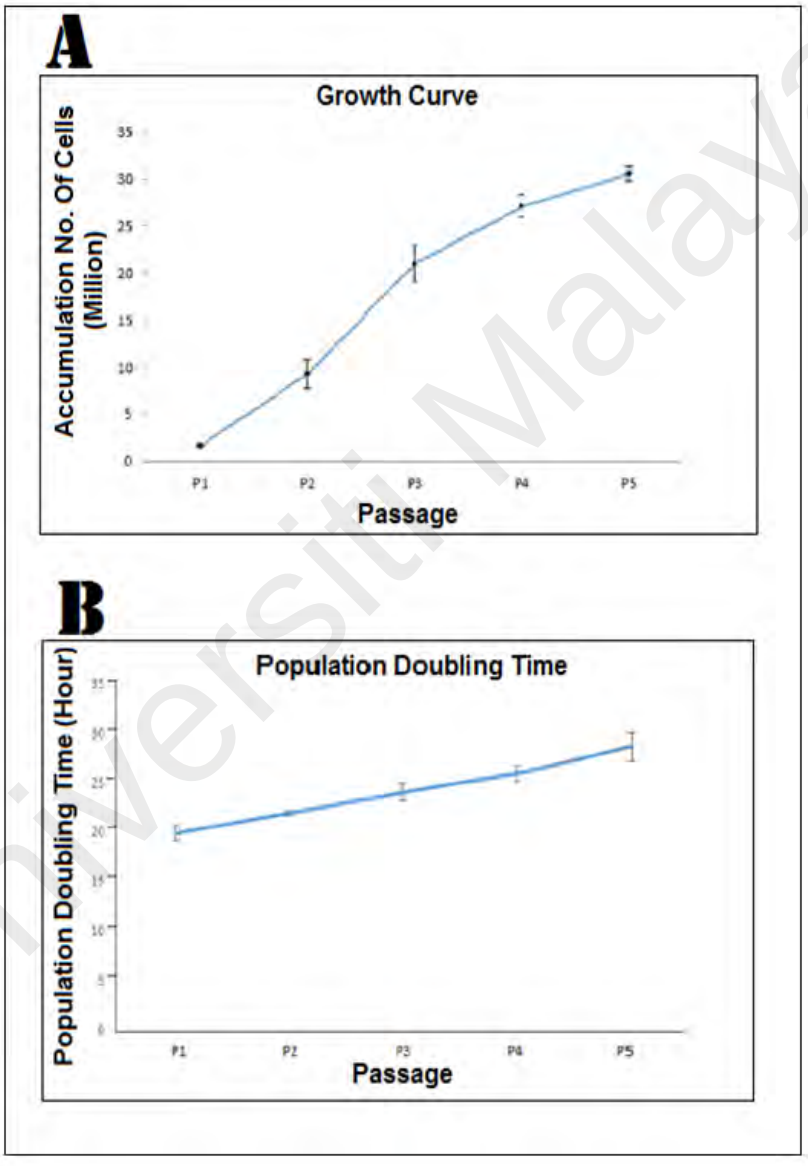


Figure 3.7: Growth kinetics of isolated PDLSCs-D. A) Cumulative cell number of PDLSCs-D from passage 1 until passage 5 (n=3). B) Population doubling time of PDLSCs-D from passage 1 until passage 5.

3.4 Discussion

MSCs derived from diverse tissues share some common properties (Dominici et al., 2006). In pursuit of MSCs-like population residing in periodontal ligament, basic assessment was carried out on the isolated PDLSCs-D. In this study, isolated PDLSCs-D displayed bona fide MSCs properties extending from morphologically, plastic-adherence up to multi-differentiation ability similar to the reported studies on PDLSCs-D (Vasandan et al., 2014; Lei et al., 2014; Silverio et al., 2005; Seo et al., 2004). Besides, the cumulative cell number indicated the potential of getting adequate cells for transplantation by passage 3. Of note, these cells are able to at least give rise to osteogenic, adipogenic and chondrogenic lineages. In addition, the presence of pluripotent markers, OCT4 and SOX2 play an important regulatory role in stem cells renewal process that might determine the cell stemness fate under *in vitro* condition (Pan et al., 2002; Sarkar et al., 2013). Expression of SSEA4 which is regarded as a specific marker for deciduous PDLSCs-D further confirm the existence of multipotent stem cells in the isolated PDLSCs-D (Fukushima et al., 2012). Aligned with these findings, our study was directed to determine the competence of PDLSCs-D in giving rise to hepatic lineage or endodermal lineage.

3.5 Summary

Dental origin stem cells are profoundly regarded as the most promising source of autologous cells with the fact that these cells are easily accessible by non-invasive methods. Moreover, isolated PDLSCs-D shares similar characteristics of MSCs comparable to other sources. In addition to the above, PDLSCs-D could also minimise the age related loss of stemness as it is isolated from young children. Therefore, PDLSCs-D can be considered to be a potential source of MSCs for cell based regenerative therapy.

CHAPTER 4: DIFFERENTIATION OF STEM CELLS FROM HUMAN DECIDUOUS PERIODONTAL LIGAMENT INTO HEPATIC LINEAGE

4.1 Introduction

Ever since the first discovery of periodontal ligament derived stem cells by Seo et al., 2004, huge amount of research have been deployed on these stem cells and yet, there is still more to uncover. The most intriguing facts about these stem cells are their multi-lineage potential which allows them to differentiate into three germ layers, and also easily accessible nature (Archarya et al., 2010). Ultimately placing them as a very good candidate for tissue regeneration. However, most avenues in deploying PDLSCs-D are directed toward the field of dentistry such as in regeneration of periodontal tissues (Zhu and Liang, 2015).

Nevertheless, studies on PDLSCs-D have shown that these cells can be differentiated into neural cells (Widera et al., 2007), pancreatic cell lineage (Lee et al., 2014), retinal cells (Huang et al., 2013), and also the ability to enhance angiogenesis (Yeasmin et al., 2014) reflecting the potential ability beyond tooth regeneration (Park et al., 2016). In the context of stem cell based regeneration therapy for liver diseases (Christ et al., 2010; Zhou et al., 2012; Tsolkai et al., 2015; Lee et al., 2018), generation of hepatocyte-like cells in non-hepatic lineage cells (Synkers et al., 2011; Yin et al., 2015; Yu et al., 2015; Xue et al., 2016) have been previously described and appeared to provide new opportunity with fewer ethical concerns and in autologous settings.

To date, generating hepatocyte-like cells can be achieved by four main protocols; exposure to cytokines and growth factors by chemically defined media (Snykers et al., 2006; Chivu et a., 2009; Liu and Wang, 2014), genetic modification using transcription factors (Ishi et al., 2008), adjustment of the micro-environment and changes in physical

parameters (Wu et al., 2012; Zhang et al., 2012). Among these protocols, the chemically defined media is the preferred method due to the effect that been considered as major contributor in differentiation towards hepatocyte-like cells.

Moreover, stem cells derived from dental origin were also shown to generate high purity hepatocyte-like cells (Iskitive et al., 2012; Han et al., 2017) along with our very own group demonstrated differentiation of dental pulp stem cells from deciduous tooth (SHEDs) under fetal bovine serum (FBS) and human platelet serum (HPL) (Vasanthan et al., 2014). Likewise, preliminary data also showed the ability of PDLSCs-D to differentiate into insulin-producing cells (Huang et al., 2009). Thus, these human deciduous PDLSCs-D could be signified that they can also differentiate into endodermal lineage.

In a nutshell, we investigate the appropriate merits of human deciduous periodontal ligament stem cells with respect to their propensity towards differentiating into hepatocyte-like cells by chemically defined media.

Experimental works carried out to meet the following objectives:

Aim II: To study the potential of PDLSCs-D differentiation into hepatic lineage. The objectives were:

1. To induce differentiation towards hepatic lineage using chemically defined media
2. To assess the morphology of the differentiated cells from PDLSCs-D
3. To ascertain gene expressions for hepatocytes of differentiated PDLSCs-D using real time polymerase chain reaction (qPCR) and immunocytochemistry techniques
4. To assess the functional properties of generated hepatocytes from differentiated PDLSCs-D

4.2 Materials and Methods

4.2.1 Ethics approval for collection of human extracted deciduous teeth

Sample collections were conducted with prior written informed consent according to the guidelines set by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number: DF CD 1411/0087 (P) (Appendix A).

4.2.2 Media preparation for hepatic differentiation

Three different types of media are required for differentiation towards hepatic lineage. Composition and procedure to prepare those media are described further.

4.2.2.1 Preparation of cell culture medium

Periodontal ligament stem cells (PDLSCs-D) culture medium was prepared by using DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen) with the composition as shown in Table 4.1.

Table 4.1: Composition of culture media

Component	Manufacturer	Volume
Australian characterised Fetal bovine serum (FBS)	Hyclone, Thermo Fisher, Scientific Inc, Waltham, MA	50.0 ml
Penicillin/Streptomycin	Invitrogen	2.5 ml
Glutamax	Invitrogen	5.0 ml
DMEM-KO	Invitrogen	442.5 ml

4.2.2.2 Preparation of Hepatic initiation media

Hepatic initiation media was prepared using DMEM-KO (Invitrogen) added with 2% FBS (Hyclone) followed by 20 ng/ml of Hepatocyte Growth factor (HGF, R&D Systems, Menniapolis, MN, USA). The complete medium was sterilised by filtration through 0.2 μm membrane filter (Thermo Scientific) and stored at 4ºC until further use.

4.2.2.3 Preparation of Hepatic maturation media

Hepatic maturation media was prepared using DMEM-KO (Invitrogen) supplemented with 10 ng/ml Oncostatin M (R&D Systems), 10 nmol/L Dexamethasone (Sigma Aldrich), and 1% Insulin- transferrin-Selenium X (ITS, Gibco). The complete medium was sterilised by filtration using a 0.2μm membrane filter (Thermo Scientific) and stored at 4ºC until further use.

4.2.3 Human hepatocellular carcinoma cell line culture

Human hepatocellular carcinoma cells (HepG2) (ATCC, Biorev) were purchased and used as a positive control cell lines in this study. These cells are highly differentiated and display many of the genotypic features of normal liver cells (Sassa et al., 1987). These cells were maintained as an adherent cell line in culture medium containing DMEM-KO (Invitrogen) as shown in Table 3.1 and incubated at 37˚C and 5% CO_2 in humidified chambers. Cells were passage as needed using 0.05% EDTA-Trypsin (Invitrogen).

4.2.4 Hepatocyte differentiation

PDLSCs-D differentiation towards hepatocyte-like cells under media induction was conducted as described previously by Ishkitiev et al., (2010) with some modifications. Confluent Passage 3(P3) PDLSCs-D culture were washed twice with DPBS (-Ca^{2+},-Mg^{2+}) (Invitrogen) and stimulated with hepatic initiation media for five consecutive days. Subsequently, the maturation process was carried out on day 6 onwards by replacing the hepatic initiation media with hepatic maturation media. For differentiation into hepatic lineage, cells were maintained in hepatic maturation media until day 21. At every three days interval, cells were provided with fresh hepatic maturation media. The morphological changes of PDLSCs-D were observed on regular basis for 21 days using phase-contrast microscope (Olympus).

4.2.5 RNA extraction and quantification

Total RNA of three different phases of PDLSCs-D compromising of undifferentiated PDLSCs-D, differentiating PDLSCs-D and differentiated PDLSCs-D (of Day 0, Day 7, Day 14 and Day 21) along with HepG2 cells and purchased adult human liver total RNA (Clontech, Takara Bio Inc, USA) were used. The total RNA were extracted using Trizol (Invitrogen) method in which the cells were washed twice with DPBS (-Ca^{2+},-Mg^{2+}) (Invitrogen) and lysed by 1ml of Trizol (Invitrogen). The lysate was then transferred into 1.5 ml micro-centrifuge tube (Axygen) and incubated for 5 minutes at 25°C. A total of 200 µl of chloroform (Sigma Aldrich) was added to the tube and mixed by inverting the tube 20 to 25 times with subsequent incubation for 2 to 3 minutes at 25°C. The mixture was then centrifuged at 12000 rpm for 15 minutes at 4°C following which the aqueous phase was transferred into a fresh micro-centrifuge (Axygen). Next, 0.5 ml of iso-proponal (Sigma Aldrich) was added and solution was mixed and incubated at 25°C for 10 minutes

to precipitate RNA. This was followed by another centrifugation step at 12000rpm for 10 minutes at 4°C. The supernatant was discarded and pellet was washed twice with 75% ethanol (Sigma Aldrich). The pellet was then air-dried, dissolved in 30 µl of RNase and DNase free water (Qiagen, Valencia, CA, USA) and incubated at 37°C. The extracted RNA was treated with DNase (Qiagen) to remove genomic DNA. Extracted RNA was quantified using 1 µl of RNA and 1 µl of ddH2O as a blank. The absorbance was recorded at 260 nm and 280 nm using Nanodrop (Thermo Fisher Scientific Inc.) prior to storage at -80°C. RNA concentration and purity were calculated using the following formula;

RNA concentration (µg/µl) = OD_{260} x (40 µg RNA/ ml),

and ration of reading of OD_{260} /OD_{280}

4.2.5.1 Conversion RNA to complementary deoxyribonucleic acid (cDNA) synthesis using Superscript II Reserve Transcriptase

Complementary DNA (cDNA) was prepared from total RNA using Superscript II Reserve Transcriptase (Invitrogen) according to manufacturer‘s protocol. A mixture composed of 1 µl of Oligo (dT) (Invitrogen), 3 µl of total RNA (1 ng to 5 µg total RNA), 1 µl of dNTP Mix (10 mM) (Invitrogen) and 7 µl of ddH_2O were put into a 0.2 ul tube (Axygen) and spinned down for a few seconds. After centrifugation, the mixture was heated at 65° C for 5 minutes and quickly chilled with ice. Next, 4 µl of 5X First Strand Buffer (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen) and 1 µl of RNaseOut (Invitrogen) were added to the mixture and heated for 2 minutes at 42° C. Finally, 1µl of Superscript II Reversed Transcriptase (Invitrogen) was added and mixed gently before they were taken for 50 minutes incubation at 42°C and followed by heating at 70°C for 15 minutes. The cDNA was ready to be used as a template for amplification in PCR and was kept immediately at -20 ° C to avoid cDNA degradation.

4.2.5.2 Reserve transcription polymerase chain reaction

The reverse transcriptase polymerase chain reaction (RT-PCR) mixture were prepared by adding 2.5 µl of 10X PCR $-Mg^{2+}$ (Invitrogen), 1.25 µl of 50 mM $MgCl_2$ (Invitrogen), 0.5 µL of 10 mM dNTP Mix (Invitrogen), 1 µl of each of the selected forward and reverse primer, 0.2 µl of Taq DNA polymerase (Invitrogen), 1 µl of cDNA and 17.55 µl of autoclaved ddH_2O into the 1.5 ml tube (Axygen). Then, the mixture was centrifuged at lower speed (500 rpm) for a few seconds. The incubation and pre-denaturation was performed at 94° C for 5 minutes in a thermal cycler. A total of 30 to 35 cycles of PCR amplification were performed according to the following phases; denaturation phase at 94° C for 45 seconds, annealing phase at 58.5° C for 30 seconds, 72° C for 45 seconds and the elongation phase at 72° C for 1 minute. Additional incubation at 72° C for 10 minutes was performed and then the samples were maintained at 4° C. The PCR samples were evaluated visually by using 1.5% agarose gel electrophoresis which can be visualized via ethidium bromide (Sigma Aldrich) staining. As for quantitative analysis (qPCR), internal calibration curves were generated by the real time software. A melting curve analysis was carried out between 60° and 95°C with a plate read every 0.5°C after holding the temperature for 20 s. The threshold cycle number (CT) 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 ΔΔCT. All calculations were normalized using 18sRNA gene as a reference control.

Table 4.2: List of primers used to characterize PDLSCs-D differentiated into hepatocytes

Gene Symbol	Primer Sequence (5'-3')	Base Pair	Annealing temperature ˚C
18 s	F: CGGCTACCATCCAAGGAA R: GCTGGAATTACCGCGGCT	186	55
AFP	F: CATCCAGGAGAGCCAAGCAT R: CGCCACAGGCCAATAGTTTG	209	59
GATA 4	F: CAGAAAACGGAAGCCCAA R: TTGCTGGAGTTGCTGGAAG	256	58
SOX17	F:CTGTAGACCAGACCGCGACA R: CTGGTCGTCACTGGCGTATC	207	58
HNF3β	F: ACTACCCCGGCTACGGTTC R: AGGCCCGTTTTGTTCGTGA	296	56
CK19	F: CCATGCGCCAGTCTGTGGAG R: GTGGTGCTCTCCTCA ATCTGCT	321	58
AAT	F: AGACCCTTTGAAGTCAAGCGACC R: CCATTGCTGAAGACCTTAGTGATGC	358	56
TDO	F: GGTTTAGAGCCACATGGATT R: ACAGTTGATCGCAGGTAGTG	424	56
TO	F: GGCAGCGAAGAAGTACAAATC R: TCGAACAGA ATCCAACTCCC	217	55
CYP3A4	F: TCACCCTGATGTCCAGCAGAAACT R: TACTTTGGGTCACGGTGAAGAGCA	251	57
CK 18	F: CCATGCGCCAGTCTGTGGAG R: GTGGTGCTCTCCTCA ATCTGCT	321	58
HNF4α	F: GCTTGGTTCTCGTTGAGTGG R: CAGGAGCTTATAGGGCTCAGAC	730	55
TAT	F: GCTAAGGACGTCATTCTGACAAG R: GTCTCCATAGATCTCATCAGCTAAG	353	56
CYP7a1	F: GAGAAGGCAAACGGGTGAAC R: ATCGGGTCAATGCTTCTGTG	275	57
ALB	F: AAGGCACCCCGATTACTCCG R: TGCGAAGTCACCCATCACCG	213	55

4.2.6 Immunofluorescence

PDLSCs-D were cultured in 2-chambers slide (BD Bioscience) with a 200 cell/cm^2 cell density in FBS media and HPL growth media until reach 70% of confluence. The samples were then briefly rinsed using DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) for 3 times and fixed using 4% PFA (Sigma Aldrich) for 20 minutes at 4˚C. The 2-chambers slide were covered using aluminum foil to avoid light. After permeabilization with 1 ml of 0.01% Triton X (Sigma Aldrich), 1 ml of 1% BSA (Sigma Aldrich) were added to the 2- chambers slide and incubated for 1 hour at room temperature (23 ± 1°C). The solution was discarded once the incubation ended and cells were washed with 1 ml of 0.5% BSA. Primary antibodies were added and cell were incubated for overnight at 4˚C.

The determination of selected hepatic markers in PDLSCs-D were done for day 0 (negative control), day 7, day 14 and day 21 of hepatic differentiation along with HepG2 cells as positive control. The primary antibodies used for the specific hepatocyte markers is listed in Table 3.4.

Table 4.3: List of primary antibodies used to determine the hepatocyte cell markers in PDLSCs-D into hepatocyte-like cell.

Primary Antibody	Dilution Ratio	Manufacturer
AFP (mouse monoclonal)	1:400	Abcam
HNF4 (mouse monoclonal)	1:400	Abcam
Cytokeratin 18 (Mouse monoclonal)	1:400	Milipore
Albumin (Mouse monoclonal)	1:400	Sigma

Secondary antibody was added after the cells were washed 3 times with 0.5% BSA for 30 seconds to remove the primary antibodies. The detection of selected hepatic markers in PDLSCs-D were done using only FITC-conjugated IgG (Abcam) as the secondary antibodies. PDLSCs-D were incubated for 90 minutes at room temperature (23 ± 1°C). After three washes using 0.5% BSA (Sigma Aldrich), PDLSCs-D were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, Millipore) and covered with glass cover slip. Cells were incubated for 20 minutes at temperature (23 ± 1°C) in a dark room. The slide was screened under fluorescence microscope (Nikon-Eclipse 90i, Nikon,Tokyo, Japan) in a dark room.

4.2.7 *In vitro* functional assay

Periodic acid-Schiff (PAS) (Sigma Aldrich) staining method was used to detect the glycogen storage in the undifferentiated PDLSCs-D (negative control), differentiating PDLSCs-D and differentiated PDLSCs-D cells at 7, 14 and 21 days of hepatic differentiation along with HepG2 cells (positive control) in the 35mm dishes (BD Bioscience) according to the manufacturer's protocol.

