CHAPTER 1

INTRODUCTION

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In recent years, human are being confronted with a wide range of chronic liver diseases that have led to the deficiency of liver functions. Hence, there is a great necessity for the therapeutic intervention to overcome this situation. Previously, allogeneic liver transplantation has been considered as an attractive therapeutic tool for the liver dysfunction (Kakinuma et al., 2009). However, this approach has certain boundaries such as serious shortage in organ donor and possibility of rejection by host immune system. In observation of these deficits, cell-based hepatocytes transplantation has been suggested as the best alternative to cure liver dysfunction since it is simpler, non-invasive and could be cryopreserved for future use (Kawashita et al., 2005). Nevertheless, several studies have reposted that the direct-transplanted hepatocytes cells have lower survival rate and thus could not accomplish enough liver population successfully (Rajvanshi et al., 1996; Gewartowska & Olszewski, 2007). Therefore, researchers are in search for other effective treatments and cell-based therapies have appeared to offer a promising alternative for treating liver dysfunction.

Stem cells are the unspecialized cell which has the capability to maintain its undifferentiated state and could differentiate into more specialized cells in response to various instructive signals (Lyssiotis et al., 2011). Stem cells were first studied by Becker et al in 1963 and they have broadly divided into embryonic stem cells and adult stem cells as reported by Bajada et al (2008). Embryonic stem cells (ESCs) can be defined as the pluripotent cells isolated from the inner cell mass (ICM) of delaying-implanting blastocysts which have been well-maintained under *in vitro* condition in undifferentiated state for an indefinite period of time, and still maintaining the ability to form to all somatic tissues in the fetus and also germlines (Fairchild et al., 2004). The

ESCs have been successfully used to treat various human diseases in animal models. Although ESCs are classified as the most flexible type of stem cells, however, their expansion and usage have brought a lot of ethical issues (Alison et al., 2002). Precisely, adult stem cells seem to offer a better choice with wider option for sources and less ethical issues as compared to ESCs.

The use of human mesenchymal stem cells (MSCs) in multiple clinical approaches has been successfully demonstrated over the past few years. The potential of MSCs to proliferate extensively while maintaining their differentiation capability under *in vivo* and *in vitro* have marked them as a particularly attractive cell type for cell-based therapies in human (Bosch et al., 2006). It has been suggested that MSCs are diversely distributed in the human body as they can be isolated from different sources including adipose tissues, synovia membrane, muscle, skin, umbilical cord, liver, amniotic fluid, peripheral blood and also the dental tissues (da Silva Meirelles et al., 2006). The recent discovery of MSCs isolated from dental tissues has been suggested as the most easily accessible stem cells source and has thus demonstrated that frequent discarded tissues may also become a valuable source of MSCs (Perry et al., 2008).

The first isolation of MSCs from dental tissues was done in 2000 (Gronthos et al. 2000) and were named as dental pulp stem cells (DPSCs). Subsequently, researchers have isolated MSCs from other dental tissues sources such as human exfoliated deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament (PDLSCs) (Seo et al., 2005a), dental follicle (DFPCs) (Morsczeck et al., 2005) and currently apical papilla (SCAPs) (Sonoyama et al., 2006). Among all the five types of dental stem cells, the DPSCs and SHED isolated from the dental pulp are the most studied and explored. Stem cells from the dental pulp have displayed multipotential differentiation ability and expressed the MSCs surface markers such as STRO-1 and CD 146 (Gronthos et al., 2000). They also have shown ability to develop colony-formation under *in vitro*

condition and showed potential to develop into dentin under *in vivo* condition (Gronthos et al., 2002). Several studies have described stem cells from deciduous dental pulp as a clonogenic cells with extensive proliferation rate and differentiation potential toward neuronal, adipocytes and odontoblast types (Huang et al., 2010). Moreover, they have been reported to have potential in generating significant amount of alveolar and orofacial bone for tissue regeneration (Gronthos et al., 2000; Miura et al., 2003). They have showed their potential in the cell-based therapies such as neurological disorders (Arthur et al., 2008), cardiac disorders (Gandia et al., 2008), ischemic disoders (Iohara et al., 2008) and liver diseases (Ikeda et al., 2008). With the advancement of technology, the clinical benefits of dental stem cells are not only applicable for the dental use, but, also for reparative and regenerative medicine (Huang et al., 2010). Undoubtedly, stem cells researches have opened future options in regenerative medicine that may positively influence the lives of thousands of patients globally. However, the path towards the development of an effective cell-based therapy involves overcoming various practical issues especially ethics and safety.

The use of fetal bovine serum (FBS) in the expansion of stem cells has brought a lot of questionable safety issue to the application of stem cells clinically. One serious concern is FBS may bear the risk of viral and pathogens transmission with xeno-immunization (Horwitz et al., 2002). Moreover, the variation composition in lot-to-lot collection of FBS has resulted phenotypical differences (van der Valk et al., 2010). From the view of animal ethics, the collection of FBS has caused a great grief among the animal lovers as it involved the killing of prenatal cows as well as calf fetus (van der Valk et al., 2004). Previous studies have documented that the application of FBS in stem cells expansion have implicated the arthus-immune response in the patient (Selvaggi et al., 1997). With all disadvantages of FBS as stated above, it is necessary to find alternative expansion medium to replace the use of FBS. Over the past few years,

researchers have discovered high contents of growth factors in human platelet lysate (HPL) that could enhance the stem cells proliferation, namely, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), platelet factor 4 (PF-4) and platelet-derived epidermal growth factor (PDEGF) (Kilian et al., 2004; Doucet et al., 2005). The medium supplemented with HPL has shown to has higher capability to sustenance the growth of stem cells and viabilities (Johansson et al., 2003). Moreover, stem cells expanded in the presence of HPL has demonstrated a similar potential of differentiation to osteogenic, chondrogenic and adipogenic lineages (Doucet et al., 2005). Therefore, HPL has been strongly suggested as the most suitable serum replacement for FBS in stem cell expansion for clinical applications.

Aim of the study

The aim of this study is to evaluate the human platelet lysate (HPL) as an alternative culture media to fetal bovine serum (FBS) in the expansion of human dental pulp stem cells from deciduous teeth (SCD) from the aspect of growth kinetic, endodermal differentiation potential, immunophenotypic and immunocytochemistry together with the gene expression.

Objectives of the study

1. To examine the effect of FBS and HPL on proliferation rate of SCD.
2. To study the characteristics of SCD expanded using FBS and HPL.
3. To examine the hepatic differentiations potential of SCD expanded in FBS and HPL.
4. To study the expression of stem cells and hepatocyte-cell markers in the SCD expanded in HPL and FBS.

Null Hypothesis

There is no difference in growth kinetic, endodermal differentiation potential, immunophenotypic and immunocytochemistry together with the gene expression of SCD cultured in FBS and HPL.

CHAPTER 2

LITERATURE REVIEW

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2. 1 Tooth development and structure

Oral cavity has a series of hard and soft tissues. The salivary glands and lining mucosa of mouth are classified as soft tissues while teeth and jawbones are the hard tissues. Koussoulakou et al. (2009) described teeth as the epithelial appendages with high mineral contents that are located in the entrance of the alimentary canal. The main function of teeth includes permitting and preparing various food for digestion, defense, for proper speech and communication (Moynihan & Petersen, 2004). The human dentition are divided into primary and secondary teeth that consists a group of specified teeth namely incisors, canines and molar, which are originated from diverse areas of oral epithelium (Figure 2.1).

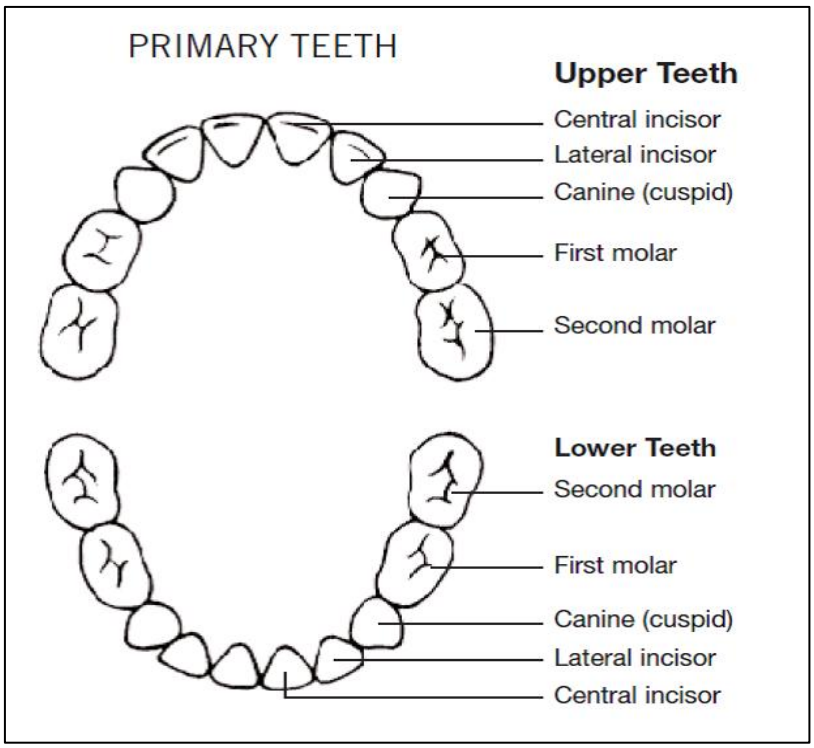

Figure 2.1: Basic human dentition (Extracted from http://www.gankowdds.com)

The tooth development in humans starts at the early embryonic stages, which involves the interactions between epithelium and mesenchymal tissues. At the early stages of morphogenesis, most of the organ share common morphological features. This is because of the fact that, all organs are developed from a series of transition process involving epithelial and mesenchyme cells (Thesleff et al., 1995) (Figure 2.2). This transition process can be classified into two types. The first process is epithelial to mesenchymal transition (EMT) while the second is a reverse interaction, which is recognized as mesenchymal to epithelial transition (MET) (Thiery et al., 2009). The importance of epithelial-mesenchymal interaction in organogenesis has been studied by a number of researchers in the past decade (Gurdon, 1992). Previous studies by embryologist have revealed that the development of epithelial tissues branching was strongly correlated with the presence of mesenchymal tissues (Sakakura et al., 1976; Nakaya & Sheng, 2008). Several other studies also reported that the mesenchymal cells were not undergoing differentiation process unless the signals from epithelial tissues are received (Kollar & Baird, 1970; Li et al., 2011).

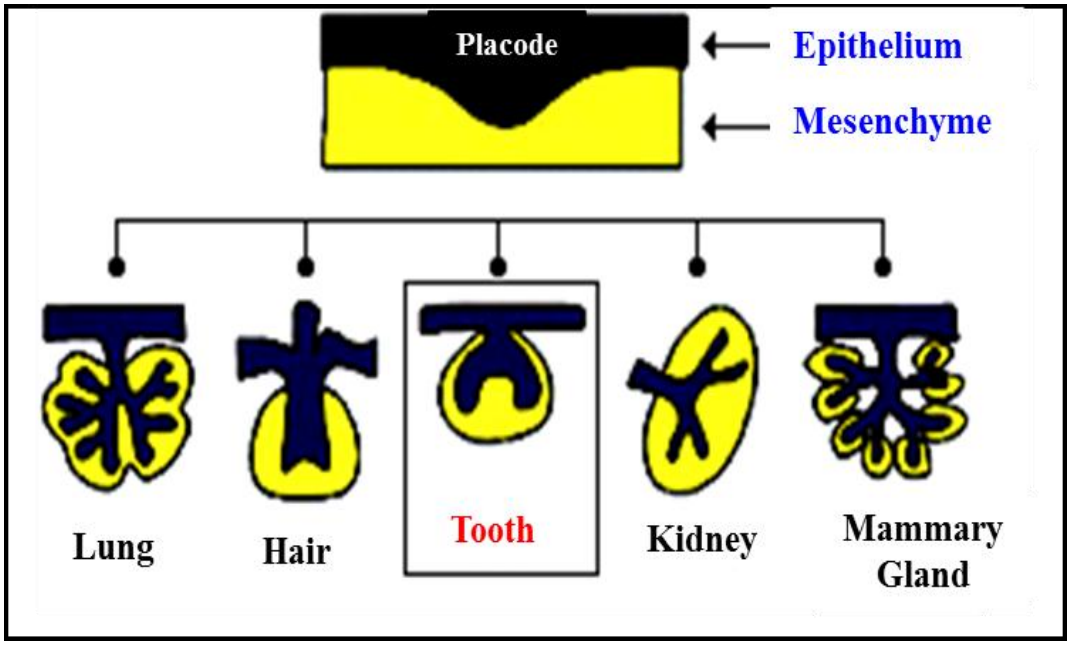

Figure 2.2: Common morphological features of the organs in the early development stages (Modified from Thesleff et al., 1995).

The first step in the tooth formation is the induction stage, where the oral epithelium tissues send signals to the neural crest-derived mesenchymal cells to initiate the development process (Figure 2.3). Previously, various studies were conducted to investigate and characterize the inductive interactions of different stages of tooth morphogenesis (MacNeil & Thomas, 1993; Thesleff, 1997). The primary morphological indication of tooth formation is presented after the induction process with the appearance of thickened oral epithelium bands known as the primary dental lamina (Smith, 2003; Stock, 2007). Subsequently, the thickened oral epithelium is converted into neural crest mesenchyme, which finally forms dental placode. The dental placode acts as a signaling center of the tooth and gives signal for the mesenchymal cells to aggregate and forms the mesenchymal condensation (Nakao et al., 2007).

Next phase is the bud stage. It is a period of extensive proliferation of dental lamina and transforms into tooth bud. The condensed mesenchymal cells that surround the tooth bud tissue then differentiate into dental papilla and dental follicle. The dental papilla forms as tooth pulp and odontoblast, whereas the dental follicle will produce the cementoblasts and periodontal tissues (Thesleff & Tummers, 2009). The process continues with the presence of enamel knot in the cap stage and the signals from the enamel knots control the tooth generation and morphology (Jernvall & Thesleff, 2000). Moreover, the formation of cervical loop are also started in the cap stages and during this stage, the lateral side of the epithelial bud are enclosed by the underlying mesenchyme. The basal epithelial layer of the cervical loop containing dental papilla is called the inner enamel epithelium, whereas the outer enamel epithelium is the region that fronting the dental follicle (Thesleff & Tummers, 2009). The interior part of the cervical loop is occupied with stellate reticulum and stratum intermedium cells. The cervical loop is preserved in the growing teeth with the epithelial part of adult stem cells niche (Tummers & Thesleff, 2009).

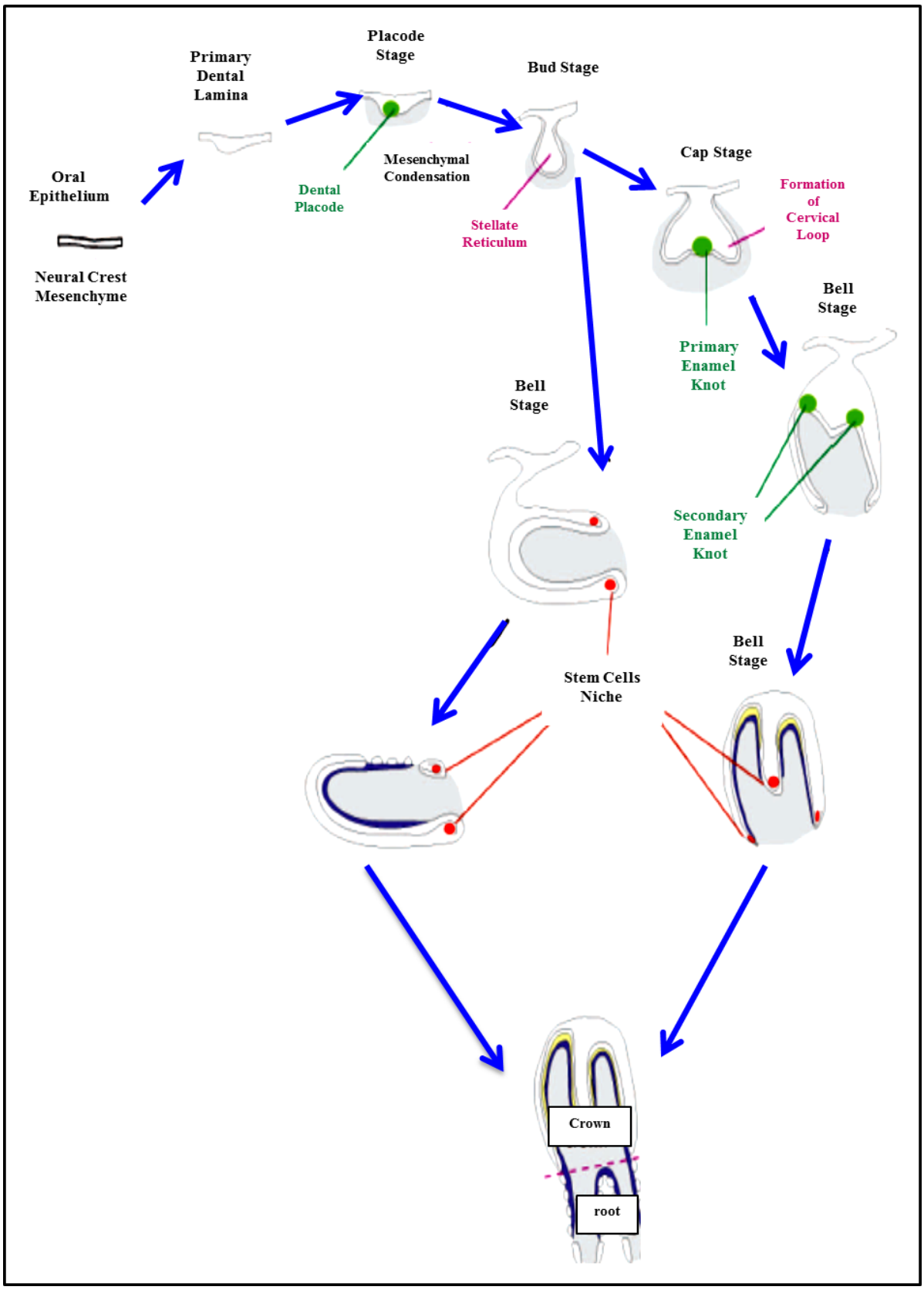

Figure 2.3: Development of early tooth morphogenesis (Modified from Thesleff & Tummers, 2009).

The anatomy of a tooth can be classified as three important structures namely; enamel, dentin and dental pulp (Figure 2.4). The observable portion of a tooth in the oral cavity is known as crown while the part that firmly supports the tooth within an alveolar sockets is referred as root.

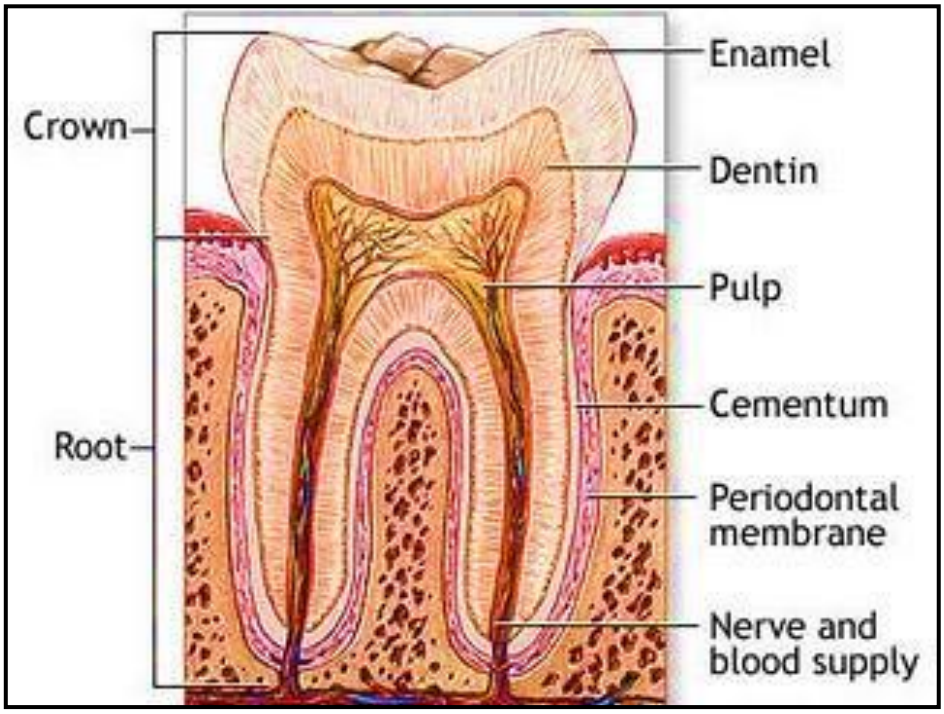

Figure 2.4: Anatomy of tooth (extracted from http://www.nlm.nih.gov/medlineplus)

Enamel is characterized as a collagen-free and most mineralized tissue in the human body (Koussoulakuo et al., 2009). Since it is located on the outermost part of tooth, it acts as a protective shell that covers dentin and other tooth structures. Enamel also acts as a primary material for mastication of food which is subjected to cyclic loading, wear and friction within a moisturized environment. Enamel consists of hydroxypatite crystals that are arranged as prismatic rods and surrounded by organic sheath. The main components of enamel are amelogenin, ameloblastin, enamelin, amelotin, tuftelin and odotogenic-ameloblast associated proteins (ODAM), which are all hydrophobic proteins (Sire et al., 2007).

The body of tooth is comprised of calcified tissues, known as dentine which is secreted by odontoblast cells. Dentine is layered underneath the enamel and functions as an elastic foundation and provides protective layer for dental pulp. Collagen, dentin sialophosphoprotein, dentin matrix protein and hydroxylapatite are the major component of dentine (Koussoulakuo et al., 2009). The types of substances present in the dentin are similar to enamel but may prevail in different quantity (Ten Cate, 1998). About 65 to 70 percent of dentin is comprised of calcium hydroxyl apatite and other minerals. The microstructure of dentine also can be divided into three major structural forms which are peritubular dentin, intertubular dentin and tubules.

2.2 Dental pulp

Dental pulp is one of the main structures in human tooth that can be considered as a soft, gelatinuous living tissues located in the center of the tooth. Dental pulp is developed from the connective tissue of dental papilla and surrounded by dentin. The quantity of dental pulp in every tooth may vary. For example, dental pulp in the molar tooth is three to four times larger than incisor pulp. Dental pulp is the most sensitive part of the tooth for mechanical and thermal stimuli and this is due to the presence of blood vessels and nerves throughout the dental pulp (Bartold, 2006).

The importance of dental pulp in the tooth can be displayed through their essential functions. The primary role of dental pulp is the initiation and development of dentin that are correlated with the formation of enamel (Lisi et al., 2007). Moreover, dental pulp also serves as a source to supply crucial nutrients for the dentin formation. Dental pulp stimulates odontoblast to form dentin as a defense barrier (Pashley et al., 2002). This barrier will decrease the entrance of irritants and delay carious penetration to protect the tooth and dental pulp.

The structural design of dental pulp can be divided into four layers of cells, starting with the outmost layer of dental pulp that made up of odontoblast cells (Figure 2.5). Odontoblasts cells are capable to form a single layer tissue at its periphery and organize the mineralization of dentin as well as synthesize the matrix (Sasaki & Garant, 1996). The second layer of dental pulp is relatively a cell-free area with abundance of extracellular matrix. Next layer is the cell-rich zone with high concentration of progenitor cells that displays plasticity and pluripotent capabilities. The main composition in this area is the fibroblast and undifferentiated mesenchymal cells which can be considered as stem cells (Jo et al., 2007).The last region of dental pulp comprises of major branches of nerves and blood vessels, known as the pulp core.

Research on dental pulp was expanded rapidly in the recent years with the discovery of stem cells niche within it. Dental pulp developed from both ectodermic and mesenchymal components that comprise of neural crest cells, which exhibits plasticity and multipotential capabilities (Sinanan et al., 2004). It remains entrapped within the mineralized crown and therefore it is preserved from environmental differentiation stimuli (Erickson & Reedy, 1998). The development of third molar is considered as the only organogenesis event that is completely performed after the birth. Thus, the embryonic tissue from the dental lamina during tooth morphogenesis remains quiescent at undifferentiated state inside the jaw until the initiation of tooth development at six years of old (Graziano et al., 2008). As a result, the structure of third molar teeth are in the immature stage and the undifferentiated cells inside the cell rich zone in the dental pulp can be used for further organ development (Graziano et al., 2008). The finding of sealed niche of undifferentiated cells within the dental pulp may explain the presence of a large number of stem cells in the tooth.

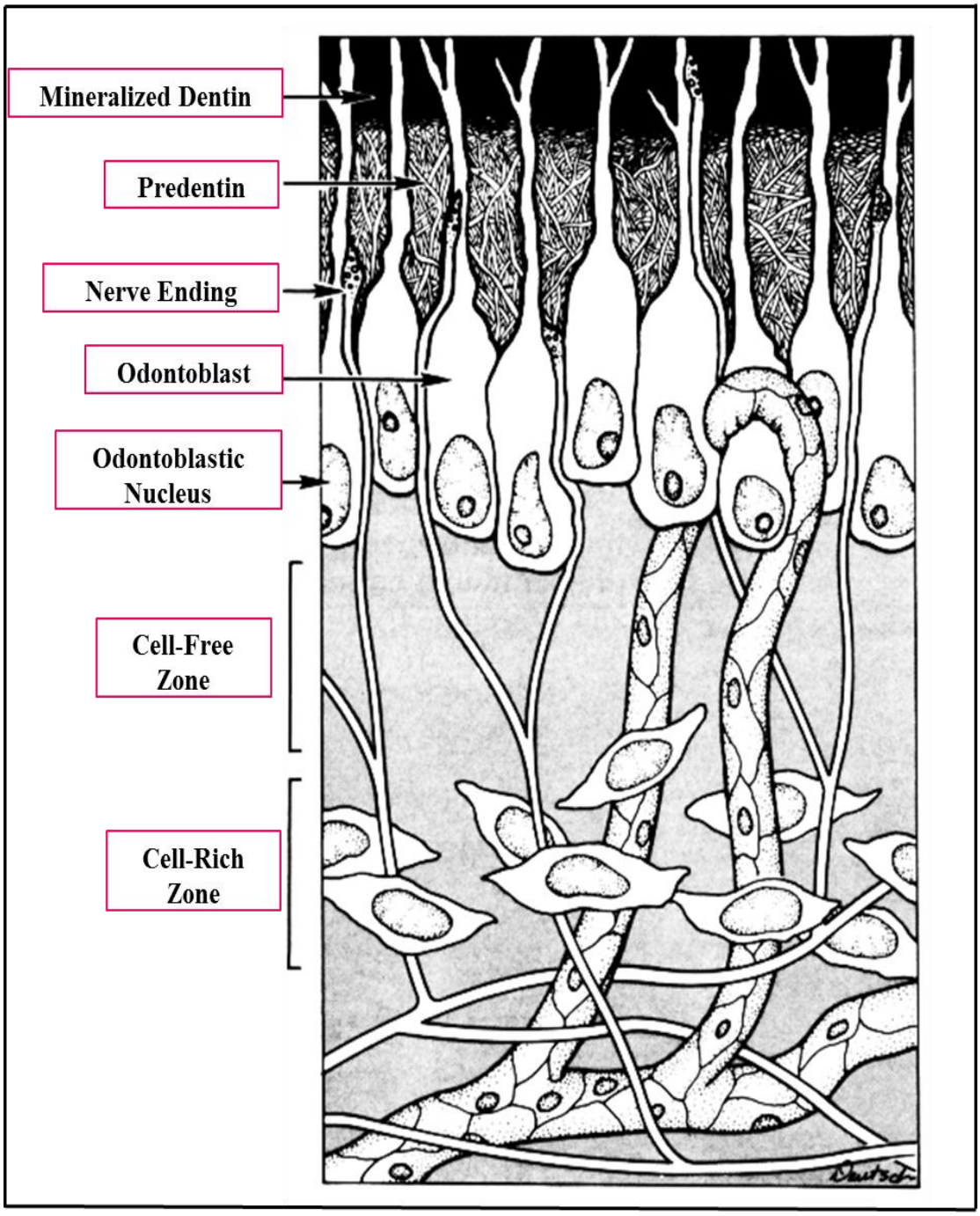

Figure 2.5: Cell layers in the dental pulp (Modified from Pashley et al., 2002).

2.3 Stem cells

Stem cells research has become a new promising field for tissue engineering and regenerative medicine. The origin of the word "stem" came from an old botanical terminology. It referred to the cells in plants at the apical root and shoot meristems that play roles in the plant regenerative (Kiessling & Anderson, 2003). Stem cells are defined as the unspecialized cells in the body that have potential to become specialized cells with new specialized cell functions (Bongso & Lee, 2005). Therefore, the term "stem cells" refers to the cells with the unique ability to self-renewal and differentiate into other lineages (de Korompay & Mayne, 2009).

The potency of stem cells usually varies and can be classified as totipotent, pluripotent, multipotent and tissue-specific stem cells (Hima Bindu & Srilatha, 2011). The definition and example for the degree of differentiation of stem cells are summarized in Table 2.1 and Figure 2.6.

Table 2.1: Definition for the degree of differentiation of stem cells

Degree of Differentiation	Definition	Reference
Totipotent	Cells are capable to differentiate into any type of specific cells in the given organism.	Bongso & Richards, 2004 Umehara et al., 2007
Pluripotent	Cells can form into three types of germ layers namely ectoderm, mesoderm and endoderm but not the whole organism.	Niwa, 2007 Lyssiotis et al., 2011
Multipotent	Cells have the same ability as pluripotent stem cell but toward more limited lineages	Wobus & Boheler, 2005 Rosenbaum et al., 2008
Unipotent	Cells that are restricted to form specific type of cell only	Alison et al., 2002 Blanpain et al., 2007

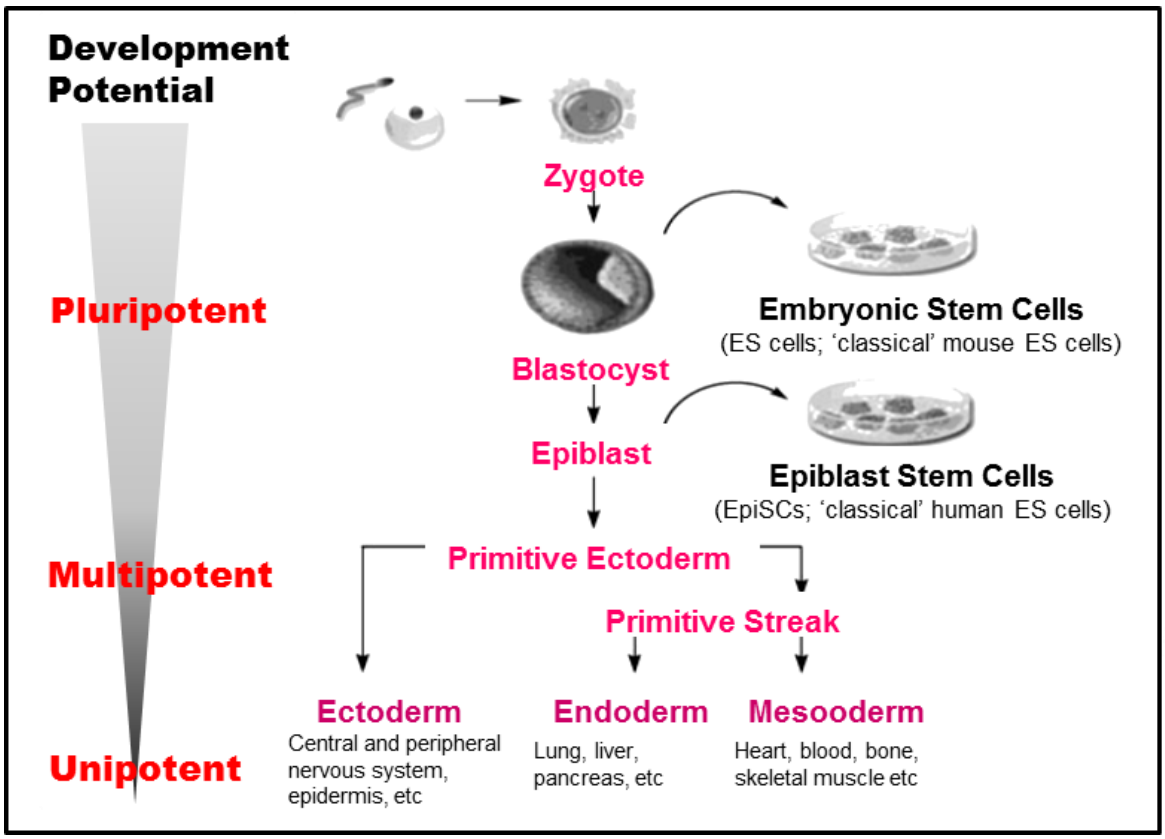

Figure 2.6: Degree of differentiation of stem cells and their examples (Modified from Lyssiotis et al., 2011).

Stem cells can be divided into two broad groups which are embryonic stem cells and adult stem cells (Rimondini et al., 2009). Human embryonic stem cells (ESCs) are the pluripotent cells derived from the inner mass of blastocysts (Thomson et al., 1998), whereas, adult stem cells are derived from several organs and tissues. All humans are developed from the union of an egg and a sperm (zygote) and subsequently results in the embryogenesis process. Ultimately the development reaches the blastocyst stage, consisting of trophoblast and inner cell mass (ICM). The ICM is selected as the source of embryonic stem cells and they are harvested through immunosurgery procedures (Heins et al., 2004). Stem cells in the adult organs are also evolved from the embryonic stem cells and remains in the stem cells niches upon for restoring injured tissues (Anderson et al., 2001). The connection between embryonic stem cells and adult stem cells are illustrated in Figure 2.7.

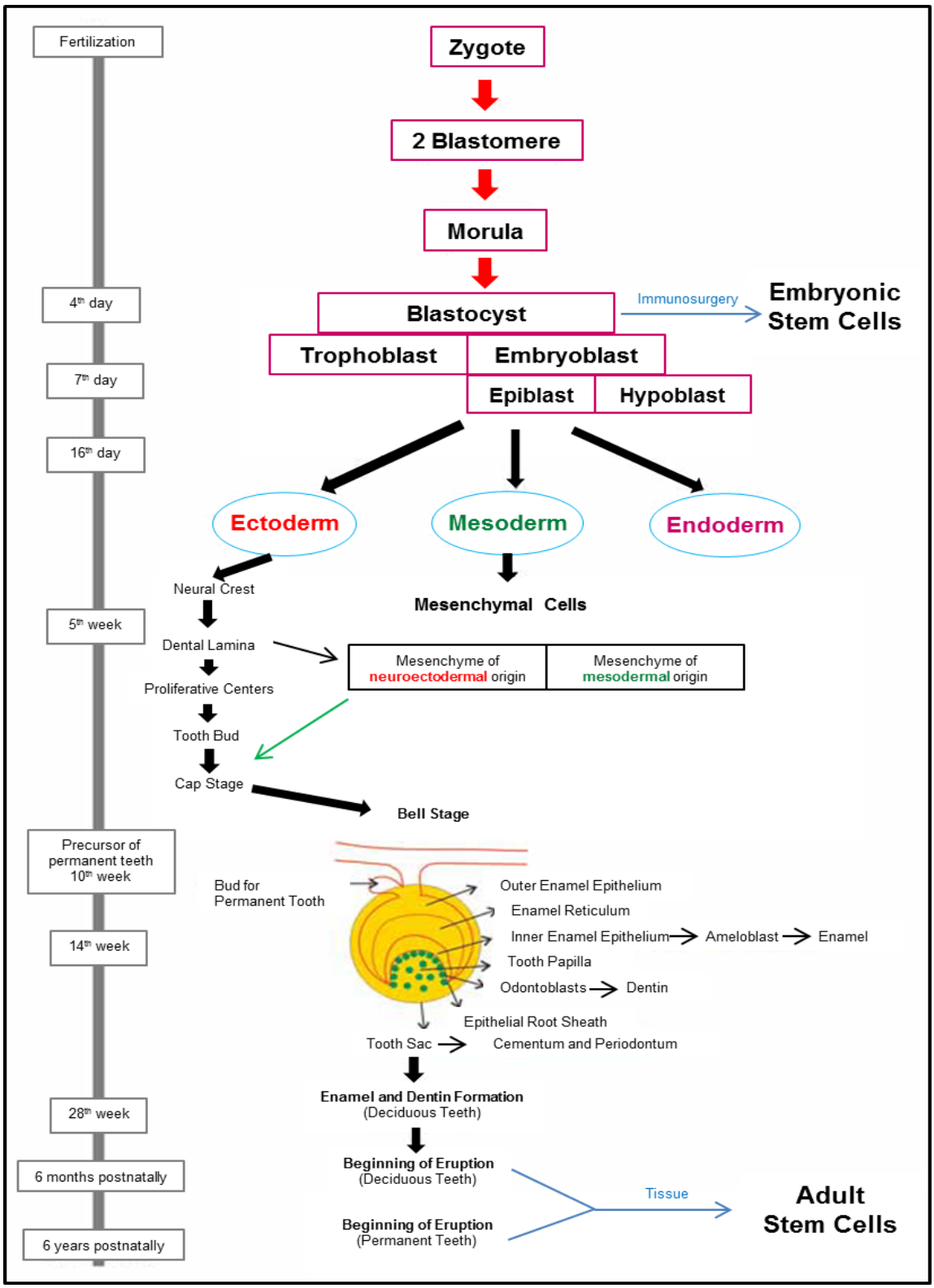

Figure 2.7: Origin of stem cells (Modified from Ulmer et al., 2010).

The first isolation of embryonic stem cells (ESCs) was done from the ICM of mouse embryo in 1981 (Evan & Kaufman, 1981). The term 'embryonic stem cells' was proposed by Gail G. Martin in order to distinguish the differences between embryo-

derived pluripotent cells and teratocarcinoma-derived pluripotent embryonal carcinoma cells (Andrew et al., 2005). The unique criteria of ESCs are their ability to renew themselves for numerous generations and can be stimulated to differentiate into multilineages cells with specialized function under certain physiological or experimental conditions (Passier & Mummery, 2003). Based on these special characteristics, ESCs have been recommended as an important source for stem cells therapies. This source is considered as the best biological tool for drug discovery and toxicology in industrial applications (Sartipy et al., 2007; Bigdeli et al., 2008). Even though ESCs have the indefinite self-replicate capacity and are easier to maintain, however, there are a lot of ethical, legal and political concern involved in the use of ESCs. Moreover, recent researches have shown that ESCs are more difficult to maintain and tend to form teratoma or tumor (Ryu et al., 2004). Reports suggested that there are chances of mutation and high risk of contamination could occur from the nutrient supplementations and thus limit the shelf life of ESCs cell lines (Maitra et al., 2005).