Further, albumin and urea detection, cell culture media were collected from undifferentiated PDLSCs-D (negative control) and differentiating PDLSCs-D at day 7, day 14 and day 21 along with HepG2 cells as positive control. Albumin production was determined by using the albumin blue fluorescent (ABF) kit from Active Motif adhering to the manufacturer's specification. As for study on urea secretion was carried out, media collected after 24 hours of exposure to 1 mmol/L NH_4Cl (Sigma Aldrich). 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 glutathione kinetic method (Fawcett and Scott 1960) and the optical densities were measured at 492 nm.

4.2.8 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, U.S.A.). The significance level was set at P=0.05. Tukey Post-hoc multiple comparisons were carried out to determine the differences between the groups.

4.3 Results

4.3.1 Acquisition of hepatocyte morphology

PDLSCs-D undergoing hepatic lineage differentiation were observed on regular basis using phase contrast light microscope. Fibroblastic morphology of the cells were maintained during the first phase of differentiation (Figure 4.1) however the cells tend to grow in circular angle. Following the second phase of differentiation, PDLSCs-D were found to modify their phenotype from spindle fibroblastic shape into polygonal shape typical of epithelial cells can be seen (Figure 4.2). At the end of differentiation process, the cells appeared in three distinct morphologies in the cell culture. Firstly, cultured cells appeared in colony with spherical shaped in a higher population number, while the second group observed were in broadened polygonal shape and lastly the non-endoderm cells still remained in the spindle-fibroblast shape resulting in a heterogeneous population (Figure 4.3). However, the increase in the number of days in differentiation (day 14 to Day 21) resulted in a higher fraction of polygonal shape with tight cell to cell interactions compared to spindle shape conformation denoting the typical morphology of a hepatocyte.

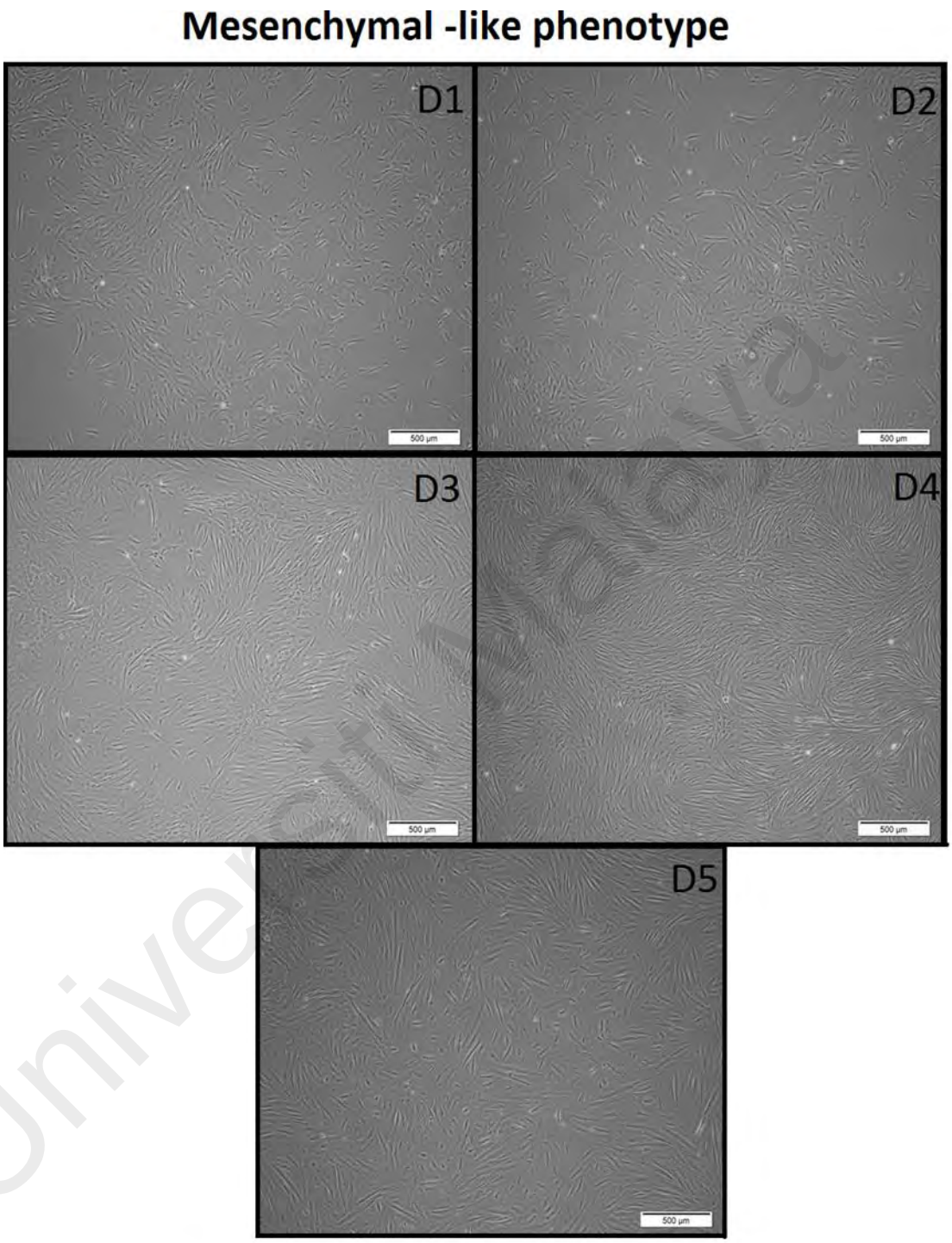


Figure 4.1: Micrograph showing the hepatic initiation phase of PDLSCs-D. PDLSCs-D were observed to be circular angle with mesenchymal phenotype during the first initiation media induction and variation of the shapes were only found towards the end of hepatic initiation period as seen in Day 5. Scale bar: 500µm.

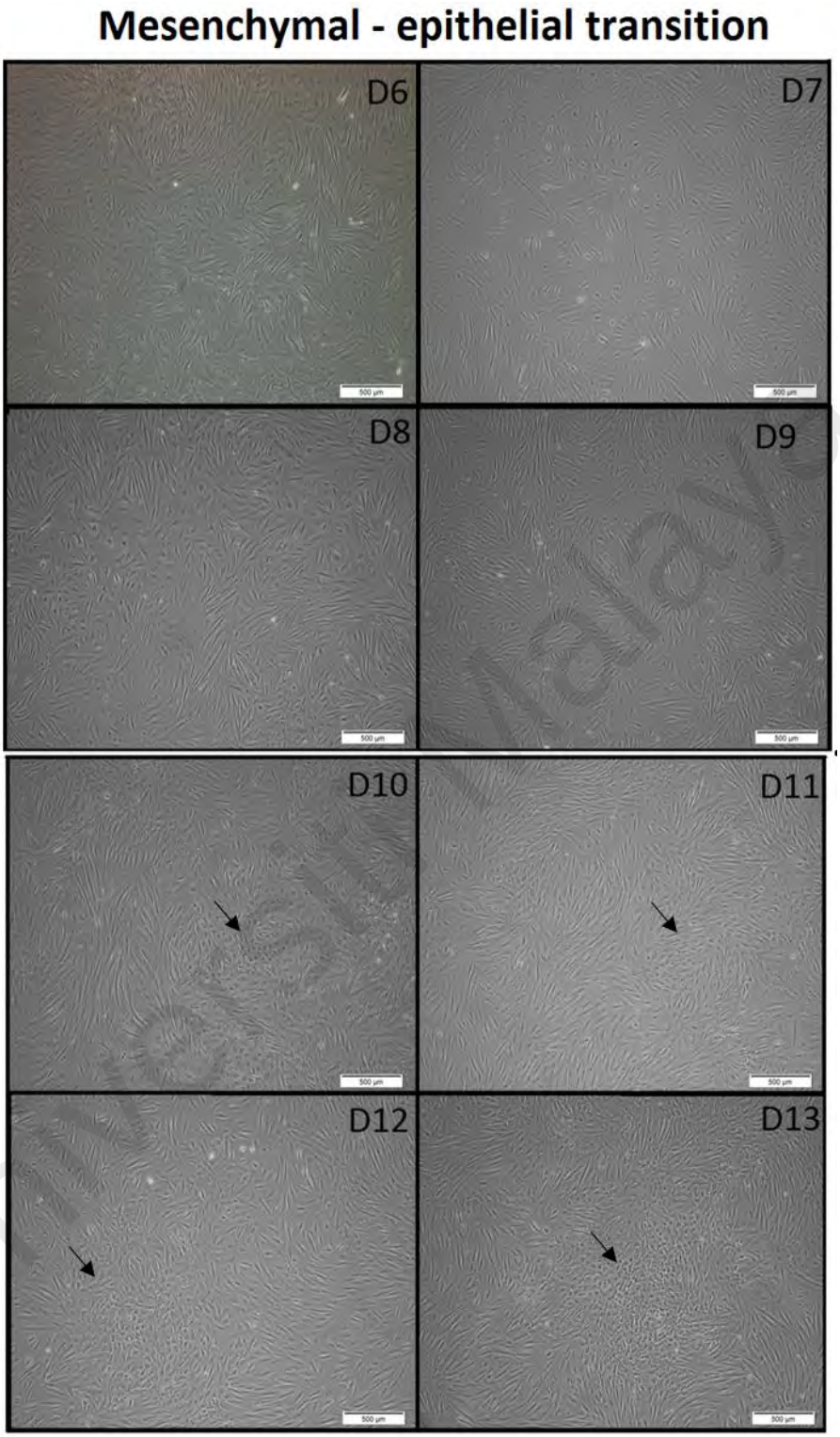


Figure 4.2: Micrograph showing first phase of hepatic maturation of PDLSCs-D. Initially, the spindle shape were observed but later stages, polygonal shape typical of of epithelial phenotype were observed as seen in Day 10 to Day 13 as shown in black arrows.

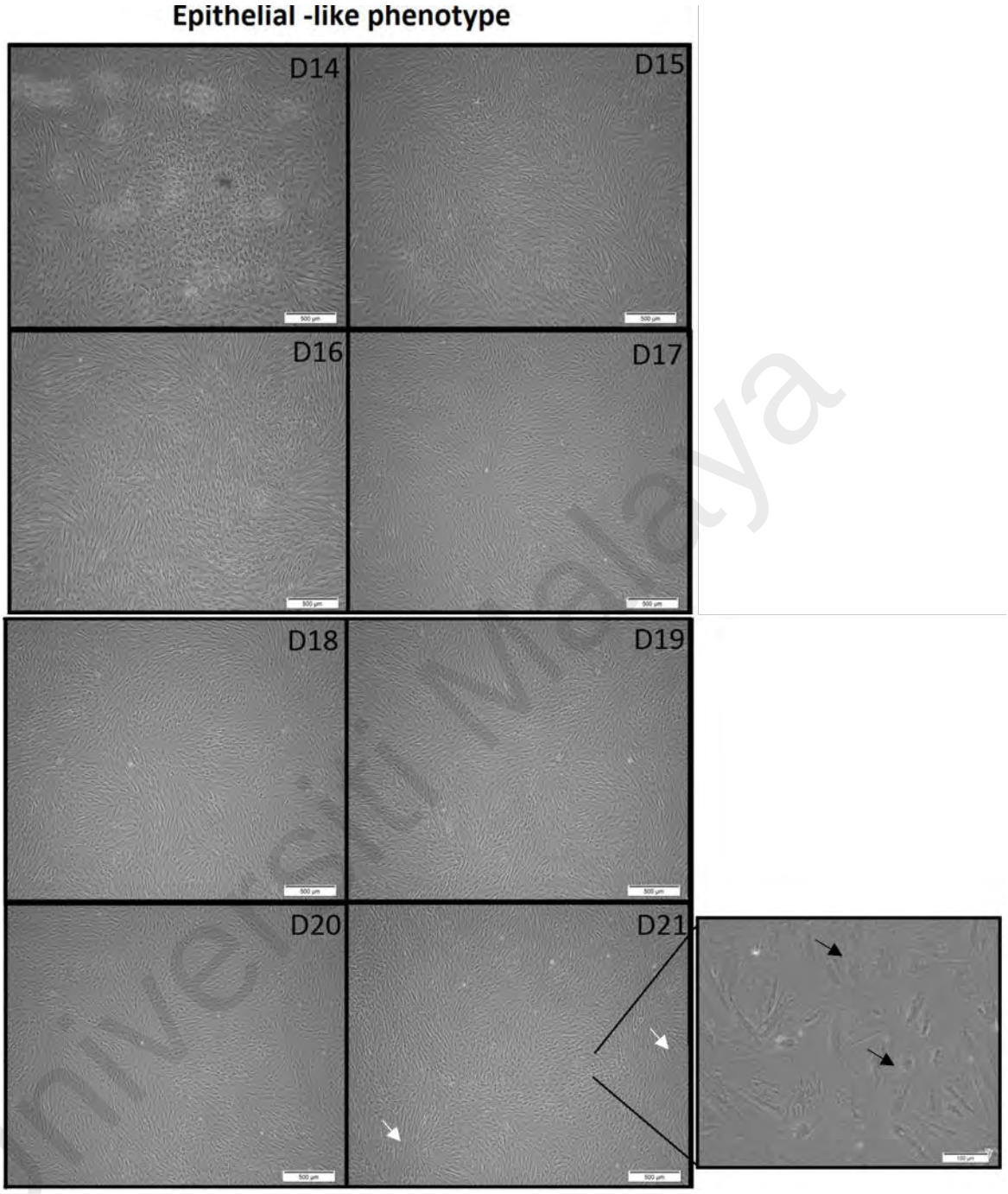


Figure 4.3: Micrograph showing the second phase of hepatic maturation of PDLSCs-D. Appearance of polygonal shape typical of epithelial cells were highly notable during this phase as seen from Day 14 up to Day 21.(Scale bar = 500µm). Attentively, magnification x20 image (scale bar = 100µm) of Day 21 differentiation of PDLSCs-D present formation of polygonal shape cells (black arrows). However, spindle shape cells still remained at the end of differentiation as seen in Day 21 (white arrows).

4.3.2 Expression of hepatic markers during differentiation

Generally, hepatic markers are categorized according to the stages of in vivo hepatogenesis as shown qualitatively in Figure 4.4 in which early endoderm markers compromising hepatoblast or hepatocyte progenitor transcripts (SOX17, HNF3β, GATA4 and AFP) were found to be expressed in PDLSCs-D on day 7 differentiation (Figure 4.5). Lower expression of these early markers were found in adult liver tissue except for HNF3β meanwhile for HepG2, all the markers were highly expressed except AFP. Down-regulation of these markers were seen toward the end of the experiment period parallel to the up-regulation of prominent hepatocyte-specific markers such as HNF4α, CK18, ALB, TAT, TO, TDO, and AAT. Cytochrome markers (Cyp3A4 and Cyp7A1) expression were likely less and negligible respectively on Day 21 of differentiation along with the absence of CK19 expression (Figure 4.6). Despite having a noticeably increased hepatic markers were found in PDLSCs-D differentiated into hepatocyte-like cells, the levels were admittedly lower than HepG2 as well as adult liver tissue.

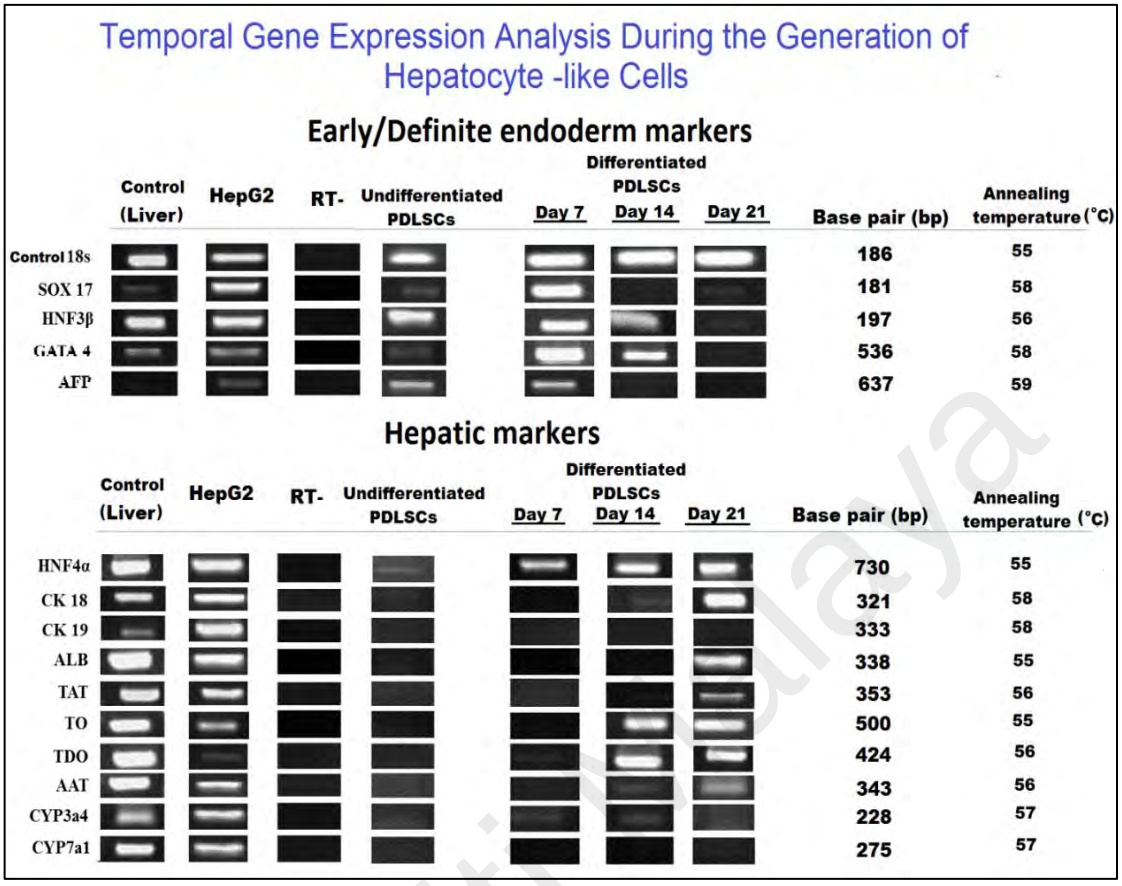


Figure 4.4: Gene expression manifestation of PDLSCs-D during hepatic differentiation. Qualitative gene expression were analysed at interval of Day 0, Day 7, Day 14 and Day 21 with HepG2 and Adult total liver RNA as negative and positive control respectively. Notably, early endodermal markers were expressed in undifferentiated PDLSCs-D. Differentiated PDLSCs-D showed expression of several hepatic markers which were absent during early stage.

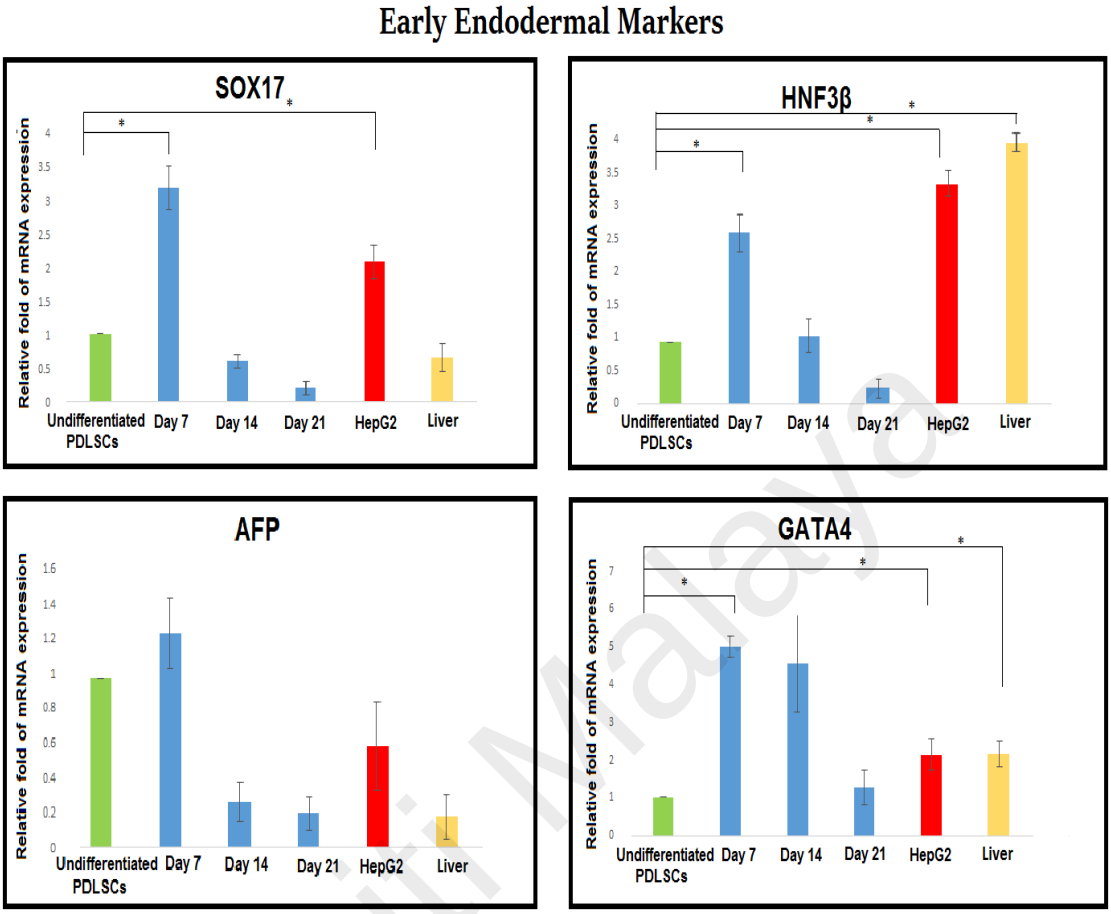


Figure 4.5: Quantitative expression of early endodermal markers by RT-PCR during PDLSCs-D differentiation into hepatic lineage. Early endodermal markers compromising hepatoblast or hepatocyte progenitor transcripts (*SOX17, HNF3β, GATA4 and AFP*) were found to be expressed at Day 7 which is after hepatic initiation period. For each marker, the expression level in undifferentiated PDLSCs-D was used as a standard respectively (i.e., relative expression = 1) and normalized against 18s RNA. The expression levels were also compared with the total of RNA from HepG2 and adult liver tissue. The expressions that were significantly different from that of undifferentiated PDLSCs-D were marked with asterisk ($p < 0.05$). Mean ± SD values were shown from six independent experiments.