Adult stem cells can be defined as the stem cells found in any developed tissues in the organisms (Bongso & Richards, 2004). The most recognized adult stem cells are hematopoietic stem cells and mesenchymal stem cells (MSCs) (Pittenger et al., 1999). The categorization of adult stem cells was proposed by Caplan in 1991. Cells that can be differentiated into mesenchymal lineages with the proposed physical supporting role to the hematopoietic stem cells niche are considered as mesenchymal stem cells (Devine & Hoffman, 2000). Hematopoietic stem cells have the capability to self-generate and differentiate into all blood cells lineages. The hematopoietic stem cells are located in the bone marrow and are arranged according to the hierarchy of progenitors (Orkin, 2000). There are several studies suggested that hematopoietic stem cells have the ability to differentiate into hepatocyes that may be applicable to therapeutic applications (Lagasse et al., 2000).

Mesenchymal stem cells (MSCs) can be defined as a unique population of non-hematopoietic stem cells that can be found in the bone marrow and other connective tissues in the human body (Augello et al., 2010). The Mesenchymal and Tissue Stem Cells Committee of the International Society for Cellular Therapy (ISCT) have projected a set of minimum criteria to define human MSCs in the laboratory, based on previous scientific investigations (Dominici et al., 2006). The first characteristic of MSCs is the adherent to the plastic surface and this primary criterion has to be maintained even by the subset of MSCs (Colter et al., 2000; Jiang et al., 2002). The next criterion for MSCs is the expression of specific antigen surface (Ag) that can be measured using flow cytometry. The crucial Ag needed for MSCs characterization is listed in Table 2.2. Next is the ability to undergo trilineage differentiation which is osteoblast, adipocyte and chondrocyte. These trilineages differentiation can be demonstrated using staining protocols or immunofluorescence staining. Based on these criteria, MSCs have been found to reside in various organs such as adipose tissues, skin, bone marrow, tooth and other locations (Pittenger et al., 1999; Caplan & Bruder, 2001).

Table 2.2: List of specific antigen surface (Ag)s for the characterization of MSCs (Modified from Dominici et al., 2006).

Status of Expression	Antigen surface (Ag)
Positive (≥ 95%)	CD 105 (also known as endoglin or MAb SH2)
	CD 73 (also known as ecto 5' nucleotidase, MAb SH3)
	CD 90 (also known as Thy-1)
Negative (≤ 2%)	CD 45 (also known as LCA, B220, T200)
	CD 34 (also known as HPCA1, gp105-120)
	CD 14 or CD11b
	CD 79α or CD19
	HLA-DR

Bone marrow-derived stem cells (BMSCs) are derived from aspirated bone marrow (Alhadlaq & Mao, 2004; Marion & Mao, 2006). The first isolation of BMSCs was done by Friedenstein et al. (1970) with the illustration as the 'colony-forming fibroblast-like cells'. The BMSCs is described as heterogeneous population of cells in human body (Colter et al., 2000). The BMSCs have ability to self-replicate and differentiate into osteoblast, chondrocytes, myoblasts, adipocytes, neuron-like cells and pancreatic islet beta cells under experimental conditions (Alhadlaq & Mao, 2004; Kim et al., 2006; Marion & Mao, 2006). Moreover, BMSCs have the potentiality to contribute to the formation of muscle, liver and also neuronal tissue (Grove et al., 2004).

Umbilical cord stem cells (UCSCs) are defined as the cells isolated from the blood of umbilical cord. There has been a tremendous interest for UCSCs in stem cells therapies for their capacity of self-renewal and ability to differentiate into various ranges of cells (Laughlin et al., 2001). Studies have shown that UCSCs were able to form into several cells types including skeletal muscle, hepatocytes, neural tissue and immune cells (Warnke et al., 2004). Their higher capacity in regards to plasticity was acknowledged by the possibility of UCSCs being chronologically very closer derivatives of embryonic stem cells compared to other adult stem cells (Moretti et al., 2010). In the *in vivo* studies using mice, UCSCs have shown high potential in curing cardiac diseases and diabetes (Kadner et al., 2002; Chao et al., 2008).

The next type of adult stem cells is adipose stem cells (ASCs). Most of the ASCs were derived from liposuction procedures and they have showed that they were able to differentiate into adipocytes, chondrocytes, myocytes, neuronal and osteoblast lineages (Zuk et al., 2002; de Ugarte et al., 2003; Peptan et al., 2006). ASCs possess the main criteria of MSCs, including high potential to self-renewal without losing the potential for further differentiation (Gimble et al., 2007). Furthermore, ASCs have several

advantages compared to other types of adult stem cells as they are present abundantly in certain individuals and are readily accessible. Moreover, ASCs is one of the most renewable sources of stem cells. However, the ability to reconstitute tissues and organs by ASCs opposed to other types of adult stem cells are not yet fully discovered.

2.4 Dental stem cells

The dental stem cells (DSCs) are isolated from several region of tooth structure such as dental pulp of primary and permanent teeth, periodontal ligament and apical area (Mao et al., 2006). Theoretically, all the DSCs were originated from the neural crest-mesenchymal cells during tooth development (Zhang et al., 2006a; Takashima et al., 2007). The DSCs also fulfill the minimum criteria for MSCs with the capacity to regenerate dentin as showed from the *in vivo* study (Yen & Sharpe, 2008). Dental stem cells can be classified into dental mesenchymal stem cells and dental epithelial stem cells (Batouli et al., 2003; Huang et al., 2010) (Figure 2.8).

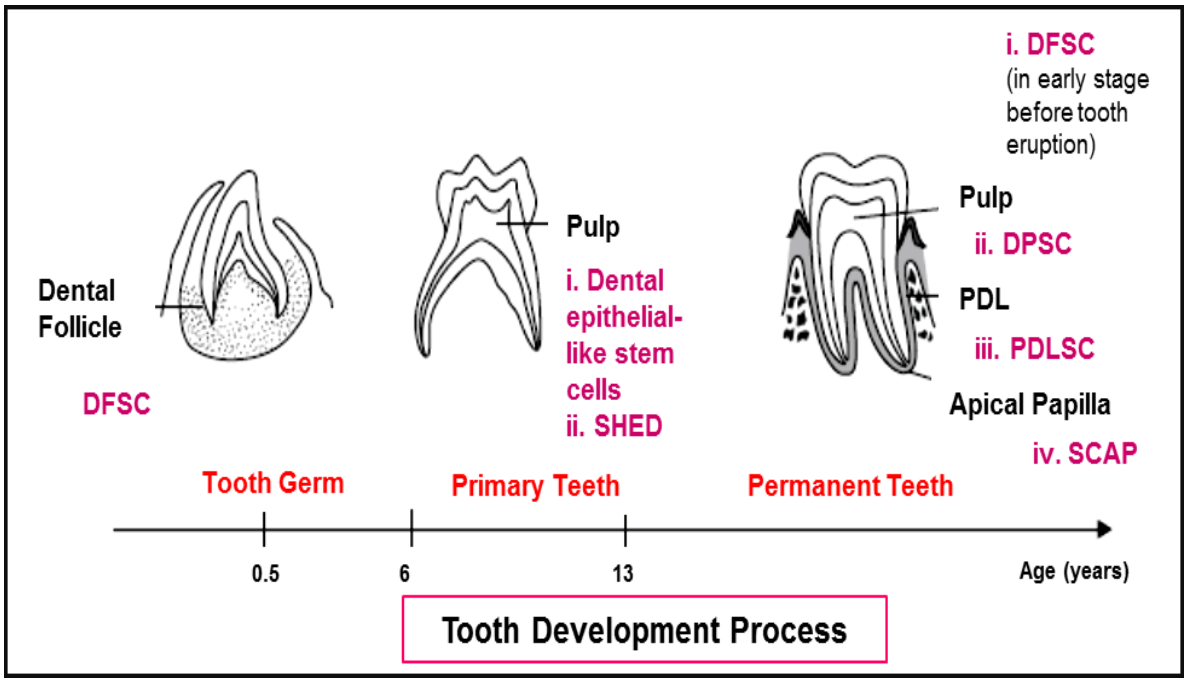

Figure 2.8: Various sources of dental stem cells (Modified from Huang et al., 2010).

There are several types of dental mesenchymal stem cells that have been studied over the past few years including the dental follicle stem cells (DFSCs) that are derived from human third molar tooth (Handa et al., 2002), dental pulp stem cells (DPSCs) from human pulp teeth (Gronthos et al., 2002), stem cells from human exfoliated deciduous teeth (SHED) (Miura et al., 2003) and the periodontal ligament stem cells (PDLSCs) (Seo et al., 2005a). In the case for dental epithelial stem cells, they have reported to be isolated from the continuously growing incisor in mice, whereas, in mammalian species it was in molar teeth (Harada et al., 1999; Morotomi et al., 2005).

2.4.1 Dental epithelial stem cells

Enamel is the most mineralized tissue in the body which is formed at the crown stages of dental development (Ten Cate, 1998). The enamel is formed from the ameloblasts that are raised from epithelial stem cells. Epithelial stem cells are important as they are the only stem cells that are originated from ectodermal and thus have major functions for odontogenesis (Ulmer et al., 2010). However, during the process of tooth eruption, the ameoblast will be broken down and thus they exhibit the self-regeneration of the enamel and leave no ectodermal stem cells for cell recovery (Frandson et al., 2007). There is a specialized structure located at the apical region of the labial cervical loop called as apical bud. The apical bud is made from the condensed epithelial stem cells and they play an important role in replenishing the dentition and differentiate into ameloblast through an interaction with mesenchymal cells (Harada et al., 1999; Morotomi et al., 2005; Yu et al., 2007).

2.4.2 Dental follicle stem cells

Dental follicles are surrounded around the developing tooth germs and consist of neural crest-mesenchymal tissues (Vollner et al., 2007). It plays a major role in the genesis of cementum, periodontal ligament and alveolar bone. The dental follicle stem

cells (DFSCs) can be isolated from the follicles of impacted third molar tooth (Yalvac et al., 2009). Several studies have shown that DFSCs play a major role in the tooth eruption process through the regulation of osteoclastogenesis and osteogenesis (Wise et al., 2005; Wise & Yao, 2006). DFSCs are known to express positive stem cell markers such as Notch1, STRO-1 and Nestin and are able to maintain it under *in vitro* environment for at least 15 passages (Morsczeck et al., 2005). They also possess the ability to differentiate into cementoblasts (Kemoun et al., 2007) and cementum *in vitro* (Handa et al., 2002) and could also regenerate periodontal ligament after *in vivo* implementation (Yokoi et al., 2007).

2.4.3 Periodontal ligament stem cell

The periodontal ligament is a specialized tissue located between the cementum and alveolar bone with the responsibility to maintain and sustain the teeth. The periodontal ligament stem cells (PDLSCs) can be harvested from the roots of extracted tooth and was reported to have capacity to differentiate into collagen-forming cell and cementoblast cells (Seo et al., 2004). They also can differentiate into adipogenic, osteogenic, and chondrogenic differentiation under *in vitro* condition (Gay et al., 2007). The expressions of specific cell surface markers of PDLSCs are also demonstrated to be positive towards the STRO-1 and CD146. Furthermore, PDLSCs could be used to develop root or the periodontal complex to support normal tooth function (Sonoyama et al., 2006).

2.4.4 Stem cells from exfoliated teeth

The isolation of post-natal stem cells from an easily accessible source is essential for tissue engineering and clinical applications. The isolation of mesenchymal progenitors from the pulp of human deciduous incisors or human exfoliated deciduous teeth (SHED) has been successfully done by various researchers. SHED was originally classified as clonogenic cells or immature cells since they showed high potential to

differentiate into neurons, adipocyte, osteoblasts and odontoblasts (Miura et al., 2003). SHED also showed a high commitment to the formation of mineralized tissues (Luisi et al., 2007; Wei et al., 2007) and dentin (Kitagawa et al., 2007) as displayed in the animal studies using immune-deficient mice (Miura et al., 2003).

2.4.5 Dental pulp stem cells

The first isolation of dental pulp stem cells (DPSCs) were done by Gronthos and co-workers in the year 2000 (Gronthos et al., 2000). Research on DPSCs expanded with the isolation of SHED (Miura et al., 2003). Despite the fact that the origin of DPSCs was not fully determined, however, a hypothesis suggests that there are two different types of populations of DPSCs in the pulp (Morsczeck et al., 2007). The first population of DPSCs was originated from the neural crest-mesencymal while another one is derived from the dental laminae (Suchanek et al., 2007). Moreover, there are diverse subpopulations of progenitor cells in the dental pulp tissues that differ in terms of their self-renewal ability, proliferation rate and the differentiation potential (Gronthos et al., 2002; Honda et al., 2007; Sumita et al., 2009). If there is any dental injury, dental pulp will be involved in the reparative dentinogenesis which is defined as a process of cell deposition into new dentin matrix to restore the injured site (Mitsiadis & Rahiotis, 2004). DPSCs can either regenerate new stem cells or can undergo differentiation process depending on the specific signals from their environment. Studies have demonstrated that adult dental pulp contains precursors that are able to develop into odontoblasts under appropriate signals (About et al., 2000; Alliot-Licht et al., 2005). The multilineages potential of DPSCs was proven to be maintained even after temporary storage in liquid nitrogen (Papaccio et al. 2006; Zhang et al., 2006b; Woods et al. 2009). Indeed, DPSCs are capable to survive for long periods of culture and can maintained up to more than 80 passages without apparent any sign of senescence (Laino et al., 2005, 2006). *In vitro* studies have also revealed that the differentiation potential

of DPSCs into odontoblasts, osteoblasts, endothelocytes, smooth muscle cells, adipocytes, chondrocytes and neurons (Ulmer et al., 2010). Therefore, DPSCs have been widely suggested as a source for the therapeutic applications based on their potential (Prescott et al., 2008).

2.5 Dental stem cells in regenerative medicine

The basic definition for regenerative medicine is the multiple discipline field of research evolved in parallel with the advacement in the biotechnological field. It involves the use of biomaterials, growth factors and cell therapy to repair or regenerate tissues and organ damaged by injuries or diseases (Guilak, 2002). The aim of regenerative medicine is to reconstruct *in vitro* mechanisms and processes which occur during the initiation and morphogenesis in the specific organs (Bluteau et al., 2008). These objectives in regenerative medicine can only be achieved by the transplantation of cells that were typically manipulated *ex vivo* into the targeted organ in sufficient numbers to restore the normal function of the organs and tissues (Levicar et al., 2007). Potential candidate to be used in regenerative medicine include autologous primary cells, cell lines, and various types of stem cells including bone marrow stem cells, cord blood stem cells and embryonic stem (ES) cells (Fodor 2003). Stem cells are the most ideal because of their ability to self-renewal and plasticity to the multiple cell lineages (Pittenger et al., 1999; Gimble et al., 2007). The selection criteria for the stem cells should be made before it can be used in the regenerative medicine as stated in Table 2.3.

Table 2.3: Selection criteria for stem cells in regenerative medicine (Gimble, 2003).

Selection criteria for stem cells in regenerative medicine
a) It can be found in abundant numbers (millions) **b)** It can be harvested by minimally invasive procedures with minimal morbidity **c)** It can be differentiated along multiple cell lineages pathways in a controllable and reproducible manner **d)** It can be safely and effectively transplanted to either autologous or allogeneic host **e)** It can be produced in accordance with current Good Manufacturing Practice guidelines (GMP)

The International Society of Stem Cell Research (ISSCR) has released specific guidelines to guarantee the participation of global scientific and ethical principles in the stem cells pre-clinical researches (Hyun et al., 2008). Advances in stem cell biology have offered huge promises for clinical therapy in the future. In spite of the unquestioned totipotency of ES cells, there are still relevant unanswered biological issues towards their growth and differentiation potential. The safety profile of unselected ESCs cells for transplantation was questionable as ESCs demonstrated unstable, rapid cell proliferation along with the formation of cancer (Reubinoff et al., 2000). In addition, there are abundant of ethical and legal concerns related to ESCs cells and thus shifted the focus of regenerative medicine towards the adult stem cells for their clinical potential. The comparison of ESCs and adult stem cells in the regenerative medicine is summarized in Table 2.4.

Table 2.4: Comparison between ESCs and adult stem cells (Modified from Ochiya et al., 2010).

Source	ESCs	Adult Stem Cells
Autologous transplantation	No	Yes
In vitro differentiation	Yes	Yes
In vivo differentiation	Yes	Yes
Differentiation potency	Very high	High (limited)
Growth in vitro	Infinite	Semi-infinite
Ethical issues	Yes	No
Avaibility in medicine	No	Yes
Government Law	Yes	No
Tumorigenesis	Yes	No
Rejection	Yes	Low / No
Trophic activity in vivo	No	Yes

Even though there are several sources of adult stem cells were likely to fulfill the minimum selection criteria, however, DSCs have several advantages due to its easy accessibility, availability and invasive procedure for cell collection. Since the existence of DPSCs have been already established (Gronthos et al., 2000), it has developed as a type of stem cells with high potential in regenerative medicine. Recent understandings of DSCs from human teeth have led to the development of new storage method to preserve tooth as a source of stem cells (Huang et al., 2010). The first commercial tooth bank was established as a venture company at National Hiroshima University in Japan in 2004 (Masato et al., 2007). With the advanced technology in tissue engineering and tooth bank, the potential of DSCs in the regenerative medicine could be explored aggressively. At present, DSCs are being investigated as a potential source to treat neurodegenerative disease such as Parkinson's disease (Su et al., 2011). The expressions of neural markers in DSCs have suggested their potential to be used to treat neurodegenerative diseases. Dental pulp and periodontal ligament have been proposed

to harbor cells that are able to differentiate into neuronal direction (d'Aquino et al., 2007; Arthur et al., 2008). In addition, Iohara et al. (2004) and Almushayt et al. (2006) have reported successful regeneration of odontoblasts and dentin from the DSCs. Furthermore, stem cells presented in dental pulp have shown immune-regulatory and immunosuppressive properties (Pierdomenico et al., 2005). The DSCs also have demonstrated a high commitment towards adipocyte and neuron cells (Arthur et al., 2008). Several researches have suggested the possibility of DSCs to be used in the treatment of myocardial infarction and liver dysfunction (Gandia et al., 2008; Ikeda et al., 2008). Hence, the therapeutic ability and clinical benefits of DSCs are not only limited to the use in dentistry area but also in the regenerative medicine as shown in Table 2.5.

Table 2.5: Benefits of using dental stem cells in regenerative medicine (Modified from Huang et al., 2010).

Benefits of dental stem cells
1. Less ethical issues **2.** Autograft - Increased the success rate in tooth autotransplantation (Chang et al., 2009) - Better proliferation and immunoregulation than bone marrow stem cells (Pierdomenico et al., 2005) **3.** Dental stem cells based tissue engineering - Oral medicine : tooth regeneration (Ohazama et al., 2004), pulp/dentin regeneration (Huang et al., 2008), periodontal ligament regeneration (Liu et al., 2008) - Other medical application: bone formation (d'Aquino et al., 2009), stroke therapy (Yang et al., 2009), heart disease (Gandia et al., 2008). **4.** General benefits - Efficient and easy to access source of MSCs - Potential for commercial banking

2.6 Liver

Liver is an important organ as it is responsible for 500 functions in the body (Desmet, 2001). The main roles of liver is to provide both endocrine functions by producing factors such as albumin and urea into the blood, and exocrine functions by secreting bile to the intestine and lastly as a hub for carbohydrate, lipid, and amino acid regulation and storage (Taub, 2004). The functions of liver are vital to maintain the body's metabolic homeostasis mechanism. Furthermore, the liver is actively involved in the systemic responses towards trauma or injury by producing acute phase proteins (Lee et al., 2003). In addition to its metabolic activity, the liver is one of the first defense systems to inactivate the toxins and xenobiotics absorbed by the intestine and clear the unknown particles from the blood (Nedredal et al., 2003; Willekens et al., 2005). The exclusive characteristic of liver is its ability to self-regenerate from any massive injuries and to restore back its original mass even with small proportion (20%) of undamaged cells (Michalopoulos & DeFrances, 1997). Generally, there are three level of liver regeneration pathway (Sell, 2001). In a mild liver injury, the mature primary cells in liver namely hepotocyte will undergo several cycles in response to the injury. The ductal progenitor cells will give a longer and limited proliferation of cells and stem cell from circulation will start to involve in the liver regeneration. These cells enter into liver in a random distribution either in isolated cells or in a portal and periportal distribution (Petersen et al., 1999). The structure of liver comprises of 70 percent of parenchymal hepatocytes while the rest are non-parenchymal cells namely Ito cells, Kupffer cells and bile duct (Table 2.6).

Table 2.6: Cellular composition of rat liver (table modified from Morin et al., 1988)

Cell Type	Diameter (µm)	Volume (% of total)	Number (% of total)	Type	Density (g/ml)
Parenchymal cells					
Hepatocytes	20-25	77.8	65	Epithelial	1.10-15
Non-parenchymal cell					
Sinusoidal endothelial cells	6.5-11	2.8	16	Endothelial	1.0-80
Kupffer cells	10-13	2.1	12	Macrophages	1.076
Stellate (Ito) cells	10.7-11.5	1.4	8	Fibroblasts	1.05
Pit cells		Minor	Minor		
Extracellular spaces					
Sinusoidal lumina		10.6			
Space of dice		4.9			
Bile canaliculi		0.43			

2.6 Liver Dysfunction

The liver dysfunction causing around 25,000 deaths per year and is the tenth most frequent cause of death in the United States (Popovic & Kozak, 2000). Cirrhosis, the end result of long-term liver damage has long been a significant cause of death in many developed country. The major causes of cirrhosis globally were hepatitis B, C and alcohol. The intakes of alcohol and obesity with diabetes will contribute to the increase of fibrosis and cirrhosis (Fallowfield & Iredale, 2004). Liver cirrohosis is an acute liver disease with continuous destruction of liver tissue while fibrosis are identified as hepatic scarring which are established after the persistent injuries and led to the termination of liver functions (Levicar et al., 2007). The cirrhotic stage in liver disease

is considered as irreversible and the only available treatment for these damages is liver transplantation. However, the increasing shortages of organ donors and high risk of organ rejection have restricted the liver transplantation for patients (Kakinuma et al., 2009). Direct transplantation of hepatocyte has been successfully carried out in the patient in two clinical trials (Fox et al., 1998). Unfortunately there were several limitations in this procedure such as limited amplification of hepatocyte, short period of functioning cells and the need of immune-suppression (Ohashi et al., 2001; Najimi & Sokal, 2005). In addition, the *in vitro* expansion of mature hepatocytes was not feasible because that would bring several effects for example hypo-function and hepatocytic metabolism (Kakinuma et al., 2009). Therefore, the treatments for the liver diseases are limited and an alternative treatment needed to be explored to open a new hope for patient with liver diseases.

2.7 Stem cells as an alternative treatment for liver dysfunction

Human ESCs represents an alternative source for hepatocytes to solve the lack of liver donor for live transplantation. Several studies have demonstrated successful differentiation of human ESCs into hepatocyte cells line using various culture systems (Cai et al., 2007; Duan et al., 2007; Basma et al., 2009). However, the approaches of using serum in culture media, matrigel or mouse embryonic fibroblasts as feeders may cause mismatched of the tissue for the use in clinical applications (Touboul et al., 2010).

Petersen et al. (1999) were first demonstrated that BMSCs could be used as a source for hepatic oval cells by using cross-sex bone marrow transplantation after the induction of liver dysfunction. The liver repopulation by bone marrow cells was slow but significant. The first hepatocytes of bone marrow origin appeared 7 weeks after transplantation. After 22 weeks of transplantation, the results showed that one-third of the liver was comprised of BMSCs. This suggested that BMSCs could contribute to

hepatocyte generation during injury situation where the regenerative potential of hepatocytes is impaired. Furthermore, the recent studies have demonstrated that BMSCs injected during liver injury can reduce the liver fibrosis (Sakaida et al., 2004; Zhao et al., 2012).

The *in vitro* studies have established the potential of BMSCs to differentiate towards many lineages including hepatic lineage. Previous studies have exposed the evidence that there were a small niche of cells that expressed the hematopoietic stem cell markers such as CD34, c-kit, Thy-1, AFP and c-met within the rat bone marrow (Oh et al., 2000; Miyazaki et al., 2002). The expression of albumin which is a marker for differentiated hepatocytes was also present after the cells were exposed to the media containing hepatocyte growth factor (HGF) and epidermal growth factor (EGF). The differentiation of BMSCs into hepatocyte cells in the *in vivo* study was also reported in rats (Petersen et al., 1999, Shu et al., 2004), mice (Theise et al., 2000a) and humans (Alison et al., 2000; Theise et al., 2000b). The isolation of BMSCs has released a new hope in regenerative medicine using bone marrow cells. However, the isolation of BMSCs required a challenging and time-consuming process. Thus, other mesenchymal stem cells sources were searched for hepaotcyte differentiation with a better and easy isolation procedure.

The first differentiation of ASCs into hepatocyte cells using the induction media containing HGF, oncostatin M and dimethyl sulfoxide was reported by Seo et al. (2005b). In addition, Lee et al. (2004) have also demonstrated the possibility of generating hepatocyte-like cells from ASCs. Many researchers have used MSCs to generate functional hepatocytes but there are still question regarding the cell fusion and poor functionality. Further researches are needed to generate fully functional hepatocyte cells from MSCs before it can be used in clinical applications (Yamamoto et al., 2008).

A preliminary research by Ishkitiev et al (2010) has reported the potentiality of DPSCs to undergo hepatocyte differentiation. Although DPSCs showed more commitment towards ectodermal differentiation for example neuronal cells (Gronthos et al., 2002; Lopez-Cazaux et al., 2006), however this finding has unleashed the potential of DPSCs towards endoderm lineages.

2.8 Serum replacement in stem cells cultivation

The selection of the tissue culture condition for the expansion of stem cell is a crucial aspect as it will affect the proliferation rate and differentiation expression of the cultured cells. According to Masters (2000) and Davis (2002), tissue culture condition for the cells must impersonate the *in vivo* physiological environment from every aspect including temperature, pH, osmolarity and oxygen supply. The main parameters involved in the preparation of a good tissue culture condition are illustrated in Figure 2.9.

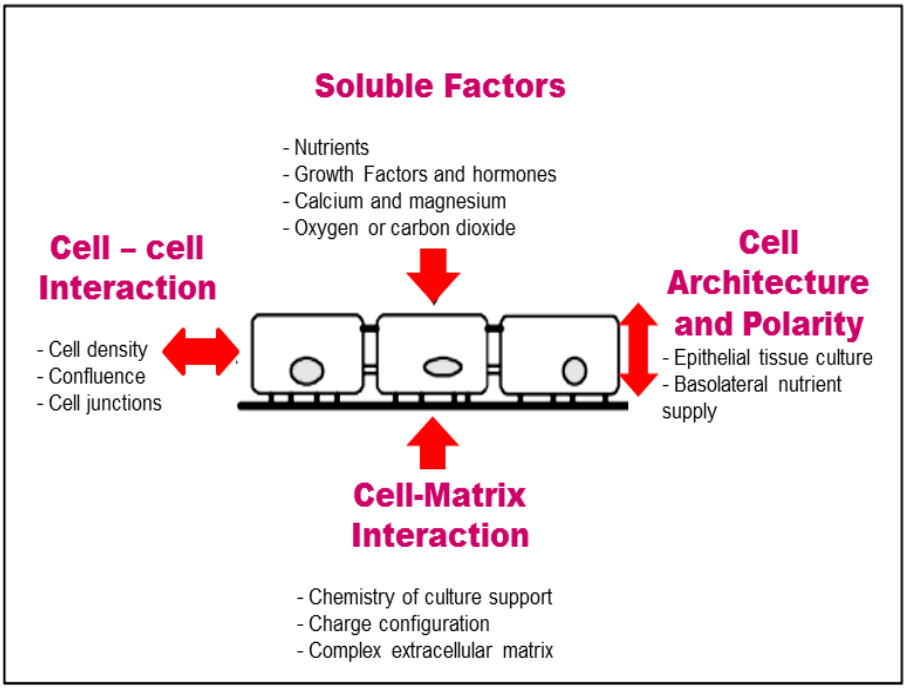

Figure 2.9: Parameters for an appropriate tissue culture condition (Modified from Gstraunhaler, 2003).

The culture medium is classified as one of the major features in the tissue culture condition. It will act as a source of essential nutrients, minerals, hormones along with growth factors as well as oxygen supply (Masters, 2000; Davis, 2002; Gstraunthaler, 2003; Freshney, 2005). Brunner et al. (2010) have stated several crucial components in the media during culture as summarized in Table 2.7.

Table 2.7: Components in the culture media and their functions (Modified from Brunner et al., 2010).

Component	Function
Biosynthetic precursors	For the cell anabolism
Catabolic substrates	For the energy metabolism
Vitamins and trace	For the primary catalytic
Electrolytes	For the catalytic and physiological function

The universal basal medium used in the stem cells cultivation is Eagle's Minimal Essential Medium (Eagle's MEM or MEM) and modified MEM by Dulbecco known as Dulbecco's Modified Eagle's Medium (DMEM). Basal media is needed to be supplemented with a serum to maintain the cell proliferation and differentiation (van der Valk et al., 2010). Serum can be defined as a blood without the presence of cells, platelets and clotting factors (Jochems et al., 2002). There are four important functions of serum in tissue culture medium. First, it supplies stimulating hormone factors to promote the cell growth and differentiation process. The second function of serum is the transportation of proteins, minerals with trace and lipid to the cells. Next, serum will plays as a germinating factor to allow better connection towards the surface of culture flask. The final function of serum is maintaining the pH and inhibits protease and also toxins that are present in the medium (Brunner et al., 2010). The most commonly used

serum is fetal bovine serum (FBS) and it have been adopted as a standard supplement for the tissue culture media (Shahdadfar et al., 2005).

2.8.1 Fetal bovine serum

Fetal bovine serum (FBS) is classified as the most effective and important supplement for cell proliferation as it contains a lot of growth factor and lower globulin level (Shah, 1999). The usage of FBS has increased the efficiency of cell production by decreasing and eliminating several phases in the formulation and optimization of the culture media (Brunner et al., 2010). A number of studies have demonstrated that cells were unable to grow without the presence nutrients in the FBS (Pawlowski et al., 2009; Kozlowski et al., 2009). However, the presence of FBS in culture media also bears some disadvantages either at biological perspective or at animal welfare level.

There are always a chance for serum to become a source for microbial contamination including fungi, mycoplasma, viruses and also prions to the cells (Dormont, 1999). The use of FBS in stem cells cultivation could also transport the immunogenicity into the cells and cause immunological reactions (Selvaggi et al., 1997; Shahdadfar et al., 2005). Spees et al. (2004) reported that a single preparation of 1 X 10^8 human MSCs cultured using FBS in a standard condition could transmit approximately 7-30 mg of FBS proteins. Thus, FBS may introduce several unknown variables into the tissue culture system (Gstraunthaler, 2003). Moreover, the lot-to-lot variation in the production of FBS can cause serious phonotypical differences in the cell culture (van der Valk et al., 2010).

The demand for FBS has increased worldwide by estimated 500,000 liters per year which will involve harvesting of more than 1 million of bovine fetus per year (Shah, 1999; Jochems et al., 2002). Moreover, the method use to collect the FBS may

cause severe pain to the animal. FBS are produced by drowning out the blood using a large-diameter needle from the prenatal cows (Brunner et al., 2010). The collections of FBS have specifically influenced the development of the fetus and cause a lot of pain to the prenatal cows. Other collection method includes the puncturing of umbilical cord or jugular vein (Jochems et al., 2002). Thus, this matter has raised dramatic ethical issues for animal welfare in the production of FBS (Shailer & Corrin, 1999; van der Valk et al., 2004).

Since the usage of FBS cause virus and prion contamination along with the ethical issue for animal, these would become a great barrier for clinical applications of stem cell therapies. Several researchers have suggested serum-free culture as a major solution to this problem (Froud, 1999; Zimmermann et al., 2000; Gstraunthaler, 2003). Moreover, wide databases of serum-free culture media have been established with the purpose of providing information and ease to the application of serum-free cells (Falkner et al., 2006). The use of serum-free culture may become as an acceptable alternative but could result in slower cell growth and required specific adaptation for the cell lines (Kadar et al., 2009). Additional methods of choice for reducing the requirement of FBS is the substitution to the bovine colostrum (Belford et al., 1995) and protein fraction from plant origin known as vegetal serum (Pazos et al., 2004). Unfortunately, the production of bovine colostrum is lower than FBS while the production of vegetal serum was stopped (Pazos et al., 2004; van der Valk et al., 2004). Therefore, it is necessary to search other alternative serum for animal serum replacement and recently serum from human origin has shown a great promise to be used in the tissue culture system.

2.8.2 Human platelet lysate

The rapid developments in stem cells therapeutic applications have demanded serum from human origin in order to accomplish a successful stem cells expansion and implantation. Recent clinical records for human MSCs cultured in FBS have possessed minor side effects and certain immunological reactions along with anti-FBS antibodies and therefore would affect the treatment (Horwitz et al., 2002; Sundin et al., 2007).

Very recently, human platelet lysates (HPL) have been shown to serve as a safe substitute for animal serum in MSC expansion (Yamada et al., 2004; Doucet et al., 2005; Muller et al., 2006). The collection of HPL were obtained from the outdated thrombocyte concentrates (Rauch et al., 2011). HPL was isolated using freeze/thaw technique as reported by Schallmoser & Strunk (2009). According to Reed (2007), the activation of platelet will release chemokines, cytokines and growth factors. A number of growth factors namely platelet-derived growth factors (PDGFs), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) that are necessary for *in vivo* wound healing are present in the platelet (Barrientos et al., 2008; Nurden et al., 2008). Yamaguchi et al. (2002) and Levy et al. (2008) had described the function of PDGF and bFGF as the growth factors for MSCs. Previous studies also revealed that the combination of PDGF, bFGF and transforming growth factors were sufficient to expand MSCs in a serum-free medium under *in vitro* condition (Ng et al., 2008). Therefore, the advantages of platelet-derived growth factors are recently examined for the therapeutics application of chronic ulcer, bone, tendon, nerve regeneration and MSCs cell therapy (Kitoh et al., 2004; Yamada et al., 2004; Anitua et al., 2006).

HPL has been proven to be equivalent to the FBS in terms of sustaining the growth and differentiation of human MSCs and thus are suitable for the clinical

application (Stute et al., 2004; Kobayashi et al., 2005). The replacement of FBS by HPL resulted in highly increased proliferation rates in MSCs derived from bone marrow and umbilical cord blood (Reinisch et al., 2007; Schallmoser et al., 2007). The cells expanded in HPL medium also demonstrated high capability to maintain their typical immunophenotype, differentiation capacity and immune regulatory potential (Reinisch et al., 2007; Bieback et al., 2009). Compared to the FBS in good manufacturing practice (GMP), the GMP-human platelet lysate (GMP-HPL) have shown better option for accelerating expansion, maintenance of phenotype stability and the absence of clonal chromosomal aberrancy in human MSCs cultures (Govindasamy et al., 2011). Compared to other serum, HPL has demonstarted the safest serum for human MSCs expansion based on molecular analysis (Crespo-Diaz et al., 2011) and there were no changes in the quality and cell potency when using HPL as serum in culture media (Bieback et al., 2009). HPL allowed the isolation of MSCs with comparable immune phenotype, *in vitro* functionality regarding T-cell suspension and differentiation potential like FBS. However, additional test for genomic stability and *in vivo* differentiation potential will be needed before HPL can be apply in therapeutic applications (Schallmoser et al., 2008). Previos report have suggested that that autologous serum may even favor genomic stability as compared to FBS (Shahdadfar et al., 2005; Schallmoser et al., 2008). Moreover, the cells in autologous serum displayed a preserved methylated and unmethylated state compared to the FBS (Dahl et al., 2008). The data as far as available suggest the biological safety of HPL even under *in vivo* condition (Doucet et al., 2005; Lange et al., 2007; Schallmoser et al., 2007, 2008). Infact, BMSCs expanded in HPL has shown to have potential to treat refactory graft versus host disease (von Bonin et al., 2009).

The selection of serum for stem cell expansion has a tremendous impact on the stem cells. Different serum may cause different cellular subset, dissimilarity in the

differentiation potential and therapeutics potential for the cells. The usage of HPL in tissue culture system has proven to increase the cell yield, maintain the same immunophenotype and degree of differentiation. Moreover, HPL not only excludes the contaminations of bovine viruses or other xenogeneic pathogens in FBS, but it also help to overcome the lot-to-lot variability problem of FBS (Bieback et al., 2009). Hence, it can be suggested that HPL is a potential nominee for the stem cells expansion (Capelli et al., 2007)

CHAPTER 3

MATERIALS & METHODOLOGY

CHAPTER 3

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3.1 Sample collection

Deciduous teeth were collected from four young donors (n = 4; Age 3–9 years old) who came for extraction at the Department of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya. Medical ethics approval was obtained prior to the commencements of this study from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya, DF CD 0907/0042 (L) as in Appendix 1.1.

3.2 Extirpation of dental pulp tissue from teeth

The extracted deciduous teeth were immediately immersed into Povidone-iodine (PVP-I, Sigma Aldrich, St. Louis, MO, USA, http://www.sigmaaldrich.com) before the sectioning procedures. This step was important to avoid any exogenous contamination from bacterial and fungal onto the surface of the tooth. The tooth was mounted on a specially designed jig and then sectioned horizontally, layer by layer using a diamond disc on a straight handpiece until the pulp chamber exposed. During this process, double distilled water (ddH_2O) was used to continuously wet the tooth to avoid dehydration of the tooth and to minimize the amount of heat generated during sectioning. Once the pulp chamber was exposed, the tissue was removed gently using a tweezers and immersed into the transportation media consisted of basal media, Dulbecco's modified Eagle's medium-knock-out (DMEM-KO) (Invitrogen, Carlsbad, CA, USA, http://www.invitrogen.com), supplemented with 1 % penicillin/streptomycin (Invitrogen) in a 1.5 ml tube (Axygen, Union City, CA, USA, http://www.axygen.com). The pulp samples were subsequently brought to the laboratory for processing. All

utensils and reagents utilized and the steps of dental pulp extraction were illustrated in Figure 3.1 and Figure 3.2.