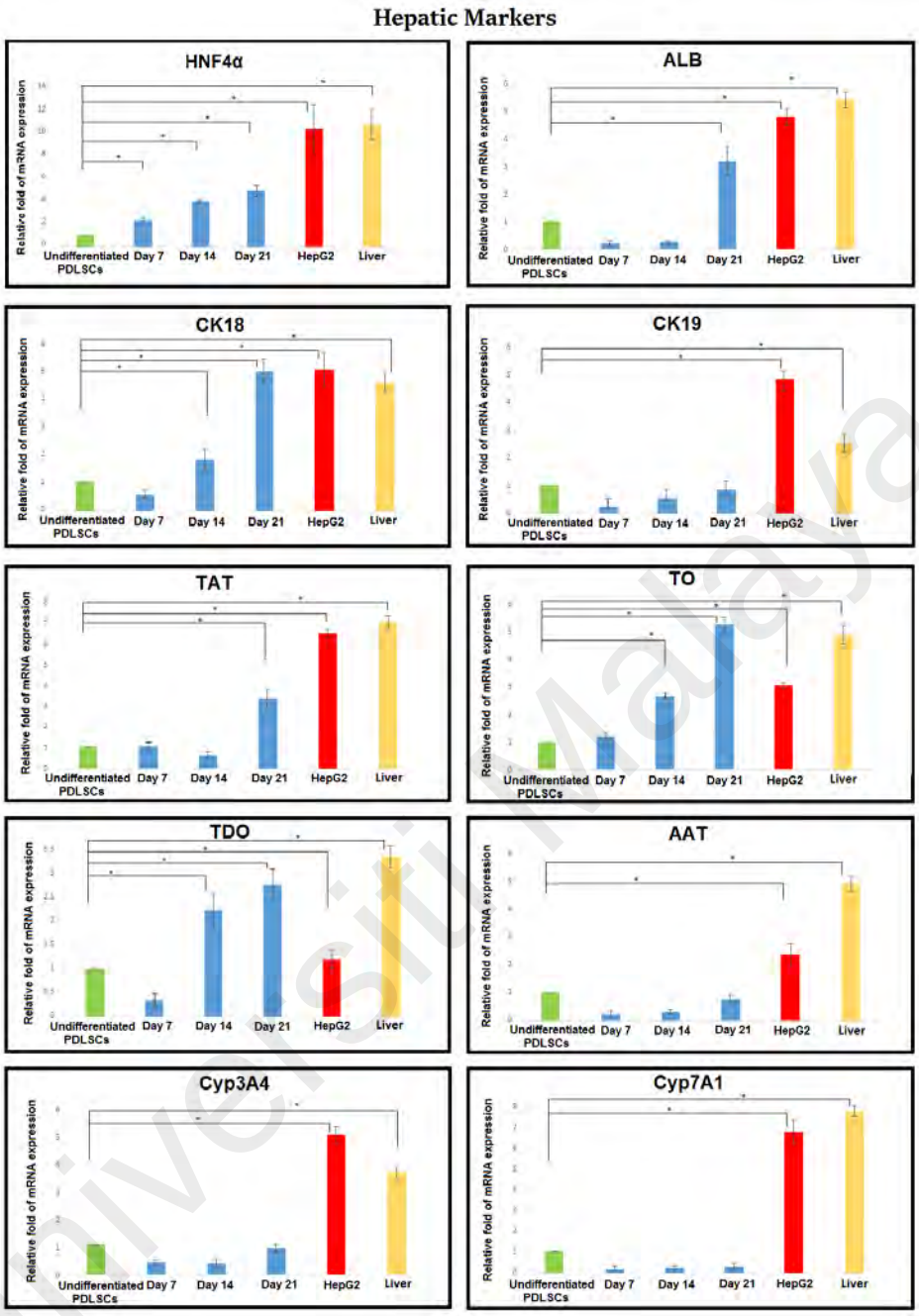


Figure 4.6: Quantitative expression of hepatic markers by RT-PCR during differentiation of PDLSCs-D into hepatic lineage. Hepatic associated transcriptional markers were detected at different levels throughout the course of differentiation. Prominent hepatocyte-specific markers such as *HNF4α, CK18, ALB, TAT, TO, TDO* and *AAT* were expressed on Day 21 of differentiated PDLSCs-D. Meanwhile, less likely expression of cytochrome markers were observed on differentiated PDLSCs-D. For each marker, the expression level in undifferentiated PDLSCs-D was used as a standard respectively (i.e., relative expression = 1) and normalised against 18s RNA. The expression levels were also compared with the total of RNA from HepG2 and adult liver tissue. The expressions that were significantly different from that of undifferentiated PDLSCs-D were marked with asterisk ($p < 0.05$). Mean ± SD values were shown from six independent experiments.

4.3.3 Immunofluorescence markers

To determine the proteomic signature of hepatocyte-like cells generated from PDLSCs-D, we assessed the immunocytochemistry along the stages of hepatic lineage. Differentiated PDLSCs-D on day 7 showed expression of endoderm marker AFP which later diminishing toward the end of differentiation period. However, the expression of hepatocyte specific markers HNF4α and CK18 gradually increased over the course of the differentiation period with the notable existence of ALB which only occurred on day 21 of differentiated PDLSCs-D (Figure 4.7).

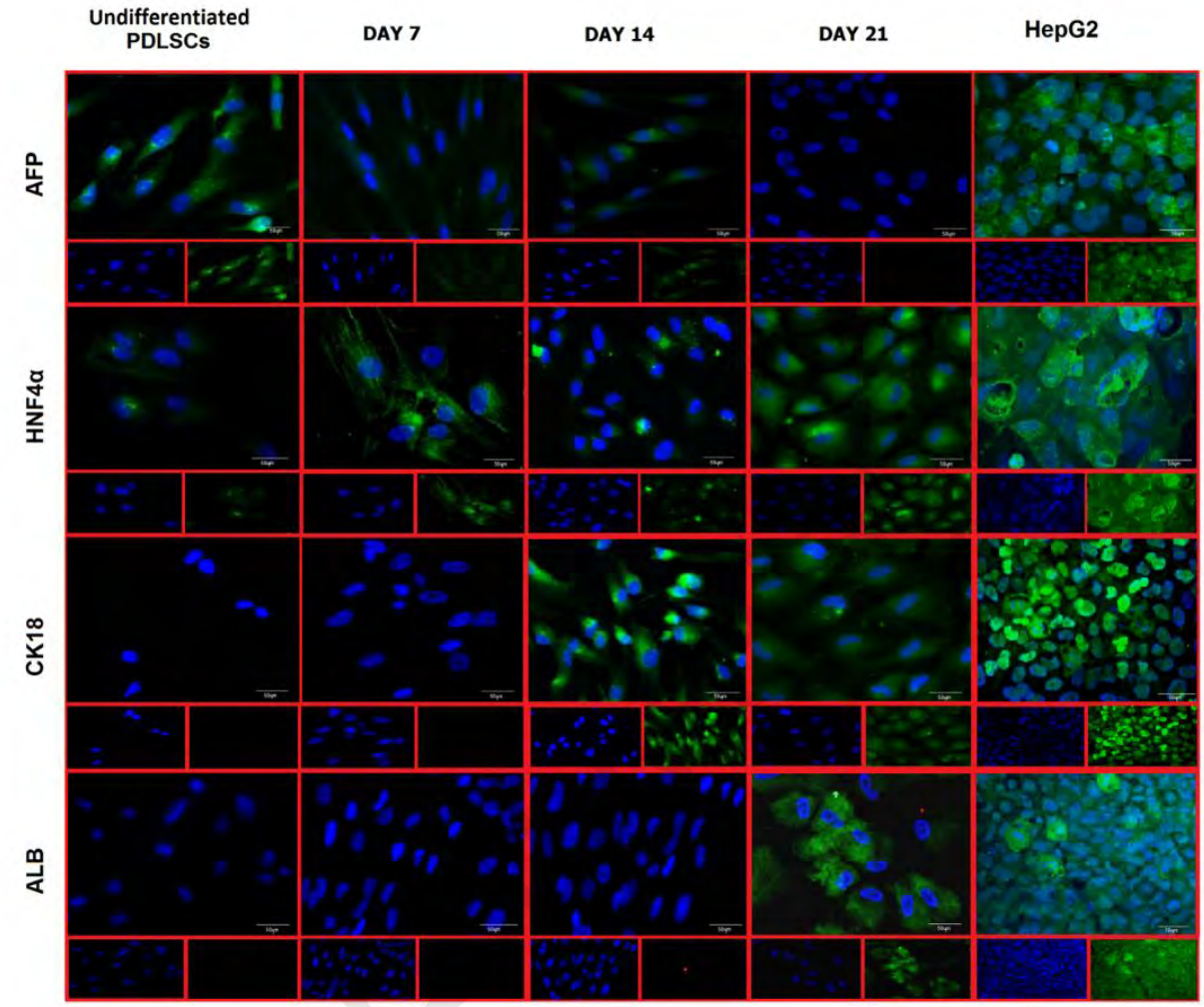


Figure 4.7: Immunocytochemistry of PDLSCs-D transformation into hepatic lineage. Representative images of immunofluorescence from selective hepatic markers at differentiation interval of Day 0, Day 7, Day 14 and Day 21. Differentiated PDLSCs-D gradually expressed hepatic nuclear factor (HNF), albumin (ALB), and cytokeratin 18 (CK18) while expression of alpha-fetoprotein (AFP) subsided during the course of differentiation. Scale bar = 50µm. Each image denoted with right lower corner representing FITC and left lower corner representing DAPI image and main image, overlay stained images. HepG2 cells has been used as positive control whereas undifferentiated PDLSCs-D were used as negative control.

4.3.4 Functionality of generated hepatocyte-like cells

Glycogen storage was observed in differentiating and differentiated PDLSCs-D by Periodic Schiff staining (PAS) (Figure 4.8A). The measurement of albumin secretion in the supernatants was only noted on the final day hepatocyte-like cells derived from PDLSCs-D with a value of 0.78 ± 0.04 ug/ml (Figure 4.8B). The assessment of urea showed significant difference between HepG2 and PDLSCs-D derived hepatocyte-like cells (Figure 4.8C). We found urea concentration gradually increased along the differentiation period from a value of 118.33 ± 1.15 ng/ml to a value of 764.67 ± 2.51 ng/ml on day 21 of differentiation.

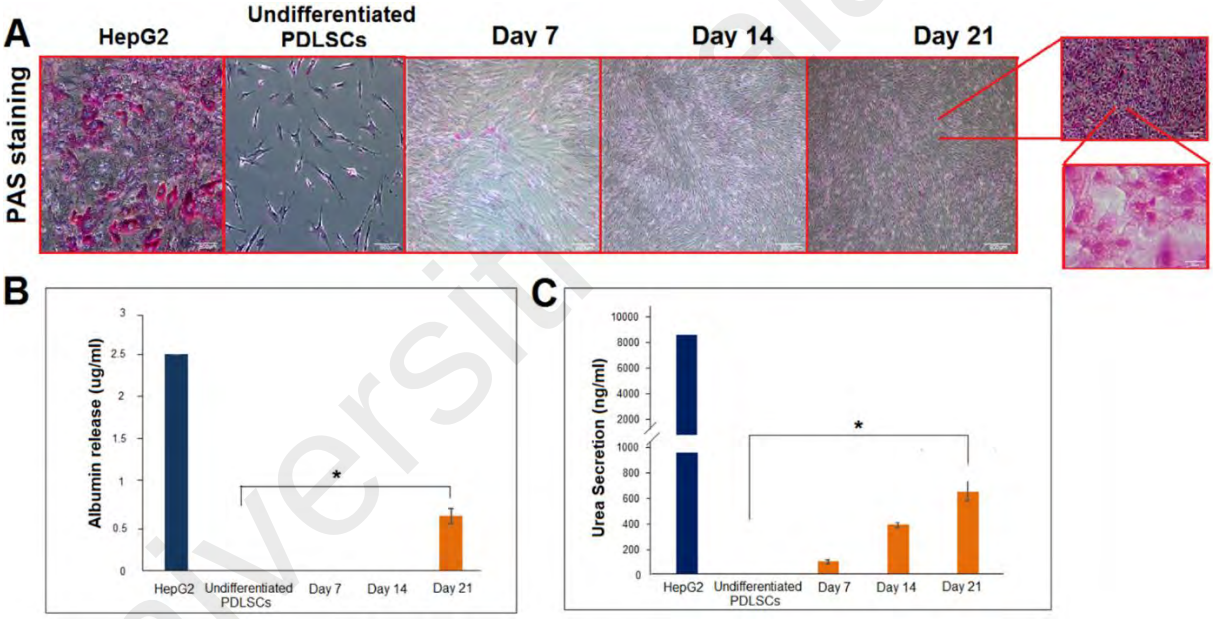


Figure 4.8: Ultimate functional affirmation of PDLSCs-D differentiation into hepatic lineage. A) PAS stained PDLSCs-D result showed the increase ability of glycogen uptake from Day 7 onwards as PDLSCs-D transformed into hepatic like cells. B) Secretion of albumin were only detected on Day 21 suggesting the presence of fully formed PDLSCs-D into hepatic like cells. C) Ureagenesis an important liver role was detected on gradual upsurge from Day 7 to Day 21. However, the levels of the aforementioned functionality of PDLSCs-D differentiation into hepatic lineage were lower compared to HepG2 cells. Asterisk* mark indicates significance with p-value to be < 0.05. Mean ± SD values are shown from four independent experiments.

4.4 Discussion

Viable curative options for liver diseases have resulted in a shift towards cell therapies and cell transplantation and as a result obtaining hepatocytes from intra and extra-hepatic origin have been extensively explored. Recently, research pertaining to generating hepatocyte-like cells are focused on many adult stem cell lineages. The present study demonstrates that putative stem cells from periodontal ligament are able to differentiate into morphologic and functionally sound hepatocyte-like cells *in-vitro*.

Under the influence of exogenous factors cocktails (hepatocyte growth factor, oncostatin M and dexamethasone), these cells display the morphological changes typical of primary hepatocyte. The cells tend to develop into polygonal shapes from spindle fibroblast morphology through cytoplasm contraction (Behbahan et al. 2011). The phenomena of phenotype modification is the first landmark on identifying the ability of PDLSCs-D to differentiate into hepatocyte-like cells.

General insights into mammalian liver development show that the whole process proceeds through several distinct steps and that many transcription factors and proteins are involved in each step (Soto-Guiterrez et al. 2008). Cells with primitive endodermic phenotypes will progress into definitive endodermic phenotypes, and then on to specific hepatic endoderms, followed by hepatoblasts, and finally mature hepatocytes in response to a series of factors, some secreted by surrounding cells (Lemaigre 2009). During the differentiation of PDLSCs-D, similar occurrence of those transcription factors and proteins were detected that depict the tangible transition of PDLSCs-D into hepatocyte-like cells. Most prominently, SOX17, GATA4 and AFP genes were detected as these are known as the

earliest markers for the detection of hepatocyte differentiation and signifying the induction of definitive endoderms (Rossi et al. 2001).

Thereafter, responsible late hepatic- specific markers such as cytokeratin (CK18) and hepatocyte nucleus factor (HNF4α) appear to accompany early endoderm markers. CK18 is considered as a simple epithelial keratin required in the maintenance of hepatocyte integrity (Loranger and Duclos 1997) while HNF4α is known as the master gene for hepatocyte differentiation responsible in exhorting reciprocal interaction with liver enriched transcription factor network and WNT/b-catenin pathway (Watt et al. 2003; Santagelo et al. 2011).

In addition, generated hepatocyte-like cells from PDLSCs-D were also able to express late hepatic enzymes such as tyrosine aminotransferase (TAT) that are observed in cellular differentiation of experimental models of liver development/ maturation *in vitro* (Shelly et al. 1989). Other late markers of expression noted were TO and TDO which denote the amino acid metabolism potential of the hepatocyte-like cells from PDLSCs-D (Alison et al. 2004). In contrast, a lower expression of secreted plasma protein gene AAT known as acute phase protein was also detected. This gene is normally stimulated by inflammatory cytokines, such as TNF-alpha and IL-6, increased its expression by not more than 100-fold in native liver (Kalsheker et al. 2002). It is possible that the low expression of alpha-1-antitrypsin in generated hepatocyte-like cells from PDLSCs-D is due to the experimental condition and absence of inflammatory cytokines.

Other relatively important markers include the cytochrome (Cyp) family marker Cyp3A4 responsible for biotransformation enzymes (Gomez-Lechan et al. 2004) and Cyp7A1, a marker otherwise noted as a hallmark of a fully differentiated hepatocyte.

Unfortunately, the low expression and absence of these genes indicated the presence of immature characteristics in our differentiated PDLSCs-D.

Surprisingly, we found that hepatocyte-like cells derived from PDLSCs-D expressed ALB only on day 21. This protein is usually synthesized by mature hepatocyte during early fetal stages and reaches the maximal level in adult hepatocytes (Donato et al 2008). This demonstrates that there is fully matured cell among hepatocyte-like cells differentiated from PDLSCs-D.

The molecular data confirms previous studies carried out on dental pulp stem cells (10) wherein the sequential expression of genes along the hepatocyte differentiation pathway were established in the course of PDLSCs-D transition into hepatocyte-like cells.

Concomitant with the gene and protein expression, generated hepatocyte-like cells from PDLSCs-D showed the potential to store glycogen which is regarded as basic but important requirement for hepatocytes' role in glucose metabolism (Wu and Tao 2012). In addition to this, production of urea and albumin measured further confirm hepatic function.

Despite being able to perform the functional activities, our data suggests that the manifestation of markers being expressed are in keeping with hepatocyte-like cells from PDLSCs-D of immature hepatic characteristics and within a heterogeneous population. We hypothesize that this could be due to absence of key regulators even though early hepatogenic transcripts are clearly present in PDLSCs-D.

Therefore, future studies should be directed toward the elucidation of the full composite of factors and culture conditions required to generate completely differentiated and mature hepatocytes like cell population from PDLSCs-D, clearly a highly complex developmental program that ultimately mediates hepatic differentiation.

At present, the potential use of the conveniently obtained PDLSCs-D beyond dental tissue regeneration might hold a promise to develop new approaches and give ways to treat liver diseases. In additional, these valuable sources of very proliferative stem cells from dental origin is a simple alternative to harvesting stem cells from other tissues that require invasive surgical procedure or ethical issues associated. However, further research on the *in vivo* behaviour of these cells are required to determine the potential to bring about regeneration in clinical conditions especially in liver associated diseases.