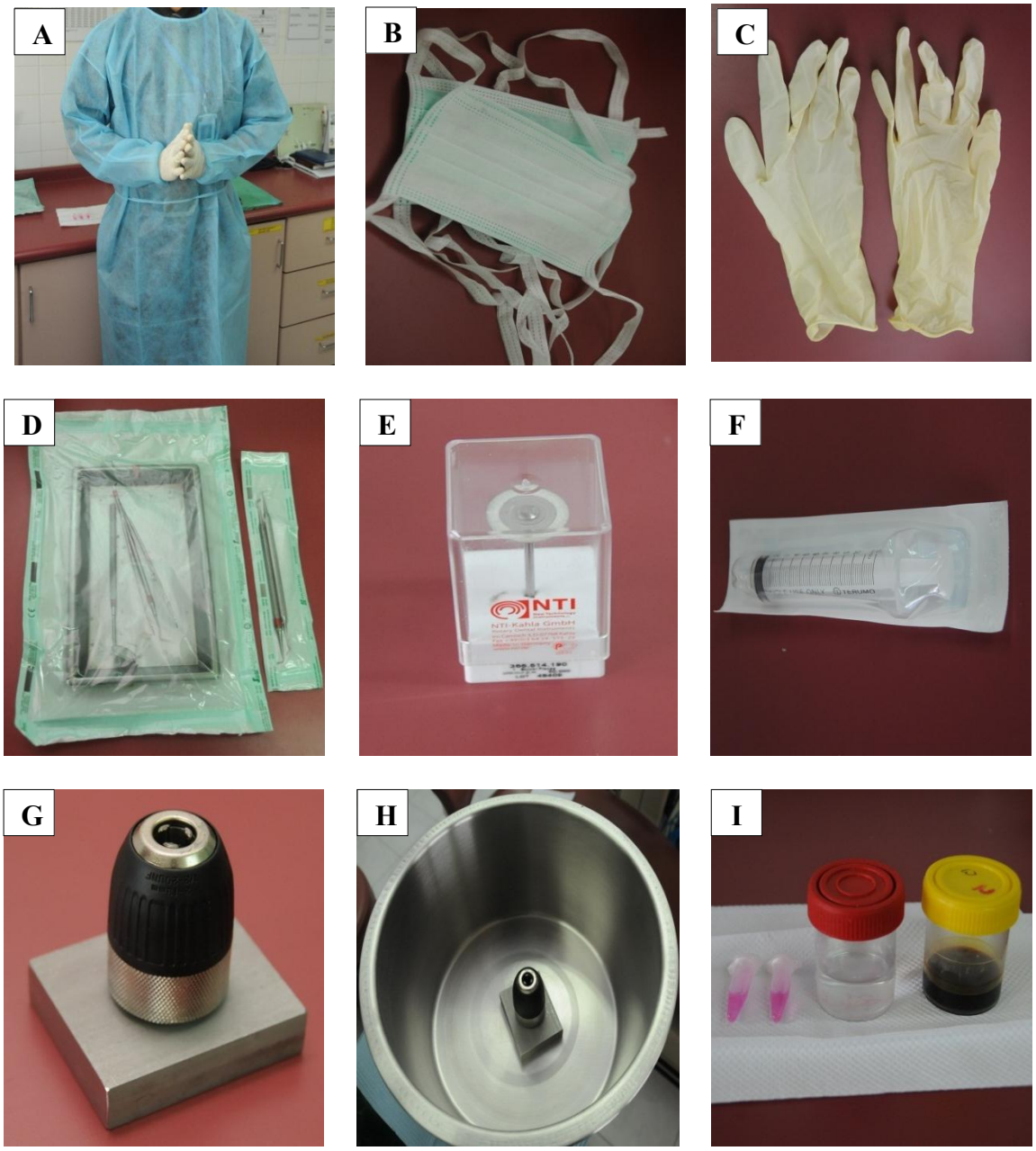

Figure 3.1: Chemical and utensils utilized in the procedures of dental pulp tissue extraction from tooth; A: medical garment, B: face mask, C: hand glove, D: tweezers and excavator, E: diamond disc, F: syringe, G: designated jig to hold tooth during the process, H: stainless steel container and I: from left to right, the transportation media, ddH_2O and PVP-I solution.

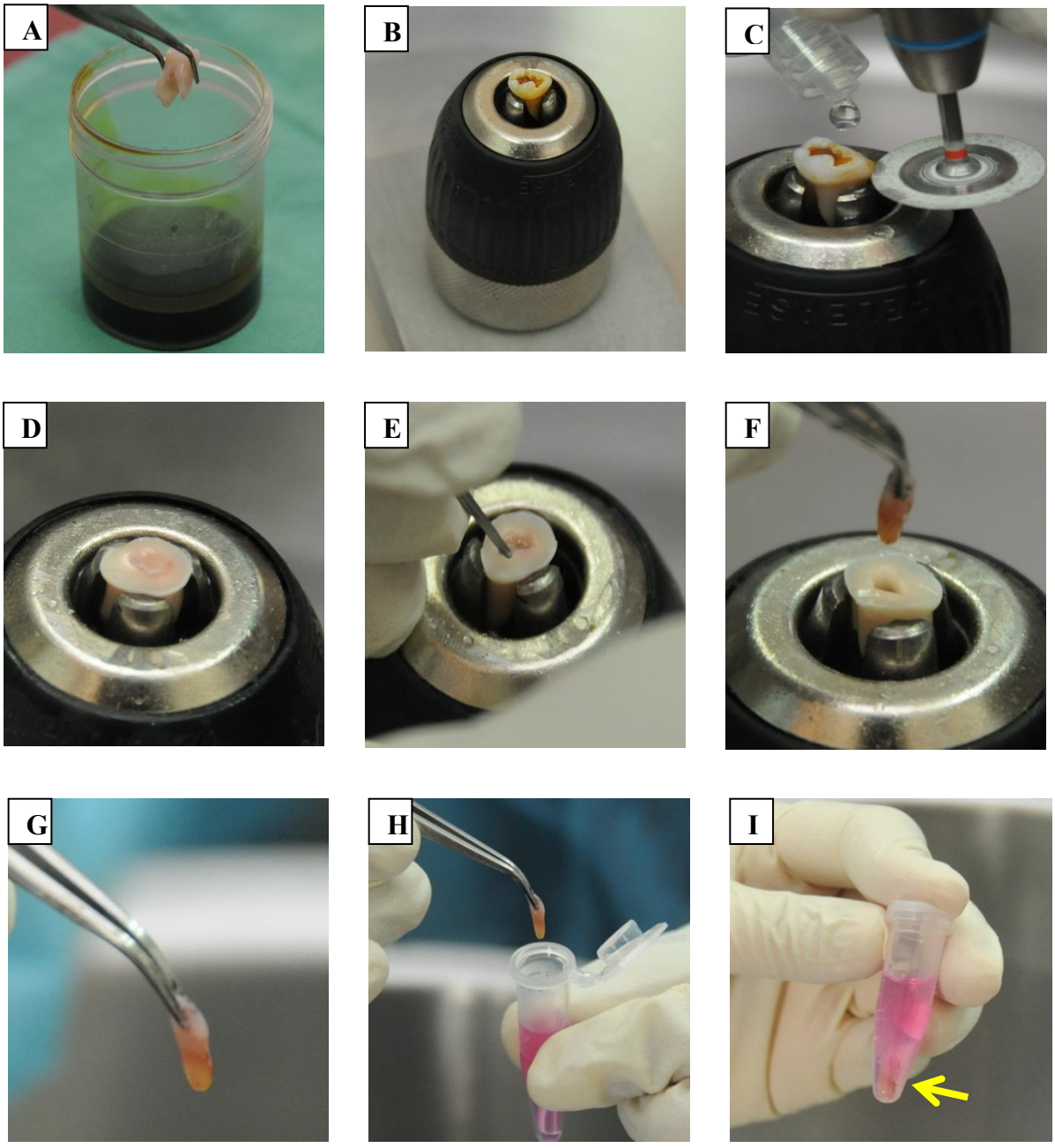

Figure 3.2: Pictures describe the step of dental pulp tissue extirpation from tooth; A: tooth were immersed in the PVP-I solution to eliminate exogenous contamination, B: tooth was placed in the designated jig, C: ddH_2O were continuously supplied during the cutting of tooth, D: tooth was cut until the dental pulp chamber was exposed, E: dental pulp chamber was opened using excavator, F, G: pulp tissues was retrieved of tooth from the pulp chamber and H, I: dental pulp tissue was immersed into the transportation media. Arrow indicated the dental pulp tissue in transportation media.

3.3 Isolation of dental pulp stem cells from deciduous teeth

The isolation of dental pulp stem cells from deciduous teeth (SCD) was done in the biosafety cabinet under sterile environment. Dental pulp tissues were washed three times by using washing buffer which consisted of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; -Ca^{2+}, -Mg^{2+}, Invitrogen) and 0.5% of penicillin/streptomycin (Invitrogen) in a 1:1 ratio. Next, the tissues were transferred into several 1.5 ml tubes (Axygen) containing DMEM-KO (Invitrogen). Mechanical destruction was carried out by mincing the tissues using a sterile scissor. Then, the remnant tissues were enzymatically digested by using 3 mg/ml of collagenase type I (Invitrogen) and incubated for 30 minutes at 37 ºC at in a humidified atmosphere containing 5% CO_2. The mixture was then neutralized by adding 6 ml of growth media (Appendix 1.2) and the whole mixture was transferred into 15 ml tubes (BD Bioscience, Franklin Lakes, NJ, USA, http://www.bdbiosciences.com) and subsequently spin down at 1100 rpm for 6 minutes. The supernatant was discarded and the pellet was gently resuspended with 3 ml of growth media. The pellet was immediately cultured into T-25 flask (BD Bioscience) containing a mixture of 0.1% gelatin (Sigma Aldrich) and supplemented with 3 ml of growth media. The cultured flask was incubated at 37°C in a humidified atmosphere containing 5% CO_2 until the colonies of cells were appeared.

3.4 Preparation of human platelet lysate

The human platelet lysate (HPL) was prepared from 30 to 40 donors who had donated at the University Malaya Blood Bank. Briefly, the whole blood (WB) was collected into a quadruple blood-bag system (Baxter Health Care Corporation, Deerfield, IL, USA, http://www.baxter.com) and was centrifuged at 4250g for 13 min at 22 º C to separate the plasma and buffy coat as illustrated in Figure 3.3. Platelet-rich

plasma (PRP) was prepared by mixing 4 units of buffy coat (from donors Group O) and 1 unit of plasma (from donors Group AB). Immediately after preparation, the PRP was frozen down to -80ºC and then thawed to 37°C to obtain platelet-released growth factors. A total of 10-12 thawed PRP units, now called HPL, were pooled to prepare a standardized pooled HPL. The pooled HPL was then centrifuged at 4000 g for 15 min at 4ºC to remove the platelet fragments. The supernatant plasma was furthered filtered using a 40 µM filter (BD Biosciences) and was then transferred into 50 ml conical tubes (BD Biosciences). A total of 2 U/ml Heparin (Heparinol, Ain Medicare Sdn Bhd, Malaysia, http://www.ainmedicare.com.my) was added into pooled HPL before stored at -20°C for further use.

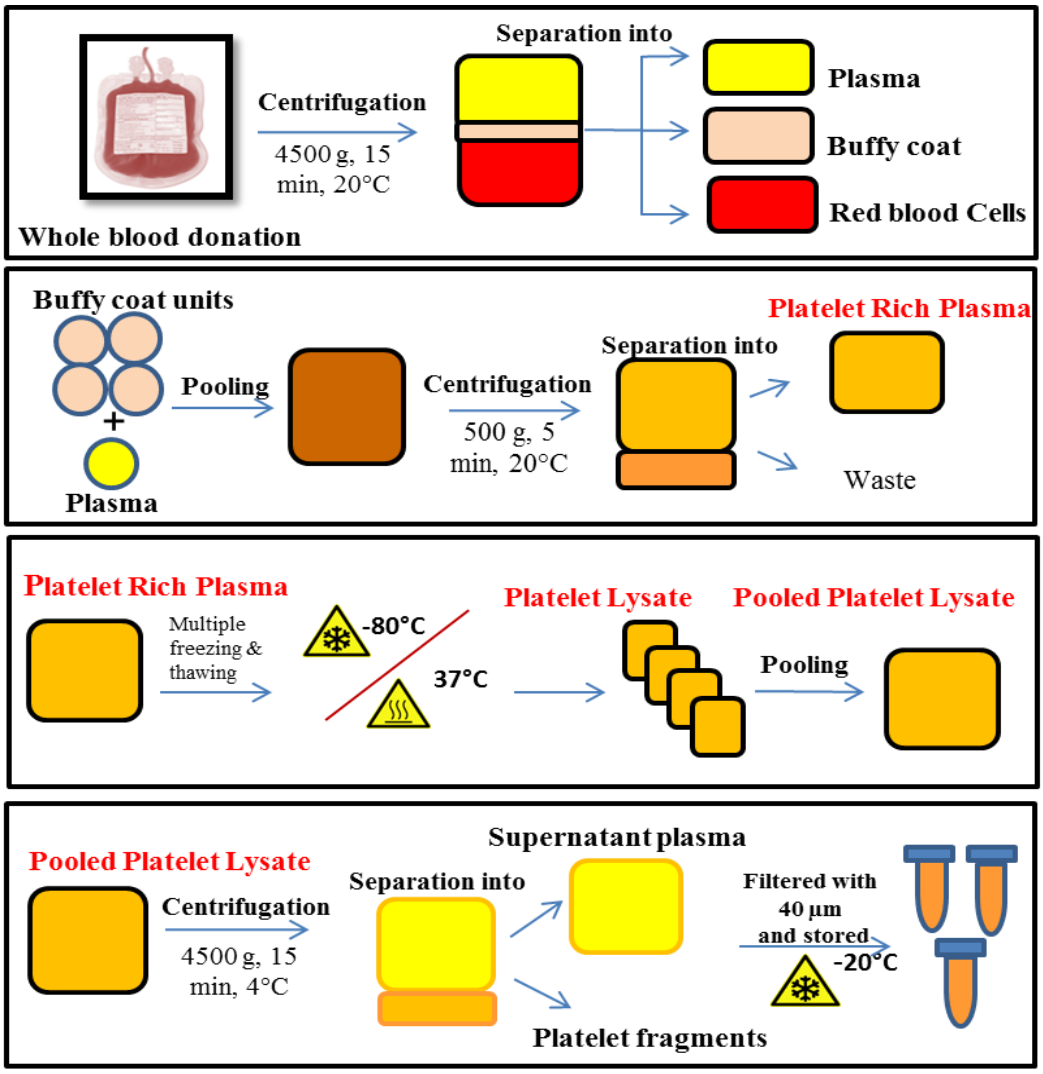

Figure 3.3: The schematic presentation of the preparation of pooled HPL done under sterilized condition.

3.5 Optimization and expansion of SCD

Optimization of SCD was done using two different types of growth media. The first growth media that was labeled as 'FBS media' comprised of basal media DMEM-KO (Invitrogen), supplemented with 10% Australian characterized fetal bovine serum (FBS, Hyclone, MA, USA, http://www.thermofisher.com), 1% glutamax (Invitrogen) and 0.5% penicillin/streptomycin (Invitrogen). The second growth media that was labeled as 'HPL media' consisted of DMEM-KO (Invitrogen), supplemented with 10% of pooled human platelet lysate (HPL), 1% glutamax (Invitrogen), 0.5% penicillin/streptomycin (Invitrogen) and 0.001 ml of heparin (Heparinol). The growth media composition is provided in Appendix 1.2. Growth medium was changed every three days of culture in order to maintain appropriate level of nutrient for cells. SCD were cultured for 4 to 7 days depending on the growth of SCD until they reach 80% of confluence. Trypsinization was done to detach all SCD and cultured for the following passage. Briefly, the SCD were washed twice by using DPBS ($-Ca^{2+}$, $-Mg^{2+}$, Invitrogen) to remove growth media and debris. Next, 0.05 % EDTA-trypsin (Invitrogen) was added to the culture flask and left for 1 minute in a humidified atmosphere containing 5% CO_2 at 37°C to allow the enzymatic reaction to take place. After the complete detachment of SCD, an immediate neutralization process using 10 ml of growth media was done to avoid cell apoptosis or stress. Suspension of SCD was centrifuged at 1200rpm for 6 minutes to separate EDTA-Trypsin from cells. The total number of cells were counted using hemocytometer (Sigma Aldrich) and the viability of SCD was monitored using Trypan Blue (Sigma Aldrich) staining. The following passage for SCD was cultured using 1000 cell/cm^2 from the newly harvested cells into T-25 flask (BD Bioscience). Fresh growth media and similar incubation condition (37°C and a humidified atmosphere containing 5% CO_2) was used for every passage. SCD culture was expanded until passage 5.

3.6 Growth kinetics

The growth kinetics of SCD cultured in media FBS and HPL were demonstrated in two ways: the population doubling (PD) rate and the population doubling time (PDT). A total of 1000 cell/cm^2 of SCD were cultured in T-25 flask with three replicates for each samples according to the type of media until 90% confluence. Then, the total harvested number of SCD in that given passage was counted using hemocytometer (Sigma Aldrich) and the viability of SCD was monitored using Trypan Blue (Sigma Aldrich) staining. The time taken for SCD to reach 90% confluence was also recorded. Then, the SCD were sub-cultured again for the next passage with the initial seeding of 1000 cell/cm^2 to T-25-flask (BD Bioscience). This cycle was repeated until the culture of SCD reached passage 5. The population doubling (PD) rate was used to determine the proliferation of SCD whereas the population doubling time (PDT) was used to demonstrate the time required for SCD to reach 90% of confluence in every passages. The PD and PDT were calculated using the following equation respectively;

PD = [$\log_{10}$ (number of cells harvested) - $\log_{10}$ (number of cells plated)] / $\log_{10}$

PDT = $\log_2$ (time) / log (initial no. of cell – final no. of cell).

3.7 Colony forming unit assay

The colony forming unit (CFU) assay was determined by re-plating 100 cells in 35-mm dish (BD Bioscience) followed by 14 days of culture in both FBS and HPL. Then, the cells were rinsed twice from growth media by using DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and fixed with 100% methanol (Mallindkrodt, Hazelwood, USA, http://pharmaceuticals.covidien.com) for 20 minutes at room temperature (23 ± 1°C) followed by 3% of crystal violet (Sigma Aldrich) staining. The composition and preparation of 3% of crystal violet (Sigma Aldrich) is included in Appendix 1.3. Next,

the blue stain was rinsed four times using tap water until the dishes become colorless. The dishes were inverted downwards on a clean cloth and allowed to air-dry for several minutes. Only the stained colonies with size larger than 2 mM (>2 mM) were counted. The CFU of SCD was calculated using the formula;

CFU% = (the total number of colonies stained / the initial number of cells) X 100.

3.8 Senescence associated β-galactosidase assay

The senescence associated β-galactosidase (SA-β-gal) assay for SCD cultured in media FBS and HPL was carried out using senescence β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA, http://www.cellsignal.com) according to the manufacturer's procedures. Briefly, SCD cells were expanded in a 35-mm tissue culture dishes (BD Bioscience) until it reached to 80% confluence. The SCD were then washed twice with DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and incubated with 1X Fixative solution for 15 minutes at room temperature (23 ± 1°C). Subsequently, the fixed SCD were rewashed using 2 ml of DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and stained with 1 ml of staining solution mixture for overnight at 37°C in a humidified atmosphere containing 5% CO_2. The staining solution mixture was prepared by mixing 1X staining solution, staining supplement A, staining supplement B and 20 mg/ml of X-gal prepared in N-N-dimethylformamide (Sigma Aldrich). Preparation of all solution used in this assay is presented in Appendix 1.4. The development of blue color cells was observed under a phase-contrast microscope using 4X and 10X magnifications while the staining solution mixture was still present in the plate. After the observation, the stained cells were washed twice with 2 ml of DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and stored at 37°C in a humidified atmosphere containing 5% CO_2. For long-term storage, the cells were

overlaid with 70% of glycerol (Sigma Aldrich) and stored at 4°C. The SA-β-gal assay of SCD was calculated using following formula;

SA-β-gal % = (number of senescence cells / total number of cell) X 100

3.9 Flow cytometry

Immunophenotyping of SCD cultured in FBS and HPL were examined by using flow cytometry at passage 5. On reaching 80% confluency, the SCD were harvested using 0.05% trypsin (Invitrogen) and resuspended into 10 ml DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen). The cell suspensions were centrifuged at 400g for 5 minutes. Next, the supernatant was discarded and the cell pellet was resuspended again with DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) at a cell density 1.5 X 10^6 cells/ml. A total of 200 µl cell suspensions were aliquoted into several test tubes and mixed well with labeled antibodies. The cells were incubated for 1 hour at 37° C under dark condition. Then, the cells were washed with 2 ml DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and centrifuged at 400g for 5 minutes. The antibodies used to mark the cell surface epitopes are listed in Table 3.1 and Table 3.2. All analyses were standardized against negative control cells incubated with isotype-specific IgG1-PE and IgG1-FITC (BD Pharmingen, BD Bioscience). At least 10,000 events were acquired on Guava Technologies flow cytometry and the results were analyzed using Cytosoft, Version 5.2, Guava Technologies.

Table 3.1: List of labeled antibodies used for immunophenotype analysis of SCD expanded in FBS and HPL media

Cell surface antigen	Manufacturer
CD34-phycoerythrin	BD Pharmingen
CD44-phycoerythrin	BD Pharmingen
CD73-phycoerythrin	BD Pharmingen
CD90-phycoerythrin	BD Pharmingen
CD166- phycoerythrin	BD Pharmingen
CD45-Fluoro-isothyocyanate	BD Pharmingen
7-AAD	BD Pharmingen
HLA-DR-FITC	BD Pharmingen

Table 3.2: List of primary antibodies used for immunophenotype analysis of SCD hepatocyte-like cells in FBS and HPL.

Primary Antibody	Manufacturer
AFP (mouse monoclonal)	Abcam
HNF4 (mouse monoclonal)	Abcam
Cytokeratin 18 (Mouse monoclonal)	Milipore
Albumin (Mouse monoclonal)	Sigma
Cytokeratin 19 (Rabbit polyclonal)	Abcam

3.10 Multilineages differentiation of SCD

3.10.1 Adipogenic differentiation

For differentiate into adipogenic lineage, the SCD were cultured at 1000 cell/cm^2 cell density in a 6-wells plate (BD Bioscience) using FBS and HPL growth medium until reach 80% of confluence. The media were then changed to adipogenic induction medium which comprised of DMEM-KO (Invitrogen), 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 1 µM dexamethasone sodium phosphate (Sigma Aldrich), 10 µg/ml insulin (Sigma Aldrich), 200 µM indomethacin (Sigma Aldrich) and 0.5 mM 3-isobuthyl-1-methyxanthine (Sigma Aldrich) for 21 days, with medium changed three times per week.

Adipogenic differentiation of SCD was tested using Oil Red O (Sigma Aldrich) staining procedure. Cell fixation was done using 4% paraformaldehyde (PFA, Sigma Aldrich) for 30 minutes at room temperature (23 ± 1°C) and rinsed with 1 ml of DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) for 2 times. Next, the differentiated cells were washed twice using diethylpyrocarbonate-treated water (DEPC-treated water, Merck, Whitehouse Station, NJ, USA, http://www.merck.com). Oil Red O working solution was gently added and incubation was done for 50 minutes at room temperature (23 ± 1°C). The stained solution was carefully removed and rinsed with DEPC-treated water for 3 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 1.5.

3.10.2 Chondrogenic differentiation

Chondrogenic induction media was prepared by adding DMEM-KO (Invitrogen) with 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 5.35 µg/ml linoleic acid (Sigma Aldrich), 10 µg/ml L-ascorbic 2 phosphate (Sigma Aldrich), 10 µg/ml insulin (Sigma Aldrich), 1.0 µg/ml dexamethasone sodium phosphate (Sigma Aldrich) and 1.25 µg/ml bovine serum albumin (BSA, Sigma Aldrich). The growth media were replaced with chondrogenic induction media for 21 days after SCD reached 80% of confluence in the growth media condition.

Alcian blue staining (Sigma Aldrich) was used to ascertain the chondrogenic differentiation of SCD. SCD were washed twice using DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and fixed using 4% PFA (Sigma Aldrich) for 10 minutes at room temperature (23 ± 1°C). A total of 1 ml of Alcian Blue working solution was added into the well and SCD were incubated for 30 minutes at room temperature (23 ± 1°C). The wells were washed using tap water for 3 times followed by another 2 times wash using ddH_2O twice. The blue stained cells were observed under microscope for the present of sulfate proteoglycan accumulation which was stained in blue colour. The preparation for Alcian Blue working solution is described in Appendix 1.6.

3.10.3 Osteogenic differentiation

A total of 1000 cell/cm^2 SCD were cultured in a 6-wells plate (BD Bioscience) using FBS and HPL growth media until reach 80% of confluence. Then, the growth media was removed and osteogenic induction media containing DMEM-KO (Invitrogen), 10% Australian characterized FBS (Hyclone), 1% glutamax (Invitrogen), 0.1 µM dexamethasone sodium phosphate (Sigma Aldrich), 0.2 mM L-ascorbic 2 phosphate (Sigma Aldrich) and 10 mM β-glycero-2-phosphate (Sigma Aldrich) were

supplied to the SCD for the next 21 days. The induction media was changed twice a week. Osteogenesis of SCD was evaluated using Von Kossa staining.

In order to detect the calcium mineralization, SCD was fixed using 4% PFA (Sigma Aldrich) for 15 minutes at room temperature (23 ± 1°C) and rinsed once with 5 ml of ddH_2O. Differentiated SCD were then incubated with 1% silver nitrate (Sigma Aldrich) for 60 minutes under a bright light. Staining procedures were finished up by rinsing once with ddH_2O and then observed under microscope. The calcium mineralization was observed as black colored clump or precipitation. The preparation of 1% silver nitrate solution are shown in Appendix 1.7.

3.11 Hepatic differentiation

The hepatic differentiation protocol was modified from the protocol described previously by Ishkitiev et al. (2010). The initial hepatic induction media comprised of DMEM-KO (Invitrogen), 2% Australian characterized FBS (Hyclone) and 25 ng/mL recombinant human hepatocyte growth factor (HGF, R&D Systems, Minneapolis, MN, USA, http://www.rndsystems.com/) and this component was added for 5 consecutive days to the SCD which were about 70% of confluence. The maturation process were done at day 6 with the following components; Knockout DMEM (Invitrogen), 15 ng/mL Oncostatin M (R&D Systems), 30 nmol/L dexamethasone (Sigma Aldrich) and 1% Insulin-Transferrin- Selenium-X (ITS, Invitrogen). Fresh media were added every 3 days up to 15 days of maturation process. The morphological changes of SCD were captured every day for 21 days using phase-contrast microscope.

3.12 Periodic acid-Schiff staining

Periodic acid-Schiff (PAS) staining (Sigma Aldrich) was used to stain the glycogen in the hepatocyte-differentiated cells according to the manufacturer's protocol. The SCD were cultured using FBS and HPL growth media in the 35mm dishes (BD Bioscience) until reached 70% of confluence. All the dishes were fixed using formalin-ethanol fixative solution and rinsed gently with running tap water for 1 minute. Then, Periodic Acid solution were added into the dishes and incubated for 5 minutes in a dark room. The dishes were then rinsed with ddH_2O for several times (2-4 times) and Schiff's reagent was added and incubated for 15 minutes in a dark room. After washing with running tap water for 5 minutes, hematoxylin solution was added to the dishes and incubated for 90 seconds. Then, the dishes were washed several times (3-5 times) under the running tap water and air-dried for 2 minutes. The SCD hepatocyte-like cells were observed under the phase-contrast microscope. This staining protocol was done for SCD cultured at day 0 (control), day 7, day 14 and day 21 in hepatocyte induction media.

3.13 Immunofluorescence

SCD 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 slides 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) was 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 cells were incubated for overnight at 4˚C.

The determination of stem cells markers in SCD expanded in both FBS and HPL media were done using co-immunufluroscence (co-IF) staining. The first primary antibodies were added in the 2-well chamber (BD Bioscience) and incubated for 30 minutes at 4˚C. Next, the second primary antibodies were added in the same chamber and incubated for overnight at 4˚C. The two types of primary antibodies were paired up based on the different source of primary antibodies were raise in. All primary antibodies used in co-IF is listed in Table 3.3.

Table 3.3: List of primary antibodies used to determine the stem cells markers in SCD cultured in FBS and HPL.

Primary Antibody	**Dilution ratio**	**Manufacturer**
Nestin (Mouse monoclonal) + Oct 4 (Rabbit polyclonal)	1:400 1:400	Millipore Abcam
Vimentin (Mouse monoclonal) + Oct 4 (Rabbit polyclonal)	1:400 1:400	Millipore Abcam
MAP 2(Mouse monoclonal) + Oct 4 (Rabbit polyclonal)	1:400 1:400	Millipore Abcam
SSEA-4 (Mouse monoclonal) + Oct 4 (Rabbit polyclonal)	1:400 1:400	Millipore Abcam

The determination of hepatocyte cell markers for SCD hepatocyte-like cells cultured in FBS and HPL was done for day 0 (control), day 7, day 14 and day 21 of hepatocyte induction media. Initially, SCD were cultured in FBS and HPL growth media until 70% of confluence. SCD were induced to SCD hepatocyte-like cells according to the day of induction stated (day 0, day 7, day 14 and day 21) before

undergoing the immunoflurescence procedures. The primary antibodies used for the specific hepatocyte markers are listed in Table 3.4.

Table 3.4: List of primary antibodies used to determine the hepatocyte cell markers in SCD hepatocyte-like cell cultured in FBS and HPL.

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
Cytokeratin 19 (Rabbit polyclonal)	1:500	Abcam

Secondary antibodies were added after the cells were washed 3 times with 0.5% BSA for 30 seconds to remove the primary antibodies. The detection of stem cells markers in SCD were done using FITC-conjugated IgG (Abcam) and Rhodamine-conjugated IgG (Milipore, Billerica, MA, USA, http://www.millipore.com) while the detection of hepatocyte cell markers was done using only FITC-conjugated IgG (Abcam) as the secondary antibodies. SCD were incubated for 90 minutes at room temperature (23 ± 1°C). After 3 washes using 0.5% BSA (Sigma Aldrich), SCD were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI, Millipore) and covered with glass cover slip. Cells were incubated for 20 minutes at room 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. All the immunocytochemistry results were evaluated using a standard indication of expression as listed in Table 3.5.

Table 3.5: Standardized indication of expression for immunocytochemistry analysis.

Level of Expression	Label
Very strong	+++++
Strong	++++
Moderate	+++
Weak	++
Very weak	+
Negative / No expression	-

3.14 RNA isolation

Total RNA of SCD was extracted by using Trizol technique. The pellets of SCD were gently mixed, transferred to a 1.5ml tubes and mixed with 1 ml of Trizol (Invitrogen). Then, the tubes were taken for incubation at room temperature (23 ± 1°C) for 1 minute. This was followed by the addition of 200 µl of chloroform (Sigma Aldrich) and the incubation was continued for another 3 minutes. The pellets were centrifuged at 1200 rpm for 10 minutes at 4° C to separate the aqueous and organic phases. The aqueous phase was carefully aspirated and transferred into a new 1.5ml tubes. A total of 500 ul of isopropanol (Sigma Aldrich) was added to the aqueous buffer and incubated for 10 minutes at room temperature (23 ± 1°C). Next, the buffer was centrifuged at 1200 rpm for 10 minutes at 4° C to obtain the pellets. Once the pellets were obtained, the buffer solution was removed and the pellets were left to air-dry for several minutes before 20 µl of DEPC-treated water was added. The pellet was heated at temperature 65° C for 5 minutes and immediately kept in -20° C for 1 hour and transferred to -80° C freezer for the long term storage.

3.15 Conversion RNA to 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 the 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 it was 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.

3.16 Reserve transcription polymerase chain reaction

The reverse transcriptase polymerase chain reaction (RT-PCR) mixture was prepared by adding 2.5 µl of 10X PCR $–Mg^{2+}$ (Invitrogen), 1.25 µl of 50 mM MgCl2 (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 was 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 analyzed by using 1.5% agarose gel electrophoresis which was visualized via ethidium bromide (Sigma Aldrich) staining. All the RT-PCR results were evaluated using a standard indication of expression as listed in Table 3.6.

Table 3.6: Standardized indication of expression for RT-PCR.

Level of Expression	**Label**
Very high	++++
High	+++
Moderate	++
Low	+
Negative / No expression	-

The primers used for the characterization of SCD cultured in FBS and HPL were listed in Table 3.7. The primer used for SCD hepatocyte-like cell cultured in FBS and HPL at day 0, day 7, day 14 and day 21 were listed in Table 3.8.

Table 3.7: List of primers used in RT-PCR for SCD cultured in FBS and HPL .

Gene Symbol	Description	Primer Sequence (5'-3')	Base Pair	Accession Number
18s	Homo sapiens RNA, 18S ribosomal 1	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGCGCT	186	NR_003286.2
OCT 4	Homo sapiens POU class 5 homeobox 1 (POU5F1)	F: CGACCATCTGCCGCTTTGAG R: CCCCCTGTCCCCCATTCCTA	534	NM_203289.3
SOX 2	Homo sapiens SRY (sex determining region Y) - box 2	F: CCCCCGGCGGCAATAGCA R: TCGGCGCCGGGGAGATACAT	411	NM_003106.2
Nanog	Homo sapiens Nanog homeobox	F: TCCTCCATGGATCTGCTTATTCA R: CAGGTCTTCACCTGTTTGTAGCTGAG	212	NM_024865.2
Nestin	Homo sapiens nestin	F: CAGCGTTGGAACAGAGGTTGG R: TGGCACAGGTGTCTCAAGGGTAG	346	NM_006617.1
βIII Tubulin	Homo sapiens tubulin, beta 3 (TUBB3)	F: AACAGCACGGCCATCCAGG R: CTTGGGGCCCTGGGCCTCCGA	204	NM_006086.2
MAP 2	Homo sapiens microtubule-associated protein 2	F: CAGCTGAAGCCCAGGCA R: TCTGGAGACTGTGCTAGGTTCCT	320	NM_002374.3
GATA 2	Homo sapiens GATA binding protein 2	F: AGCCGGCACCTGTTGTGCAA R: TGACTTCTCCTGCATGCACT	205	NM_032638.3
MSX 1	Homo sapiens msh homeobox 1	F: CCTTCCCTTTAACCCTCACAC R: CCGATTTCTCTGCGCTTTTC	245	NM_002448.3
Hand 1	Homo sapiens heart and neural crest derivatives expressed 1	F: TGCCTGAGAAAGAGAACCAG R: AGGATGAACAAACAC	235	NM_004821.1
AFP	Homo sapiens alpha-fetoprotein	F: AGAACCTGTCACAAGCTGTG R: GACAGCAAGCTGAGGATGTC	637	NM_001134.1
HNF3β	Hepatocyte nuclear factor 3-beta	F: GACAAGTGAGAGAGCAAGTG R: ACAGTAGTGGAAACCGGAG	197	NM_153675.1

Abbreviation: F-forward, R-reverse.

Table 3.8: List of primers used in RT-PCR and real time-PCR for SCD hepatocyte-like cell cultured in FBS and HPL.

Gene Symbol	Description	Primer Sequence (5'-3')	Base Pair	Accession Number
18s	Homo sapiens RNA, 18S ribosomal 1	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGCGCT	186	NR_003286.2
Sox 17	Homo sapiens SRY (sex determining region Y), box 17	F: CGCACGGAATTTGAACAGTA R: GGATCAGGGACCTGTCACAC	181	NM_022454.3
HNF3β	Hepatocyte nuclear factor 3-beta	F: GACAAGTGAGAGAGCAAGTG R: ACAGTAGTGGAAACCGGAG	197	NM_153675.1
GATA 4	Homo sapiens GATA binding protein 4	F: CTCCTTCAGGCAGTGAGAGC R: GAGATGCAGTGTGCTCGTGC	536	NM_002052.2
AFP	Homo sapiens alpha-fetoprotein	F: AGAACCTGTCACAAGCTGTG R: GACAGCAAGCTGAGGATGTC	637	NM_001134.1
HNF4α	Homo sapiens hepatocyte nuclear factor 4, alpha	F: GCTTGGTTCTCGTTGAGTGG R: CAGGAGCTTATAGGGCTCAGAC	730	NM_000457.3
CK19	Homo sapiens keratin 19	F: TGAGGTCATGGCCGAGCAGAAC R: CATGAGCCGCTGGTACTCCTGA	333	NM_002276.4
Albumin	Homo sapiens albumin	F:CTGCTTGAATGTGCTGATGACAGGGCGG R: GGCATAGCATTCATGAGGATCTG	338	NM_000477.5
CK 18	Homo sapiens keratin 18 (KRT18), transcript variant 1	F: CCATGCGCCAGTCTGTGGAG R: GTGGTGCTCTCCTCAATCTGCT	321	NM_000224.2
TO	Tryptophan-oxygenase	F: GGCAGCGAAGAAGTACAAATC R: TCGAACAGAATCCAACTCCC	500	NM_019911
TDO	Homo sapiens tryptophan 2,3-dioxygenase	F: GGTTTAGAGCCACATGGATT R: ACAGTTGATCGCAGGTAGTG	424	NM_005651.2

AAT	Homo sapiens serpin peptidase inhibitor	F: AGACCCTTTGAAGTCAAGCGACC R: CCATTGCTGAAGACCTTAGTGATGC	343	NM_000295.4
CYP3a4	Homo sapiens cytochrome P450, family 3, subfamily A, polypeptide 4	F: TCACCCTGATGTCCAGCAGAAACT R: TACTTTGGGTCACGGTGAAGAGCA	228	NM_017460.5
TAT	Homo sapiens tyrosine aminotransferase (TAT)	F: GCTAAGGACGTCATTCTGACAAG R: GTCTCCATAGATCTCATCAGCTAAG	353	NM_000353.2.
CYP7a1	Homo sapiens cytochrome P450, family 7, subfamily A, polypeptide 1	F: GAGAAGGCAAACGGGTGAAC R: ATCGGGTCAATGCTTCTGTG	275	NM_000780.3

Abbreviation: F-forward, R-reverse.

3.17 Real time-PCR

Complementary DNA was diluted with the ratio of 1:4 (25 ng/sample) and mixed with SYBR Green QPCR (Applied Biosystems). The selected forward and reverse primers were added to the reaction mix as a preparation for the real time-PCR. The reaction mix was placed in an Applied Biosystems Prism 7900HT sequence detection system that ran at 95° C for 10 minutes to denature the cDNA, followed by the cycling phase at 95° C for 15 seconds and annealing step at 60° C for 1 minute with the amplification of 40 cycles. The final phase consisted of a dissociation phase at 95° C for 15 seconds, 60° C for 15 seconds and 95° C for 15 seconds to ensure the melting temperature of the PCR fragments was reached and to avoid the presence of primer-dimer pairs. The relative quantification of gene expressions was assessed using ΔΔCT method. All real time-PCRs were performed in triplicate reaction and the results were analyzed using SDS v2.1 software. All measurements were normalized by 18s rRNA. The real time-PCR was done for the SCD hepatocyte-like cell cultured in FBS and HPL at day 0 (control), day 7, day 14 and day 21 in hepatocyte media induction. The primers used in real time-PCR are listed in Table 3.8.

3.18 Urea assay

In order to ascertain the functionality of SCD hepatocyte-like cells, a preliminary study on urea secretion was carried out. The cell culture media were collected from hepatocyte differentiated cells on day 0, day 7, day 14 and day 21 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 and the optical densities were measured at 492 nm.

3.19 Statistical analysis

All values are given as mean and standard deviation. Data were analyzed using SPSS statistical software, version 19.0 (SPSS Inc, Chicago, IL, U.S.A.). The differences of FBS and HPL in the population doubling and population doubling time of SCD were carried out using independent t-tests. A probability of $p < 0.05$ is considered as a significant

CHAPTER 4

RESULTS

CHAPTER 4

RESULT

4.1 Isolation of dental pulp stem cells from deciduous teeth

The initial outgrowth of dental pulp stem cells from deciduous teeth (SCD) for all the four samples were first detected three days after onset of culture as shown in Figure 4.1. Based on the observation, the pulp tissues adhered to the surface of the culture flask within 24 hours of culture due to the presence of gelatin. All the samples showed rapid growth of fibroblast-like cell from the pieces of the pulp tissue and indicated migration towards the media. The SCD covered approximately 80% of the surface of the culture flask on day 14.

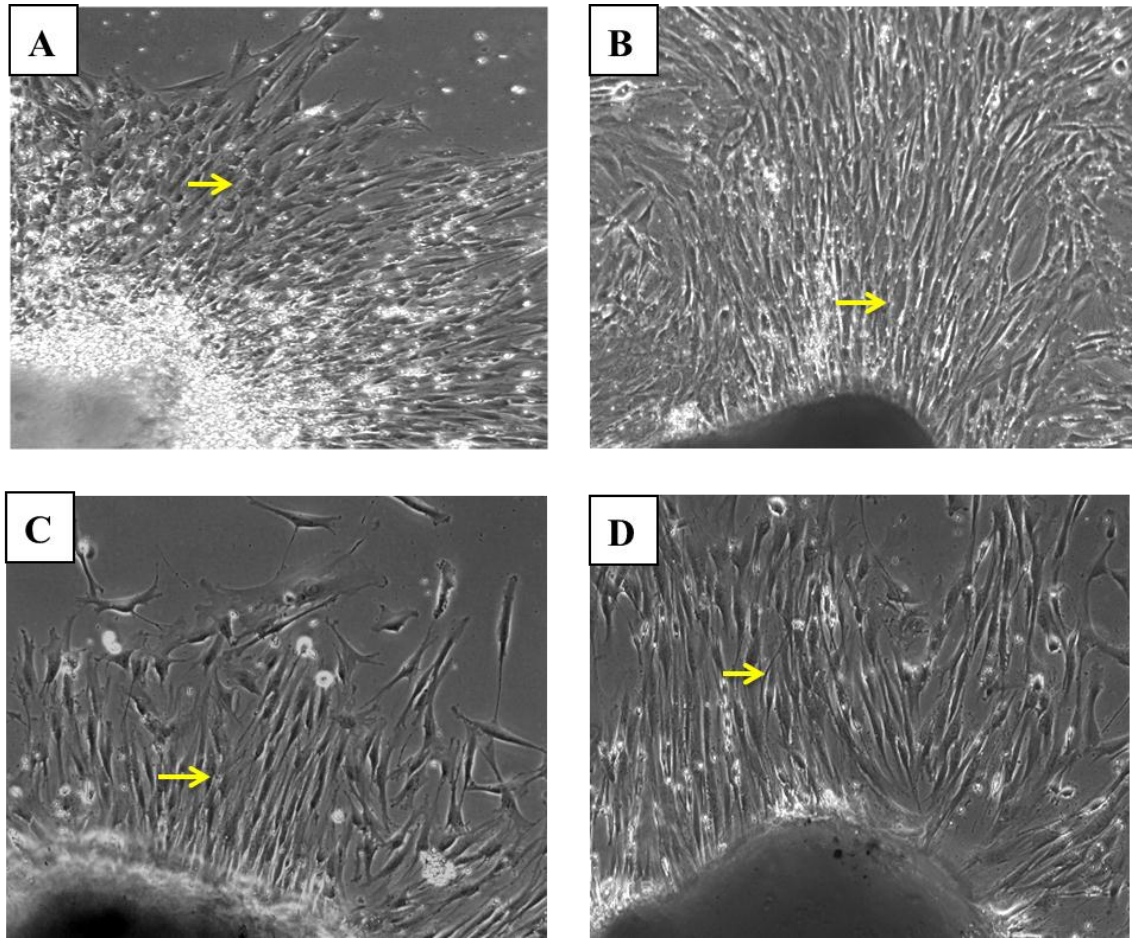

Figure 4.1: Representative images of primary culture from four samples; A: UMDP001; B: UMDP002; C: UMDP003; and D: UMDP004 (Magnification 10X; phase contrast images). Arrows indicate cells migration towards the media.

4.2 Morphology of SCD

The morphology of SCD expanded in FBS formed thicker flattened cells compared to HPL, where smaller and long spindle-shaped cells was observed (Figure 4.2). The SCD in HPL also formed highly condensed colonies and cells tended to overlap with each other while in FBS, the cells were loosely formed and segregated.

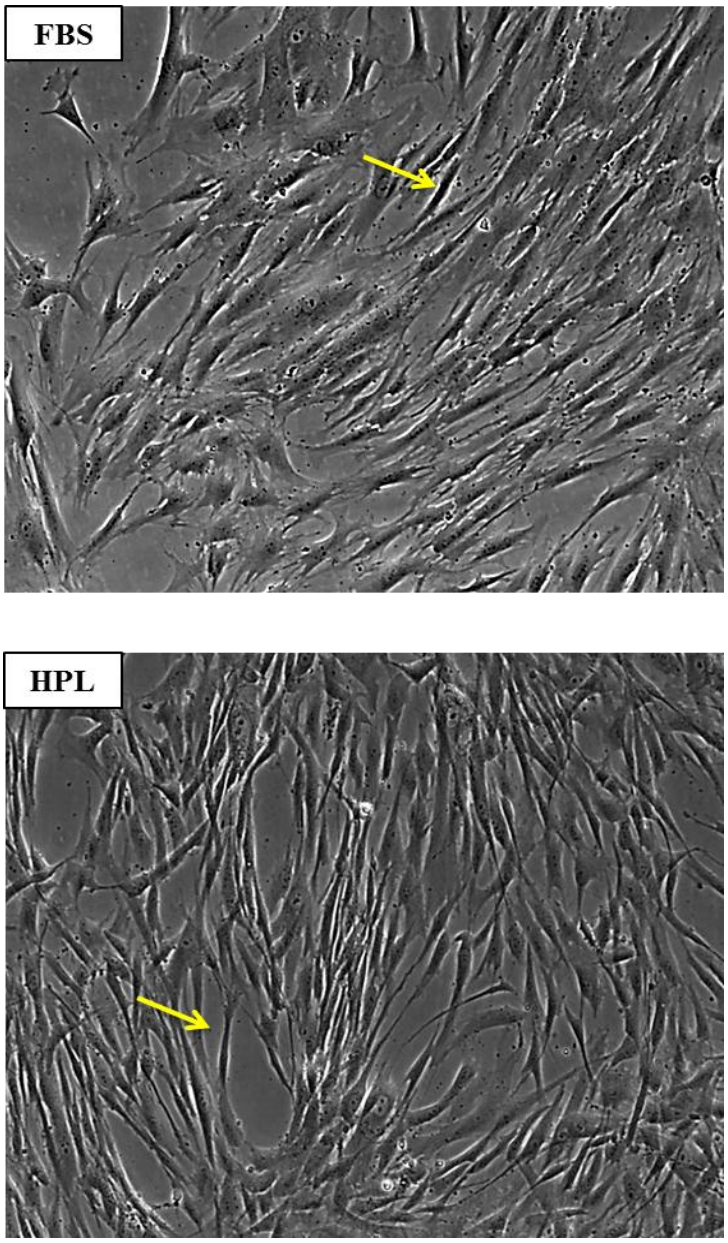

Figure 4.2: Representative images of SCD expanded in FBS and HPL at passage 4 (Magnification 10X; phase contrast images). More spaces in SCD expanded in HPL compared to the FBS, as indicated by the arrows.

4.3 Growth kinetics

The growth kinetics of SCD expanded in FBS and HPL comprised of the population doubling (Figure 4.3) and the population doubling time (Figure 4.4). The population doubling of SCD in HPL showed a higher proliferation rate as compared to FBS with the yield of cells for passage 1, 1.71 ± 0.10 in million and 1.11 ± 0.07 in million and 2.36 ± 0.21 in million and 1.57 ± 0.12 in million for passage 2 respectively. Moreover, SCD in HPL demonstrated a gradual increase in population doubling rate with 3.34 ± 0.19 in millions of cells for passage 3, 3.97 ± 0.27 in millions of cells for passage 4 and 4.42 ± 0.20 in millions of cells for passage 5. Although the SCD expanded in FBS showed tremendous proliferation rate from passage 3 (2.41 ± 0.14 in million) to passage 4 (3.05 ± 0.21 in million), the increase in passage 5 with 3.17 ± 0.18 in millions of cells was not as high as in HPL. The results represented were the means and standard deviations of four independent experiments. All the data is presented in Appendix 2.1.

The population doubling time for SCD cultured in FBS was increased from passage 1 to passage 5, with 21.93 ± 0.35 hours for passage 1, 24.15 ± 0.46 hours for passage 2, 29.16 ± 0.37 hours for passage 3, 34.64 ± 0.47 hours for passage 4 and 37.81 ± 0.44 hours for passage 5. SCD cultured in HPL demonstrated a gradual increase with the increase of passages, with 19.70 ± 0.29 hours for passage 1, 21.97 ± 0.43 hours for passage 2, 23.80 ± 0.29 hours for passage 3, 26.27 ± 0.36 hours for passage 4 and 28.94 ± 0.26 hours in passage 5. The results represented were the means and standard deviations of four independent experiments. All data is presented in Appendix 2.2.

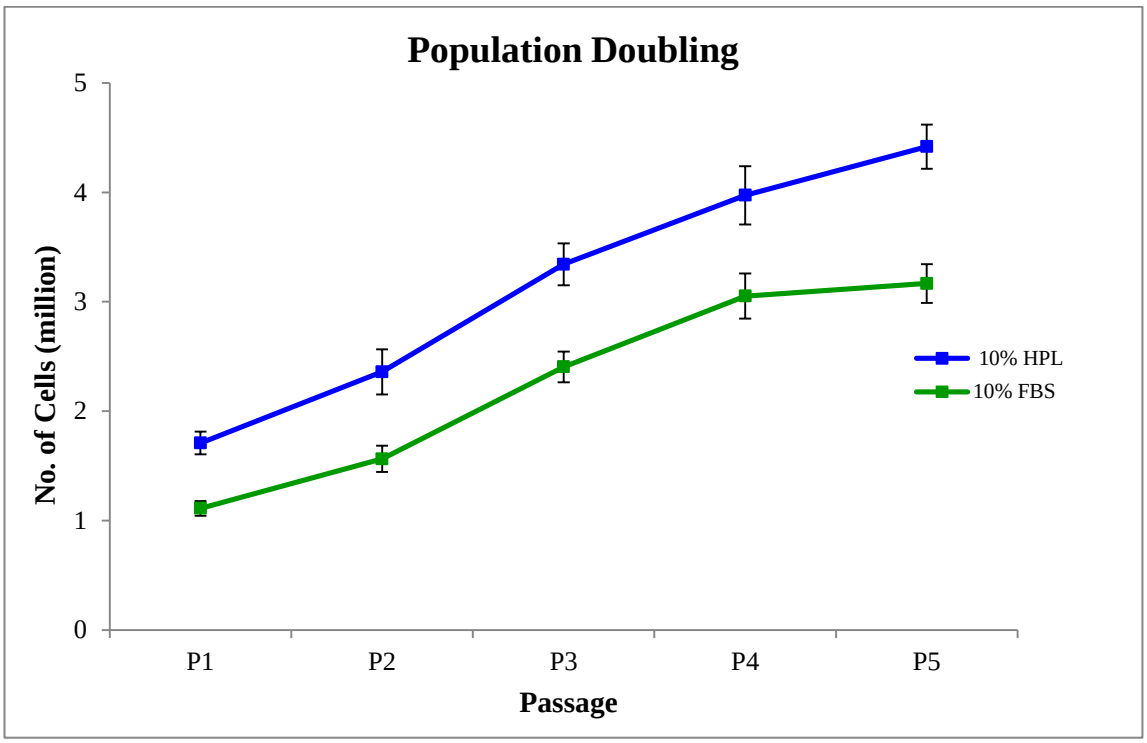

Figure 4.3: Population doubling of SCD cultured in FBS and HPL ($p<0.05$).

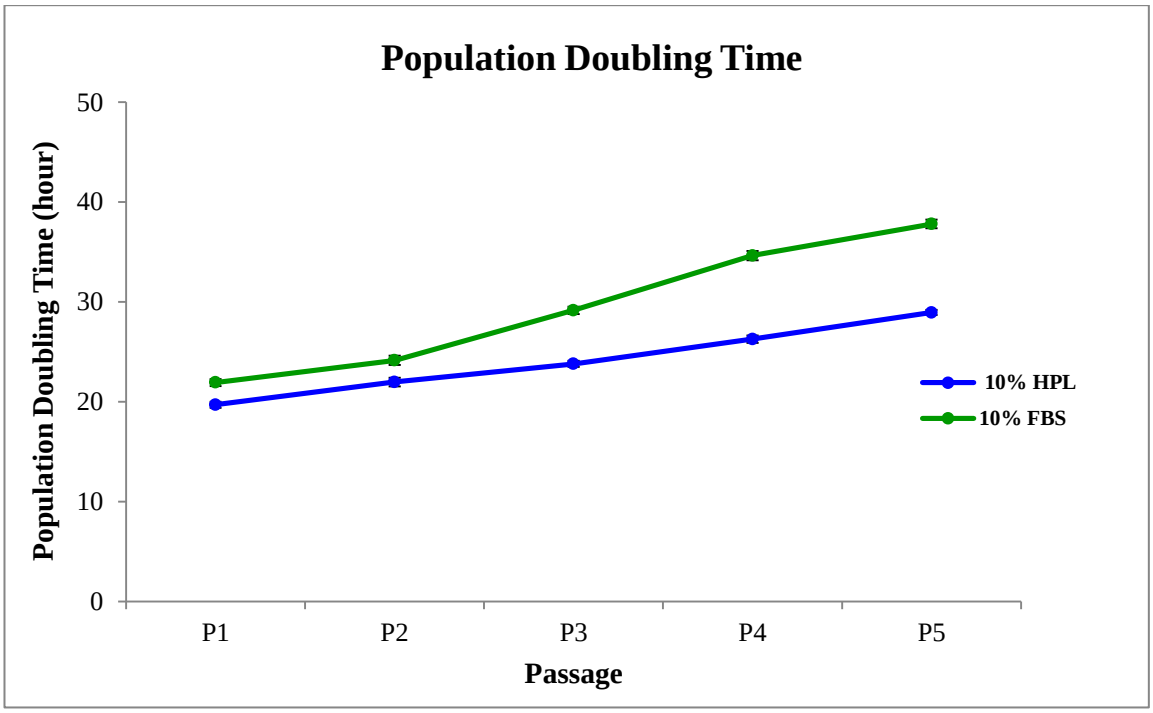

Figure 4.4: Population doubling time of SCD cultured in FBS and HPL ($p<0.05$).

4.4 Colony forming unit

The colony forming unit (CFU) assays of SCD expanded in FBS demonstrated a lower rate of cellular proliferation, 67.50 ± 6.45% compared to HPL with 83.75 ± 4.79% as shown in Figure 4.5. The results represented were the means and standard deviations of four independent experiments. Based on the observation, SCD in both media also showed heterogeneity in the formation of colony size and cell morphology as illustrated in Figure 4.6. The SCD in FBS formed moderate size colonies compared to HPL where larger and smaller scattered colonies were formed. The data for colony forming unit assay is in Appendix 2.3.

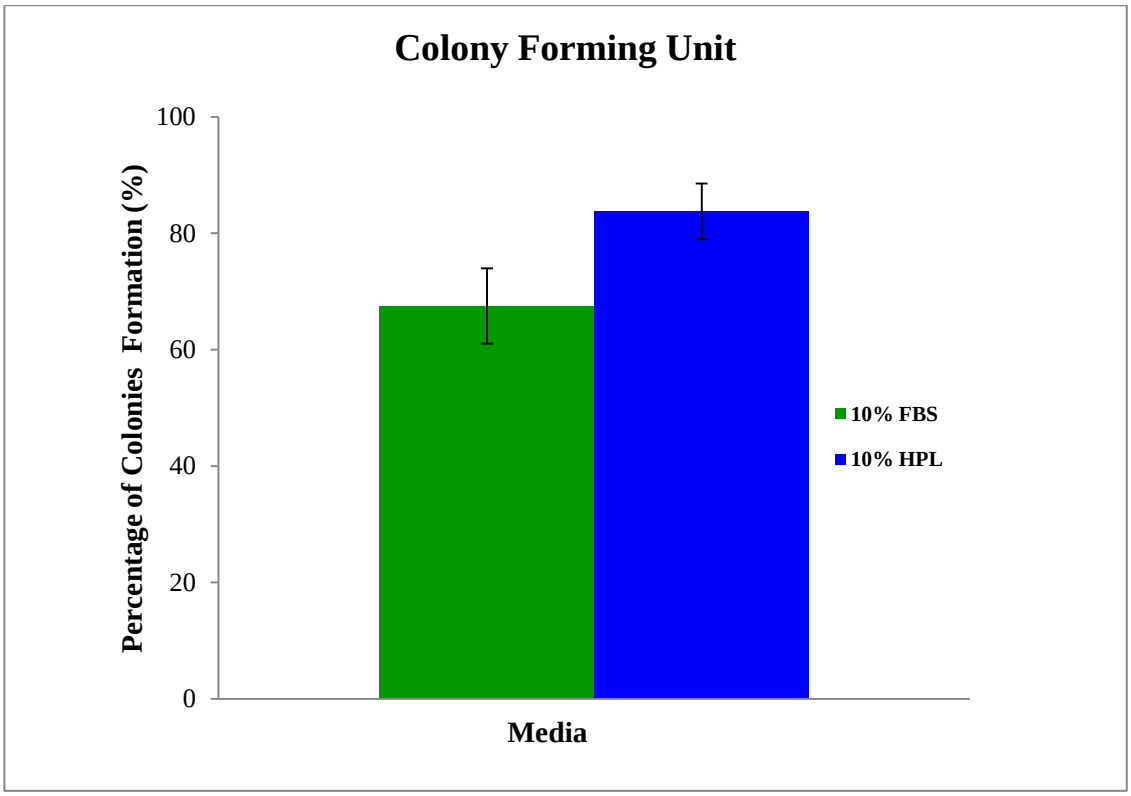

Figure 4.5: Percentage of colonies formation of SCD cultured in FBS and HPL.

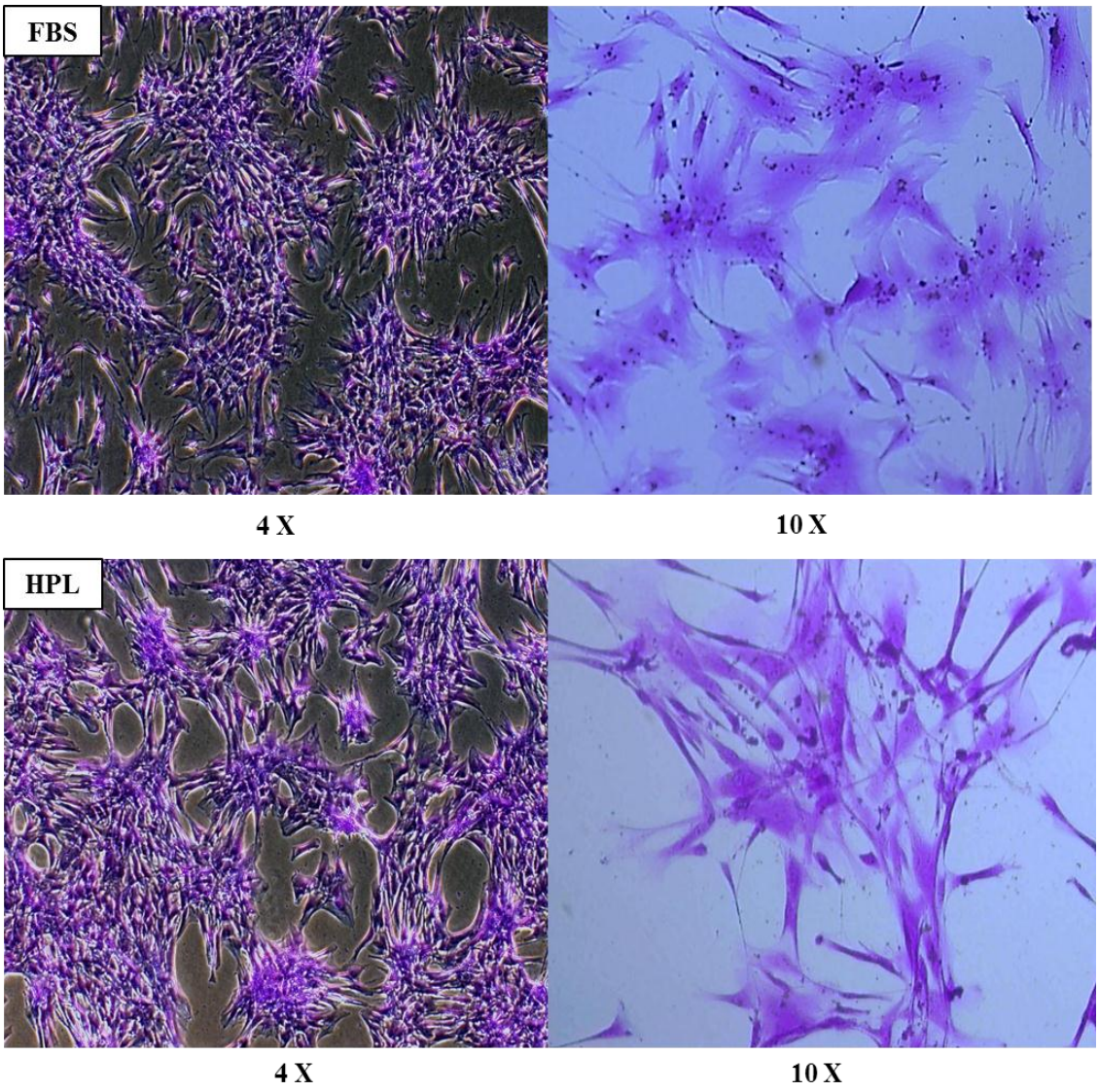

Figure 4.6: Representative images of SCD expanded in FBS and HPL stained with crystal violet in passage 4 (Magnification 4X and 10X; phase contrast images).

4.5 Senescence assay

The senescence associated β-galactosidase assay (SA-β-gal) of SCD cultured in FBS and HPL displayed low tendency towards apoptosis with 29.5 ± 6.85 % and 21.5 ± 2.08 % senescence cells respectively (Figure 4.7). The results represented were the means and standard deviations of four independent experiments. All the data are in Appendix 2.4. The SCD in both media stained negative for SA-β-gal, however some cells were intensely blue while others were moderately blue as shown in Figure 4.8. The SCD in FBS demonstrated more blue stained cells compared to HPL. The blue stained cells together with the presence of gross enlargement were reflected as the senescent SCD.

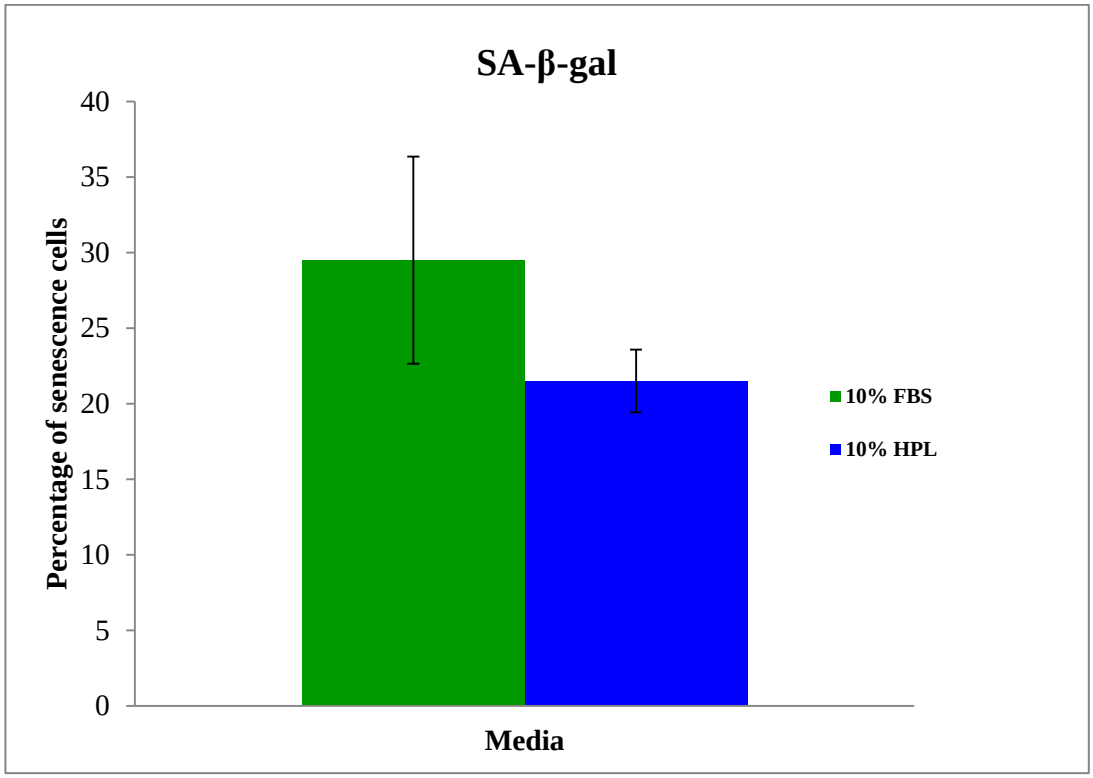

Figure 4.7: SA-β-gal staining of SCD expanded in FBS and HPL.

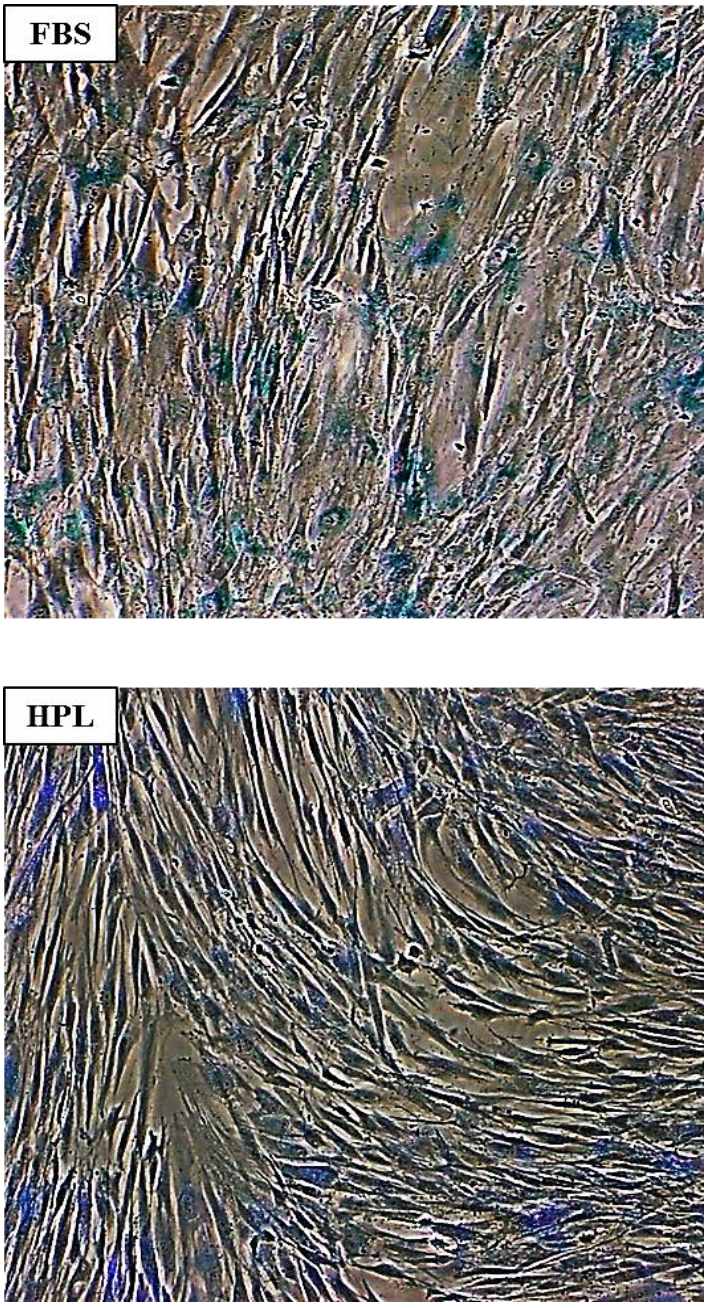

Figure 4.8: Representative images of qualitative SA-β-gal assay by X-gal staining of SCD expanded in FBS and HPL in passage 4 (Magnification 10X; phase contrast images).

4.6 Immunophenotype analysis of SCD

According to the phenotype analysis, SCD expanded in HPL demonstrated highly positive results for the CD 44 (94.11 ± 2.56), CD73 (99.52 ± 0.31) and CD166 (99.03 ± 1.30) expressions compared to the FBS. The cell surface marker for CD90 was higher in SCD cultured in FBS (93.97 ± 5.62) compared to the HPL (92.13 ± 8.17). However, lower negativity was observed in the expression of CD34 in HPL (0.11 ± 0.16) compared to FBS (0.26 ± 0.22). In addition, the expressions of CD 45 were also low for HPL compared to the FBS with 0.03 ± 0.02 and 0.11 ± 0.11, respectively. Both SCD showed negative result for the HLA-DR expression with 0.07 ± 0.11 for FBS and 0.03 ± 0.04 for HPL. The graph represented the phenotype analysis of SCD is shown in Figure 4.9. The results represented the means and standard deviations of four independent experiments. All the data is presented in Appendix 2.5.

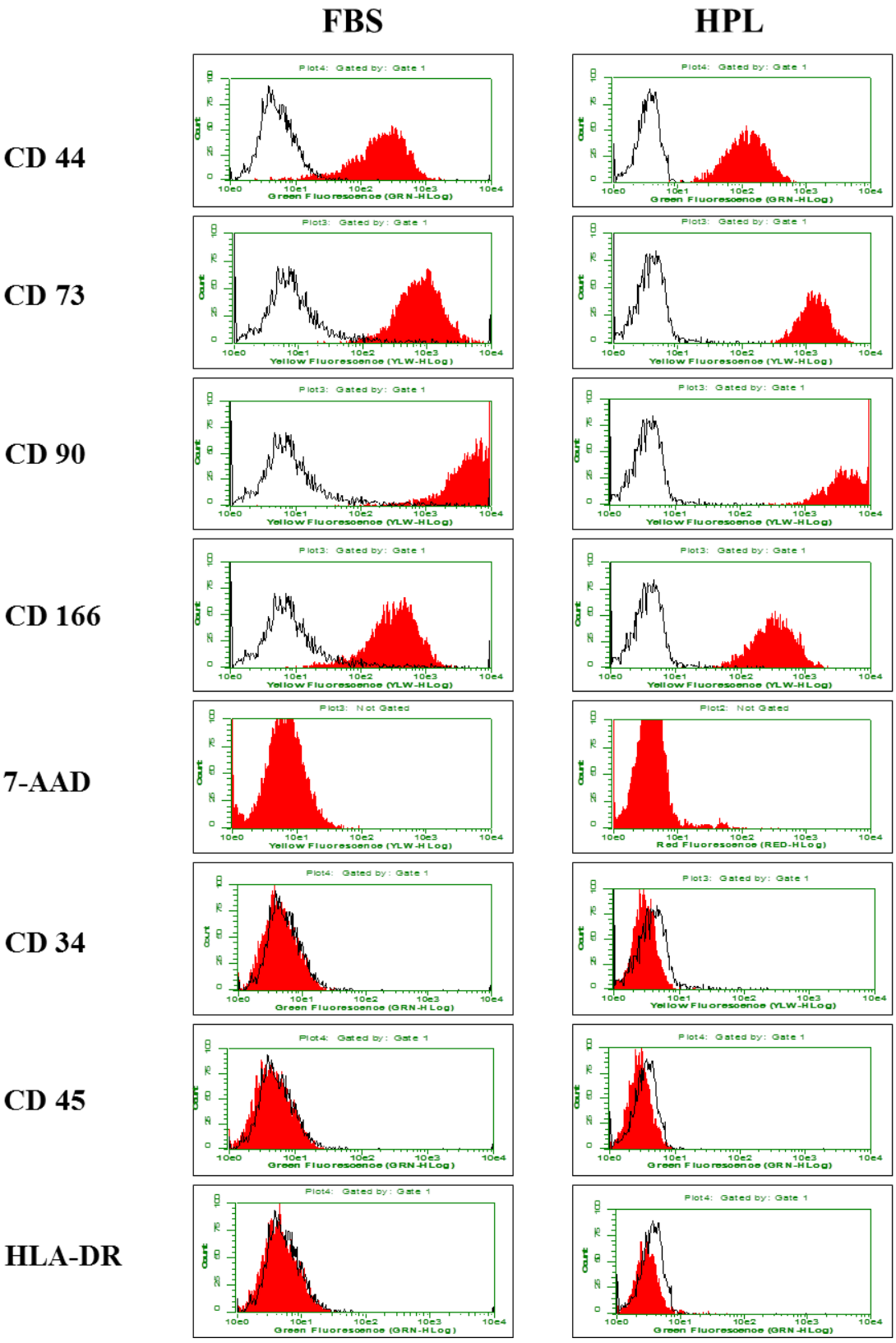

Figure 4.9: Representative results of the cell phenotype analysis of SCDs expanded in FBS and HPL using flow cytometry.

4.7 Trilineage differentiation of SCD

4.7.1 Adipogenic differentiation

The adipogenesis of both SCD cultured in FBS and HPL was visualized by the formation of lipid droplet stained by Oil Red O after 21 days of induction (Figure 4.10). The SCD expanded in HPL showed higher formation of lipid droplet in clumps compared to the FBS. The staining procedure was done based on the principle that the dye was more soluble in lipid compared to other vehicular solvent. Thus, the dye was absorbed into the lipid droplet formed in the adipogenic differentiation

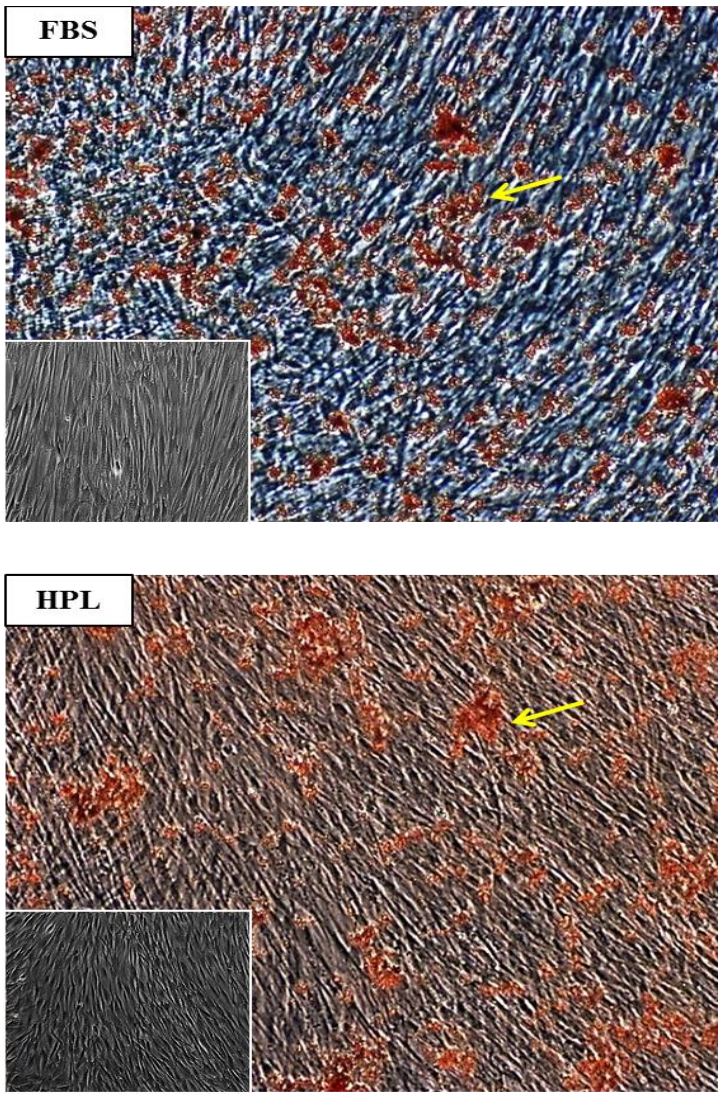

Figure 4.10: Representative images of Oil Red O staining of SCD expanded in FBS and HPL in passage 4 compared to the undifferentiated SCD as the control (Magnification 10X; phase contrast images). Arrows indicate the stained lipid droplet.

4.7.2 Chondrogenic differentiation

In vitro chondrogenesis differentiation of SCD cultured in FBS and HPL was detected by the presence of proteoglycan stained using Alcian Blue (Figure 4.11). The SCD cultured in HPL showed less stained cells compared to the FBS. Alcian blue dye stained proteoglycan as it is a copper phthalcynin dye that contains positively charged group to bind to proteoglycan.