4.5 Summary

Based on the findings of this study, PDLSCs-D were able to differentiate along a hepatic lineage. Presence of polygonal shape cells typical of epithelial cells further acknowledge the ability of PDLSCs-D to form hepatic-like cells. In addition to the morphological attributes, molecular changes associated with hepatic features were found in the differentiating as well differentiated PDLSCs-D. Likewise, detection of early endodermal markers as well as hepatic markers. Although, the functions of the differentiated PDLSCs-D resemble similar to that of the hepatic cells, the level of gene expression together with the functional assay was lower than comparable to the control cells (HepG2 and adult liver tissue). Hence, PDLSCs can be deliberated as potential MSCs for usage in liver associated therapy.

CHAPTER 5: ISOLATION OF EXOSOMES DERIVED FROM HUMAN DECIDUOUS PERIODONTAL LIGAMENT STEM CELLS

5.1 Introduction

Customarily, emergences of other source of cells besides intra-organ cells such as in the case of liver regeneration wherein MSCs differentiated into hepatocytes have been assisting along the way in the field of regenerative medicine (Fausto, 2004; Kuo et al., 2008; Yi et al., 2012; Liu et al., 2015). While regenerative medicine are undertaking the quest in finding the most suitable type of MSCs for regenerative therapy purpose, the currently observed final outcomes of cell based therapies are often similar with the fact and lacking of convincing evidence on donor –recipient in most of the studies indicate differentiation of transplanted cells may not be the main mechanism involved during regeneration. Conversely, recent developments in the latter field have put forward other possible mechanisms that could actually involve with MSCs-mediated regeneration of damaged tissues (Spees et al., 2016). Even though Pittenger and team revealed that the possible mechanism is merely from the cytokines and growth factor production by the MSCs rather than the cell replacement and differentiation activity (Pittenger et al., 2015). However, the findings of exosomes released by MSCs have been found to be the main factor mediating regeneration (van Poll et a., 2008; Yeo et al., 2013; Wen et al., 2016; Derkus et al., 2017; Phinney and Pittenger, 2017). Therefore, great number of studies have shown significant progress of research that unwrapped new perspectives for cell free based regeneration therapies. In this new field, focus has been shifted toward paracrine mediated regeneration in replacement to the existing cell mediated regeneration. DSCs are of particular interest which have many superiority extending from conveying preeminence in terms of ethic and safety issues to unlimited and

minimally invasive source of MSCs, (Gronthos et al., 2000; Seo et al., 2004; Miura et al., 2003; Petrovic and Stefanovic, 2009; Bakkar et al., 2017).

The influence of the paradigm shift from cell mediated regeneration to cell free mediated regeneration leads to the aim of the current work. Having this in mind, under the scope of the present review, we would address the existence of exosomes derived from PDLSC-D in which isolation and characterization being the main aim of this study.

Experimental works were carried out to meet the following objectives:

Aim III: To obtain exosomes from PDLSC-D conditioned medium. The objectives were:

1. To isolate exosomes from PDLSCs-D conditioned medium
2. To quantify and characterize exosomes derived from PDLSC-D
3. To determine the correlation between cell confluence and exosome release

5.2 Materials and Methods

5.2.1 Ethics approval for collection of human extracted deciduous teeth

Sample collections were conducted with prior written informed consent according to the guidelines set by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number: DF CD 1411/0087 (P) (Appendix A).

5.2.2 Preparation of cell culture medium

5.2.2.1 Preparation of culture media

Periodontal ligament stem cells (PDLSCs-D) culture medium was prepared by using DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen) with the composition as shown in Table 5.1

Table 5.1: Composition of culture media

Component	Manufacturer	Volume
Australian characterised Fetal Bovine Serum (FBS)	Hyclone, Thermo Fisher, Scientific Inc, Waltham, MA	50.0 ml
Penicillin/Streptomycin	Invitrogen	2.5 ml
Glutamax	Invitrogen	5.0 ml
DMEM-KO	Invitrogen	442.5 ml

5.2.3 Culture of periodontal ligament derived stem cells (PDLSCs-D)

PDLSCs-D (n = 3) were isolated as previously outlined in Section 3.2.3. The cells were then maintained and expanded in culture media containing DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen). Once cells reached 80 – 90 % confluence, cells were rinsed twice using 5 ml DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove culture media and debris. Subsequently, 2 ml of 0.05% EDTA-Trypsin (Invitrogen) were added to into the culture flask and incubated at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 to detach cells via enzymatic reaction. Immediately after complete detachment, 8 ml of culture media were added to neutralise as to avoid cell apoptosis. Next, the cells in the culture media were transferred into 15 ml centrifuge tube (Axygen) and were centrifuged at 1250 rpm for 6 minutes at 25 ºC in which supernatant was discarded and cells re-suspended into culture media. Aliquot of solution was used to calculate total cell count. A total of 10 µl from the solution was mixed with 90 µl of Trypan Blue stain (Sigma Aldrich) and 10 µl of the mixture was transferred to a haemocytometer (Sigma Aldrich) and total cell count was determined by viewing under microscope (Olympus). Cells not stained with Trypan Blue stain were counted based on the following formula: [Number of cells x dilution factor x 10^4 x volume of media used for re-suspending the cells] /4. After cell count was conducted, some of the newly harvested cells were re-suspended in 7 ml of culture media, seeded into a new T-25 cm^2 culture flask (BD Pharmingen) and incubated at 37 ºC in humidified atmosphere of 95% air and 5% CO_2 at a cell seeding density of 1000 cells/ cm^2. The culture media was replenished every three days until cells reached 80 – 90% confluence for subsequent study.

5.2.3.1 Preparation of xenogenic free culture media for PDLSCs-D

In order to obtain xenogenic free culture media as FBS contains extremely high level of exosomes that might contaminate stem cell derived exosomes, PDLSCs-D were introduced to culture media without FBS 24 hours prior to exosome isolation from PDLCSs culture media.

5.2.4 Relationship between cell confluence and exosome release

Prior to exosome isolation, a titration assay was conducted to determine the optimal cell confluence and exosome release relationship. PDLSCs-D of were maintained and expanded as outlined in Section 5.2.2 in culture media. PDLSCs-D of passage 3 were then seeded at a density of 5000 cells/cm^2 in 6-well cell culture plates and kept in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37˚C. Cell culture was washed with DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove culture media and debris and then media was substituted with culture media without FBS, 24 hours before exosome isolation and cell count. Cell culture media that were exposed to cells were known as conditioned media. Three different time intervals were chosen for harvesting conditioned media approximately 2, 4 and 6 days after initial seeding. These three different time interval represent, respectively, an approximate cell confluence of 30%, 60% and 80%. The number of cells was determined using Trypan Blue method outlined in Section 5.2.2. Meanwhile exosome amount from these sample were quantified according to the AChE activity (EXOCET quantification kit, System Biosciences) according to the manufacturer's protocol (Salomon et al., 2014) as described later on.

5.2.5 Exosomes isolation from culture media

In order to proceed with exosome isolation, PDLSCs-D of passage 3 were maintained in culture media until they reach 30-40 % confluence (ideal relationship between cell confluence and exosome release determined by titration as outlined in Section 5.2.3). The culture media was then removed, and cell culture were washed gently with DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove debris. Subsequently, culture media was replaced with culture media without FBS for additional period of 24 hours before harvesting conditioned media for exosome isolation. Exosomes were isolated from conditioned media using Total Exosome Isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, the conditioned media was pre-cleared by centrifugation at 2,000 x g for 30 minutes to remove cells debris and other contaminants. The pellets were removed while the supernatant was passed through a 0.22 μm filter before being transferred to a new centrifuge tube. Conditioned media was added with Total Exosome Isolation reagent at the ration of 1: ½ volume and incubated overnight at 2-8˚C. Later on, the mixture were centrifugation at 10,000 x g at 2-8˚C for one hour. The supernatant was aspirated and discarded. The exosomes were pelleted at the bottom of the tubes. Finally, pellets were re-suspended in 100 μl of phosphate buffered saline (PBS) and stored at – 20 ˚C for the downstream analysis.

5.2.6 Exosomes quantification

Exosome concentration was estimated using colorimetric particle quantification by EXOCET assay (System Biosciences, Mountain View, CA) according to the manufacturer's protocol (Salomon et al., 2014). This method was based on the measurement of the Acetyl-CoA Acetylcholinesterase enriched activity in exosome. Briefly, each preparation condition was

prepared by diluting 10 µl of exosomal in PBS in 90 µl lysis buffer. The mixture was then incubated at 37 C for 5 minutes followed by vortex and centrifugation at 1500 x g at room temperature for 5 minutes. Subsequently, steps of exosomal quantification were conducted according to the manufacturer's instruction and absorbance was measured at 405nm using spectrometric microplate reader. The assay was calibrated using a standard exosome preparation (System Biosciences).

5.2.7 Characterisation of exosome by CD81 and CD9 detection

Briefly, each pellet of exosomes was added with 200 ml exosome binding buffer, and incubated at 37 ˚C for 20 min. Each sample was added to the wells of the micro-titer plate, and incubated at 37 ˚C for overnight. After washing, the samples were incubated with diluted CD81 antibody (1:100) (ExoELISA, System Biosciences, Mountain View, CA), and subsequently with exosome validated secondary antibody a Horseradish Peroxide enzyme (Goat anti Rabbit) is used for signal amplification and increase assay sensitivity. (1:5000). After adding colorimetric substrate super-sensitive TMB ELISA, reaction terminated using Stop Buffer solution. Finally, CD81- positive exosome levels were quantified with a spectrometric microplate reader at 450 nm absorbance. Similar protocol used for CD9 detection.

5.2.8 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, U.S.A.). The significance level was set at P=0.05. Tukey Post-hoc multiple comparisons were carried out to determine the differences between the groups.

5.3 Results

5.3.1 PDLSCs-D confluence morphology at three different level

PDLSCs-D of passage 3 at different cell confluence are presented as in Fig. 5.1 prior to exosome isolation from conditioned media. Cell viability were 95% using Tryhan blue and no contamination was observed during the collection of conditioned media.

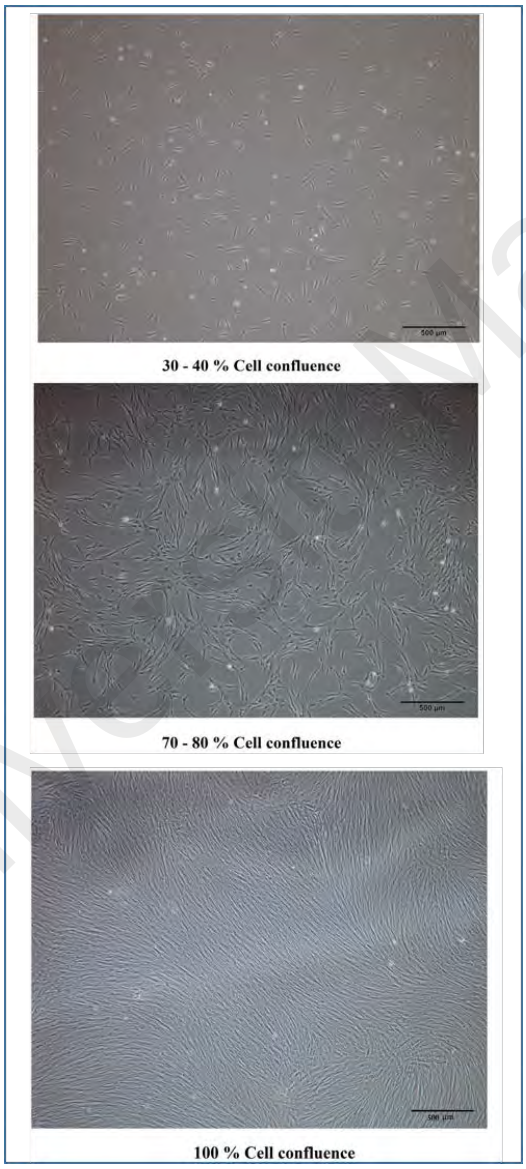


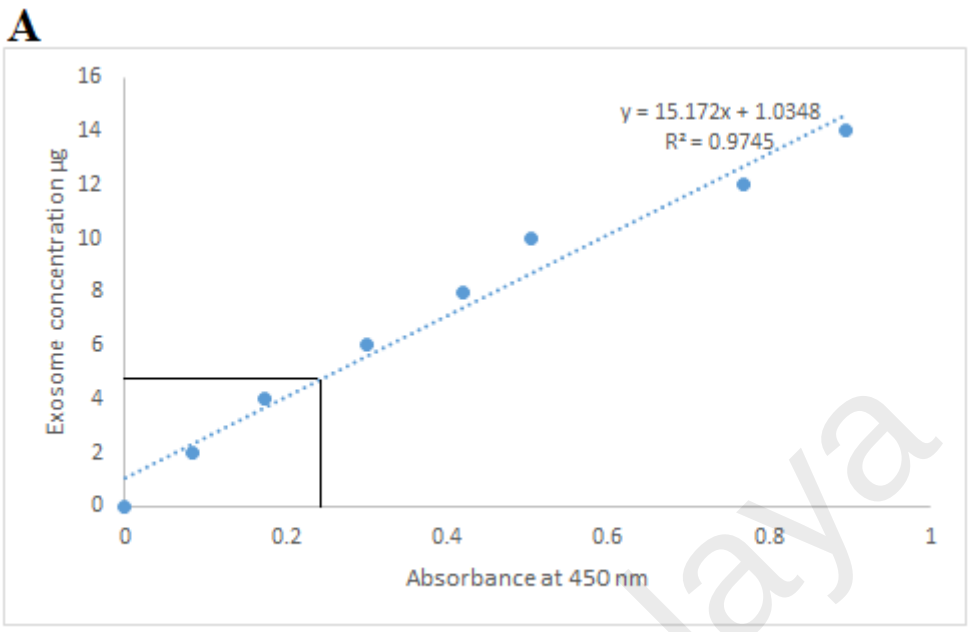
Figure 5.1: Micrographs showing cell confluence morphology: Representative images of PDLSCs-D at confluence level 30-40%, 70-80% and 100% of PDLSCs-D respectively.

5.3.2 Exosome Quantification

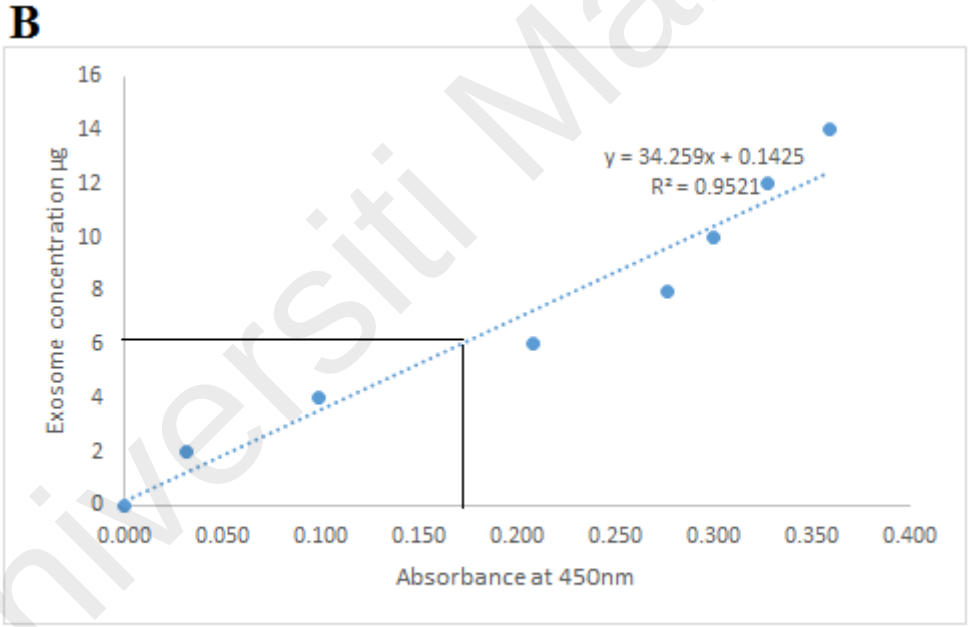
Exosome isolation was conducted using polymeric precipitation by commercially available isolation kit. Following quantification of exosome using ExoCET, amount of PDLSCs-D derived exosome was 42.59 ± 19.63 µg / ml, 128.27 ± 11.05 µg / ml, 72.41 ±15.92 µg / ml for 30%, 70% and 100% cell confluence respectively. Values given were corrected to the background by FBS free culture media used for expansion of PDLSCs-D. Downstream analysis require the quantification value of the exosome isolated from PDLSCs-D.

5.3.3 Expression of surface markers CD9 and CD81 on exosome derived from PDLSCs-D

Fig. 5.2 display the surface marker that are crucial for exosomes. Exosomes isolated from conditioned media of PDLSCs-D were shown to express protein marker specific for exosomes. Presences of surface markers CD9 and CD81 on PDLSCs-D-Exo were confirmed by sandwich ELISA. Both markers were detected at a level between 4-6 µg to 6-8 µg of exosomes for CD9 and CD81 respectively.



[CD9] = 4-6µg



[CD81] = 6-8µg

Figure 5.2: Characterisation of PDLSCs-D-Exo using surface markers. Standard curve of sandwich ELISA for determination of surface markers confirmed the presence of CD9 (A) and CD81 (B) in the exosome isolated from PDLSCs-D. Both surface markers were present in PDLSCs-D derived exosomes.

5.3.4 Correlation between PDLSCs-D cell confluence and PDLSCs-D derived exosome release

Relationship between cell confluence and exosome release were illustrated by Fig. 5.4. The number of exosomes released are correlated with the number of cells in the culture plate during exosome isolation from conditioned media. It was noted that the higher the number of cells during conditioned media collection, the less amount of exosomes being isolated. Exosomes yielded highest when cell confluence were between 70% setting optimal cell confluence for exosome isolation. On contrary, cell with less confluence 30% showed low yield of exosomes.

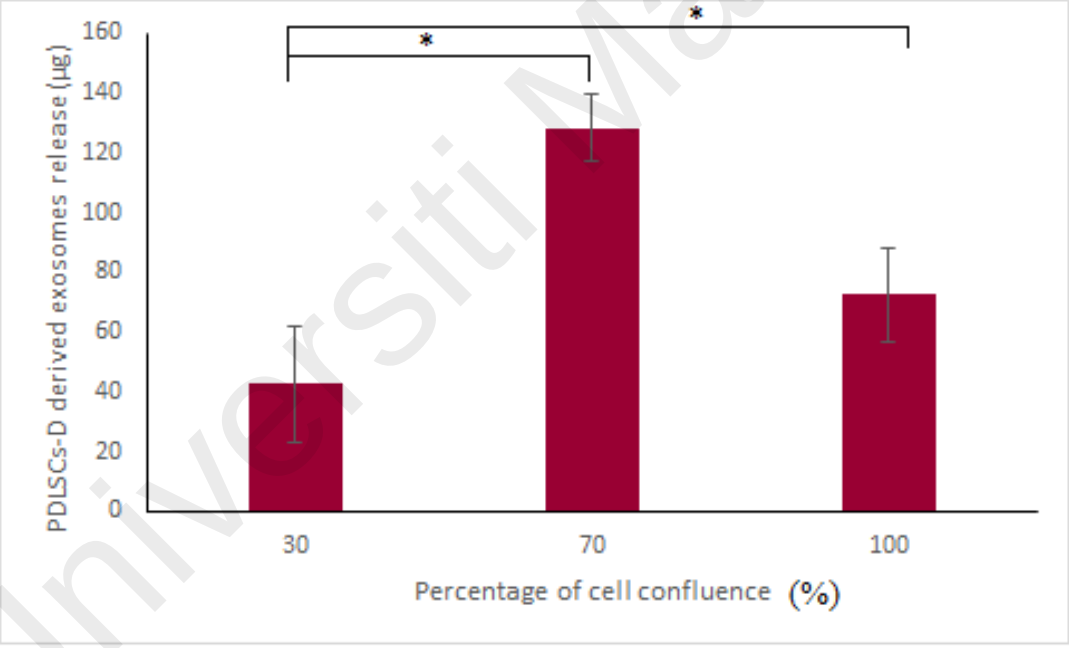


Figure 5.3: Relationship between cell confluence and exosome release. The production of exosome by polymeric precipitation was correlated to three different levels of cell confluence (30%, 70% and 100%). The values that were significantly different were marked as asterisk ($p < 0.05$). Mean ± SD values shown were from six independent experiments.