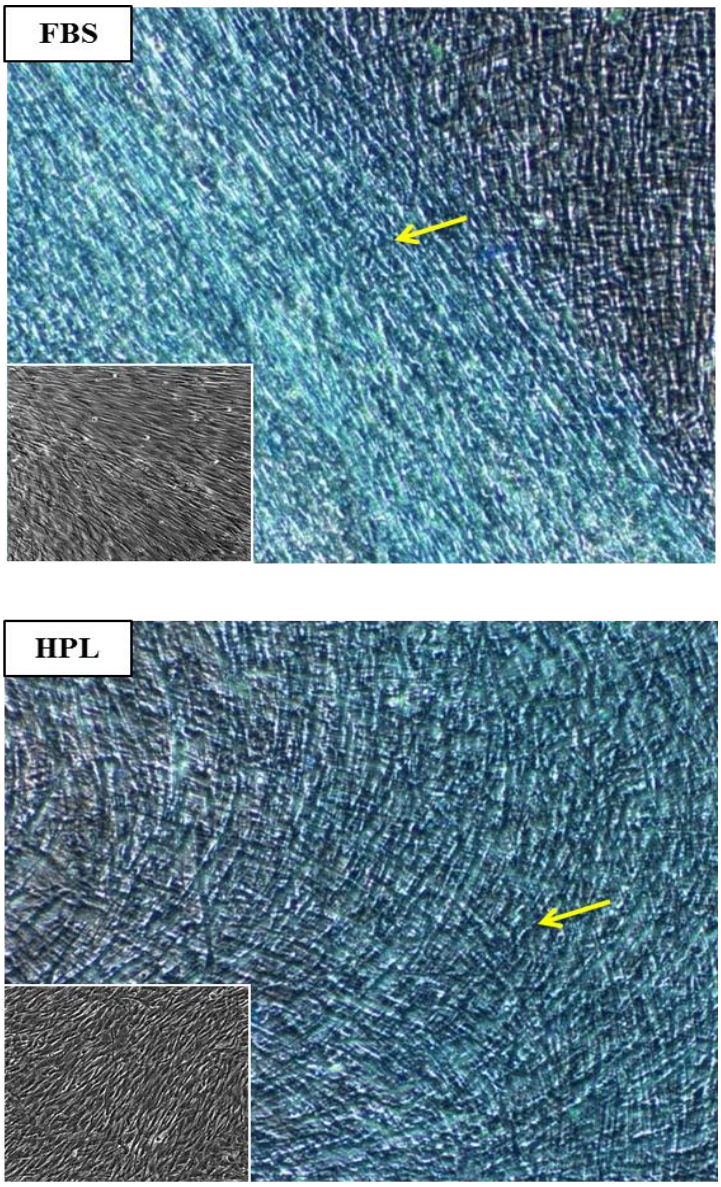

Figure 4.11: Representative images of Alcian Blue staining of SCD expanded using FBS and HPL in passage 4 compared to the undifferentiated SCD as a control (Magnification 10X; phase contrast images). Arrows showed the stained proteoglycan.

4.7.3 Osteogenic differentiation

Differentiation to osteoblast for SCD cultured in FBS and HPL showed positive as calcium precipitation was detected by Von Kossa staining as shown in Figure 4.12. Even though SCD in HPL produced bigger clumps of calcium precipitate, there was no obvious difference in the distribution of these clumps observed between the SCD cultured in FBS and HPL. The precipitation of calcium after 21 days of induction was visualized when silver ion reacted with phosphate in the presence of acidic materials under light illumination.

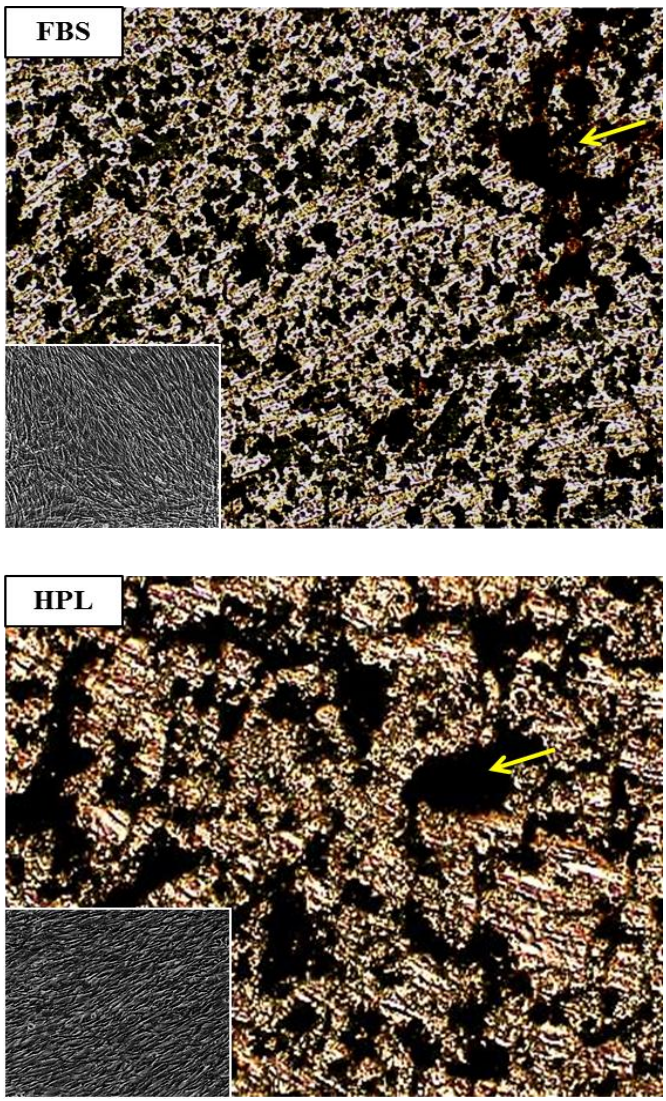

Figure 4.12: Representative images of Von Kossa staining of SCD expanded using FBS and HPL in passage 4 compared to the undifferentiated SCD as a control (Magnification 10X; phase contrast images). The arrows showed the calcium precipitation.

4.8 Immunocytochemistry analysis of SCD

The immunocytochemistry of SCD was performed using immunofluorescence technique that was implemented based on the specificity of the antibodies to the antigen on the specific molecule and visualized using fluorescent dye. Based on the observations, SCD cultured in FBS and HPL expressed positive expression for Oct 4, Nestin, Vimentin, Sox 2 and SSEA4. A very strong expression (+++++) of Oct 4 and Nestin was observed for the SCD expanded in HPL compared to the FBS (Figure 4.13). The expression of Vimentin in SCD cultured in FBS was detected as moderate expression (+++) compared to the HPL (Figure 4.14). However, SCD cultured in FBS and HPL expressed very strong expression (+++++) for Sox 2 (Figure 4.15) and SSEA4 (Figure 4.16). The expressions of these 5 specific antibodies revealed that SCD cultured in FBS and HPL had specific antigen for specific proteins. SCD cultured in FBS and HPL demonstrated the presence of Oct4, Nestin, Vimentin, Sox 2 and SSEA4 and thus indicating positive characterization for stem cell under the *in vitro* condition.

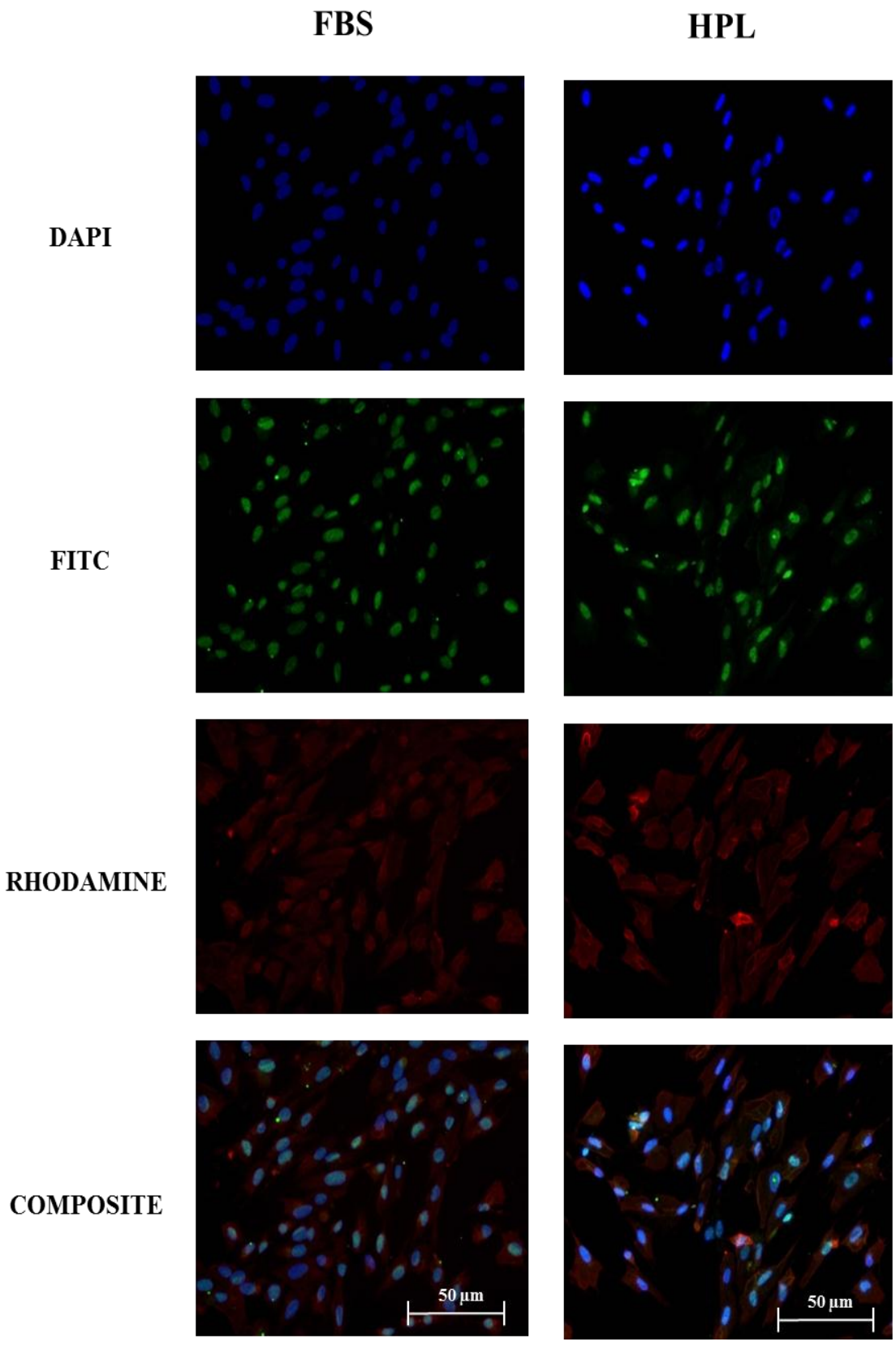

Figure 4.13: Representative images for the expression of Oct 4 and Nestin for SCD cultured in FBS and HPL. The green fluorescence (Oct4) and red fluorescence (Nestin) stained cell cytoplasmic while DAPI stained the nucleus (Magnification 20X; phase contrast images).

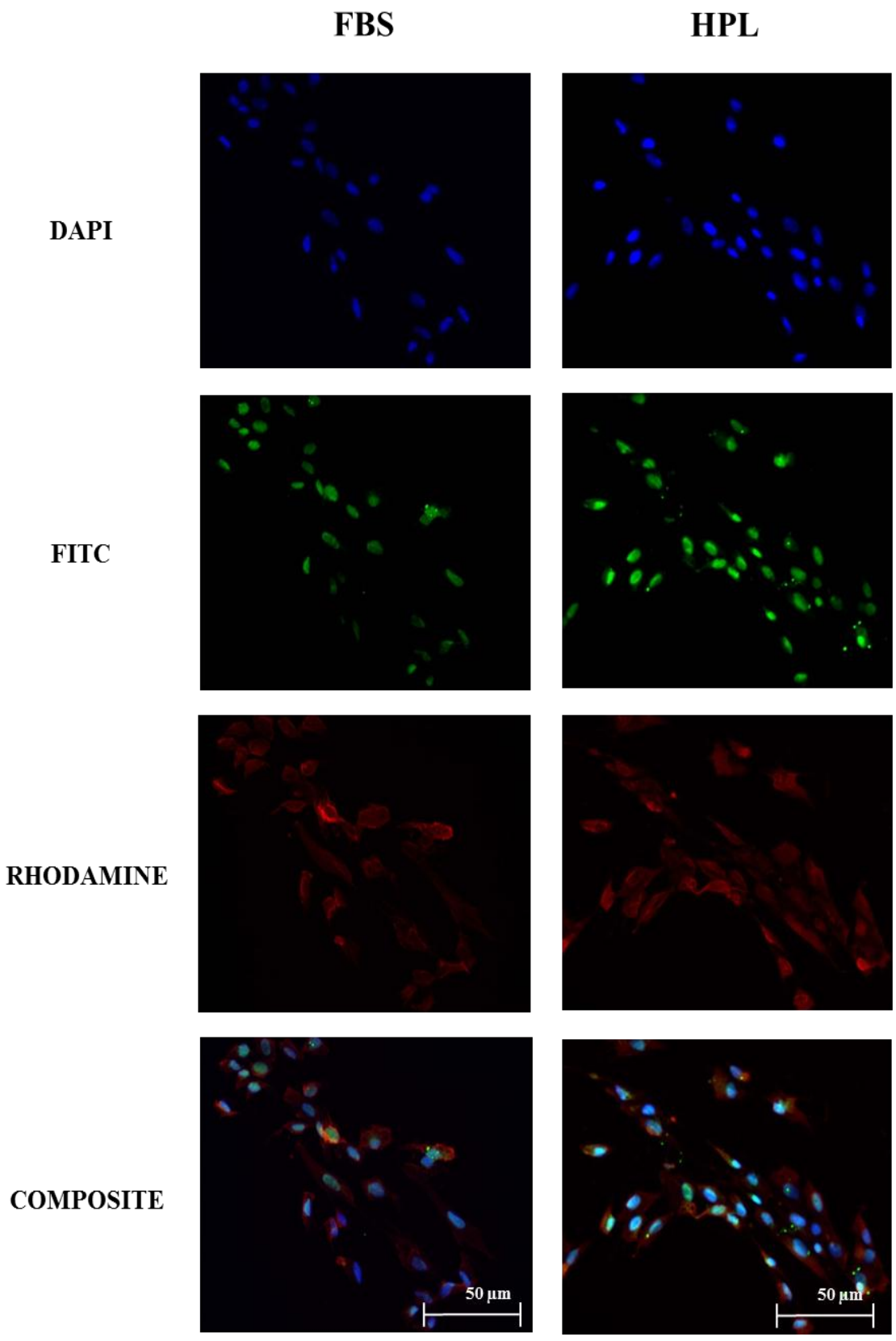

Figure 4.14: Representative images for the expression of Oct 4 and Vimentin for SCD cultured in FBS and HPL. The green fluorescence (Oct4) and red fluorescence (Vimentin) stained cell cytoplasmic while DAPI stained the nucleus (Magnification 20X; phase contrast images).

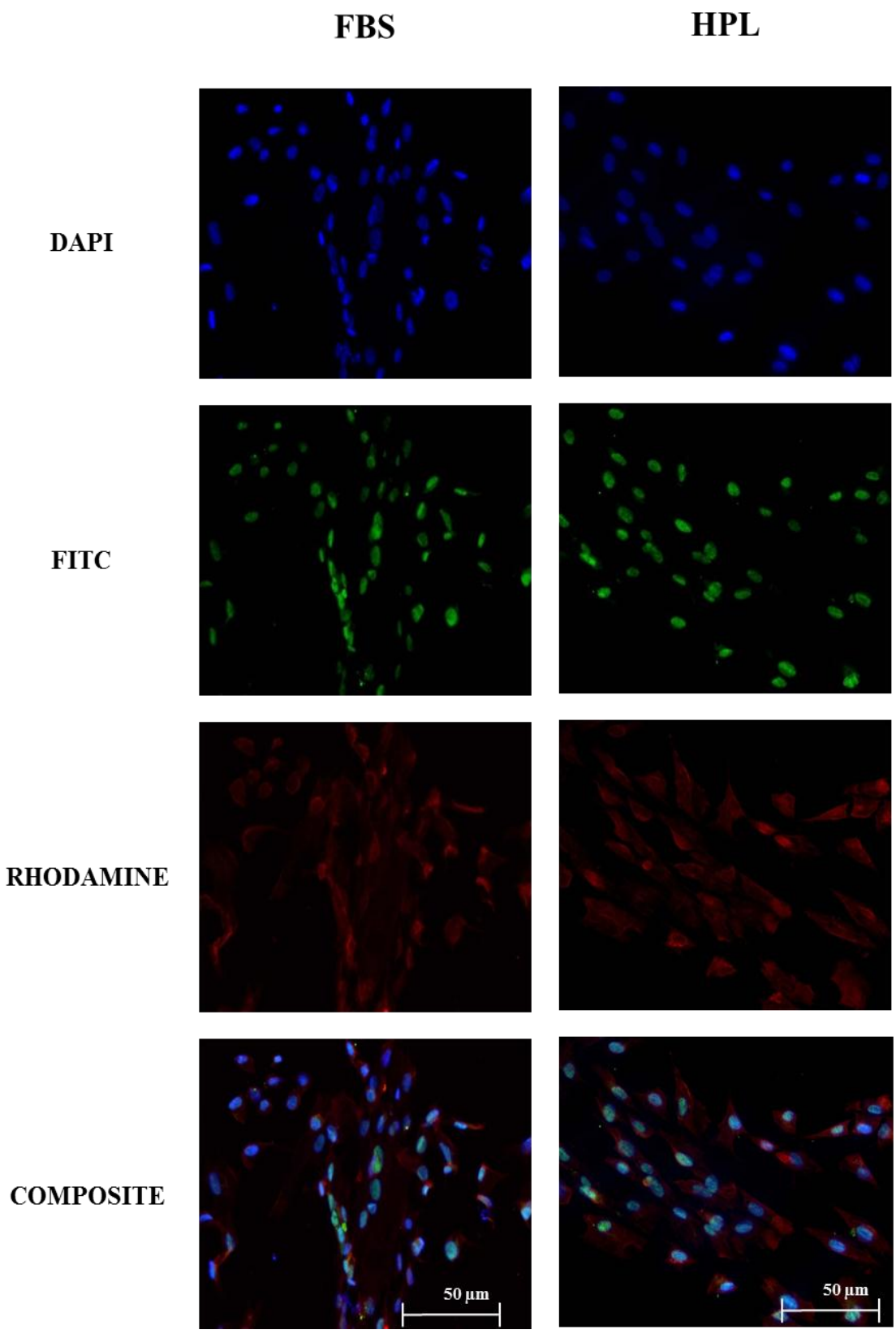

Figure 4.15: Representative images for the expression of Oct 4 and Sox2 for SCD cultured in FBS and HPL. The green fluorescence (Oct4) and red fluorescence (Sox 2) stained cell cytoplasmic while DAPI stained the nucleus (Magnification 20X; phase contrast images).

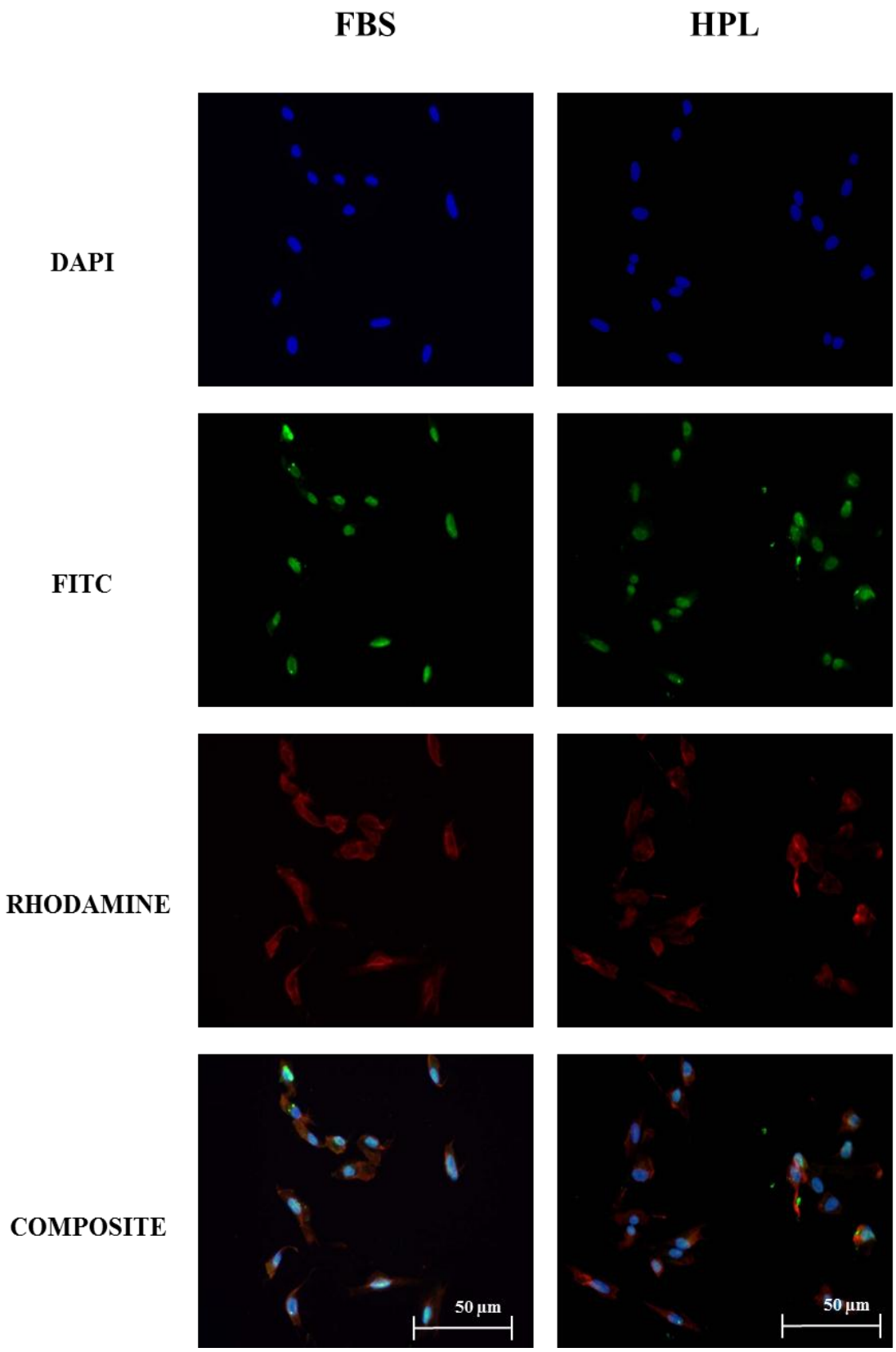

Figure 4.16: Representative images for the expression of Oct 4 and SSEA4 for SCD cultured in FBS and HPL. The green fluorescence (Oct4) and red fluorescence (SSEA4) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

4.9 RT-PCR of SCD

RT-PCR analyses were performed to verify expressions of several markers in SCD cultured in FBS and HPL (Figure 4.17). Based on the observations, SCD cultured in both media showed high expression (+++) of pluripotent stem cells markers namely OCT 4, Sox 2 and Nanog. The expression of ectoderm markers (Nestin, βIII Tubulin and MAP 2) were very high (++++) in both cells. SCD cultured in FBS and HPL also displayed high (+++) expression of mesoderm markers (GATA 2, MSX 2 and Hand1). The presence of endoderm markers (HNF3β and AFP) in both cells were demonstarted as very high (++++) and high (+++) expression, respectively.

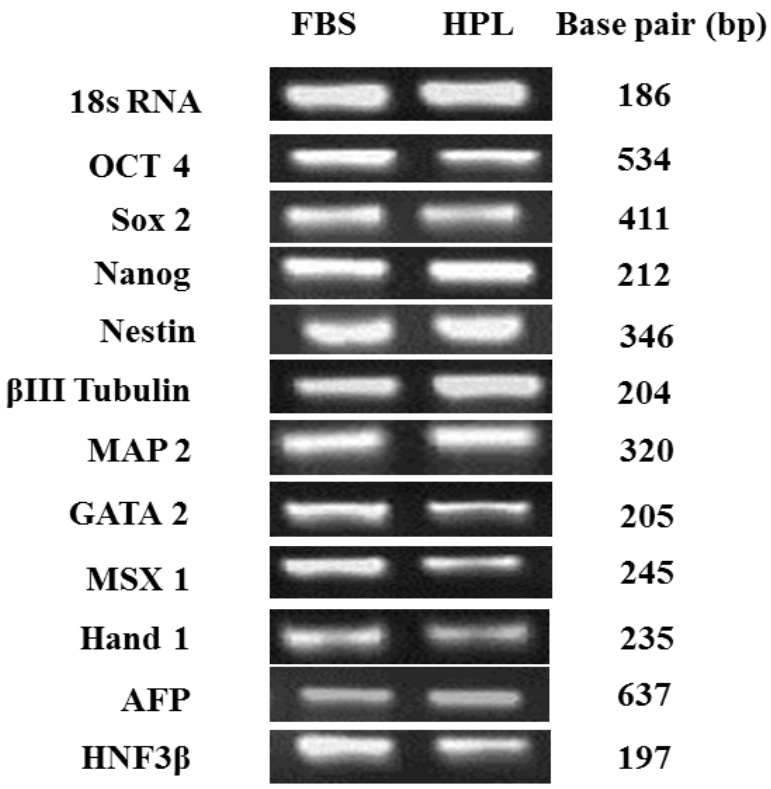

Figure 4.17: Expression of pluripotent and germ-layer markers of SCD cultured in FBS and HPL.

4.10 Hepatocyte differentiation of SCD

The first evidence of the hepatocyte differentiation from SCD cultured in FBS and HPL was the morphological changes. Microscopic examination revealed that SCD in FBS and HPL started to show a remarkable transition from elongated fibroblast-like cells to rounded epithelial-shaped of cells on day 5 of the initiation step for hepatic differentiation (Figure 4.18 and Figure 4.19). However, these changes appeared only in several parts of the cell population and the cells were mainly surrounded by the spindle-shaped cells. The contraction of the cytoplasm progressed further during the maturation stage on day 6. SCD expanded in FBS and HPL became densed and rounded with the presence of clear nuclei in some of the differentiated cells. These changes were observed on day 13 for SCD cultured in FBS and day 16 for SCD in HPL. The morphology of SCD continued to change until hexagonal-shape cells were visualized at day 16 of the induction for SCD cultured in FBS and at day 18 for HPL. Most of the differentiated SCD cultured in both media displayed double-nuclei in the late stage of hepatocyte differentiation. The presence of double nuclei in the hexagonal-shaped cells resembled primary human hepatocyte.

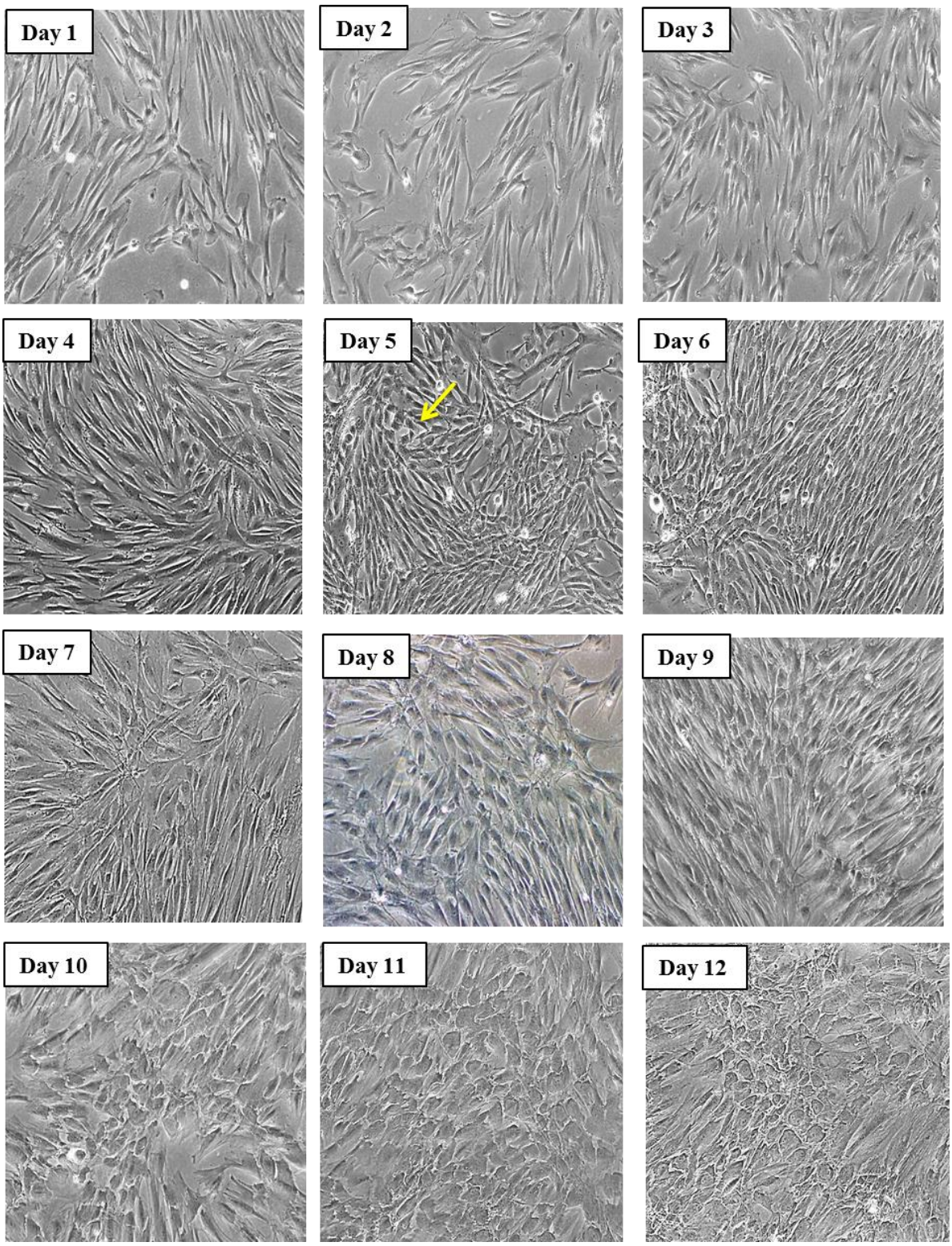

Figure 4.18: Representative images showing the morphological changes of SCD cultured in FBS towards hepatocyte-like cells for 21 days in hepatocyte induction media. Arrows indicate the morphological changes (Magnification 10X; phase contrast images).

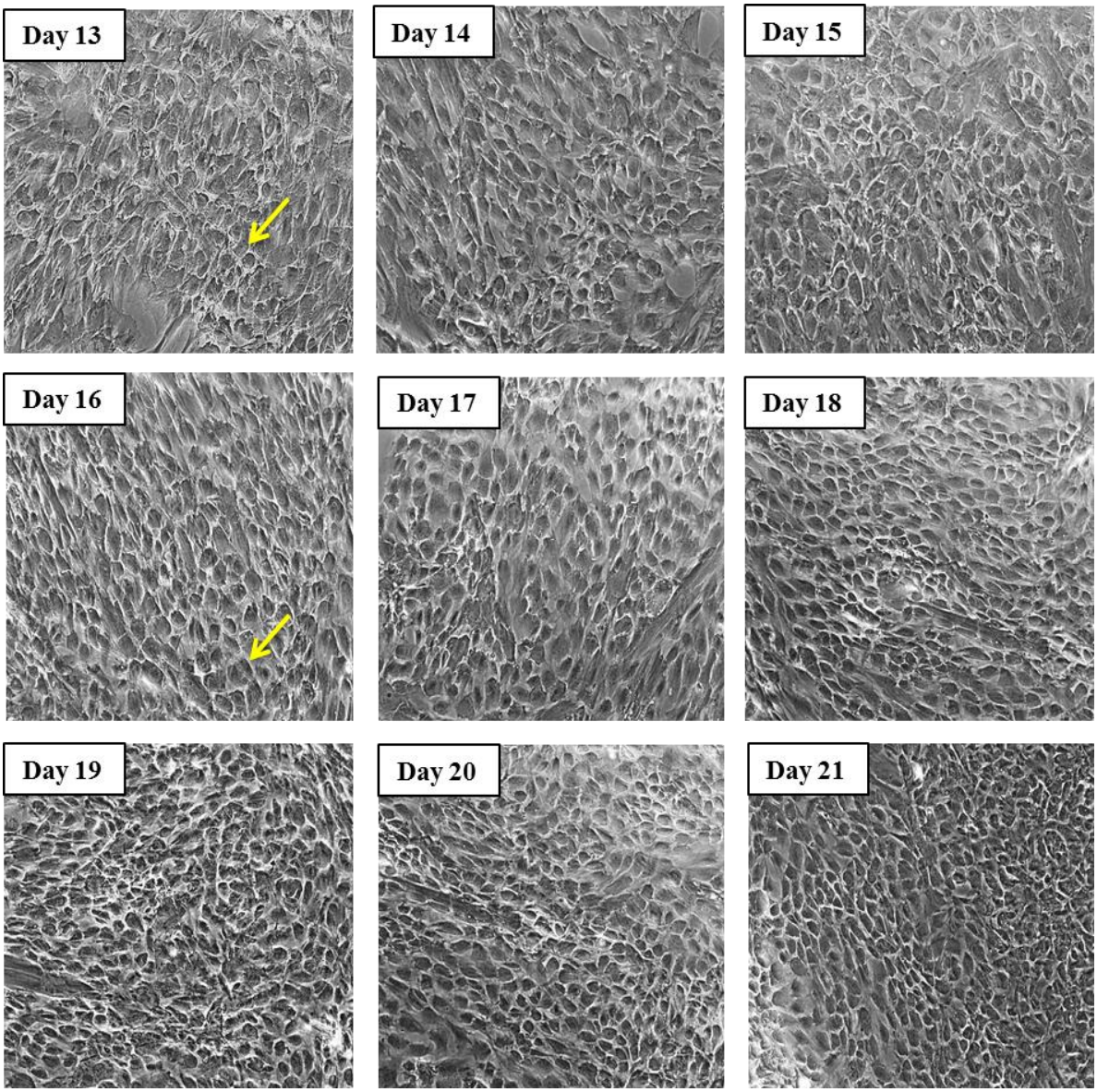

Figure 4.18, continued: Representative images showing the morphological changes of SCD cultured in FBS towards hepatocyte-like cells for 21 days in hepatocyte induction media. Arrows indicate the morphological changes (Magnification 10X; phase contrast images).

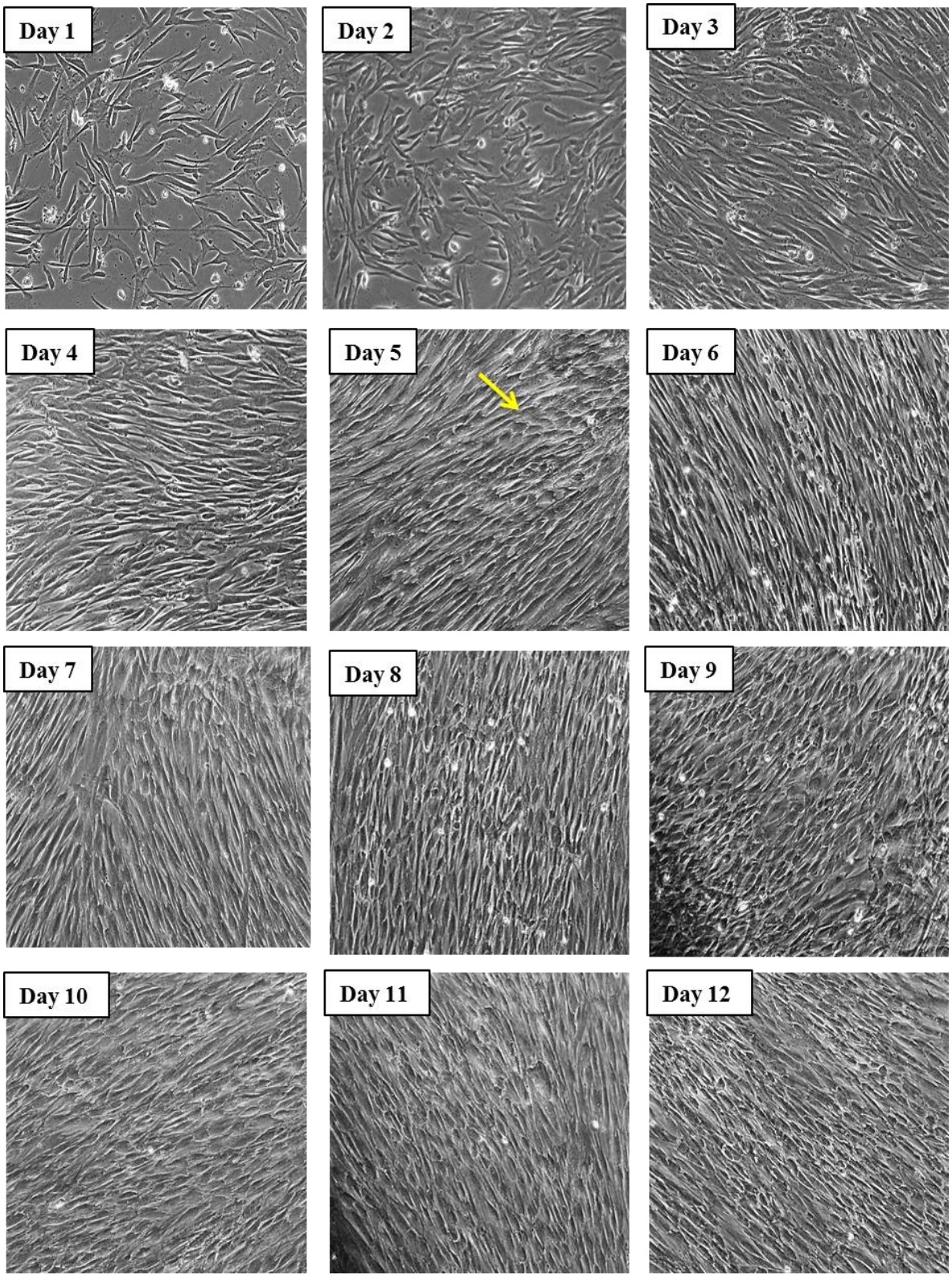

Figure 4.19: Representative images showing the morphological changes of SCD cultured in HPL towards hepatocyte-like cells for 21 days in hepatocyte induction media. Arrows indicate the morphological changes (Magnification 10X; phase contrast images).

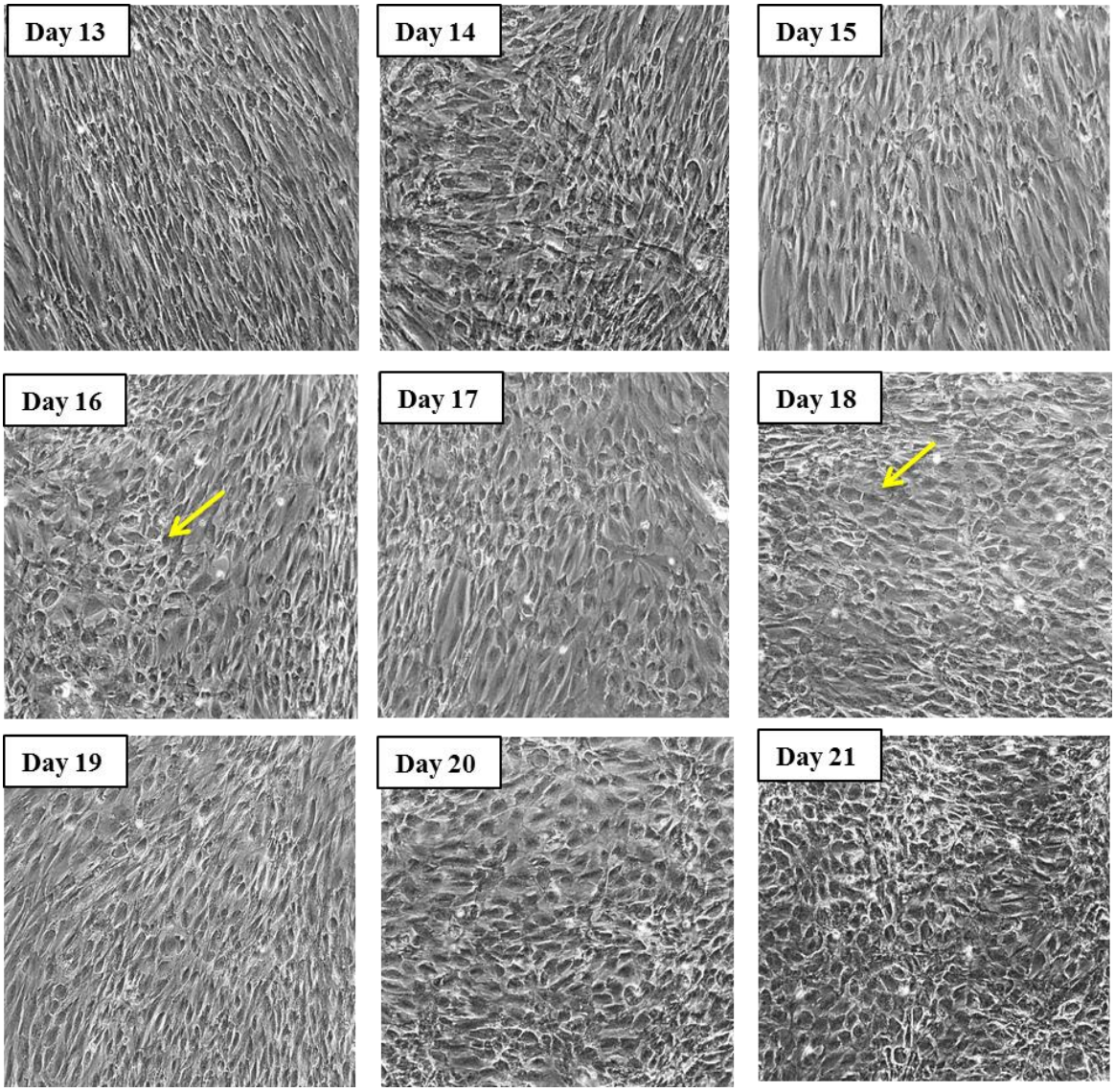

Figure 4.19, continued: Representative images showing the morphological changes of SCD cultured in HPL towards hepatocyte-like cells for 21 days in hepatocyte induction media. Arrows indicate the morphological changes (Magnification 10X; phase contrast images).

4.11 Periodic acid Schiff staining

The Periodic Acid Schiff (PAS) staining was performed to determine the presence of stored glycogen in the SCD hepatocyte-like cells cultured in FBS and HPL (Figure 4.20). Based on the observations, PAS staining was negative indicating no glycogen on day 0 (control) for SCD in both media. On day 7, both cells displayed slightly purple-pinkish staining suggesting a low amount of glycogen in the cells. By day 14 in the hepatocyte induction media, approximately 60% of the differentiated SCD cultured in FBS and HPL showed the ability to store glycogen as indicated by the increased number of stained cells and higher intensity of purple staining. Ninety five percent of the SCD hepatocyte-like cells cultured in FBS and HPL displayed positive staining on day 21. SCD cultured in FBS and HPL has shown to have potential to differentiate into hepatocyte-like cells.

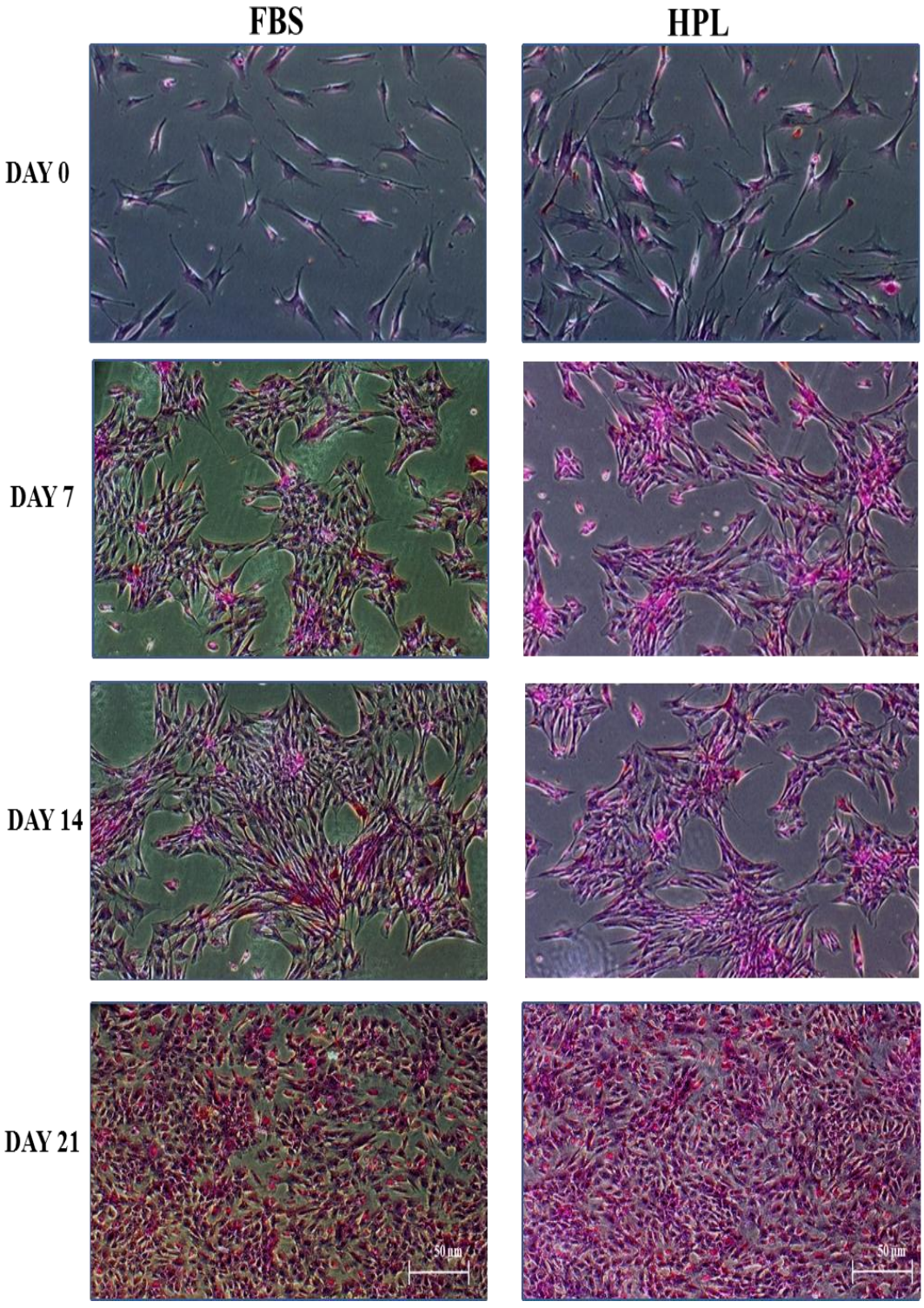

Figure 4.20: Representative images showing PAS staining of SCD hepatocyte-like cells cultured in FBS and HPL at day 0 (control), day 7, day 14, day 21 in hepatocyte induction media (Magnification 10X; phase contrast images).

4.12 Immunocytochemistry analysis of SCD hepatocyte-like cells

The immunocytochemistry of SCD hepatocyte-like cells showed positive expressions for AFP, HNF4α, CK 19, albumin and CK 18. Based on these observations, the SCD hepatocyte–like cells in FBS and HPL displayed weak expression (++) of AFP at day 0 and moderate expression (+++) at day 7. The expression of AFP in both cells decreased into weak expression (++) at day 14. On day 21, very weak expression (+) of AFP was observed for the SCD hepatocyte-like cells in FBS (Figure 4.21) whereas there was negative expression (-) for the SCD hepatocyte-like cells in HPL (Figure 4.22). The HNF4α expressions for the SCD hepatocyte-like cells in FBS was moderate (+++) at day 0, day 7 and day 14 in hepatocyte induction media. The expression became strong (++++) at day 21 (Figure 4.23). Although very weak expression (+) of HNF4α was observed for the SCD hepatocyte-like cells in HPL at day 0 and day 7, weak (++) and moderate (+++) expressions were evident at day 14 and day 21, respectively (Figure 4.24). The expression of CK 19 for the SCD hepatocyte-like cells cultured in FBS and HPL were negative (-) at day 0 and day 7, however, they began to show weak expression (++) at day 14 and moderate expression (+++) at day 21 (Figure 4.25, 4.26). Similarly, the expressions of albumin and CK 18 were also negative (-) at day 0 and day 7 for SCD hepatocyte-like cells in both media. Moderate expression (+++) of albumin were evident after the SCD in FBS and HPL had been induced to hepatocyte-like cell for 14 days (Figure 4.27, 4.28). The expression of CK 18 was very weak (+) for SCD hepatocyte-like cells in FBS, whereas negative expression (-) was observed for cells in HPL at day 14 (Figure 2.29, 4.30). The SCD hepatocyte-like cells in both media demonstrated strong expression (++++) of albumin and moderate expression (+++) of CK 18 at day 21.

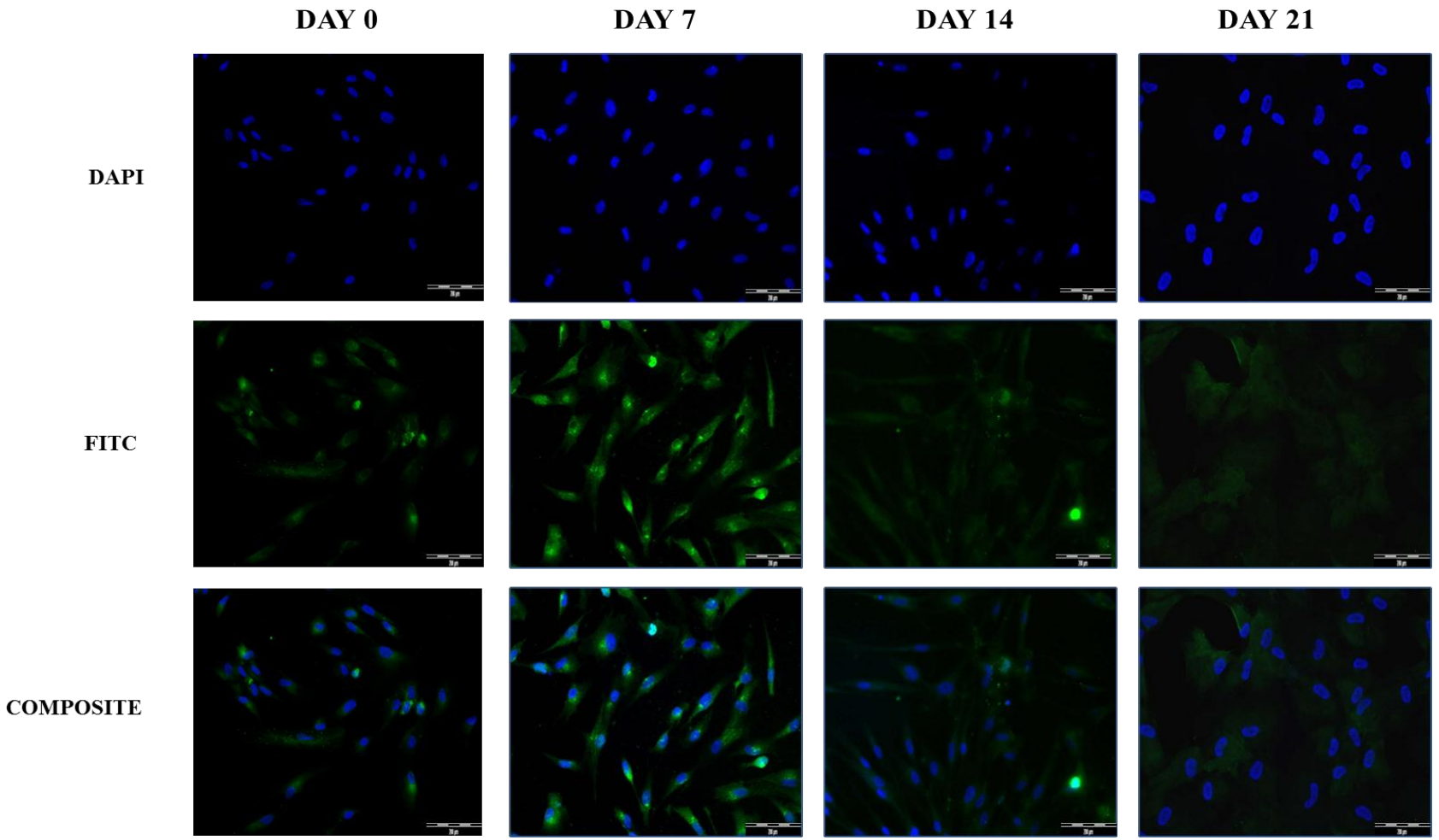

Figure 4.21: Representative images for the expression of AFP for SCD hepatocyte-like cells cultured in FBS. The green fluorescence (AFP) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

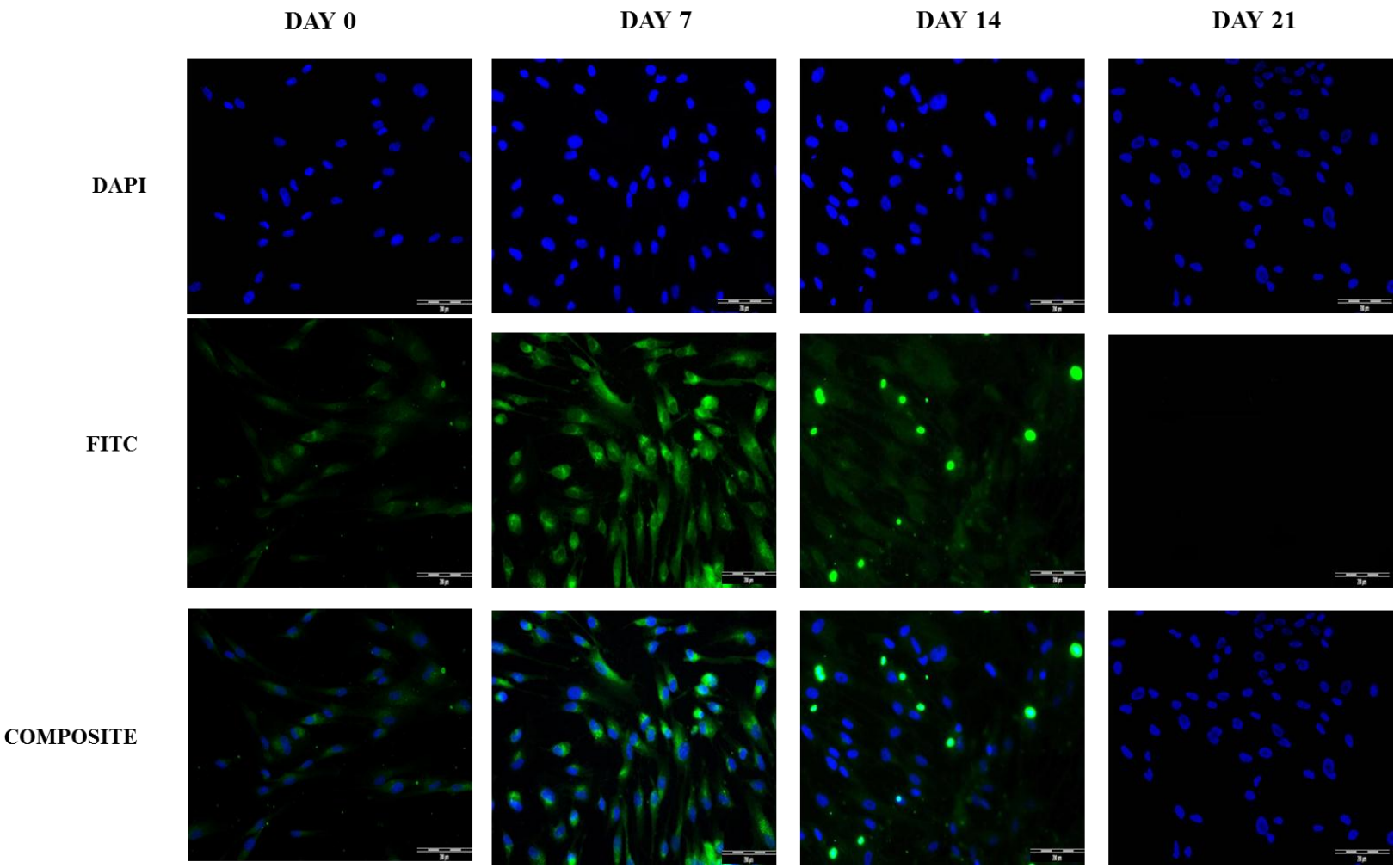

Figure 4.22: Representative images for the expression of AFP for SCD hepatocyte-like cells cultured in HPL. The green fluorescence (AFP) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

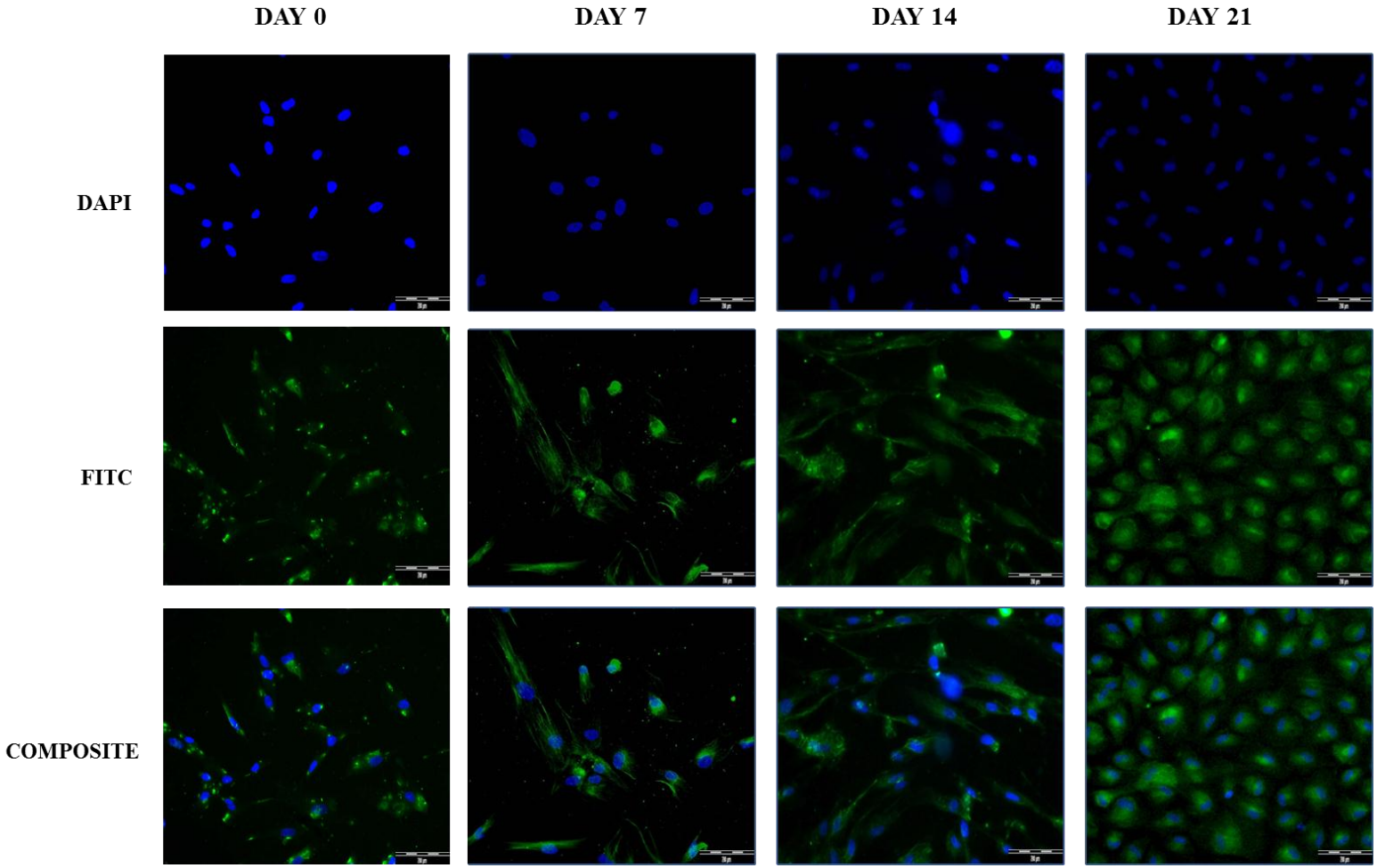

Figure 4.23: Representative images for the expression of HNF4α for SCD hepatocyte-like cells cultured in FBS. The green fluorescence (HNF4α) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

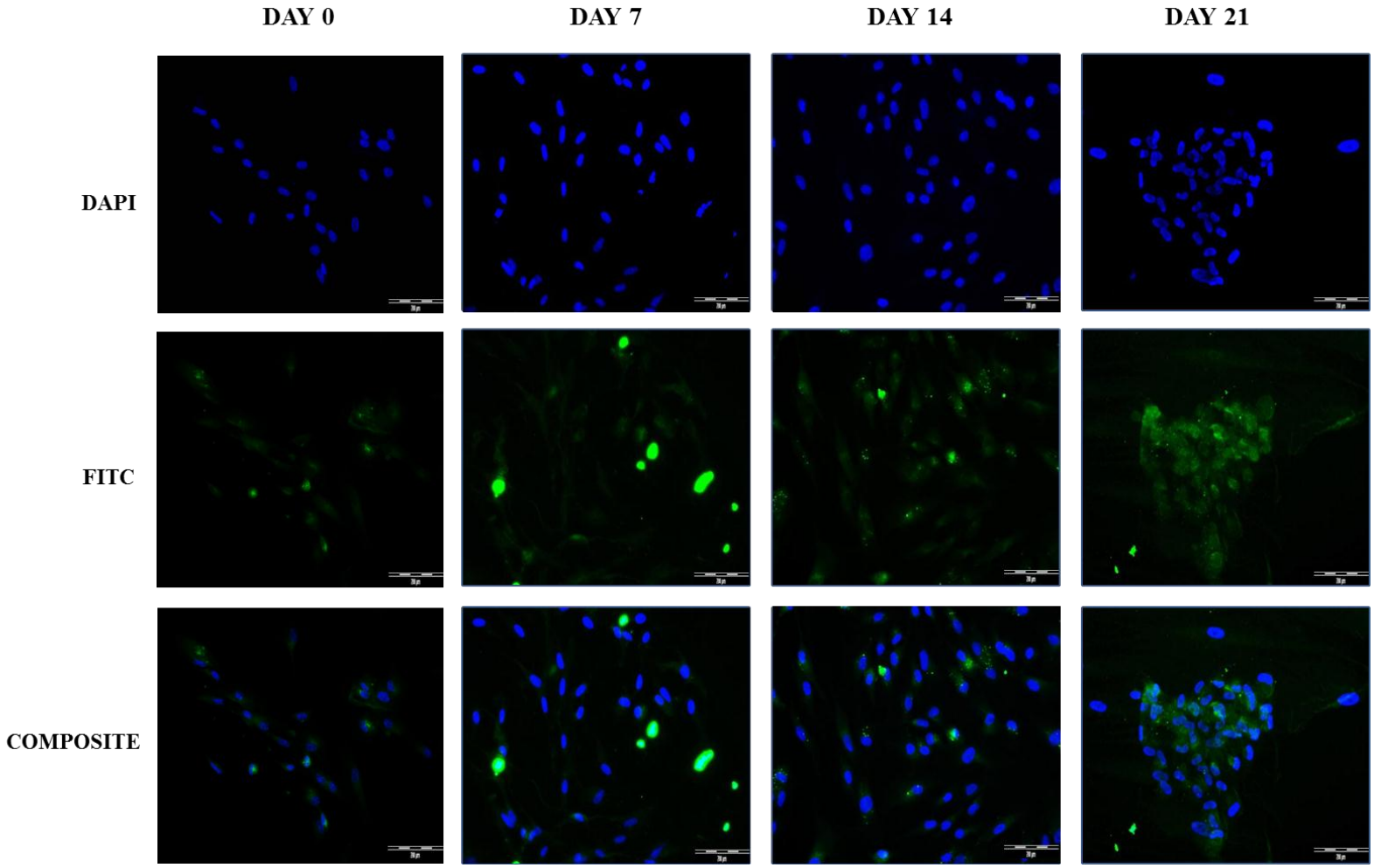

Figure 4.24: Representative images for the expression of HNF4α for SCD hepatocyte-like cells cultured in HPL. The green fluorescence (HNF4α) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

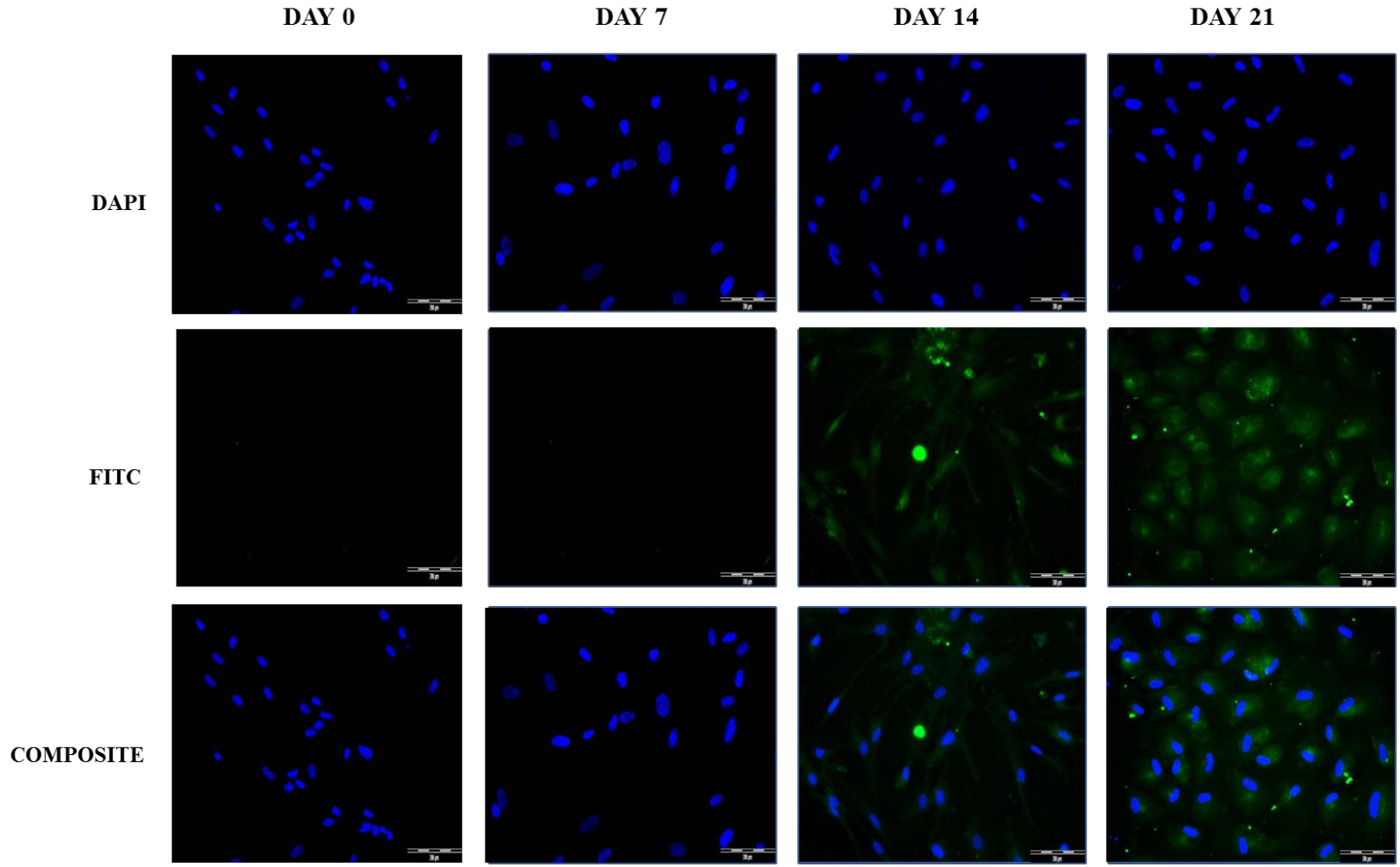

Figure 4.25: Representative images for the expression of CK 19 for SCD hepatocyte-like cells cultured in FBS. The green fluorescence (CK 19) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

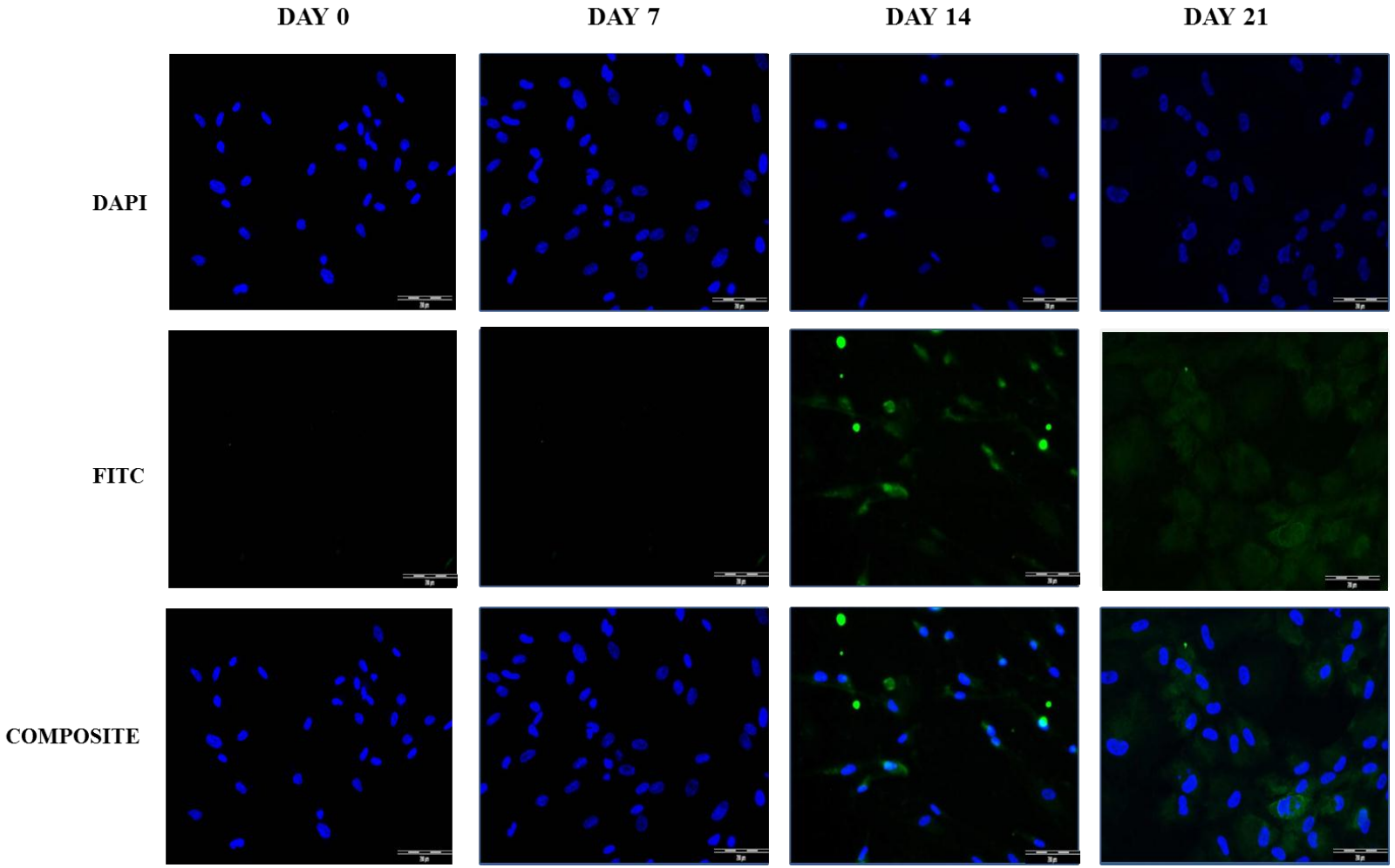

Figure 4.26: Representative images for the expression of CK 19 for SCD hepatocyte-like cells cultured in HPL. The green fluorescence (CK 19) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

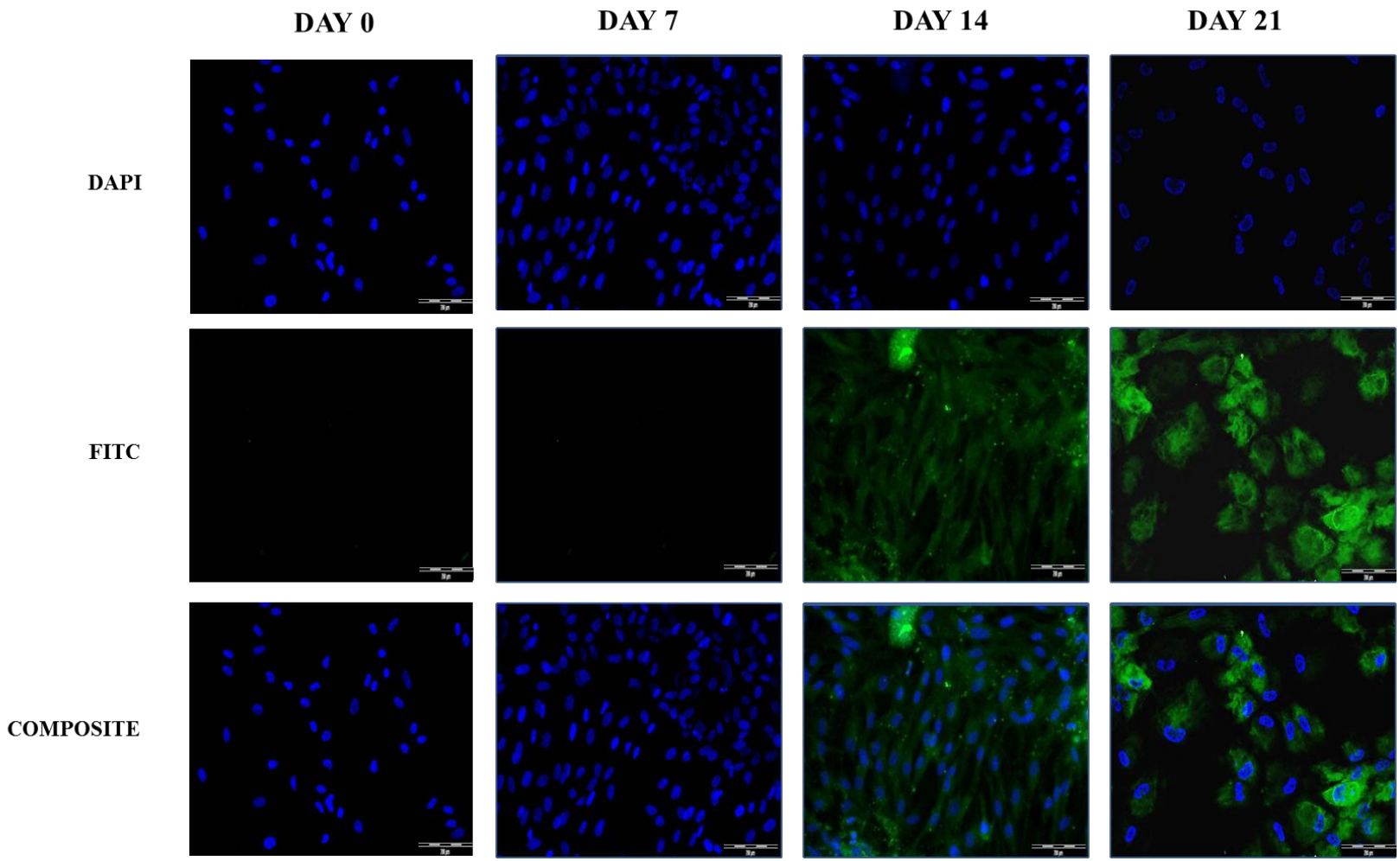

Figure 4.27: Representative images for the expression of Albumin for SCD hepatocyte-like cells cultured in FBS. The green fluorescence (Albumin) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

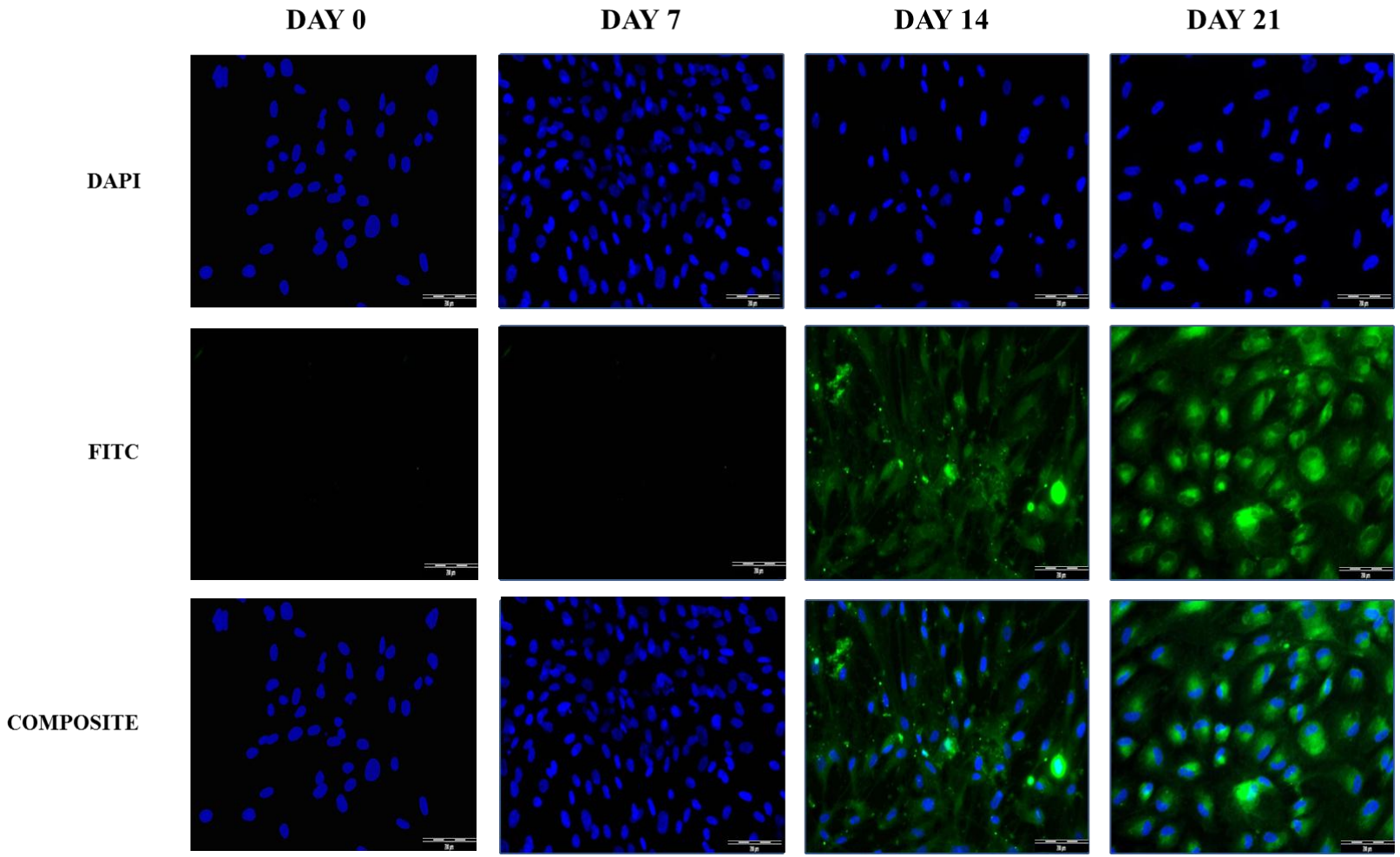

Figure 4.28: Representative images for the expression of Albumin for SCD hepatocyte-like cells cultured in HPL. The green fluorescence (Albumin) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