5.4 Discussion

As previously mentioned in the review, MSCs regenerative potential were postulated to be driven by many factors and mainly by exosomes (Liu et al., 2015, Lou et al., 2017; Phinney et al., 2017). Studies have confirmed MSCs-derived exosomes preserve not only the therapeutic benefits but also the immunosuppressive phenotype of the parent cells (Zhang et al., 2014; Zhou et al., 2019). As a proof of concept, we in current investigation were able to isolate exosomes from PDLSCs-D. Polymeric based precipitation were employed to obtain better exosomal retrieval in comparison to the gold standard exosome isolation through ultracentrifugation (Brown, 2017). Furthermore, isolated exosomes were characterised on the basis of protein surface markers that are known as tetraspanins CD9 and CD81 (Andreu and Yanez-Mo, 2014). PDLSCs-D derived exosomes in this study exhibited positively these tetraspanin. Generally, culture medium is supplemented with fetal bovine serum or human platelet lysate that contain extracellular vesicles. These would some or rather influence the biological behavior of cells (Shelke et al., 2014; Pachler et al., 2017; Lehrich et al., 2018). Therefore, in this study we exposed the PDLSCs-D on serum free media prior to exosome isolation. This step is crucial in avoiding the bias and interference on downstream analysis of exosomes as to exclude other foreign particles involvement (Gstraunthaler, 2003; Mochizuki and Nakahara, 2018)). Besides isolation and characterization of exosomes, we also look into the correlation between cell confluence and exosome release. PDLSCs-D derived exosomes were found to produce more yield in the 70-80 % cell confluence compared to higher cell confluence. Of note, confluent cells are triggered to enter quiescence state when in contact inhibition, thus may decrease exosome release and alter their characteristic compared to actively dividing cells (Steinman et al., 2003; Hayes et al., 2005; Patel et al., 2017).

5.5 Summary

As mentioned in the review earlier, PDLSCs-D in this investigation were successfully shown to produce exosomes using commercially available kit by polymeric precipitation. Moreover, assessments using quantification and surface marker identification confirmed the presences of exosomes. The amount of exosomes release from conditioned media were positively corresponding to the number of cells in the culture plate. Nevertheless, at a 100% confluent cells, the exosome release reported to be reduced. Thus, concluding the fact that to maximize exosome yield, harvesting conditioned media should be done when cells are at an optimal confluence. Another important aspect to be considered is the application xenogenic free culture media, as in this study PDLSCs-D were exposed to culture media without exogenous serum (FBS) and this would prevent possible bias in the production of exosomes by excluding foreign exosomes. Hence, based on these results, further studies can be paved into the functionality of exosomal in *in vitro* or *in vivo* condition comprising the regenerative potential.

CHAPTER 6: HEPATOXICITY AND HEPATOPROCTETIVE POTENTIAL OF PDLSCS-D EXOSOMES ON *IN VITRO* MODEL OF LIVER INJURY

6.1 Introduction

Liver an organ known for its special ability to recuperate due to injury via regeneration, though the degree of injury limits this ability especially when the rate of injury exceeds the rate of repair (Fausto et al, 2006; Michalopoulos, 2007). In such cases, orthotropic liver transplantation or hepatocytes transplantation were regarded as the only curative treatments found to be applicable. Nevertheless, the scarcity of suitable donors, invasive methods involved, immune-rejection and inevitable side effects continue to be the enormous burden (Dhawan et al., 2010; Zarrinpar and Busuttil, 2013). Over the past decades, the advancement in employing MSCs for cellular replacement became an alternative cell based regenerative treatment (Volarevic et al., 2014; Eom et al., 2015; Kholodenko and Yarygin, 2017). Likewise, numerous studies have demonstrated the ability of these cells to differentiate into hepatocyte-like cells *in vitro* (Banas et al., 2007; Snykers et al., 2011; Pournasr et al., 2011; Hang et al., 2014; Vasanthan et al., 2014) and also to the extent in which these cells able to improve *in vivo* liver injuries (Yin et al., 2015; Hu and Li., 2015; Ishkitiev et al., 2015; Zhang et al., 2017). Initially, MSCs were thought to engraft and replace at cellular level during liver injury (Yoshida et al., 2007; Liu et al., 2015), later on research have put forward other factors that might take place during liver regenerative treatment rather than engraftment and differentiation of cells (van Poll et al., 2008; Li et al., 2013; Xagorari et al., 2013; Tan et al., 2014). These recent developments have shed light on the role of extracellular vesicles

produced by MSCs by zooming into one of the crucial subsets termed exosomes (Lai et al., 2015; Phinney et al., 2017).

Of note, exosomes have advantages over corresponding MSCs as they are non-viable, smaller in size with less complexity and most importantly potential to avoid some regulatory issues face by MSCs (Katsuda et al., 2013; Lou et al., 2017).

With the above mentioned concerns, this has lead us to the aim of current work in which the hepatoprotective and hepatoregenerative potential of PDLSC-D derived exosomes were assessed on *in vitro* model of liver injury. This may provide insights on and perspectives for future development of DSCs secretome with respect to the release of exosomes that would have certain advantages over injection or administration of living DSCs. Ultimately, covering the current knowledge on creating cell free therapy for liver diseases.

Aim IV: To determine the efficacy of exosomes derived from PDLSCs-D in the treatment of *in vitro* hepatic injuries. The objectives were:

1. To create an *in vitro* model of hydrogen peroxide (H_2O_2) induced- hepatic injury using HepG2 cells
2. To optimize and evaluate the cytotoxicity of exosomes derived from PDLSCs-D
3. To investigate the potential of exosomes derived from PDLSCs-D in treating *in vitro* hepatic injuries

6.2 Materials and Methods

6.2.1 Ethics approval for collection of human extracted deciduous teeth

Sample collections were conducted with prior written informed consent according to the guidelines set by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Medical Ethics Clearance Number: DF CD 1411/0087 (P) (Appendix A).

6.2.2 Preparation of cell culture medium

6.2.2.1 Preparation of culture media

Periodontal ligament stem cells (PDLSCs-D) culture medium was prepared by using DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen) with the composition as shown in Table 6.1

Table 6.1: Composition of culture media

Component	Manufacturer	Volume
Australian characterised Fetal Bovine Serum (FBS)	Hyclone, Thermo Fisher, Scientific Inc, Waltham, MA	50.0 ml
Penicillin/Streptomycin	Invitrogen	2.5 ml
Glutamax	Invitrogen	5.0 ml
DMEM-KO	Invitrogen	442.5 ml

6.2.3 Culture of periodontal ligament derived stem cells (PDLSCs-D)

PDLSCs-D (n = 3) were isolated as previously outlined in Section 3.2.3. The cells were then maintained and expanded in culture media containing DMEM-KO (Invitrogen) supplemented with 10% (v/v) Australian Characterised Fetal Bovine Serum (FBS) (Hyclone, Thermo Scientific), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen). Once cells reached 80 – 90 % confluence, cells were rinsed twice using 5 ml DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove culture media and debris. Subsequently, 2 ml of 0.05% EDTA-Trypsin (Invitrogen) were added to into the culture flask and incubated at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 to detach cells via enzymatic reaction. Immediately after complete detachment, 8 ml of culture media were added to neutralise as to avoid cell apoptosis. Next, the cells in the culture media were transferred into 15 ml centrifuge tube (Axygen) and were centrifuged at 1250 rpm for 6 minutes at 25 ºC in which supernatant was discarded and cells re-suspended into culture media. Aliquot of solution was used to calculate total cell count. A total of 10 µl from the solution was mixed with 90 µl of Trypan Blue stain (Sigma Aldrich) and 10 µl of the mixture was transferred to a haemocytometer (Sigma Aldrich) and total cell count was determined by viewing under microscope (Olympus). Cells not stained with Trypan Blue stain were counted based on the following formula: [Number of cells x dilution factor x 10^4 x volume of media used for re-suspending the cells] /4. After cell count was conducted, some of the newly harvested cells were re-suspended in 7 ml of culture media, seeded into a new T-25 cm^2 culture flask (BD Pharmingen) and incubated at 37 ºC in humidified atmosphere of 95% air and 5% CO_2 at a cell seeding density of 1000 cells/ cm^2. The culture media was replenished every three days until cells reached 80 – 90% confluence for subsequent study.

6.2.3.1 Preparation of xenogenic free culture media for PDLSCs-D

In order to obtain xenogenic free culture media as FBS contains extremely high level of exosomes that might contaminate stem cell derived exosomes, PDLSCs-D were introduced to culture media without FBS 24 hours prior to exosome isolation from PDLCSs-D culture media.

6.2.4 Exosomes isolation from culture media

In order to proceed with exosome isolation, PDLSCs-D of passage 3 were maintained in culture media until they reach 30-40 % confluence (ideal relationship between cell confluence and exosome release determined by titration as outlined in Section 5.2.3). The culture media was then removed and cell culture were washed gently with DPBS (- Ca^{2+}, - Mg^{2+}) (Invitrogen) to remove debris. Subsequently, culture media was replaced with culture media without FBS for additional period of 24 hours before harvesting conditioned media for exosome isolation. Exosomes were isolated from conditioned media using Total Exosome Isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, the conditioned media was pre-cleared by centrifugation at 2,000 x g for 30 minutes to remove cells debris and other contaminants. The pellets were removed while the supernatant was passed through a 0.22 μm filter before being transferred to a new centrifuge tube. Conditioned media was added with Total Exosome Isolation reagent at the ration of 1: ½ volume and incubated overnight at 2-8˚C. Later on, the mixture was centrifugation at 10,000 x g at 2-8˚C for one hour. The supernatant was aspirated and discarded. The exosomes were pelleted at the bottom of the tubes. Finally, pellets were re-suspended in phosphate

buffered saline (PBS) and stored at – 20 ˚C for the downstream analysis. Exosomes isolated from PDLSCs-D were labelled as PDLSCs-D-Exo thereafter.

6.2.5 Exosomes quantification

Exosome concentration was estimated using colorimetric particle quantification by EXOCET assay (System Biosciences, Mountain View, CA) according to the manufacturer's protocol (Salomon et al., 2014). This method was based on the measurement of the Acetyl-CoA Acetylcholinesterase enriched activity in exosome. Briefly, each preparation condition was prepared by diluting 10 µl of exosomal in PBS in 90 µl lysis buffer. The mixture was then incubated at 37 C for 5 minutes followed by vortex and centrifugation at 1500 x g at room temperature for 5 minutes. Subsequently, steps of exosomal quantification were conducted according to the manufacturer's instruction and absorbance was measured at 405nm using spectrometric microplate reader.

6.2.6 Culture and seeding of hepatocellular carcinoma cell line (HepG2)

Human hepatocellular carcinoma cell line (HepG2) (ATCC, Biorev) were purchased. These cells are highly differentiated and display many of the genotypic features of normal liver cells (Sassa et al., 1987). These cells were seeded and maintained as an adherent cell in culture media as in Table 6.1 and incubated at 37˚C and 5 % CO_2 in humidified chambers. Once cells reached 70 – 80 % confluence, cells were seeded at density of 1500 cells/ cm^2 after dissociation with 0.05 % EDTA-Trypsin (Invitrogen). Media were changed every alternate days.

6.2.7 *In vitro* study on the cytotoxicity of PDLSCs-D-Exo on HepG2 cells

For cytotoxicity test, the cells were seeded at a density of 1500 cells/ cm^2 and allowed to adhere for 24 hours prior to exposure to various concentration (0, 0.05, 0.1, 1, 5 and 10 µg) of PDLSCs-D-Exo and monitored over the period of 48 hours. The cells were then assessed for cell viability using MTT assay.

HepG2 cells trypsinised and plated into 96 well plates at specific seeding densities of 6×10^3 cells per well, incubated overnight at 37 °C and 5% CO2. The medium was then removed and fresh culture media was added. Extracts as in the case PDLSCs-D-Exo were then added in triplicate and incubated for 24 and 48 hours respectively. At the end of the incubation period, test samples were removed from the wells and replaced with 200 µl fresh culture media to which 30 µl of 5 mg/ml MTT was added and incubated for 4 h. After incubation with MTT, the media in each well was removed and the formazan crystals that formed were dissolved by adding 50 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich) solubilizing reagent to each well of the plates. The plates were gently shaken until the crystals dissolved. The amount of MTT was measured immediately by detecting the absorbance at 570 nm (Mosmann, 1983). The percentage of cell viability was calculated using the formula below: The intensity of the MTT formazan produced by living metabolically active cells is directly proportional to the number of live cells present. Standard graph was plotted by taking concentration of PDLSCs-D-Exo as axis X and relative cell viability on axis Y.

$$\% \text{ cell viability} = \frac{\text{Mean absorbance of sample}}{\text{Mean absorbance of control}} \times 100$$

6.2.8 Preparation of *in vitro* model of hepatic injury

In vitro model of hepatic injury were created by exposure with various concentrations (60 µM, 100 µM and 250 µM of hydrogen peroxide (H_2O_2) on HepG2 cells to induce oxidative stress injury for 24 hours and the concentration is adjusted to achieve 50% cell viability (IC50) (lethal concentration in which 50% of the cells are killed) in order to establish the hepatic injury model. HepG2 cells were allowed to adhere 24 hours prior to treatment of 250 µM of H_2O_2 (ideal concentration achieved by IC50) in culture media supplemented with 0.5 µg/ml of PDLSCs-D-Conditioned media (CM) or 0.5 µg/ml of PDLSCs-D-Exo. There were four groups as described below:

Group A : HepG2 cells with no treatment

Group B : HepG2 cells exposed to H_2O_2

Group C : HepG2 cells exposed to H_2O_2 and treated with 0.5 µg/ml of PDLSCs-D-CM

Group D : HepG2 cells exposed to H_2O_2 and treated with 0.5 µg/ml of PDLSCs-D-Exo

All the above treatment groups were monitored over 48 hours then were subjected to following assessments.

6.2.8.1 Effect of PDLSCs-D-Exo on lactate dehydrogenase (LDH) activity in H_2O_2 induced hepatic injury

Lactate dehydrogenase (LDH) (Abcam) activity were assessed over the period of 24 hours before and after H_2O_2- induced HepG2 injury treatment with PDLSCs-D-Exo as according to manufacturer's instruction.

6.2.8.2 Effect of PDLSCs-D-Exo on superoxide dismutase (SOD) activity in H_2O_2 induced hepatic injury

After exposure cells were harvested and mixed with 1 ml of cold 1 x Lysis Buffer containing (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1 mM EDTA). Subsequently, the cells then lysed by centrifugation at 12,000 x g for 10 minutes. The supernatant containing cell lysate was transferred to a new 1.5 ml centrifuge tube and quantified using OxiSelect™ Superoxide Dismutase activity assay (Cell Biolabs, Inc, San Diego, CA). Samples together with blank were prepared in a 96 well microtiter plate and 10 µl of prediluted 1 x Xanthine oxidase solution were added into each well. The solutions were mixed thoroughly and incubated at 37˚C for one hour. Then, SOD content were measured at 490 nm absorbance in a microplate reader. SOD activity were calculated using equation as below:

$$\text{SOD activity Inhibition (\%)} = \frac{\text{OD Blank - OD Sample}}{\text{OD Blank}} \times 100$$

6.2.8.3 Effect of PDLSCs-D-Exo on Malondialdehyde (MDA) activity H_2O_2 induced hepatic injury

The cells were harvested and resuspended in PBS and then homogenized by centrifugation at 12, 000 x g for 10 minutes. The MDA assay was quantified using OxiSelect™ TBARS Assay Kit (MDA Quantitation) Activity Assay (Cell Biolabs Inc). The standard protein solution provided which was a series of standard solutions ranging from 0 µM-125 µM was prepared to construct a standard curve. 100 µL of SDS Lysis Solution was added to both 100 µL unknown samples and the MDA standards. The well-mixed samples were incubated for

5 minutes at 25˚C (room temperature) in microcentrifuge tubes. A 250 µL of TBA Reagent was added to each sample and standard to be tested. The tubes were closed and incubated at 95°C for 45- 60 minutes using a heat block, cooled at room temperature for 5 minutes, and centrifuged at 3000 rpm for 15 minutes. The supernatant were used for analysis of MDA content by measurement of absorbance at 532 nm on a microplate reader.

6.2.9 Statistical analysis

All values are given as mean and standard deviation. Statistical comparison were made and data analysed using analysis of variance (ANOVA) with significance level set at P= 0.05. Tukey post hoc multiple comparison test were carried out to determine the differences between the groups.

6.3 Results

6.3.1 PDLSCs-D-Exo as a non-toxic compound on HepG2 cells

Cell viability of HepG2 after exposure to PDLSCs-D-Exo were assessed by MTT assay after 24 hours. As illustrated in Fig.6.1, there were no intrinsic cytotoxicity on the cell viability percentage following exposure with PDLSCs-D-Exo suggesting that PDLSCs-D-Exo is not cytotoxic. Subsequent analysis were conducted with exposure of 0.05 µg/ml of PDLSCs-D-Exo.

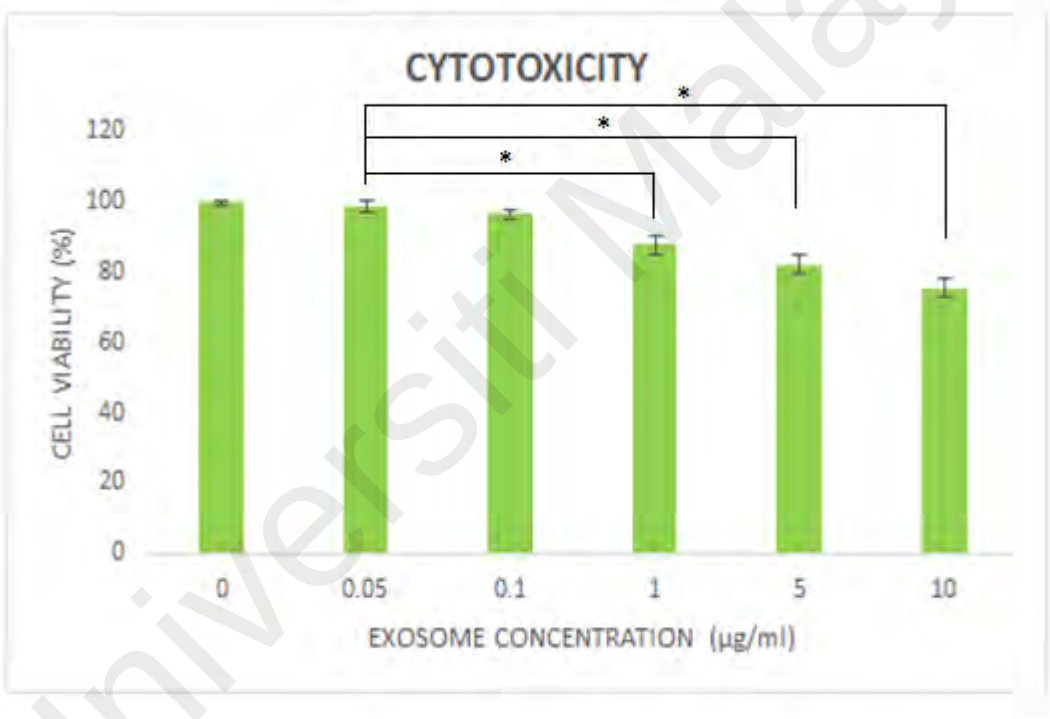


Figure 6.1: Effect of PDLSCs-D-Exo on cell viability of HepG2 cells at various concentration. Mean ± SD values were shown from six independent experiments. The cell viability that were significantly different from that of 0.05 µg/ml exosome concentration were marked with asterisk ($p < 0.05$).

6.3.2 Cell viability after treatment of PDLSCs-D-Exo on H_2O_2 induced hepatic injury

Initial MTT assay (Fig 6.2) showed lower cell viability percentage on H_2O_2 induced hepatic injury which is common as the cells undergo apoptosis due to injury. However, the cell viability observed in treated cells with PDLSCs-D-Exo increase over time period indicating the positive effect of exosome on cell injury.