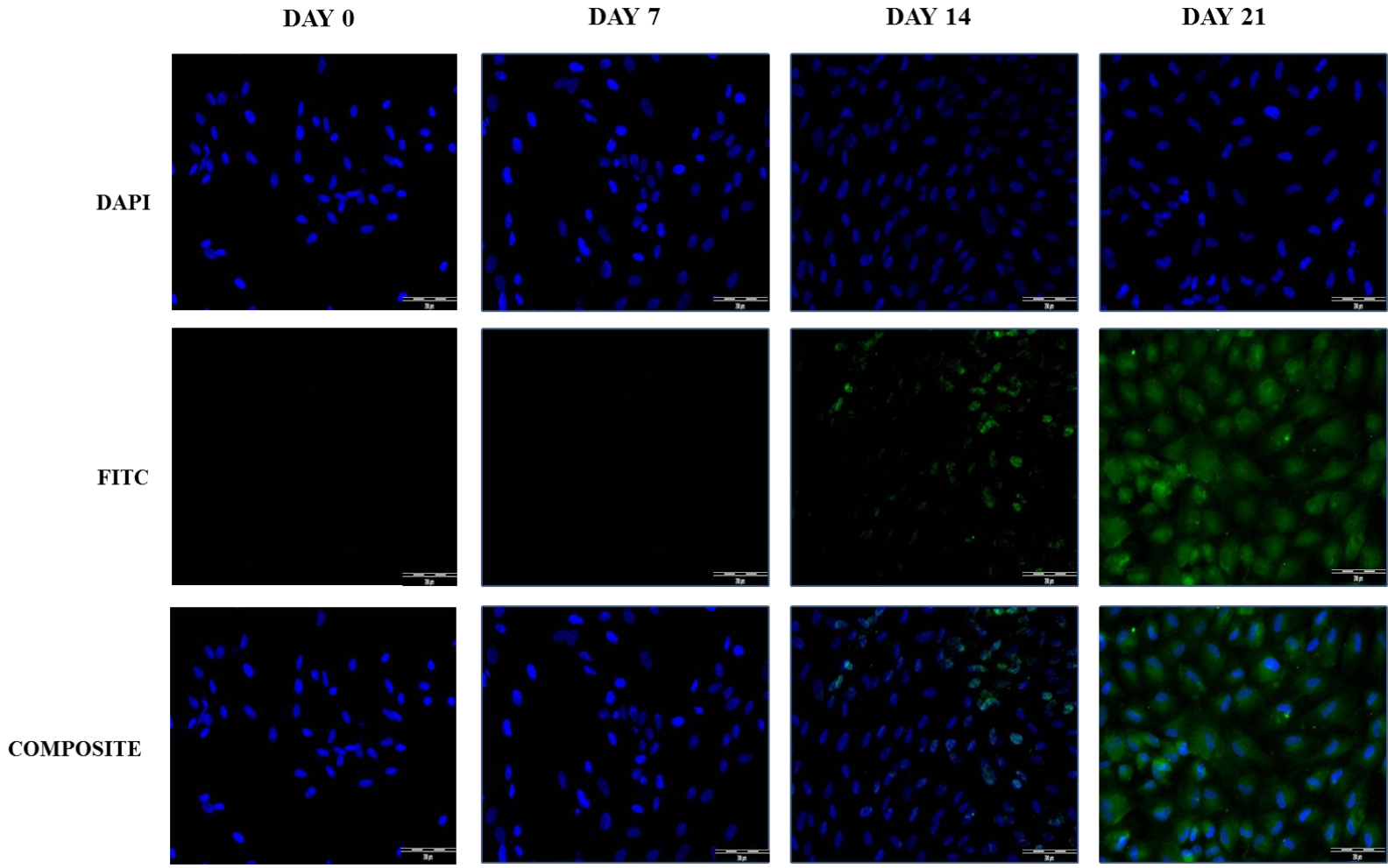

Figure 4.29: Representative images for the expression of CK 18 for SCD hepatocyte-like cells cultured in FBS. The green fluorescence (CK 18) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

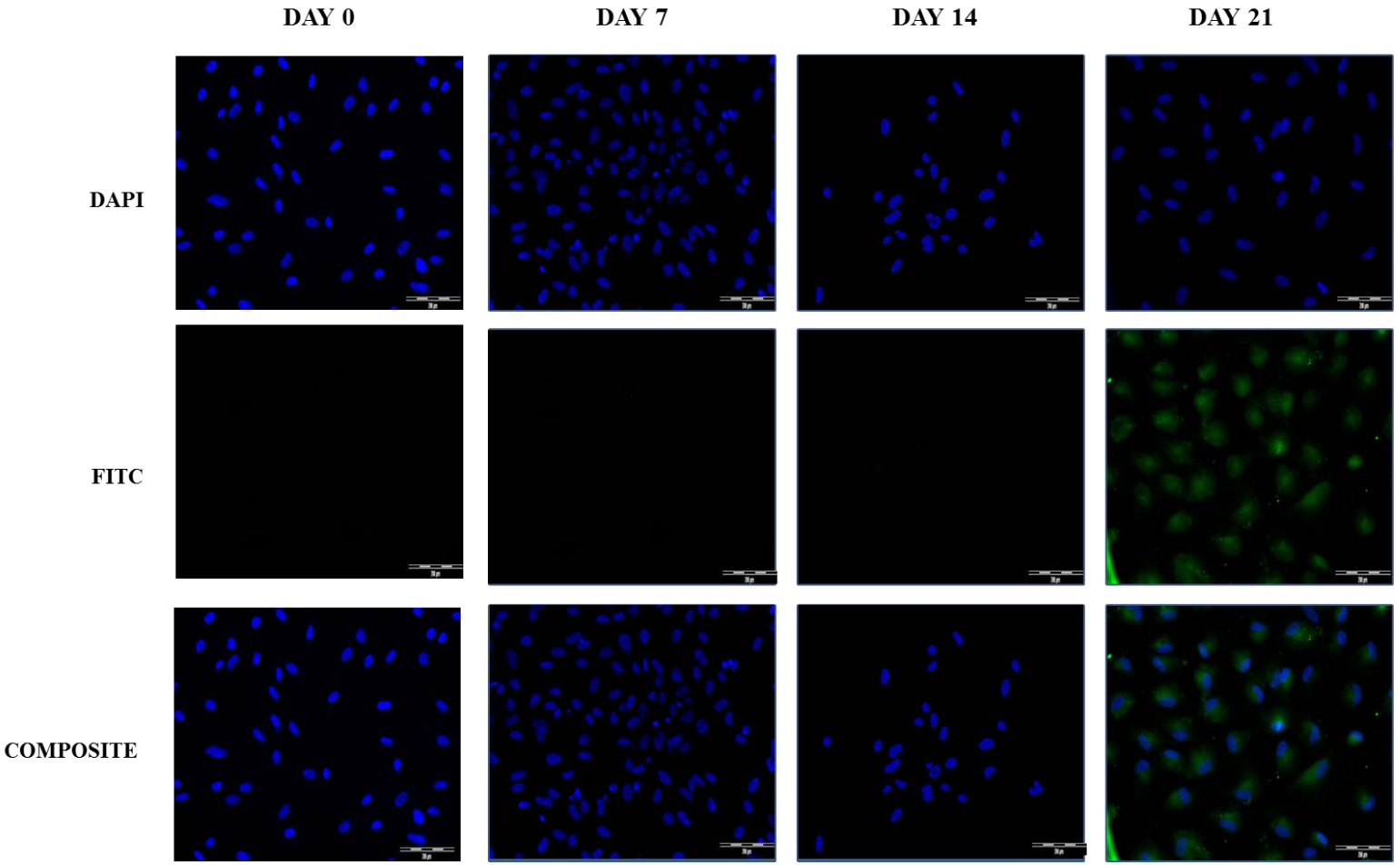

Figure 4.30: Representative images for the expression of CK 18 for SCD hepatocyte-like cells cultured in HPL. The green fluorescence (CK 18) stained cell cytoplasmic while DAPI stained the nucleus. (Magnification 20X; phase contrast images).

4.13 Immunophenotype analysis of SCD hepatocyte-like cells

The surface phenotypes of SCD hepatocyte-like cells showed positive expression of hepatocyte cell markers. The expression of AFP marker for the SCD hepatocyte-like cells in FBS and HPL displayed high value at day 7, 82.37 ± 1.95 for FBS and 84.32 ± 1.47 for HPL (Figure 4.31). However, the expression of AFP later decreased to 18.68 ± 0.74 for SCD hepatocyte-like cells in FBS and 18.88 ± 0.45 for HPL at day 14. Futhur reduction of AFP expression for both cells was observed at day 21, 5.81 ± 2.80 (FBS) and 6.05 ± 3.93 (HPL). The HNF4α expression for the SCD hepatocyte-like cells expanded in FBS showed a lower expression at day 7 (9.00 ± 1.54) but increased tremendously at day 14 (81.91 ± 2.47) and day 21 (98.06 ± 0.37). These observations were similar for SCD hepatocyte-like cells cultured in HPL at day 7 (7.93 ± 0.49), day 14 (86.20 ± 3.67) and day 21 (97.76 ± 0.04) as shown in Figure 4.32. A higher expression of CK 19 was demonstrated at day 7 for the SCD hepatocyte-like cells in FBS (12.34 ± 1.95) compared to the HPL (8.57 ± 1.01). However, the CK 19 expression became similar after SCD in FBS and HPL were induced for 14 days, 31.73 ± 2.10 and 31.18 ± 2.36, respectively (Figure 4.33). There was a remarkable increase in CK 19 expression at day 21, 87.07 ± 1.62 for the SCD hepatocyte-like cells in FBS and 89.92 ± 3.52 for HPL. The SCD hepatocyte-like cells in both media also showed very low expression of albumin at day 7, 7.35 ± 0.31 for FBS and 15.33 ± 0.62 for HPL (Figure 4.34). Nevertheless, the expression of albumin increased for the SCD hepatocyte-like cells in FBS (35.47 ± 0.81) and HPL (45.38 ± 1.88) at day 14 and reaches the highest expression at day 21. The expression of albumin was higher in the SCD hepatocyte-like cells in HPL (92.82 ± 0.81) compared to the FBS (90.71 ± 0.83). The final cell surface marker tested was CK 18, 29.96 ± 3.09 for SCD hepatocyte-like cells in FBS and 54.18 ± 4.27 for HPL at day 7. At day 14, the expression of CK 18 increased to 83.74 ± 2.29 for FBS and 86.51 ± 1.61 for HPL (Figure 4.35). The highest

expression of CK 18 for both cells was observed at day 21, 96.07 ± 0.51 (FBS) and 97.93 ± 1.17 (HPL). The results represented the means and standard deviations of two independent experiments. All the data is presented in Appendix 2.6.

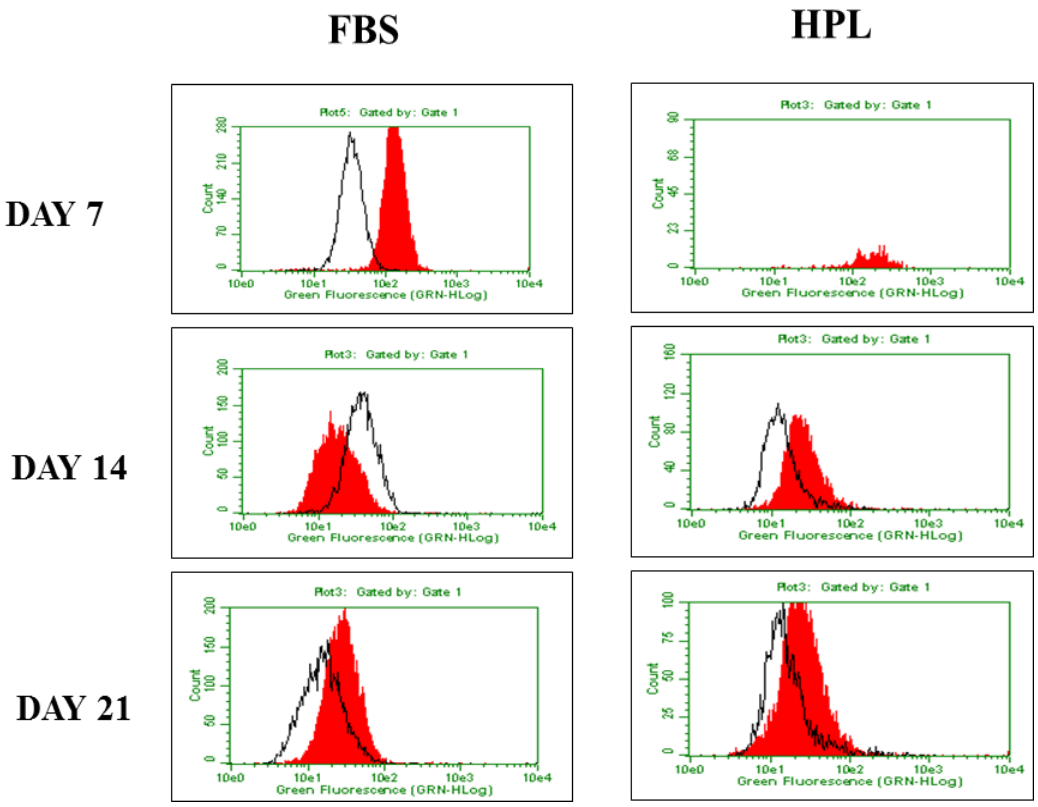

Figure 4.31: Representative results of the cell phenotype analysis of SCD hepatocyte-like cells expanded in FBS and HPL for AFP marker.

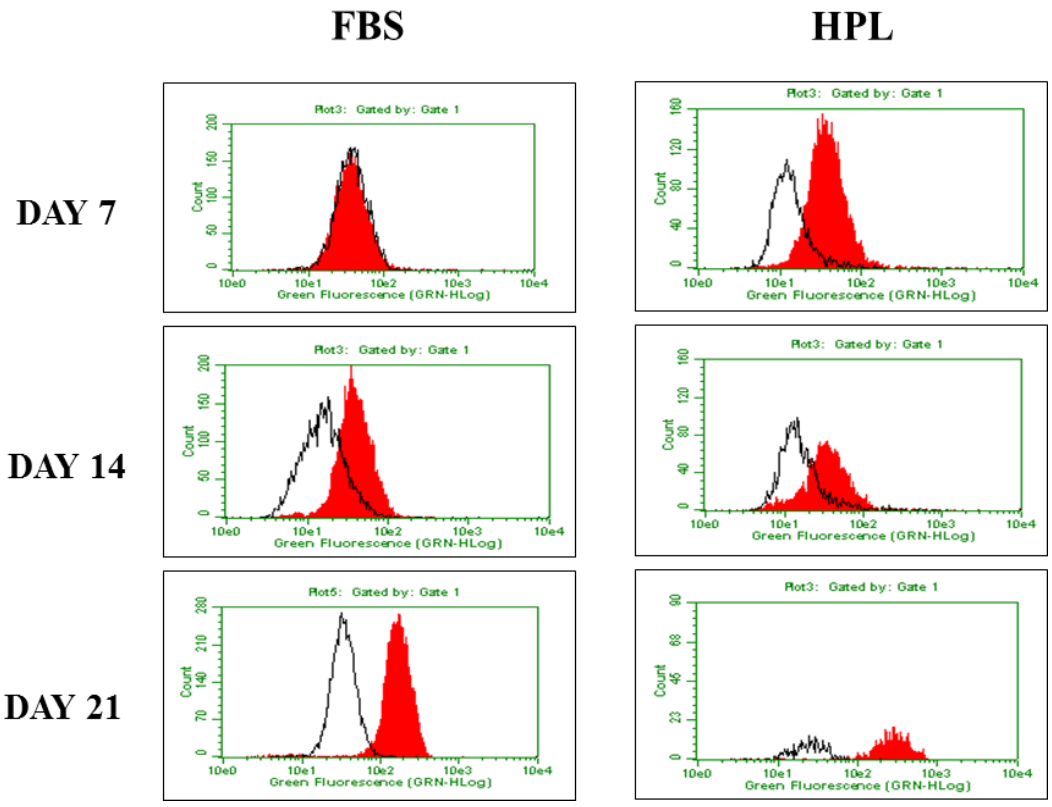

Figure 4.32: Representative results of the cell phenotype analysis of SCD hepatocyte-like cells expanded in FBS and HPL for HNF4α marker.

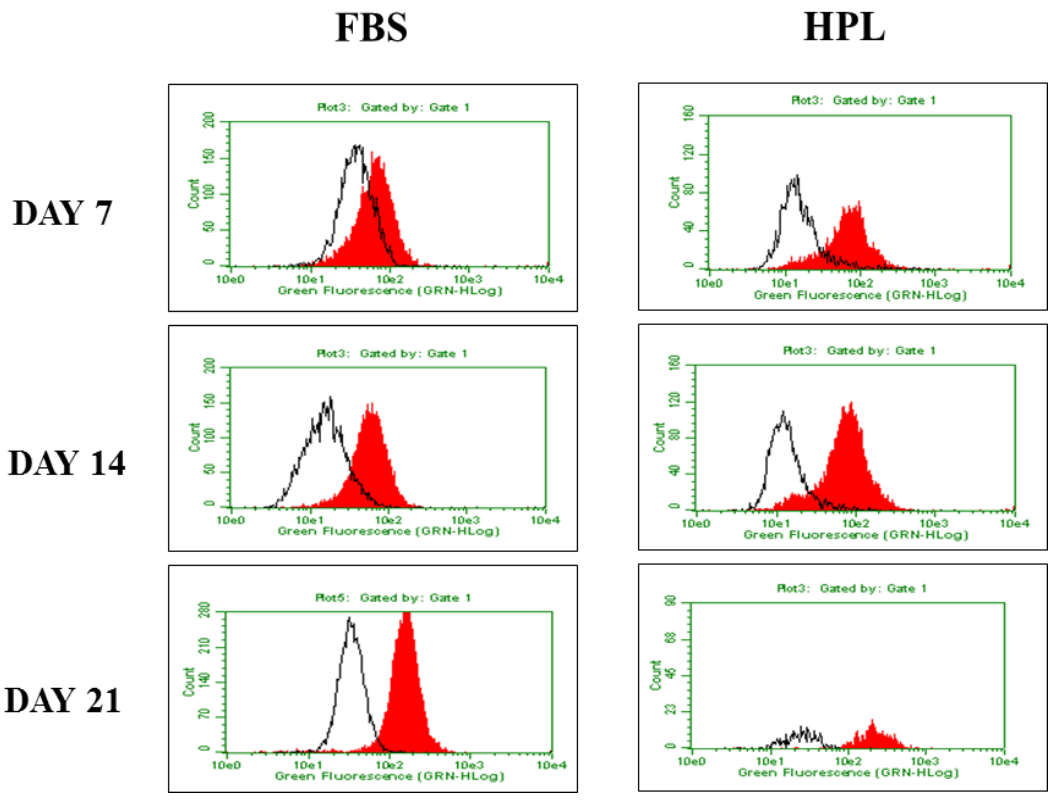

Figure 4.33: Representative results of the cell phenotype analysis of SCD hepatocyte-like cells expanded in FBS and HPL for CK 19 marker.

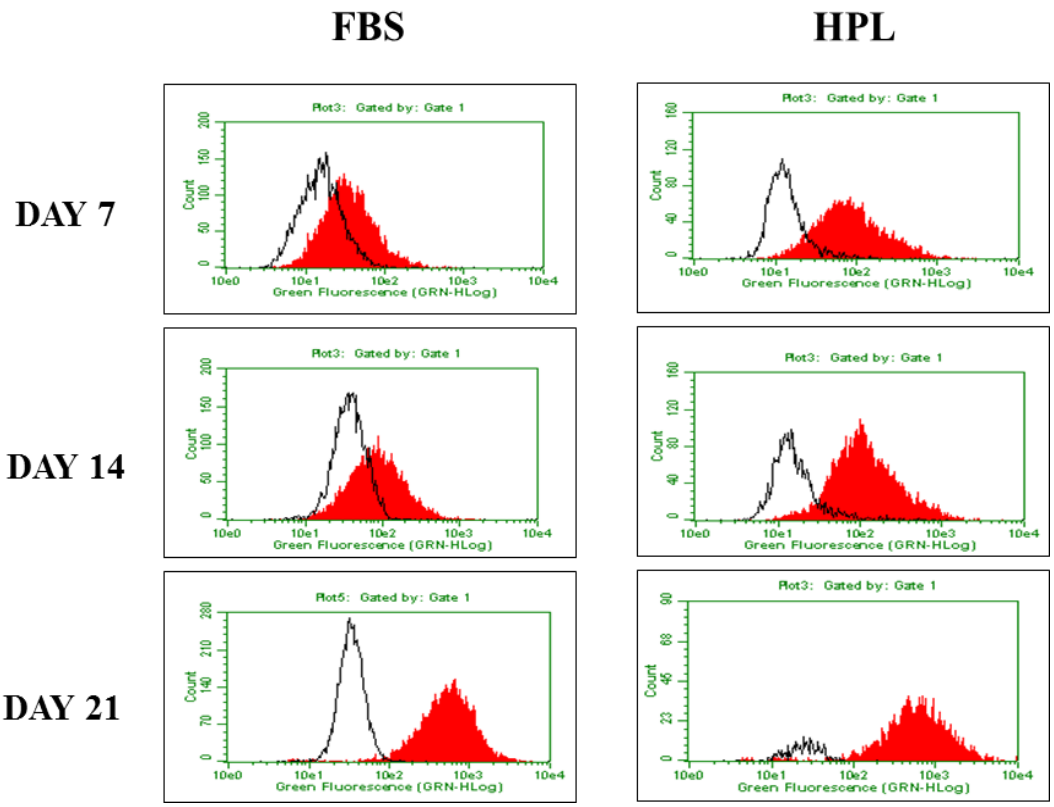

Figure 4.34: Representative results of the cell phenotype analysis of SCD hepatocyte-like cells expanded in FBS and HPL for albumin marker.

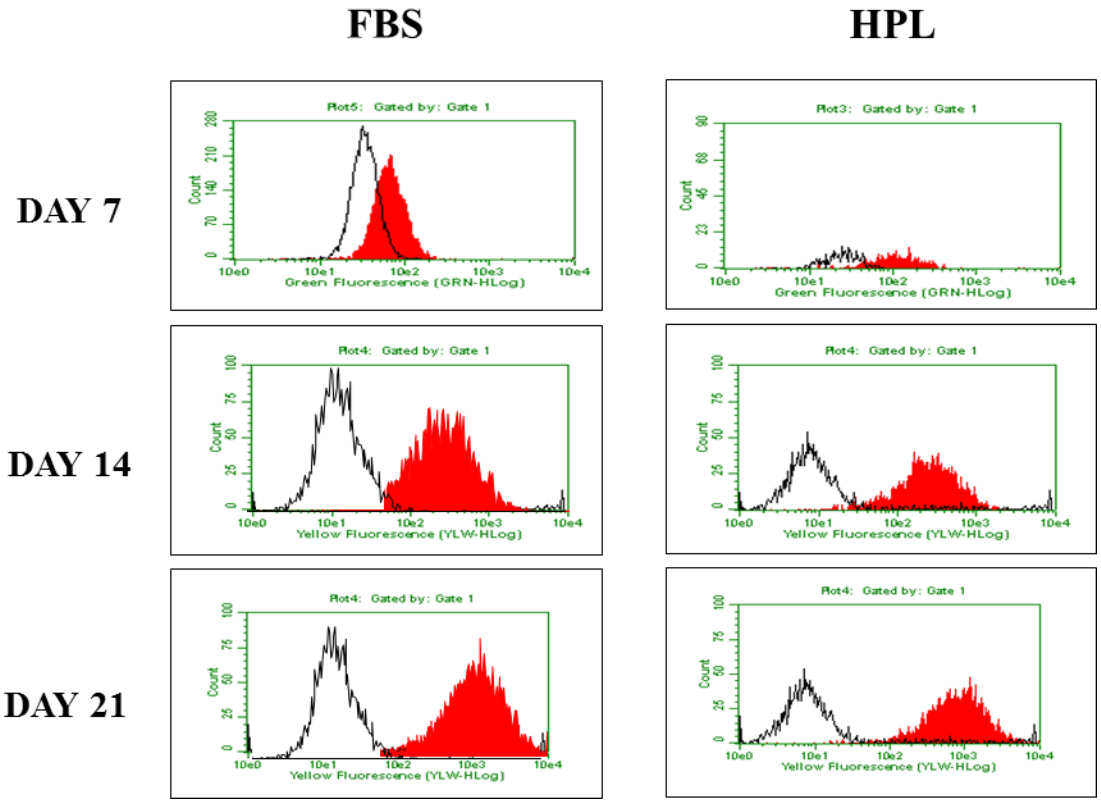

Figure 4.35: Representative results of the cell phenotype analysis of SCD hepatocyte-like cells expanded in FBS and HPL for CK 18 marker.

4.14 RT-PCR of SCD hepatocyte-like cells

The SCD hepatocyte-like cells in FBS and HPL were tested for the presence of primitive and definitive hepatocyte marker expressions using RT-PCR (Figure 4.36). Based on the observations, the primitive hepatocyte cells markers namely AFP, HNF3β, GATA 4 and Sox 17 showed moderate expression (++) for both cells at day 0. The HNF3β, GATA 4, Sox 17 showed very high (++++) expressions whereas AFP demonstrated low expression (+) at day 7 for SCD hepatocyte-like cell in both media. However, the expressions of these primitive hepatocyte markers were not detected (-) at day 14 and day 21 except for the GATA 4. Low (+) and high expressions (+++) of GATA 4 were observed for the SCD hepatocyte-like cells in FBS and HPL at day 14, respectively. There were no expressions (-) of definitive hepatocyte markers (HNF4α, CK 19, albumin, CK 18, TO, TDO, AAT, CYP3a4, TAT and CYP7a1) for both cells at day 0. The SCD hepatocyte-like cells in both media showed no expression (-) of albumin, AAT, CYP3a4 and CYP7a1 at day 7, whereas, low (+) and moderate expression (++) for TO and TAT, respectively. In addition, the SCD hepatocyte-like cells in both media expressed high expression (+++) of HNF4α at day 7. The SCD hepatocyte-like cells in FBS exhibited very high expression (++++) for CK 19, high expression (+++) for CK 18 while no expression (-) for TDO compared to the HPL at day 7. On day 14, SCD hepatocyte-like cells in HPL displayed high expression (+++) for CK 18, TO and TDO while no expression (-) for other definitive hepatocyte markers when compared to FBS. The SCD hepatocyte-like cells in both media displayed high expression (+++) of albumin whereas very high expressions (++++) of CK 18, TO and TDO at day 21. However, only moderate expression (++) of TAT was observed and no expression (-) was visualized for HNF4α, AAT and CYP7a1 for the SCD hepatocyte-like cells in both media at day 21. The CYP3a4 was only expressed (+++) for the SCD hepatocyte-like cells in HPL compared to FBS at day 21 in hepatocyte induction media.

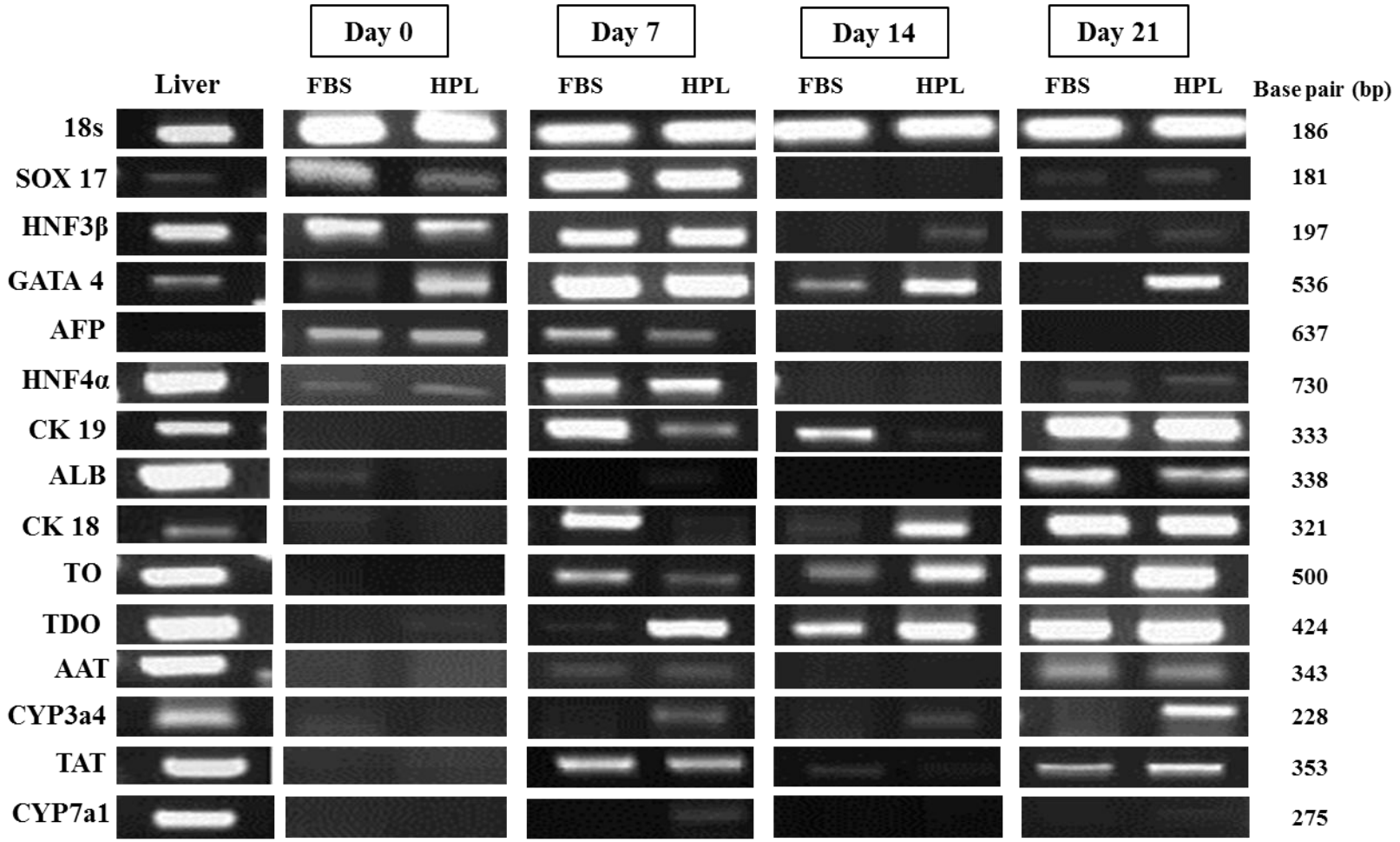

Figure 4.36: Expression of primitive and definitive hepatocyte markers of SCD hepatocyte-like cells cultured in FBS and HPL at day 0, day 7, day 14 and day 21 of hepatocyte-media induction.

4.15 Real time-PCR of SCD hepatocyte-like cells

Further analysis using real time-PCR were done to examine the presence of hepatocyte primitive and definitive markers quantitatively in the SCD hepatocyte-like cells cultured in FBS and HPL. The expression of Sox 17 (Figure 4.37) and HNF3β (Figure 4.38) displayed marked decrease of expression from day 7 to day 21 in hepatocyte induction media. The expression of primitive hepatocyte markers namely GATA 4 (Figure 4.39) and AFP (Figure 4.40) in the SCD hepatocyte-like cells in FBS and HPL were high at day 7, however these expressions gradually decreased at day 14 and day 21. A remarkable upturns of expression for the definitive hepatocyte markers (HNF4α, CK 19, albumin, CK 18, TO, TDO, AAT, CYP3a4, TAT and CYP7a1) were demonstrated by the SCD hepatocyte-like cells in both media. The expressions of HNF4α (Figure 4.41), CYP3a4 (Figure 4.48), TAT (Figure 4.49) CYP7a1 (Figure 4.50) showed gradual increase from day 7 to day 21 in hepatocyte induction media. On day 14, the expression of CK19 (Figure 4.42), CK 18 (Figure 4.44), TO (Figure 4.45), TDO (Figure 4.46) and AAT (Figure 4.47) increased approximately 2-fold compared to the day 7 for SCD hepatocyte-like cell in FBS and HPL. These markers continued to show increased expression at day 21. The highest upturn was shown by the expression of albumin in the SCD hepatocyte-like cells in both media, increase tremendously from day 7 to day 21 (Figure 4.43). The results represented the means and standard deviations of three independent experiments. All the data is presented in Appendix 2.7

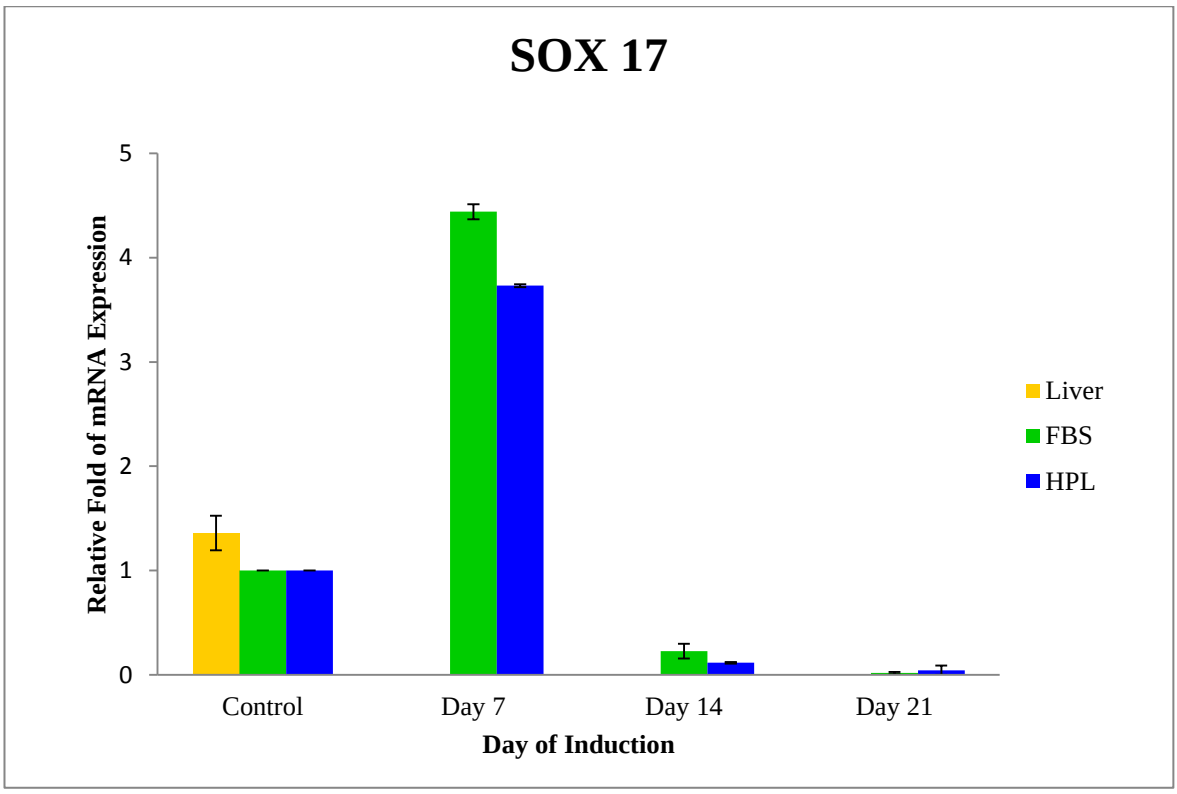

Figure 4.37: Expression of SOX 17 in the SCD hepatocyte-like cells expanded in FBS and HPL.