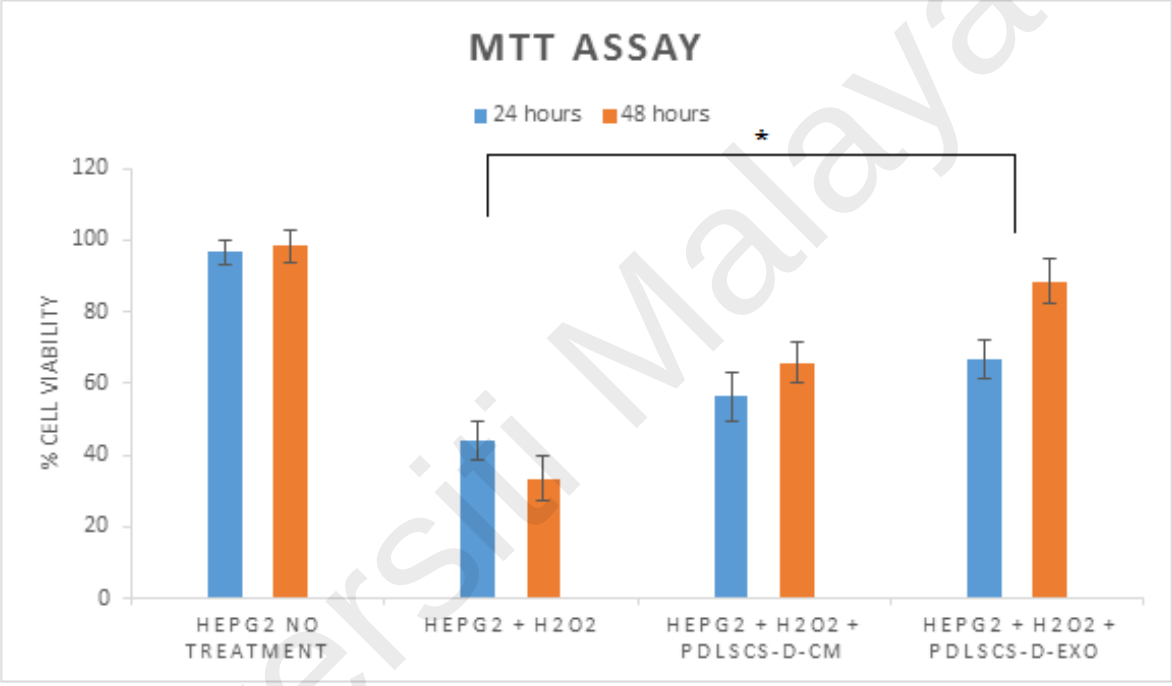


Figure 6.2: MTT assay conducted to investigate effect of PDLSCs-D-Exo treatment on H_2O_2 induced hepatic injury. Prior to treatment with PDLSCs-D-Exo, cell viability was low but increased following treatment. Mean ± SD values were shown from six independent experiments. The values that were significantly different from that of untreated HepG2 groups were marked with asterisk ($p<0.05$).

6.3.3 Effect of Lactate dehydrogenase activity on H_2O_2 induced hepatic injury

Lactate dehydrogenase which is an indicator for cell injury was observed to be increased in H_2O_2 induced hepatic injury as shown in Fig. 6.3. Nevertheless, the LDH activity shown to be decreased significantly after treatment with PDLSCs-D-Exo comparable to the conditioned media exposure.

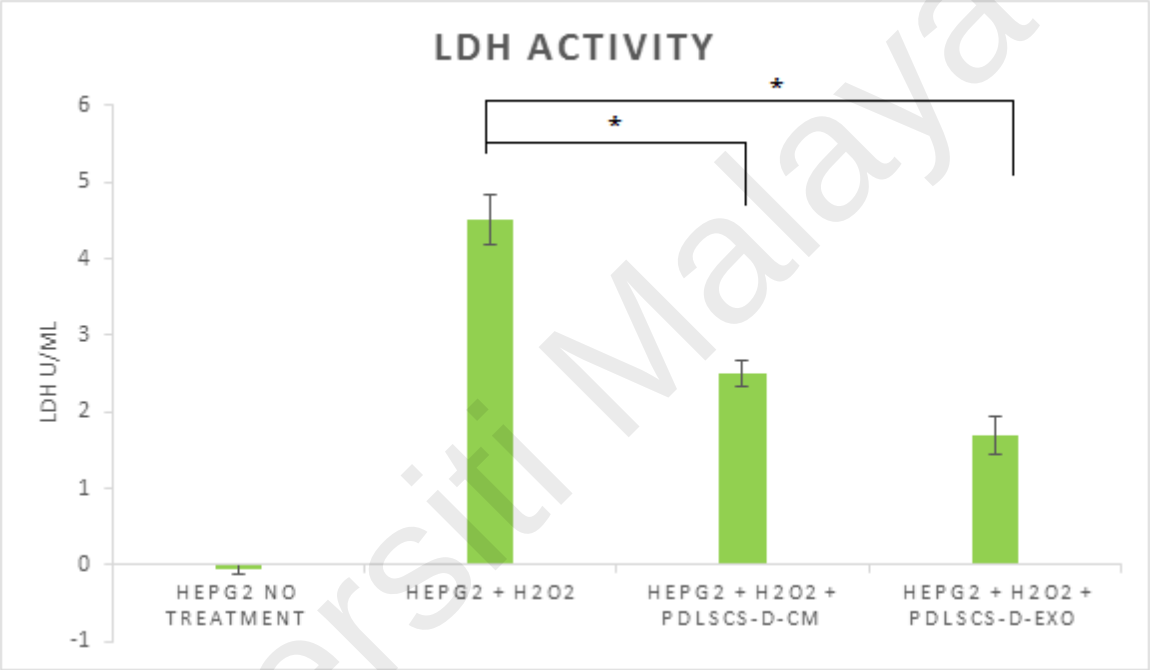


Figure 6.3: Lactate dehydrogenase activity on H_2O_2 induced hepatic injury when treated with PDLSCs-D-Exo for 24 hours. Highest level of LDH activity observed in H_2O_2 induced hepatic injury, however after exposure to PDLSCs-D-Exo treatment LDH level were found to be decreased.Mean ± SD values were shown from six independent experiments. The values that were significantly different from that of untreated HepG2 groups were marked with asterisk ($p<0.05$).

6.3.4 SOD enzyme level on H_2O_2 induced hepatic injury after treatment with PDLSCs-D-Exo

Based on Fig 6.4, SOD activity were noted to be highest in control cells while lower level were observed after being induced with H_2O_2 induced hepatic injury. Nevertheless, SOD activity were measured at higher level after being treated with PDLSCs-D-Exo. Similar effect was observed in conditioned media treated cells. Additionally, SOD level in exosomes treated cells increased over time period of 48 hours compared to the other groups.

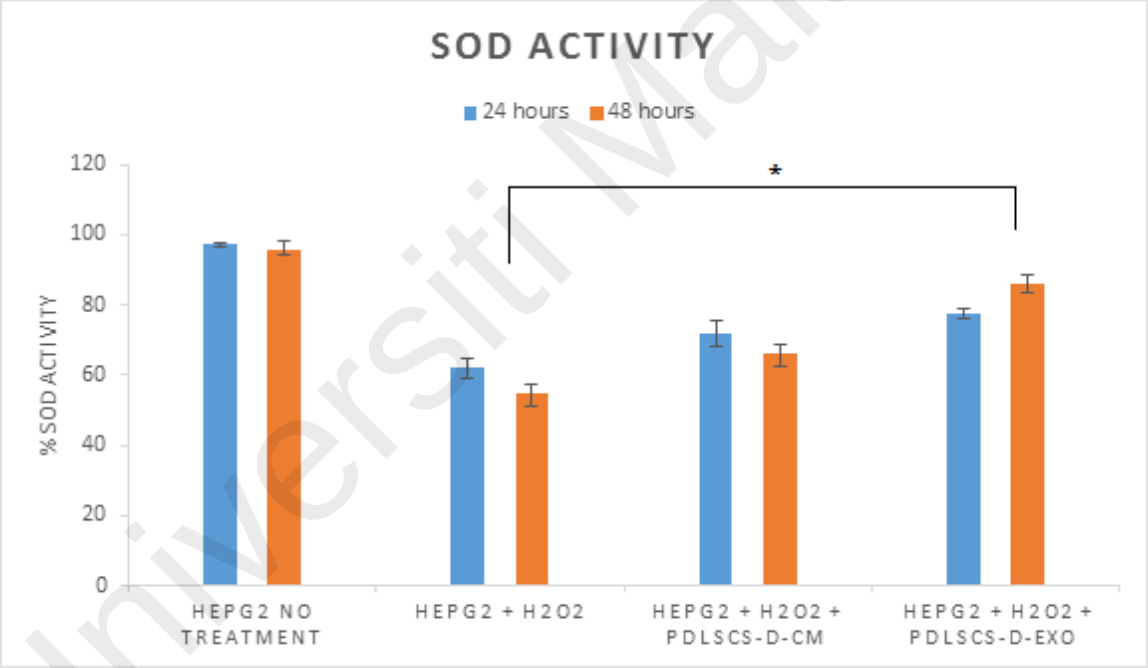


Figure 6.4: Effect of PDLSCs-D-Exo on SOD activity in H_2O_2 induced hepatic injury. Elevated activity of SOD after treatment with PDLSCs-D-Exo was observed.Mean ± SD values were shown from six independent experiments. The values that were significantly different from that of untreated HepG2 groups were marked with asterisk ($p < 0.05$).

6.3.5 MDA content determine lipid peroxidation on H_2O_2 induced hepatic injury before and after treatment with PDLSCs-D-Exo

Lipid peroxidation is correspondingly measured in terms of MDA content. Higher level of MDA content were detected in H_2O_2 induced hepatic injury cells. As shown in Fig. 6.5, this level were measured at a lower level less than 1µM in PDLSCs-D-Exo with no significant different compared to conditioned media exposure after prolong treatment exposure.

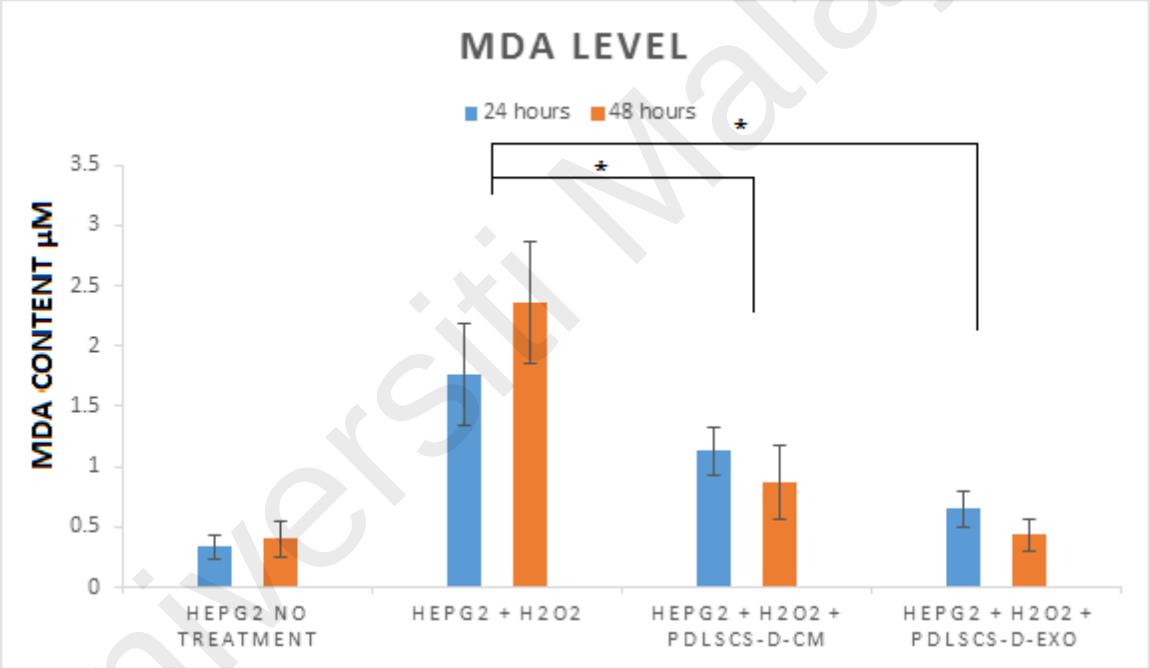


Figure 6.5: MDA content expressed at different level in H_2O_2 induced hepatic injury before and after treatment of PDLSCs-D-Exo. Cells induced with H_2O_2 induced hepatic injury notably had higher level of MDA content when compared to other groups. Mean ± SD values were shown from six independent experiments. The values that were significantly different from that of untreated HepG2 groups were marked with asterisk ($p<0.05$).

6.4 Discussion

Liver injury encompasses many factors especially oxidative stress which are known to be the major pathogenic occurrence that eventually causes liver damage due to ischemic/ reperfusion during liver transplantation (Garcia-Ruiz et al., 2015). Oxidative stress were then contribute to the dysfunction of mitochondrial in liver cells that leads to ROS activity being increased. Eventually altering the protein, lipid, DNA and to the extent of modulating pathways that control biological functions (Peralta et al., 2013; Cichoz-Lach and Michalak, 2014). In that context, H_2O_2 were documented to mimic the oxidative stress generated during early phase of liver injury (Tan et al., 2014). Similarly, in this study H_2O_2 employed to create *in vitro* hepatic injury in HepG2 cells and investigated the effect of PDLSCs-D- derived exosome ability to improve the injury. As previously reported, exosomes from MSCs were shown to express cytoprotective attributes towards H_2O_2 induce hepatic injury. Likewise, PDLSCs-D-Exo treated cells favours cell viability bringing about the role of antioxidant effect along with synergistic effect. Reduced level of LDH activity suggest that PDLSCs-D derived exosomes might play a role in protecting cell apoptosis or necrosis. It was reported that imbalance of oxidant and antioxidant results in oxidative stress and as in this study antioxidant effect were conferred by the elevated level SOD detected in the cells treated in comparison to the untreated. Besides antioxidant property that might involve in improving hepatic injury, lipid peroxidation were taken into consideration as well. Based on the study, the level of lipid peroxidation determined by MDA content that are formed due to instability were also reduced after being treated with PDLSCs-D-Exo It was noteworthy that PDLSCs-D-Exo conferred better cytoprotective agent as compared to conditioned media exposure. Therefore, postulating the amount of trophic factors and other components encapsulated in the exosomes mediating similar effects as exerted by MSCs themselves. The present study

provided information on constituting exosomes from MSCs as a compelling alternative with main advantage over the MSCs as a non-viable cells (Lou et al., 2017). Nevertheless, there are multifarious hepatotropic networks involved in tissue regeneration and regulation. Therefore, it is necessary to further explore the mechanism and also pathways on how PDLSCs-D exerting hepatoprotective properties.

6.5 Summary

Based on this study, exosome from PDLSCs-D were shown to decrease the level of oxidative stress on HepG2 cells induced by H_2O_2. The above findings suggest that PDLSCs-D-Exo contains potential regenerative attributes in hepatoprotectivity manner shown in HepG2. Thus, exosomes from PDLSCs-D may circumvent certain challenges and surrogate cell based therapy mainly due to the fact of viable cells without compromising the benefits reflected by the cells themselves. Eventually providing the paradigm shift of cell mediated regeneration to cell free regeneration in the treatment of liver injury.

CHAPTER 7: CONCLUSION

Within the limitations of this study, periodontal ligament stem cells from deciduous dental origin which are collectively regarded as dental stem cells have been shown to display ability similar to other dental stem cells particularly dental pulp stem cells. On the pretext of these easily available source in an autologous settings, these cells were also shown to be suitable alternative for regenerative purposes beyond tooth regeneration. Besides that, cell free therapy can be achieved with the current approach of MSCs secretome such as exosomes for a better regenerative potential. Ultimately unraveling the drawbacks of cell based therapy for regeneration mainly liver related diseases. This conclusion was drawn on the basis of the followings:

i. Stem cells from PDLSCs-D were confirmed MSCs based on the proposed criteria of the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. PDLSCs-D were able to differentiate into the gold standard differentiation pattern namely osteocytes, chondrocytes, adipocytes and also displayed positive MSCs protein markers.

ii. PDLSCs-D were shown to differentiate into endodermal lineage particularly towards hepatic cells in this study. During the differentiation phase, these cells displayed progress transformation from a fibroblastic cell to a polygonal cell. In addition to that, the differentiated cells were also able to perform functional hepatic role such as albumin secretion, glycogen uptake and urea secretion.

iii. Exosomal isolation from conditioned media of PDLSCs-D were conducted based on commercially available isolation kit. Prior to exosome isolation, stem cells were exposed in serum free media to allow xenogenic free growth environment

that could interfere with downstream exosome analysis. Presence of surface protein CD9 further confirms exosome retrieval from stem cells.

iv. An important aspect was displayed in this study wherein the relation between cell density and exosome release were shown and highlighted the requisite of harvesting conditioned medium at appropriate low cell confluence for better exosome yield.

v. Hepatoxicity and hepatoprotective of exosome derived from PDLSCs-D were assessed on *in vitro* models of liver injury. Acute toxicity test of exosomes derived from PDLSCs-D on HepG2 cells were negative upon exposure to the culture media. Besides that, exosome exposure *in vitro* model of liver injury showed reduction of oxidative stress by improving SOD and GSH levels. Results showed exosomes application can be convened as new cell free approach for liver disease related regeneration.

7.1 Study limitations

Current study encountered following limitations:

i. All the above experiments were carried out at passage 3 of PDLSCs-D due to the fact of low yield from the primary source isolation. However, the drawback of losing homogeneity over time period could be resolved by employing cells from passage 1 itself.

ii. As cell population in the periodontal ligament are heterogeneous, more complex analysis and additional markers are essential to distinguish these PDLSCs-D types.

iii. Additional gene signaling pathway could have been helpful to analyze the propensity of PDLSCs-D towards hepatic lineage.

iv. Limitation of time and budget restricted the more complex analysis particularly concerning the mechanism and pathways involved and conducting *in vivo* studies on both PDLSCs-D and their exosomes.

7.2 Significance and Novelty of the study

Considering the applications of human periodontal ligament stem cells, this study evaluated the potential of the stem cells population from periodontal ligament of a deciduous tooth. Foremost, stem cells from both dental pulp and periodontal ligament can be isolated from single donor tooth, which substantially increases the number of extractable primary cell populations per tooth. Of note, these easily accessible and expandable periodontal tissue may become important future source of stem cells. In addition, the study explored the ability of these stem cells to differentiate into endodermal lineage. Thus, providing additional scientific information on dental stem cells that could be applied beyond tooth regeneration. Furthermore, this study yielded useful information on novel therapeutic strategies by looking into MSCs secretome in particular exosome with the advance of cell free based therapy that could be developed aiming at regeneration of injured or loss tissues. With this in mind, banking of these cells in a similar manner to the umbilical cord will be perfectly suit for future tailor made medicine.

7.3 Future studies

i. Usage of differentiated PDLSCs-D into *in vivo* studies to determine the viability and potential regeneration capacity in liver injury.

ii. Conventional method of exosome isolation which is regarded as gold standard could be considered for side-by-side comparison to determine the exosome release based on quantity and quality.

iii. Incorporating exosomes into *in vivo* study could comprehend the effect of exosome on complex environment prior to clinical approach particularly on liver injury related regeneration.

iv. Validating gene expression and protein analysis need to be considered to elucidate the possible mechanism by which the exosome exerts their therapeutic potential.

REFEENCES

Alison MR, Poulsom R, Forbes S and Wright NA. (2002). An introduction to stem cells. *J. Pathol*., 197: 419-423

Alison MR, Vig P, Russo F et al. (2004) Hepatic stem cells: from inside and outside liver? *Cell Prolif.* 37:1-21.

Anderson DJ1. (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron*. Apr; 30(1):19-35.

Andreu Z, Yáñez-Mó M. (2014) Tetraspanins in extracellular vesicle formation and function. *Front Immunol*. 5:442

Anghileri E, Marconi S, Pignatelli A, Cifelli P, Galié M, Sbarbati A, Krampera M, Belluzzi O, Bonetti B. (2008) Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. *Stem Cells Dev.* Oct; 17(5):909-16.

Appaix, F., Nissou, M. F., van der Sanden, B., Dreyfus, M., Berger, F., Issartel, J. P., & Wion, D. (2014). Brain mesenchymal stem cells: The other stem cells of the brain?. *World journal of stem cells*, 6(2), 134–143.

Banas A, Teratani T, Yamamoto Y, et al. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology*. 2007; 46(1):219-228.

Bansal R, Jain A, Mittal S. (2015). Current overview on challenges in regenerative endodontics. *J Conserv Dent*. 18(1):1-6.

Bartold, P. M., Shi, S. and Gronthos, S. (2006), Stem cells and periodontal regeneration. *Periodontology* 2000, 40: 164-172.

Behbahan IS, Duan Y, Lam A et al. (2011). New approaches in the differentiation of human embryonic stem cells and induced pluripotent stem cells toward hepatocytes. *Stem Cell Rev*. 7:748-759.

Beertsen W, McCulloch CA. and Sodek J. (1997). The periodontal ligament: a unique, multifunctional connective tissue. *Periodontology.* 13: 20-40.

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa. (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* Sep 19; 114(6):763-76.

Berardis S, Dwisthi Sattwika P, Najimi M. & Sokal EM. (2015). Use of mesenchymal stem cells to treat liver fibrosis: current situation and future prospects. *World journal of gastroenterology*, 21(3), 742–758.

Bianco P, Robey PG, & Simmons PJ. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell stem cell*, 2(4), 313–319.

Cai J1, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H. (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology*. May; 45(5):1229-39.

Calabrese D, Roma G, Bergling S, Carbone W, Mele V, Nuciforo S, Fofana I, Campana B, Szkolnicka D, Hay DC., Tchorz J, Bouwmeester T, Wieland S, & Heim MH. (2019). Liver biopsy derived induced pluripotent stem cells provide unlimited supply for the generation of hepatocyte-like cells. *PloS one*, 14(8), e0221762.

Campard D, Lysy PA, Najimi M, Sokal EM. (2008) Native umbilical cord matrix stem cells express heaptic markers and differentiate into hepatocyte-like cells. *Gastroenterology*. 134:833-848.

Caplan AI (1991) Mesenchymal stem cells. *J Orthop Res*. Sep; 9(5):641-50.

Carpenter, L., Carr, C., Yang, C. T., Stuckey, D. J., Clarke, K., & Watt, S. M. (2012). Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. *Stem cells and development*, 21(6), 977–986.

Chang KA, Lee JH, Suh YH. (2014) Therapeutic potential of human adipose-derived stem cells in neurological disorders. *J Pharmacol Sci*.; 126(4):293-301.

Chen X, Armstrong MA. and Li G. (2006). Mesenchymal stem cells in immunoregulation. Immunol Cell Biol, 84: 413-421.

Choi SS, & Diehl AM. (2009). Epithelial-to-mesenchymal transitions in the liver. *Hepatology* (Baltimore, Md.), 50(6), 2007–2013.

Chong J J, Chandrakanthan V, Xaymardan M, Asli N S, Li J, Ahmed I, Harvey RP. (2011). Adult cardiac-resident MSC-like stem cells with a proepicardial origin. *Cell stem cell*, 9(6), 527–540.

Choudhery MS, Badowski M, Muise A, Harris DT. (2013) Comparison of human mesenchymal stem cells derived from adipose and cord tissue. *Cytotherapy*. Mar; 15(3):330-43.

Chuang JH, Tung LC, & Lin Y. (2015). Neural differentiation from embryonic stem cells *in vitro*: An overview of the signaling pathways. *World journal of stem cells*, 7(2), 437–447.

Cicchini C, Amicone L, Alonzi T, Marchetti A, Mancone C, Tripodi M. (2014) Molecular mechanisms controlling the phenotype and the EMT/MET dynamics of hepatocyte. *Liver Int*. 35:302-310.

Cichoż-Lach H, Michalak A. (2014) Oxidative stress as a crucial factor in liver diseases. *World J Gastroenterol*. 20(25):8082-8091

Cocucci E, Meldolesi J. (2015) Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol*. Jun; 25(6):364-72.

Damani A, Jaiman D, Teotia AK., & Kumar A. (2018). Mesenchymal stromal cell-derived exosome-rich fractionated secretome confers a hepatoprotective effect in liver injury. *Stem cell research & therapy,* 9(1), 31.

d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V. and Papaccio G. (2009), Human dental pulp stem cells: from biology to clinical applications. *J. Exp. Zool*., 312B: 408-415.

da Silva Meirelles L, Chagastelles PC, Nardi NB. (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci.* Jun 1; 119(Pt 11):2204-13.

Denham M, Dottori M. (2009). Signals involved in neural differentiation of human embryonic stem cells. *Neurosignals* 17(4):234-41.

Dhara SK, & Stice SL. (2008). Neural differentiation of human embryonic stem cells. *Journal of cellular biochemistry*, 105(3), 633–640.

Dhawan A, Mitry RR, Hughes RD et al. (2004) Hepatocyte transplantation for inherited factor VII deficiency. *Transplantation*. 78:1812-1814.

Dhawan A, Puppi J, Hughes RD, Mitry RR. Human hepatocyte transplantation: current experience and future challenges. *Nat Rev Gastroenterol Hepatol*. 2010;7(5):288-298.

Dominici M1, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*; 8(4):315-7.

Donato MT, Lahoz A, Montero S et al. (2008) Functional assessment of the quality of human hepatocyte preparations for cell transplantation. *Cell Transplant*. 17:1211-1219.

Drukker M, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, …Benvenisty N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 99(15), 9864–9869.

Einsiedel E1, Premji S, Geransar R, Orton NC, Thavaratnam T, Bennett LK.(2009). Diversity in public views toward stem cell sources and policies. *Stem Cell Rev Rep*. Jun;5(2):102-7.

Eom YW, Shim KY, Baik SK. (2015). Mesenchymal stem cell therapy for liver fibrosis. *Korean J Intern Med.* 30(5):580-589.

Estrela C1, Alencar AH, Kitten GT, Vencio EF, Gava E. (2011). Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration *J. Braz Dent*. 22(2):91-8.

Evans MJ, M.H. Kaufman. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature, 292, pp. 154-156F

Fausto N. (2004), Liver regeneration and repair: Hepatocytes, progenitor cells, and stem cells. Hepatology, 39: 1477-1487

Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology. 2006;43(2 Suppl 1):S45-S53.

Fausto N, Campbell JS, Riehle KJ. (2012) Liver regeneration. *J Hepatol*. 2012 Sep; 57(3):692-4.

Fawcett JK and Scott JE. (1960) A rapid and precise method for the determination of urea. *J. Clin. Pathol*. 13:156-159

Feng, J., Mantesso, A., De Bari, C., Nishiyama, A., & Sharpe, P. T. (2011). Dual origin of mesenchymal stem cells contributing to organ growth and repair. *Proceedings of the National Academy of Sciences of the United States of America,* 108(16), 6503–6508.

Fortier LA1. (2005) Stem cells: classifications, controversies, and clinical applications. *Vet Surg*. Sep-Oct; 34(5):415-23.

García-Ruiz I, Solís-Muñoz P, Fernández-Moreira D, Muñoz-Yagüe T, Solís-Herruzo JA. (2015) *In vitro* treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis. *Dis Model Mech*. 8(2):183-191.

Gay I, Chen S and MacDougall M. (2007), Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthodontics & Craniofacial Research*, 10: 149-160. doi:10.1111/j.1601-6343.2007.00399.x

Gnanasegaran N, Govindasamy V, Mani V. and Abu Kasim NH. (2017), Neuroimmunomodulatory properties of DPSCs in an *in vitro* model of Parkinson's disease. *IUBMB Life,* 69: 689-699.

Gomez-Lechon MJ, Donato MT, Castell JV, Jover R. (2004) Human hepatocyte in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab.* 5:443-462.

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.* Sep; 36 (9):1504-15.

Govindasamy V, Ronald VS, Abdullah AN et al. (2011) Differentiation of dental pulp stem cells into islet-like aggregates. *J Dent Res.* 90:646-652.

Gramignoli R, Vosough M, Kannisto K, Srinivasan RC, Strom SC. (2015) Clinical hepatocyte transplantation: practical limits and possible solutions. Eur Surg Res54 (3-4):162-77.

Grompe M. (2002) Adult versus embryonic stem cells: it's still a tie. *Mol Ther*. Sep; 6 (3):303-5.

Grompe M.(2003) Pancreatic-hepatic switches *in vivo*. *Mech Dev*.Jan; 120(1):99-106.

Gronthos S, Mankani M, Brahim J, Robey PG, & 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.

Gronthos S1, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi (2002) Stem cell properties of human dental pulp stem cells. *J Dent Res*. Aug;81(8):5315S.

Gstraunthaler G. (2003) Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX*. 20(4):275-281.

Gurdon JB. (1962). The transplantation of nuclei between two species of Xenopus. *Dev Biol*. Aug; 5:68-83.

Han Y, Li X, Zhang Y, Han Y, Chang F, & Ding J. (2019). Mesenchymal Stem Cells for Regenerative Medicine. *Cells,* 8(8), 886

Handa K, Saito M, Tsunoda A, Yamauchi M, Hattori S, Sato S, Toyoda M, Teranaka T, Narayanan AS. (2002) Progenitor cells from dental follicle are able to form cementum matrix *in vivo*. *Connect Tissue Res.* 43(2-3):406-8.

Hang H, Yu Y, Wu N, Huang Q, Xia Q, Bian J. (2014). Induction of highly functional hepatocytes from human umbilical cord mesenchymal stem cells by HNF4α transduction. *PLoS One*. 2014; 9(8):e104133

Hayes O, Ramos B, Rodríguez LL, Aguilar A, Badía T, Castro FO. (2005) Cell confluency is as efficient as serum starvation for inducing arrest in the G0/G1 phase of the cell cycle in granulosa and fibroblast cells of cattle. *Anim Reprod Sci*; 87(3-4):181-192.

Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, Bussolati B, Camussi G.(2006) Isolation and characterization of a stem cell population from adult human liver. *Stem Cells. Dec*; 24(12):2840-50.

Hilkens P, Fanton Y, Martens W, Gervois P, Struys T, Politis C, Lambrichts I, Bronckaers A.(2014) Pro-angiogenic impact of dental stem cells *in vitro* and *in vivo*. *Stem Cell Res*. May; 12(3):778-90.

Hirata M, Ishigami M, Matsushita Y, Ito T, Hattori H, Hibi H, … Yamamoto A. (2016). Multifaceted Therapeutic Benefits of Factors Derived From Dental Pulp Stem Cells for Mouse Liver Fibrosis. *Stem cells translational medicine,* 5(10), 1416–1424.

Hu C, Li L. (2015). *In vitro* and *In vivo* Hepatic Differentiation of Adult Somatic Stem Cells and Extraembryonic Stem Cells for Treating End Stage Liver Diseases. *Stem Cells Int.*:871972.

Huang GT, 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 L, Liang J, Geng Y et al. (2013) Directing adult human periodontal ligament –derived stem cells to retinal fate. Invest Ophthalmol Vis Sci. 54:3965-3974. DOI: 10.1167/iovs.13-11910

Hyun I. (2010). The bioethics of stem cell research and therapy. *The Journal of clinical investigation*, 120(1), 71–75

Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell,* 142(3), 375–386.

Ikeda E, Yagi K, Kojima M, Yagyuu T, Ohshima A, Sobajima S, Tadokoro M, Katsube Y, Isoda K, Kondoh M, Kawase M, Go MJ, Adachi H, Yokota Y, Kirita T, Ohgushi H. (2008) Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease. *Differentiation,* 76: 495–505.

Ishibashi H, Nakamura M, Komori A, Migita K, Shimoda S. (2009) Liver architecture, cell function, and disease. *Semin Immunopathol*. Sep; 31(3):399-409.

Ishkitiev N, Yaegaki K, Calenic B et al. (2010) Deciduous and permanent dental pulp mesenchymal stem cells acquire hepatic morphology and functional features *in vitro*. *J Endod*. 36:469-474.

Ishkitiev N, Yaegaki K, Imai T, Tanaka T, Nakahara T, Ishikawa H, Mitev V, Haapasalo M. (2012) High-purity hepatic lineage differentiated from dental pulp stem cells in serum-free medium. *J Endod*. 38:475-480.

Ishkitiev N, Yaegaki K, Imai T, Tanaka T, Fushimi N, Mitev V, Okada M, Tominaga N, Ono S, Ishikawa H. (2015) Novel management of acute or secondary biliary liver conditions using hepatically differentiated human dental pulp cells. *Tissue Eng Part* A. 21: 586-593.

Jadlowiec, C. C., & Taner, T. (2016). Liver transplantation: Current status and challenges. *World journal of gastroenterology*, 22(18), 4438-4445.

Jayaraman, P., Nathan, P. , Vasanthan, P. , Musa, S. and Govindasamy, V. (2013), Stem cells conditioned medium: a new approach to skin wound healing management. *Cell Biol Int*, 37: 1122-1128.

Ji K , Liu Y , Lu W , Yang F , Yu J , Wang X, Ma Q , Yang Z, Wen L. and Xuan, K. (2013), Periodontal tissue engineering with stem cells from the periodontal ligament of human retained deciduous teeth. *Journal of Periodontal Research*, 48: 105-116.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. Jul 4; 418(6893):41-9.

Jiang Z, Yu B, & Li Y. (2016). Effect of Three Statins on Glucose Uptake of Cardiomyocytes and its Mechanism. *Medical science monitor: international medical journal of experimental and clinical research*, 22, 2825–2830.

Johnson B V, Shindo N, Rathjen P D, Rathjen J, Keough R A. (2008). Understanding pluripotency—how embryonic stem cells keep their options open, *Molecular Human Reproduction,* Volume 14, Issue 9, September: Pages 513–520.

Kabir R, Gupta M, Aggarwal A, Sharma D, Sarin A, Kola MZ. (2014) Imperative role of dental pulp stem cells in regenerative therapies: a systematic review. *Niger J Surg*. 20(1):1-8.

Kalani A, Tyagi A, & Tyagi N. (2014). Exosomes: mediators of neurodegeneration, neuroprotection and therapeutics. *Molecular neurobiology*, 49(1), 590–600.

Kalsheker N, Morley S, Morgan K. (2002) Gene regulation of the serine proteinase inhibitor alpha1-antitrypsin and alpha1-antichymotrypsin. *Biochem Soc Trans*. 30:93-98.

Katsuda T, Kosaka N, Takeshita F, Ochiya T. (2013). The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Proteomics*.13 (10-11):1637-1653

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.

Kholodenko IV, & Yarygin KN. (2017). Cellular Mechanisms of Liver Regeneration and Cell-Based Therapies of Liver Diseases. *BioMed research international*, 8910821.

Kim E, Lim S, Park J, Seo J K, Kim JH, Kim KT, Ryu SH. and Suh P. (2012). Human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by AMP-activated protein kinase. *J. Cell. Physiol.,* 227: 1680-1687.

Kim DS, Ryu JW, Son MY, Oh JH., Chung KS, Lee S, … Cho HS. (2017). A liver-specific gene expression panel predicts the differentiation status of *in vitro* hepatocyte models. *Hepatology* (Baltimore, Md.), 66(5), 1662–1674.

Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL. (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol*. 21:759-806.

Kwak KA, Cho HJ, Yang JY, & Park YS. (2018). Current Perspectives Regarding Stem Cell-Based Therapy for Liver Cirrhosis. *Canadian journal of gastroenterology & hepatology*, 2018, 4197857.

Lai CP, & Breakefield XO. (2012). Role of exosomes/microvesicles in the nervous system and use in emerging therapies. *Frontiers in physiology*, 3, 228.

Lai RC, Yeo RW, Lim SK. (2015). Mesenchymal stem cell exosomes. *Semin Cell Dev Biol*.40:82-88.

Lee JS, An SY, Kwon IK, Heo JS. (2014) Transdifferentiation of human periodontal ligament stem cells into pancreatic cell lineage. *Cell Biochem Funct*. 32:605-611.

Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. (2004a) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood*. Mar 1; 103(5):1669-75.

Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK. (2004b) *In vitro* hepatic differentiation of human mesenchymal stem cells. *Hepatology*. Dec; 40 (6):1275-84.

Lehrich BM., Liang Y, Khosravi P, Federoff HJ, & Fiandaca MS. (2018). Fetal Bovine Serum-Derived Extracellular Vesicles Persist within Vesicle-Depleted Culture Media. *International journal of molecular sciences*, 19(11), 3538.

Lemaigre FP. (2009) Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology*. 137:62-67.

Li W, Li K, Wei W, & Ding S. (2013). Chemical approaches to stem cell biology and therapeutics. *Cell stem cell*, 13(3), 270–283.

Li T, Yan Y, Wang B, Qian H, Zhang X, Shen L, … Xu W. (2013). Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem cells and developmen*t, 22(6), 845–854.

Li C, Wu X, Tong J et al. (2015). Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. *Stem Cell Res Ther* 6, 55

Liu WH, Song FQ, Ren LN, Guo WQ, Wang T, Feng Y X., … Li K. (2015). The multiple functional roles of mesenchymal stem cells in participating in treating liver diseases. *Journal of cellular and molecular medicine*, 19(3), 511–520.

Lojewski X , Srimasorn S, Rauh J, Francke S, Wobus M, Taylor V, Araúzo-Bravo MJ, Hallmeyer-Elgner S, Kirsch M, Schwarz S, Schwarz J, Storch A and Hermann A. (2015), Perivascular Mesenchymal Stem Cells From the Adult Human Brain Harbor No Instrinsic Neuroectodermal but High Mesodermal Differentiation Potential. *Stem cells Translational Medicine*, 4: 1223-1233.

Loo ZX, Kunasekaran W, Govindasamy V, Musa S, & Abu Kasim NH. (2014). Comparative analysis of cardiovascular development related genes in stem cells isolated from deciduous pulp and adipose tissue. *TheScientificWorldJournal*, 2014, 186508.

Loranger A, Duclos S. (1997) Simple epithelium keratins are required for maintenance of hepatocyte integrity. *Am J Pathol*. 151:1673-1683.

Lou G, Chen Z, Zheng M, & Liu Y. (2017). Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Experimental & molecular medicine*, 49(6), e346.

Ma X, Duan Y, Tschudy-Seney B, Roll G, Behbahan IS, Ahuja TP, … Zern MA. (2013). Highly efficient differentiation of functional hepatocytes from human induced pluripotent stem cells. *Stem cells translational medicine*, 2(6), 409.

Mahla RS. (2016) Stem Cells Applications in Regenerative Medicine and Disease Therapeutics. *Int J Cell Biol*. 6940283.

McCulloch CA, Bordin S. (1991) Role of fibroblast subpopulations in periodontal physiology and pathology. *J Periodontal Res*. May; 26(3 Pt 1):144-54.

Meregalli M, Farini A and Torrente Y. (2011). Mesenchymal Stem Cells as Muscle Reservoir. *J Stem Cell Res Ther* 2011, 1:2

Metcalf D. (2007).Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. *Stem Cells*. Oct; 25(10):2390-5

Michalopoulos GK. (2007). Liver regeneration. *J Cell Physiol*; 213(2):286-300.

Mitrano TI, Grob M S, Carrión F, Nova-Lamperti E, Luz PA, Fierro FS, Quintero A, Chaparro A and Sanz A. (2010), Culture and Characterization of Mesenchymal Stem Cells From Human Gingival Tissue. *Journal of Periodontology*, 81: 917-925.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, & 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.

Mochizuki M, & Nakahara T. (2018). Establishment of xenogeneic serum-free culture methods for handling human dental pulp stem cells using clinically oriented in-vitro and in-vivo conditions. *Stem cell research & therapy*, 9(1), 25.

Morsczeck C1, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, Sippel C, Hoffmann KH. (2005). Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol*. Apr; 24(2):155-65.

Müller G. (2012). Microvesicles/exosomes as potential novel biomarkers of metabolic diseases. *Diabetes, metabolic syndrome and obesity: targets and therapy*, 5, 247–282.

Muraca M, Gerunda G, Neri D et al. (2002) Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet*. 359:317-318. DOI: 10.1016/S0140-6736(02)07529-3

Nagatomo K, Komaki M, Sekiya I, Sakaguchi Y, Noguchi K, Oda S, Muneta T and Ishikawa I. (2006), Stem cell properties of human periodontal ligament cells. *Journal of Periodontal Research*, 41: 303-310.

Najimi M1, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, Sokal EM. (2007) Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant*. 16(7):717-28.

Nakagawa M1, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. Jan; 26(1):101-6.

Nakashima M, Iohara K. Mobilized dental pulp stem cells for pulp regeneration: initiation of clinical trial. *J Endod*. 2014 Apr; 40(4 Suppl):S26-32.

Narita Y, Yamawaki A, Kagami H, Ueda M, Ueda Y. (2008) Effects of transforming growth factor-beta 1 and ascorbic acid on differentiation of human bone-marrow-derived mesenchymal stem cells into smooth muscle cell lineage. *Cell Tissue Res*. Sep; 333(3):449-59.

Okita K, Ichisaka T, Yamanaka S. (2007) Generation of germline-competent induced pluripotent stem cells. *Nature*. Jul 19; 448(7151):313-7.

Orciani M, Di Primio R. (2013) Skin-derived mesenchymal stem cells: isolation, culture, and characterization. *Methods Mol Biol*. 989:275-83.

Osman A, Gnanasegaran N, Govindasamy V et al. (2014) Basal expression of growth-factor-associated genes in periodontal ligament stem cells reveals multiple distinctive pathways. *Int Endod J.* 47:639-651.

Ostrovidov S, Shi X, Sadeghian RB, Salehi S, Fujie T, Bae H, Ramalingam M, Khademhosseini A. (2015) Stem Cell Differentiation Toward the Myogenic Lineage for Muscle Tissue Regeneration: A Focus on Muscular Dystrophy. *Stem Cell Rev Rep*. Dec;11(6):866-84.

Pachler K, Ketterl N, Desgeorges A, Dunai ZA, Laner-Plamberger S, Streif D, Strunk D, Rohde E, & Gimona M. (2017). An In vitro Potency Assay for Monitoring the Immunomodulatory Potential of Stromal Cell-Derived Extracellular Vesicles. *International journal of molecular sciences*, 18(7), 1413.

Palakkan AA, Drummond R, Anderson RA, Greenhough S, Tv K, Hay DC, & Ross JA. (2015). Polarisation and functional characterisation of hepatocytes derived from human embryonic and mesenchymal stem cells. *Biomedical reports*, 3(5), 626–636.

Pan BT, Johnstone RM. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983;33(3):967-978

Park S, Greco V, & Cockburn K. (2016). Live imaging of stem cells: answering old questions and raising new ones. *Current opinion in cell biology*, 43, 30–37.

Patel DB, Gray KM, Santharam Y, Lamichhane TN, Stroka KM, & Jay SM. (2017). Impact of cell culture parameters on production and vascularization bioactivity of mesenchymal stem cell-derived extracellular vesicles. *Bioengineering & translational medicine*, 2(2), 170–179.

Paul G, Özen I, Christophersen NS, Reinbothe T, Bengzon J, Visse E, … Brundin P (2012). The adult human brain harbors multipotent perivascular mesenchymal stem cells. *PloS one*, 7(4), e35577.

Pavlova G, Lopatina T, Kalinina N, Rybalkina E, Parfyonova Y, Tkachuk V, Revishchin A. (2012) *In vitro* neuronal induction of adipose-derived stem cells and their fate after transplantation into injured mouse brain. *Curr Med Chem*.19 (30):5170-7.

Pellegrini S, Ungaro F, Mercalli A, Melzi R, Sebastiani G, Dotta F, Broccoli V, Piemonti L, Sordi V. (2015) Human induced pluripotent stem cells differentiate into insulin-producing cells able to engraft *in vivo*. *Acta Diabetol*. Dec; 52(6):1025-35.

Peleman RR, Gavaler JS, Van Theil DH et al. (1987) Orthotopic liver transplantation for acute and subacute hepatic failure in adults. *Hepatology*. 7:484-489.

Pera M.F., Reubinoff B., Trounson A. Human embryonic stem cells. (2000) *J Cell Sci* 113, 5-10.

Pera M.F., Dottori M. (2005) Stem cells and their developmental potential. In: Bongso A., Lee E.H. Stem cells: from bench to bedside. Singapore: *World Scientific Publishing Co. Pte. Ltd*. 55-70.

Peralta C, Jiménez-Castro MB, Gracia-Sancho J. (2013) Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. *J Hepatol*. 59(5):1094-1106.

Phinney DG. and Pittenger MF. (2017), Concise Review: MSC-Derived Exosomes for Cell-Free Therapy. *Stem Cells*, 35: 851-858.

Pittenger MF1, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science*. Apr 2; 284(5411):143-7.

Pittenger MF, Le Blanc K, Phinney DG, Chan JK. (2015). MSCs: Scientific Support for Multiple Therapies. *Stem Cells Int*. 280572.

Pournasr B, Mohamadnejad M, Aghdami N, Shahsavani M, Malekzadeh R, Baharvand H. (2011) *In vitro* differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. Arch Iran Med. 14:244-249.

Raimondo F, Morosi L, Chinello C, Magni F. and Pitto M. (2011), Advances in membranous vesicle and exosome proteomics improving biological understanding and biomarker discovery. *Proteomics*, 11: 709-720.

Ranganath SH, Levy O, Inamdar MS, & Karp JM. (2012). Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell stem cell*, 10(3), 244–258.

Rani S, Ryan AE, Griffin MD, Ritter T. (2015). Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. *Mol Ther*.23(5):812-823.

Raposo G, & Stoorvogel W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology*, 200(4), 373–383.

Ren K. (2019). Exosomes in perspective: a potential surrogate for stem cell therapy. Odontology, 107(3), 271–284.

Riekstina U, Muceniece R, Cakstina I, Muiznieks I, & Ancans J. (2008). Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions. *Cytotechnology*, 58(3), 153–162.

Rohwedel J1, Guan K, Hegert C, Wobus AM. (2001). Embryonic stem cells as an *in vitro* model for mutagenicity, cytotoxicity and embryotoxicity studies: present state and future prospects. *Toxicol In vitro*. Dec; 15(6):741-53.

Romanov YA1, Darevskaya AN, Merzlikina NV, Buravkova LB. (2005) Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. *Bull Exp Biol Med*. Jul; 140(1):138-43.

Rossi JM, Dunn NR, Hogan BL, Zaret KS. (2001). Distinct mesodermal signals, including BMPs from septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev*. 15:1998-2009.

Sassa S, Sugita S, Galbraith RA, Kappas A. (1987) Drug metabolism of human hepatoma cell, HepG2. *Biochem Biophys Res Commun*. 25:52-57.

Santangelo L, Marchetti A, Cicchini C, et al. (2011). The stable repression of mesenchymal program is required for hepatocyte identity: a novel role for hepatocyte nuclear factor 4α. *Hepatology*. 53(6):2063-2074

Schiesser, J. V., Micallef, S. J., Hawes, S., Elefanty, A. G., & Stanley, E. G. (2014). Derivation of insulin-producing beta-cells from human pluripotent stem cells. *The review of diabetic studies: RDS*, 11(1), 6–18.

Schneuwly, S., Kuroiwa, A., & Gehring, W. J. (1987). Molecular analysis of the dominant homeotic Antennapedia phenotype. *The EMBO journal*, 6(1), 201–206.

Segev H, Fishman B, Ziskind A, Shulman M, Itskovitz-Eldor J. (2004) Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells*. 22(3):265 74.

Seo BM, Miura M, Gronthos S et al. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*. 364:149-155.

Seo BM, Miura M, Sonoyama W, Coppe C, Stanyon R, Shi S. (2005) Recovery of stem cells from cryopreserved periodontal ligament. *J Dent Res*. 84(10):907-12

Sharpe PT1. (2016) Dental mesenchymal stem cells. *Development*. Jul 1; 143(13):2273-80.

Shelly LL, Tynan W, Schwid W, Schutz G, Yeoh GC. (1989) Hepatocyte differentiation in vitro: initiation of tyrosine aminotransferase expression in cultured fetal rat hepatocytes. *J Cell Biol*. 109:3403-3410.

Shelke GV, Lässer C, Gho YS, & Lötvall J. (2014). Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. Journal of extracellular vesicles, 3, 10.3402/jev.v3.24783.

Shi S, Bartold P, Miura M, Seo B, Robey P. and Gronthos S. (2005). The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthodontics & Craniofacial Research,* 8: 191-199.

Shimono M, Ishikawa T, Ishikawa H, Matsuzaki H, Hashimoto S, Muramatsu T, Shima K, Matsuzaka K. and Inoue T. (2003), Regulatory mechanisms of periodontal regeneration. *Microsc. Res. Tech*., 60: 491-502.

Sloan A. and Smith A. (2007), Stem cells and the dental pulp: potential roles in dentine regeneration and repair. *Oral Diseases*, 13: 151-157.

Silvério KG, Rodrigues TL, Coletta RD, Benevides L, Da Silva JS, Casati MZ, Sallum EA, Nociti FH Jr. (2010) Mesenchymal stem cell properties of periodontal ligament cells from deciduous and permanent teeth. *J Periodontol*. 81(8):1207-15

Si-Tayeb, K., Noto, F. K., Nagaoka, M., Li, J., Battle, M. A., Duris, C., Duncan, S. A. (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* (Baltimore, Md.), 51(1), 297–305.

Smith AG1, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature*. Dec 15; 336(6200):688-90.

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

Song JS, Kim SO, Kim SH, Choi HJ, Son HK, Jung HS, Kim CS, Lee JH. (2012) *In vitro* and in vivo characteristics of stem cells derived from the periodontal ligament of human deciduous and permanent teeth. *Tissue Eng Part A*. 18(19-20):2040-51

Soto-Guiterrez A, Basma H, Navarro-Alvarez N et al. (2008) Differentiating stem cells into liver. *Biotechnol Genet Eng Rev*. 25:149-163. DOI: 10.5661/bger-25-149

Snykers, S., De Kock, J., Rogiers, V., & Vanhaecke, T. (2009). *In vitro* differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem cells* (Dayton, Ohio), 27(3), 577–605.

Snykers S, De Kock J, Tamara V, Rogiers V. (2011) Hepatic differentiation of mesenchymal stem cells: *in vitro* strategies. *Methods Mol Biol*. 698:305-14.

Steinman RA, Wentzel A, Lu Y, Stehle C, Grandis JR. (2003) Activation of Stat3 by cell confluence reveals negative regulation of Stat3 by cdk2. *Oncogene*. 22(23):3608-3615.

Stephenne X,Najimi M, Sibille C, Nassogne MC, Smets F, Sokal EM. (2006) Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. *Gastroenterology*. 130:1317-1323.

Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, … Zhang HG. (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is

enhanced when encapsulated in exosomes. *Molecular therapy: the journal of the American Society of Gene Therapy*, 18(9), 1606–1614.

Syed-Picard, F. N., Du, Y., Lathrop, K. L., Mann, M. M., Funderburgh, M. L., & Funderburgh, J.L. (2015). Dental pulp stem cells: a new cellular resource for corneal stromal regeneration. *Stem cells translational medicine*, 4(3), 276–285.

Tahan AC, & Tahan V. (2014). Placental amniotic epithelial cells and their therapeutic potential in liver diseases. *Frontiers in medicine*, 1, 48.

Takahashi K1, Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006 Aug 25; 126(4):663-76.

Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, Nishikawa S. (2007) Neuroepithelial cells supply an initial transient wave of MSC differentiation. *Cell*. Jun 29; 129(7):1377-88.

Tan CY, Lai RC, Wong W, Dan YY, Lim SK, & Ho HK. (2014). Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem cell research & therapy*, 5(3), 76.

Taub R. (2004) Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Bio*l. 2004 Oct; 5(10):836-47.

Teixeira FG, Carvalho MM, Sousa N, Salgado AJ. (2013) Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? *Cell Mol Life Sci*. 2013 Oct; 70(20):3871-82.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel SS, Marshall VS, Jones JM. (1998) Embryonic stem cell lines derived from human blastocysts. *Science*, 282 pp. 1145-1147

Thomson, M., Liu, S. J., Zou, L. N., Smith, Z., Meissner, A., & Ramanathan, S. (2011). Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell*, 145(6), 875–889. doi:10.1016/j.cell.2011.05.017

Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Müller B, Zulewski H.(2006) Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem Biophys Res Commun.* Mar 24; 341(4):1135-40.

Tomokiyo A, Maeda H, Fujii S, Wada N, Shima K, Akamine A. (2008) Development of a multipotent clonal human periodontal ligament cell line. *Differentiation*. 76:337-347.

Tsai P, Fu T, Chen YA, Ko T, Chen T, Shih Y, Hung S and Fu Y. (2009), The therapeutic potential of human umbilical mesenchymal stem cells from Wharton's jelly in the treatment of rat liver fibrosis. *Liver Transpl*, 15: 484-495.

Unzu C., Friedli M., Bosman A., Jaconi M E., Wildhaber B E., & Rougemont A L. (2016). Human Hepatocyte-Derived Induced Pluripotent Stem Cells: MYC Expression, Similarities to Human Germ Cell Tumors, and Safety Issues. *Stem cells international*, 4370142.

van Poll D, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW. and Yarmush ML. (2008), Mesenchymal stem cell–derived molecules directly modulate hepatocellular death and regeneration *in vitro* and *in vivo*. *Hepatology*, 47: 1634-1643.

Vasanthan P, Gnanasegaran N, Govindasamy V et al. (2014) Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. *Biochem Eng J*. 88:142-153.

Vlassov AV, Magdaleno S, Setterquist R, Conrad R.(2012) Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta*. 2012 Jul; 1820(7):940-8.

Vizoso FJ, Eiro N, Cid S, Schneider J, & Perez-Fernandez R. (2017). Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *International journal of molecular sciences*, 18(9), 1852.

Volarevic V, Bojic S, Nurkovic J, Volarevic A, Ljujic B, Arsenijevic N, … Stojkovic M. (2014). Stem cells as new agents for the treatment of infertility: current and future perspectives and challenges. *BioMed research international*, 2014, 507234.

Volponi AA, Sharpe PT. (2013). The tooth -- a treasure chest of stem cells. *Br Dent J*. 2013 Oct; 215(7):353-8.

Wada N, Menicanin D, Shi S, Bartold PM. and Gronthos S. (2009), Immunomodulatory properties of human periodontal ligament stem cells. *J. Cell. Physiol*., 219: 667-676.

Wagers AJ, Weissman IL. (2004). Plasticity of adult stem cells. *Cell*. Mar 5; 116(5):639-48.

Wang X, Sha XJ, Li GH, Yang FS, Ji K, Wen LY, Liu SY, Chen L, Ding Y, Xuan K. (2012) Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. *Arch Oral Biol*.Sep; 57(9):1231-40.

Wang Y, Yu X, Chen E, & Li L. (2016). Liver-derived human mesenchymal stem cells: a novel therapeutic source for liver diseases. *Stem cell research & therapy*, 7(1), 71.

Wang H, Ren Y, Hu X, Ma M, Wang X, Liang H, & Liu D. (2017). Effect of Wnt Signaling on the Differentiation of Islet β-Cells from Adipose-Derived Stem Cells. *BioMed research international*, 2017, 2501578.

Wang YH, Wu DB, Chen B, Chen EQ, & Tang H. (2018). Progress in mesenchymal stem cell-based therapy for acute liver failure. Stem cell research & therapy, 9(1), 227.

Watt AJ, Garrison WD, Duncan SA. (2003) HNF4: a central regulator of hepatocyte differentiation and function. *Hepatology*. 37:1249-1253.

Wei X, Yang X, Han, Z P, Qu FF, Shao L, & Shi YF. (2013). Mesenchymal stem cells: a new trend for cell therapy. *Acta pharmacologica Sinica*, 34(6), 747–754.

Weissman IL. (2000) Stem cells: units of development, units of regeneration, and units in evolution. *Cell*. 100(1):157-168.

Wu XB, Tao R. (2012) Hepatocyte differentiation of mesenchymal stem cells. *Hepatob Pancreat* Dis. 11:360-371.

Wystrychowski, W., Patlolla, B., Zhuge, Y., Neofytou, E., Robbins, R. C., & Beygui, R. E. (2016). Multipotency and cardiomyogenic potential of human adipose-derived stem cells from epicardium, pericardium, and omentum. *Stem cell research & therapy*, 7(1), 84.

Xagorari A, Siotou E, Yiangou M, Tsolaki E, Bougiouklis D, Sakkas L, … Anagnostopoulos A. (2013). Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury. *International journal of clinical and experimental pathology*, 6(5), 831–840.

Yamaza T, Alatas FS, Yuniartha R, Yamaza H, Fujiyoshi J K, Yanagi Y, … Taguchi T. (2015). *In vivo* hepatogenic capacity and therapeutic potential of stem cells from human exfoliated deciduous teeth in liver fibrosis in mice. *Stem cell research & therapy*, 6(1), 171.

Yin L, Zhu Y, Ni Y, Zhou Z, Chen Y, Wen L. (2015) Adipose tissue-derived mesenchymal stem cells differentiated into hepatocyte like cells *in vivo* and *in vitro*. *Mol Med Rep*. 11:1722-1732.

Yin F, Wang W Y, & Jiang W H. (2019). Human umbilical cord mesenchymal stem cells ameliorate liver fibrosis *in vitro* and *in vivo*: From biological characteristics to therapeutic mechanisms. *World journal of stem cells*, 11(8), 548–564.

Young HE1, Duplaa C, Romero-Ramos M, Chesselet MF, Vourc'h P, Yost MJ, Ericson K, Terracio L, Asahara T, Masuda H, Tamura-Ninomiya S, Detmer K, Bray RA, Steele TA, Hixson D, el-Kalay M, Tobin BW, Russ RD, Horst MN, Floyd JA, Henson NL, Hawkins KC, Groom J, Parikh A, Blake L, Bland LJ, Thompson AJ, Kirincich A, Moreau C, Hudson J, Bowyer FP 3rd, Lin TJ, Black AC Jr. (2004) Adult reserve stem cells and their potential for tissue engineering. *Cell Biochem Biophys*. 40(1):1-80.

Young MA, Larson DE, Sun CW, et al.(2012) Background mutations in parental cells account for most of the genetic heterogeneity of induced pluripotent stem cells. *Cell Stem Cell*. 10(5):570-582.

Yu B, Zhang X, & Li X. (2014). Exosomes derived from mesenchymal stem cells. *International journal of molecular sciences,* 15(3), 4142–4157.

Yuan T, Liao W, Feng NH, Lou YL, Niu X, Zhang AJ, … Deng ZF. (2013). Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurologic function in a rat model of middle cerebral artery occlusion. *Stem cell research & therapy,* 4(3), 73.

Zarrinpar A, Busuttil RW. Liver transplantation: past, present and future. *Nat Rev Gastroenterol Hepatol*. 2013;10(7):434-440.

.

Zhang YM, Hartzell C, Narlow M, Dudley SC Jr. (2002) Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation. Sep 3; 106(10):1294-9.

Zhang W, Frank X W, Shi S, Fan W, and Jansen JA (2006) Multilineage Differentiation Potential of Stem Cells Derived from Human Dental Pulp after Cryopreservation. *Tissue Engineering* 2006 12:10, 2813-2823

Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, … Kamp TJ. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation research*, 104(4), e30–e41.

Zhang S, Chen L, Liu T, et al. (2012). Human umbilical cord matrix stem cells efficiently rescue acute liver failure through paracrine effects rather than hepatic differentiation. *Tissue Eng Part* A. 2012; 18(13-14):1352-1364.

Zhang Z, Wang FS.(2013) Stem cell therapies for liver failure and cirrhosis. *J Hepatol*. Jul; 59(1):183-5.

Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. (2014) Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev*. 23(11):1233-1244

Zhang GZ, Sun HC, Zheng LB, Guo JB, Zhang XL. (2017). In *vivo* hepatic differentiation potential of human umbilical cord-derived mesenchymal stem cells: Therapeutic effect on liver fibrosis/cirrhosis. *World J Gastroenterol*. 23(46):8152-8168.

Zhang K, Zhang L, Liu W, Ma X, Cen J, Sun Z, Wang C, Feng S, Zhang Z, Yue L, Sun L, Zhu Z, Chen X, Feng A, Wu J, Jiang Z, Li P, Cheng X, Gao D, Peng L, Hui L (2018) *In vitro* Expansion of Primary Human Hepatocytes with Efficient Liver Repopulation Capacity. *Cell Stem Cell*. 2018 Dec 6; 23(6):806-819.e4.

Zhao Y, Glesne D, Huberman E. (2003) A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc Natl Acad Sci U S A*. 100(5):2426-2431

Zhou R, Li Z, He C, et al. (2014).Human umbilical cord mesenchymal stem cells and derived hepatocyte-like cells exhibit similar therapeutic effects on an acute liver failure mouse model. *PLoS One*. 2014; 9(8):e104392.

Zhou Y, Yamamoto Y, Xiao Z, Ochiya T. (2019) The Immunomodulatory Functions of Mesenchymal Stromal/Stem Cells Mediated via Paracrine Activity. *J Clin Med*. ; 8(7):1025

Zhu W, Liang M. (2015) Periodontal ligament stem cells: current status, concerns, and future prospects. *Stem Cells Int*. 2015:972313

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. Apr; 7(2):211-28

LIST OF PUBLICATIONS

1. Vasanthan, P., Jayaraman, P., Kunasekaran, W., Lawrence, A, Gnanasegaran, N., Govindasamy, V., Musa, S., Abu Kasim N.H. (2016). Generation of functional hepatocyte-like cells from human deciduous periodontal ligament stem cells. Naturwissenschaften. 103(7-8):62.

2. Vasanthan P, Gnanasegaran N, Govindasamy V et al. (2014) Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. *Biochem Eng J*. 88:142-153.

3. Vasanthan, O., Govindasamy, V., Gnanasegaran, N., Kunasekaran, W., Musa, S, Abu Kasim, N.H.(2015) Differential expression of basal microRNAs' patterns in human dental pulp stem cells. J Cell Mol Med. 19 (3):566-80.

4. Jayaraman, P., Nathan, P., Vasanthan, P., Musa, S, Govindasamy, V. (2013) Stem cells conditioned medium: a new approach to skin wound healing management. Cell Biol Int. 37 (10):1122-8.