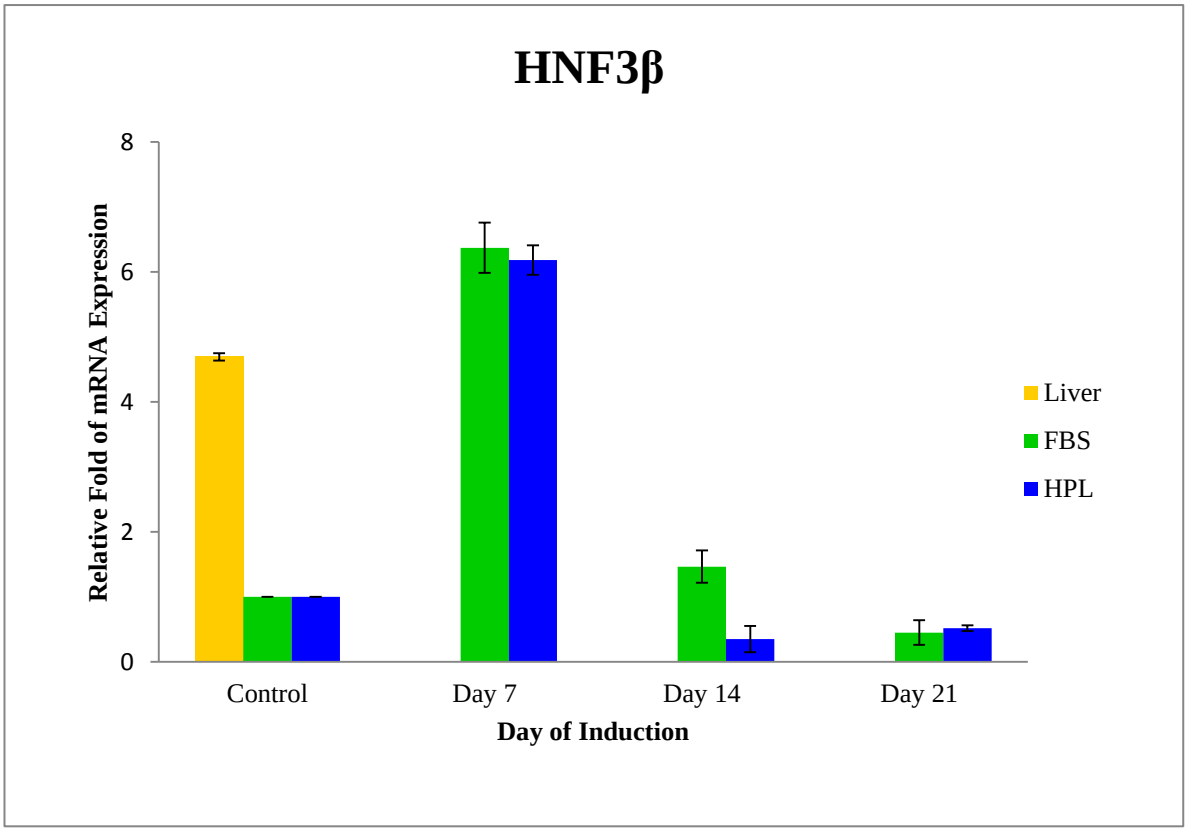

Figure 4.38: Expression of HNF3β in the SCD hepatocyte-like cells expanded in FBS and HPL.

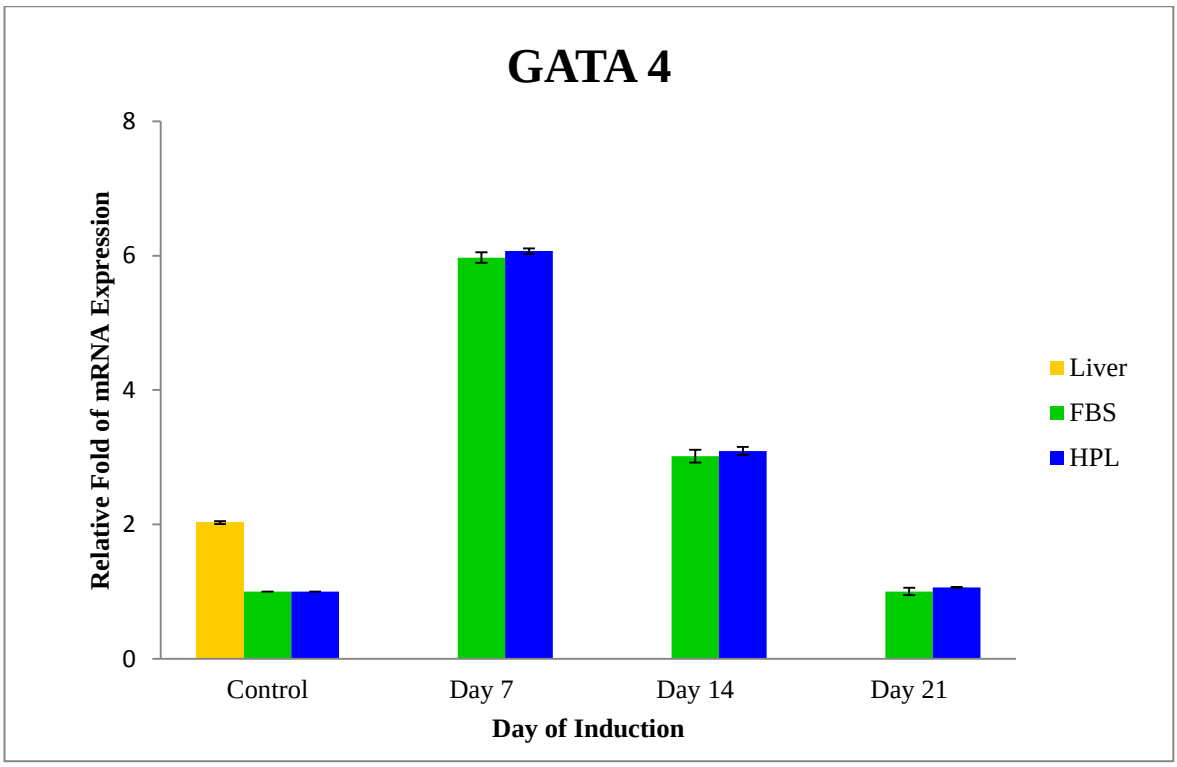

Figure 4.39: Expression of GATA 4 in the SCD hepatocyte-like cells expanded in FBS and HPL.

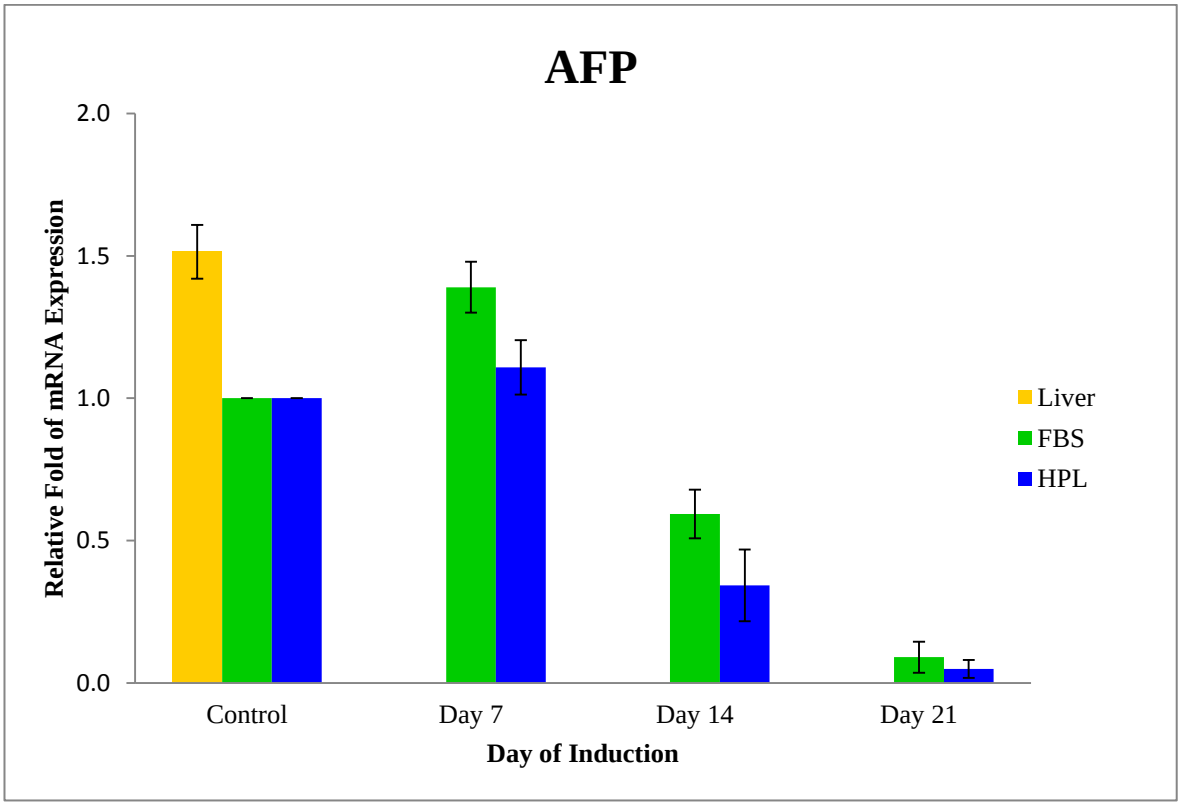

Figure 4.40: Expression of AFP in the SCD hepatocyte-like cells expanded in FBS and HPL.

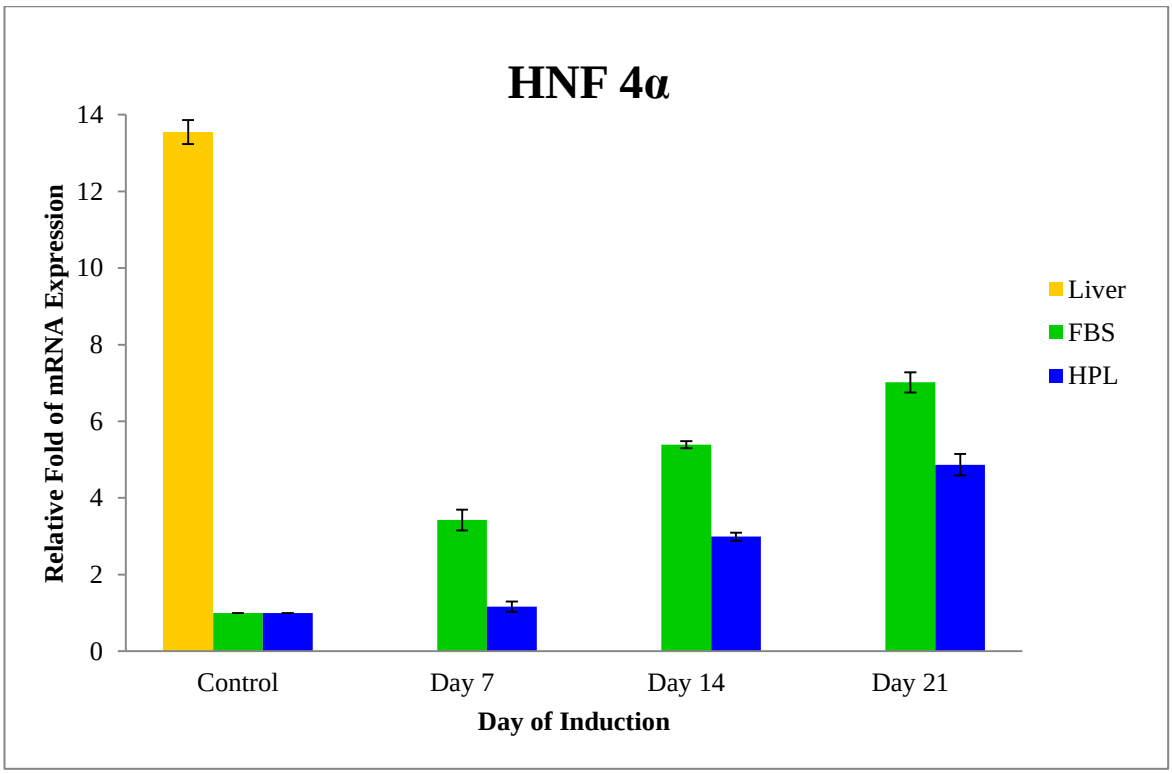

Figure 4.41: Expression of HNF4α in the SCD hepatocyte-like cells expanded in FBS and HPL.

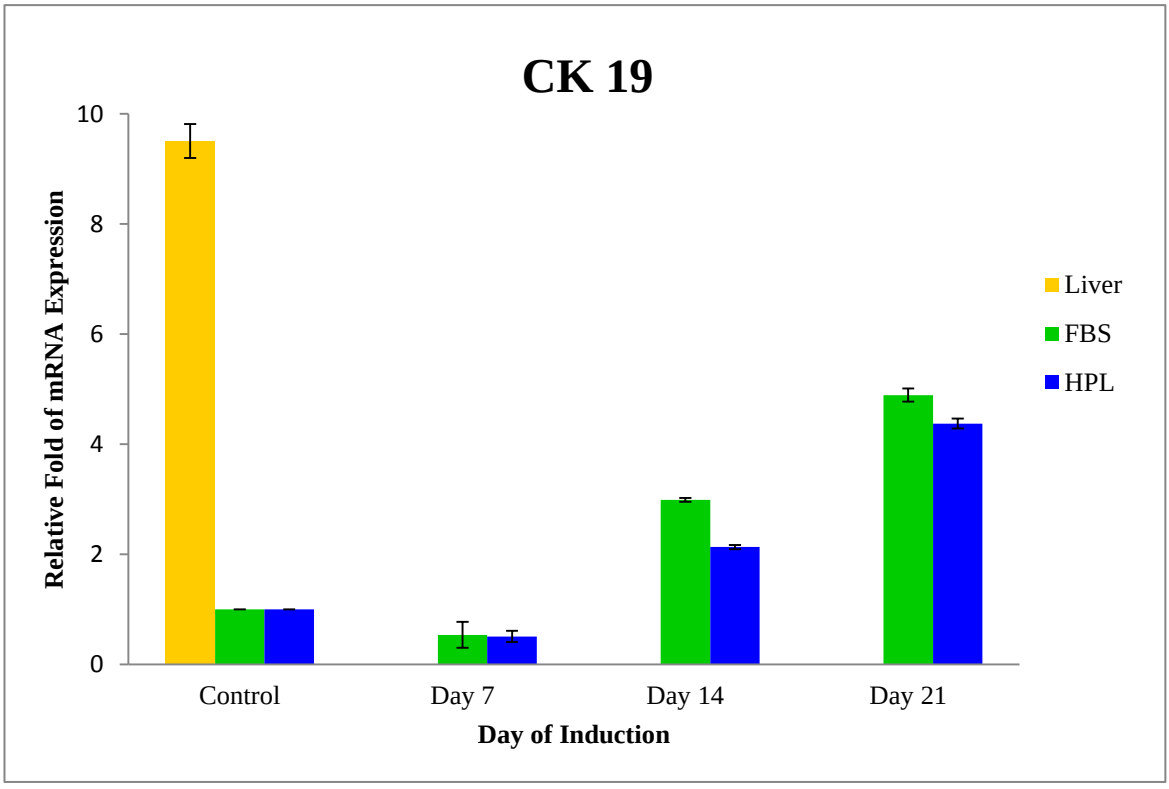

Figure 4.42: Expression of CK 19 in the SCD hepatocyte-like cells expanded in FBS and HPL.

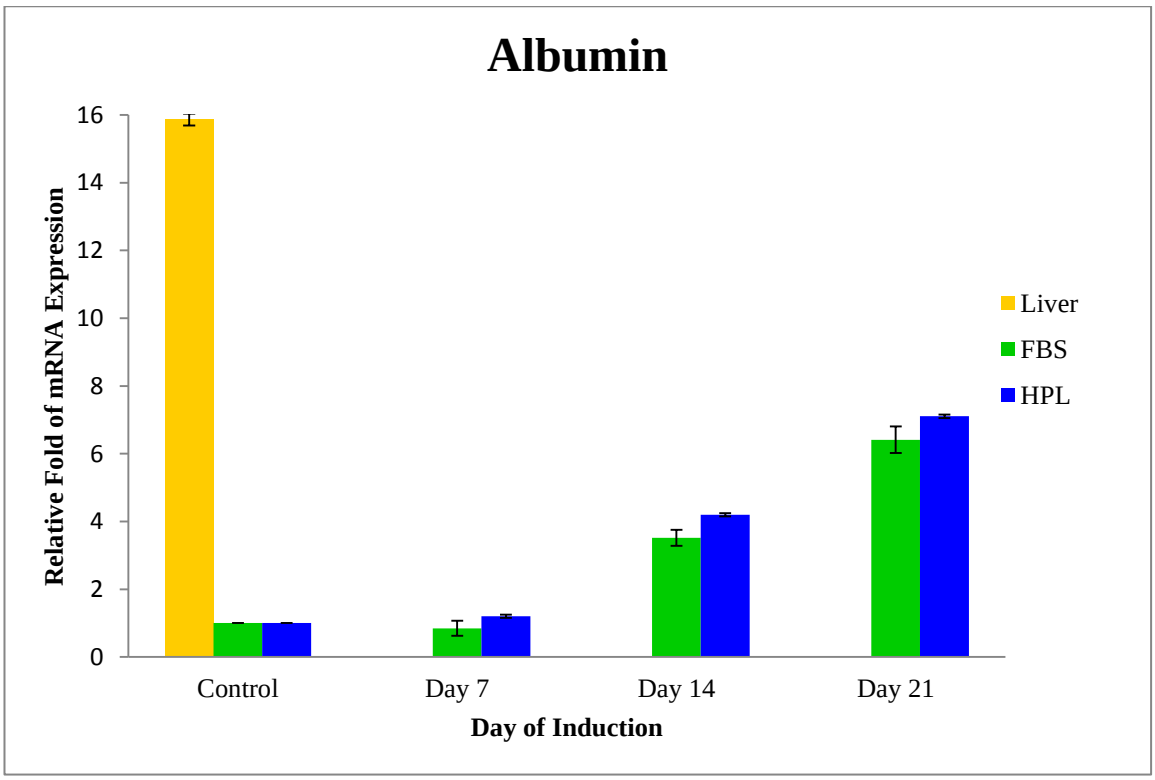

Figure 4.43: Expression of albumin in the SCD hepatocyte-like cells expanded in FBS and HPL.

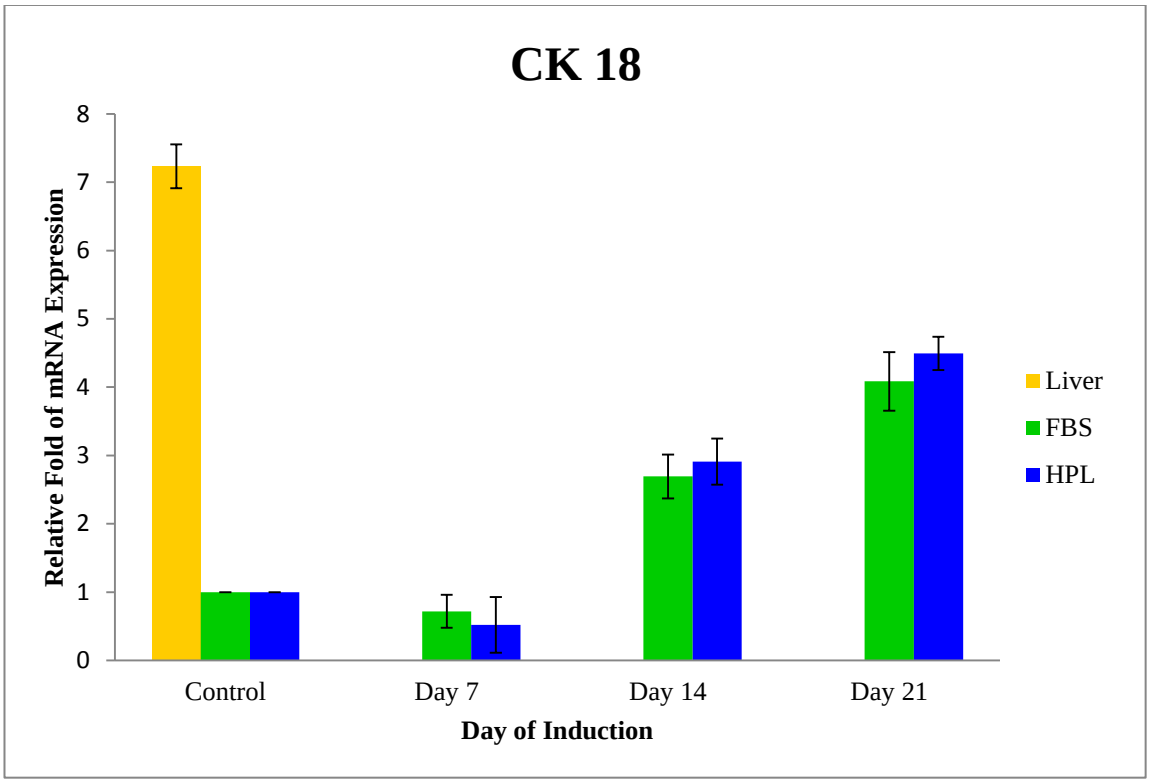

Figure 4.44: Expression of CK 18 in the SCD hepatocyte-like cells expanded in FBS and HPL.

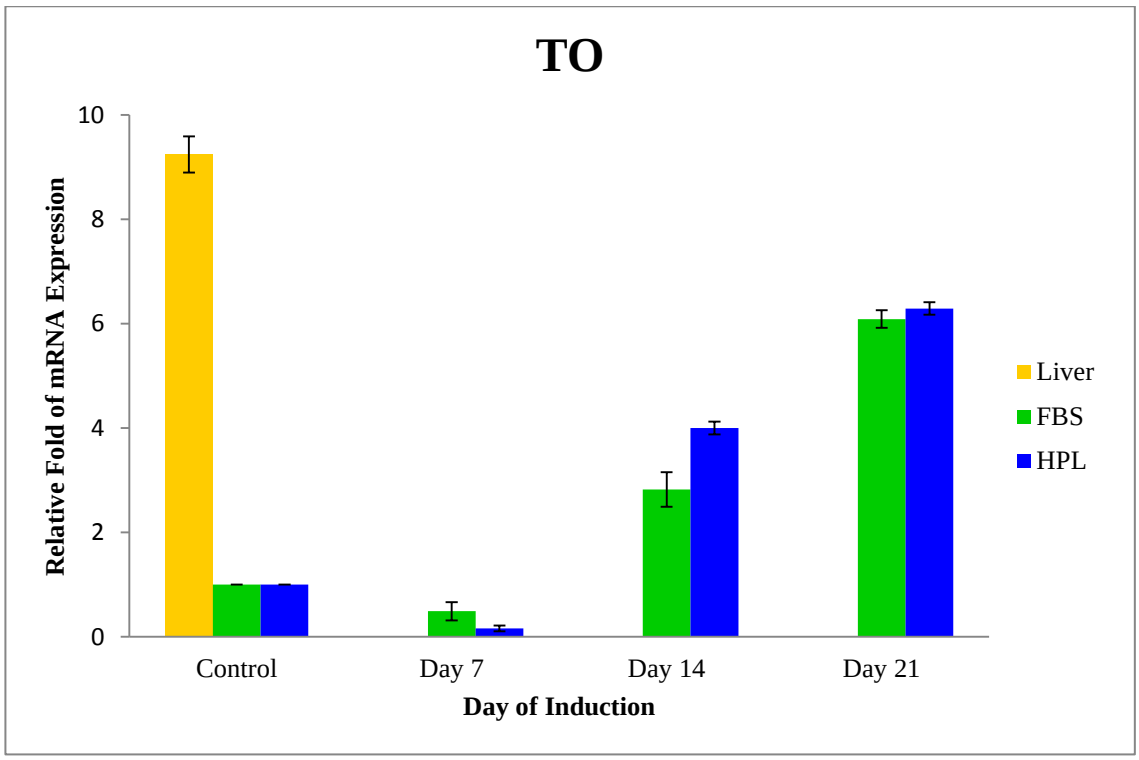

Figure 4.45: Expression of TO in the SCD hepatocyte-like cells expanded in FBS and HPL.

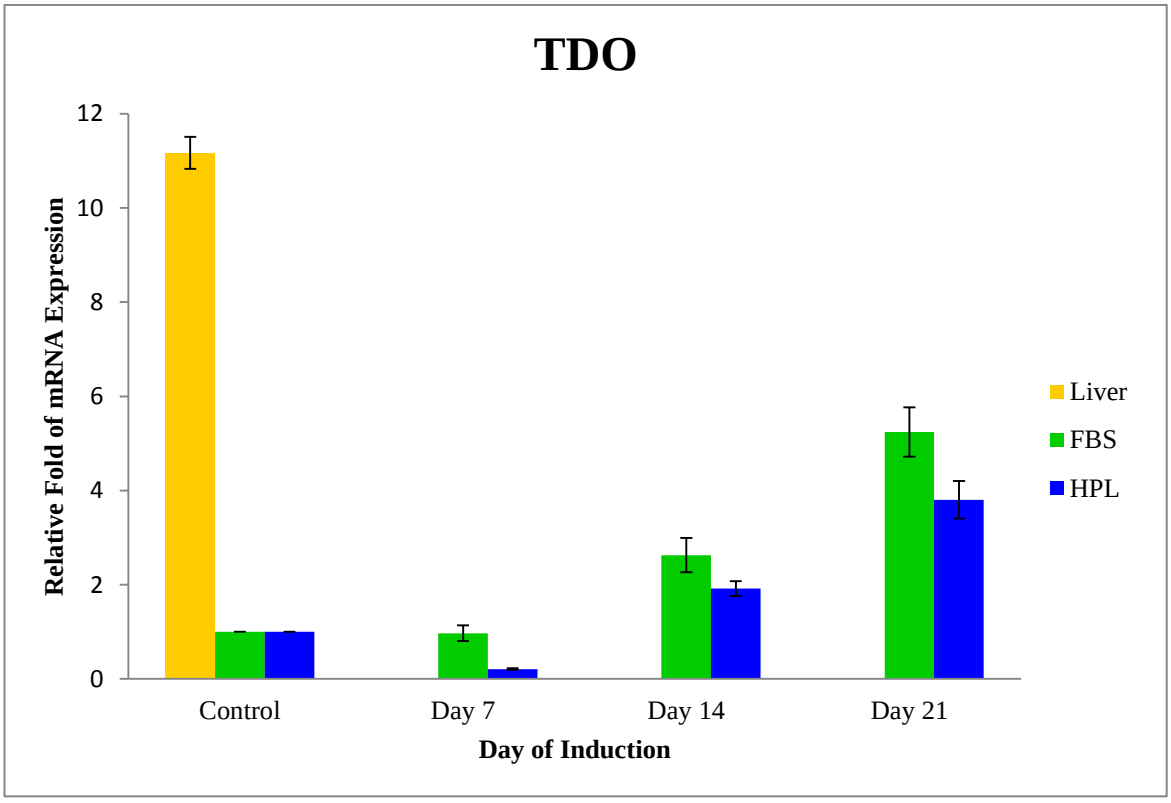

Figure 4.46: Expression of TDO in the SCD hepatocyte-like cells expanded in FBS and HPL.

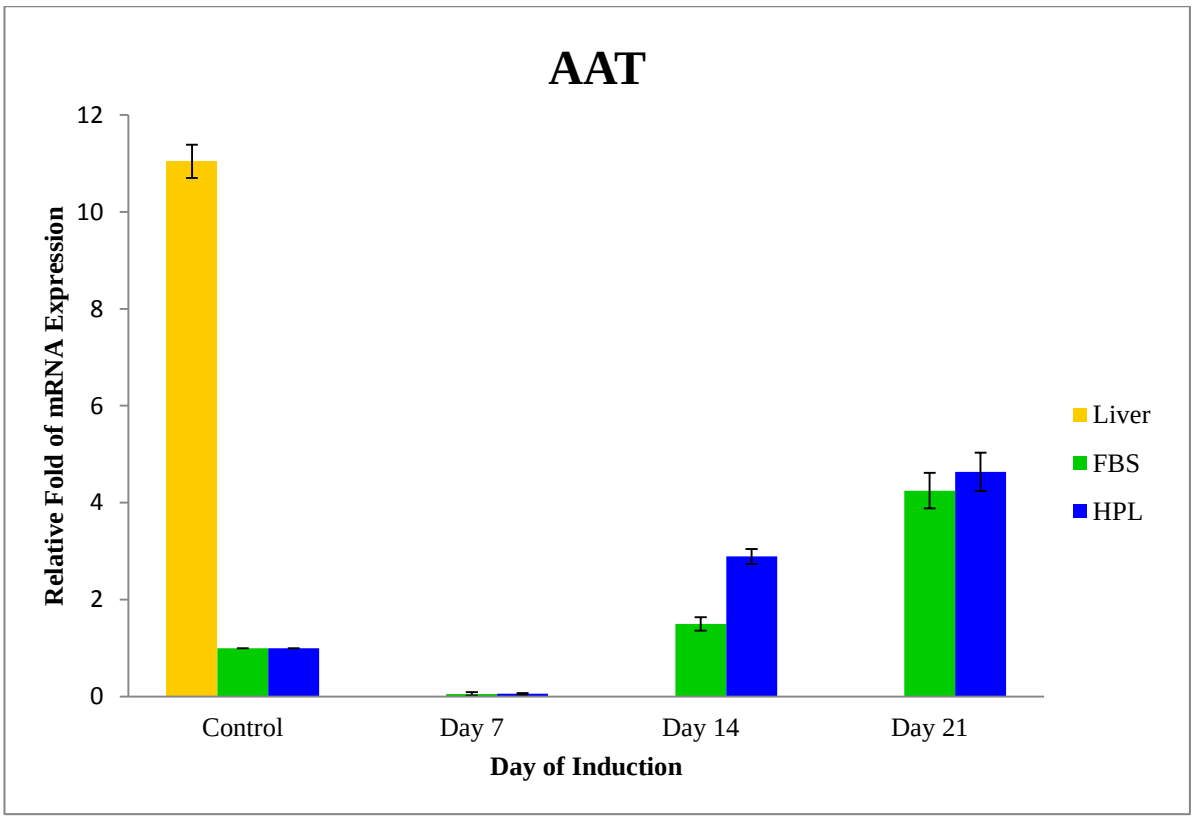

Figure 4.47: Expression of AAT in the SCD hepatocyte-like cells expanded in FBS and HPL.

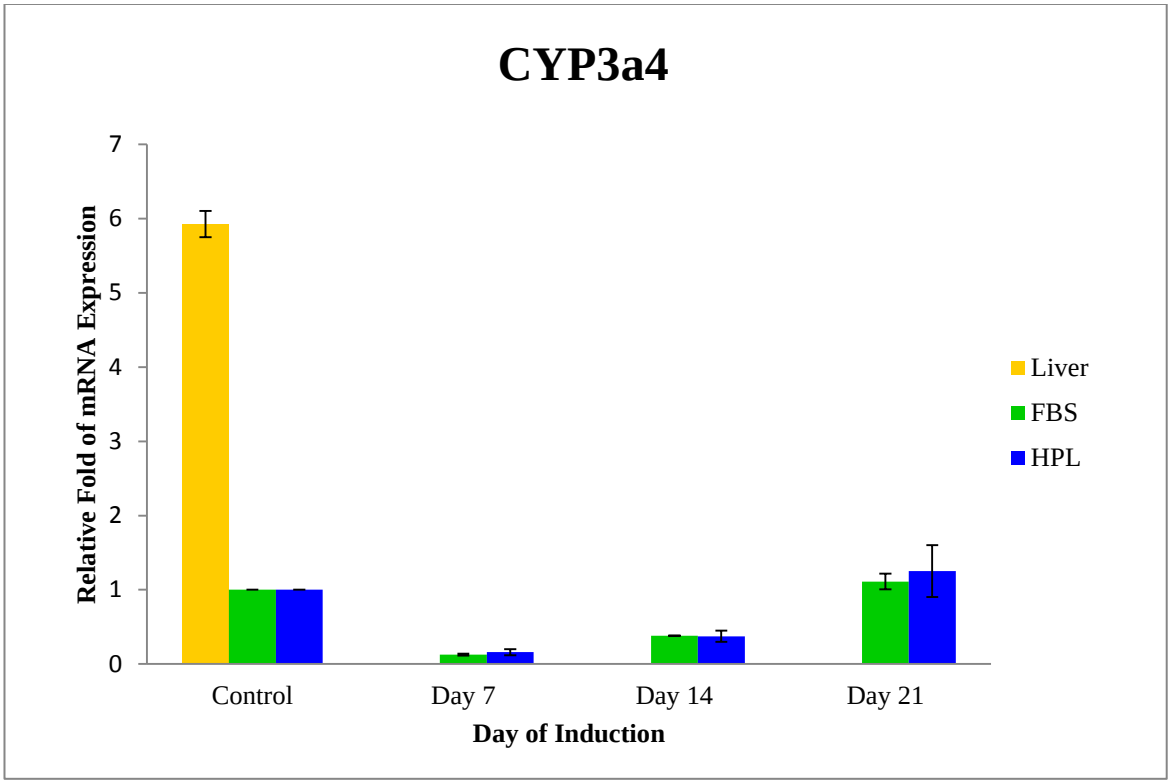

Figure 4.48: Expression of CYP3a4 in the SCD hepatocyte-like cells expanded in FBS and HPL.

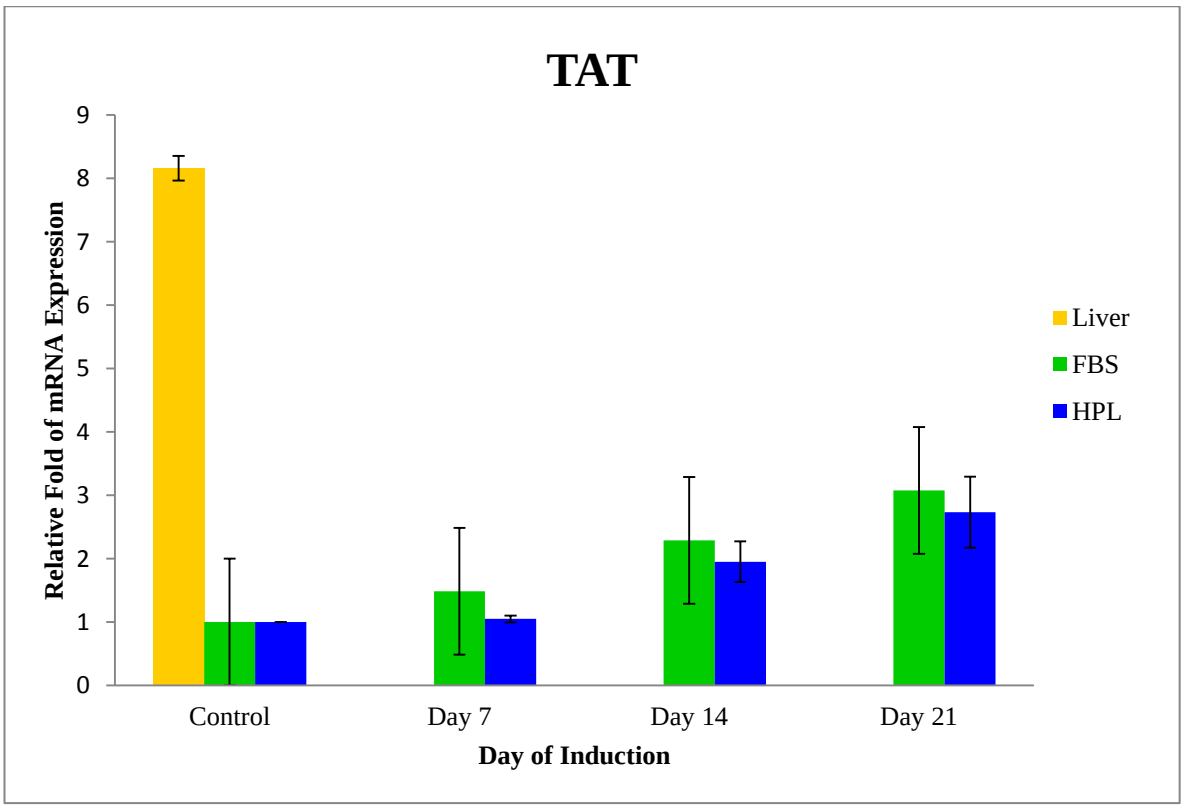

Figure 4.49: Expression of TAT in the SCD hepatocyte-like cells expanded in FBS and HPL.

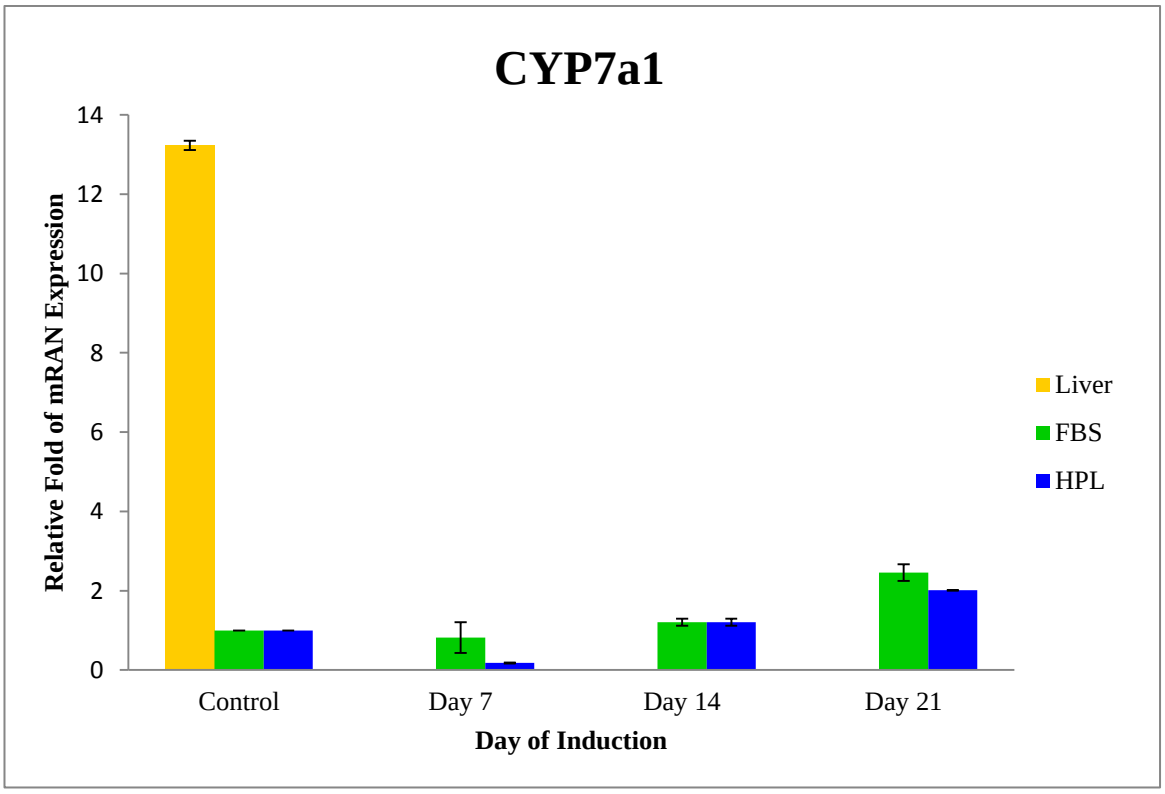

Figure 4.50: Expression of CYP7a1 in the SCD hepatocyte-like cells expanded in FBS and HPL.

4.16 Urea secretion of SCD hepatocyte-like cells

The urea secretion analysis is a functional evaluation of hepatocyte differentiation. The level of urea secretion of SCD hepatocyte-like cells cultured in FBS was higher (191.66 ± 2.08 ng/cell) compared to HPL (123.33 ± 2.51 ng/cell) at day 7. A gradual increase was observed at day 14 and day 21 for SCD hepatocyte-like cells cultured in both media (Figure 4.51). However, the SCD hepatocyte-like cells expanded in HPL showed lower urea production at day 14 (522.33 ± 1.15 ng/cell) and day 21 (794.66 ± 3.51 ng/cell) compared to FBS. All the data is in Appendix 2.8.

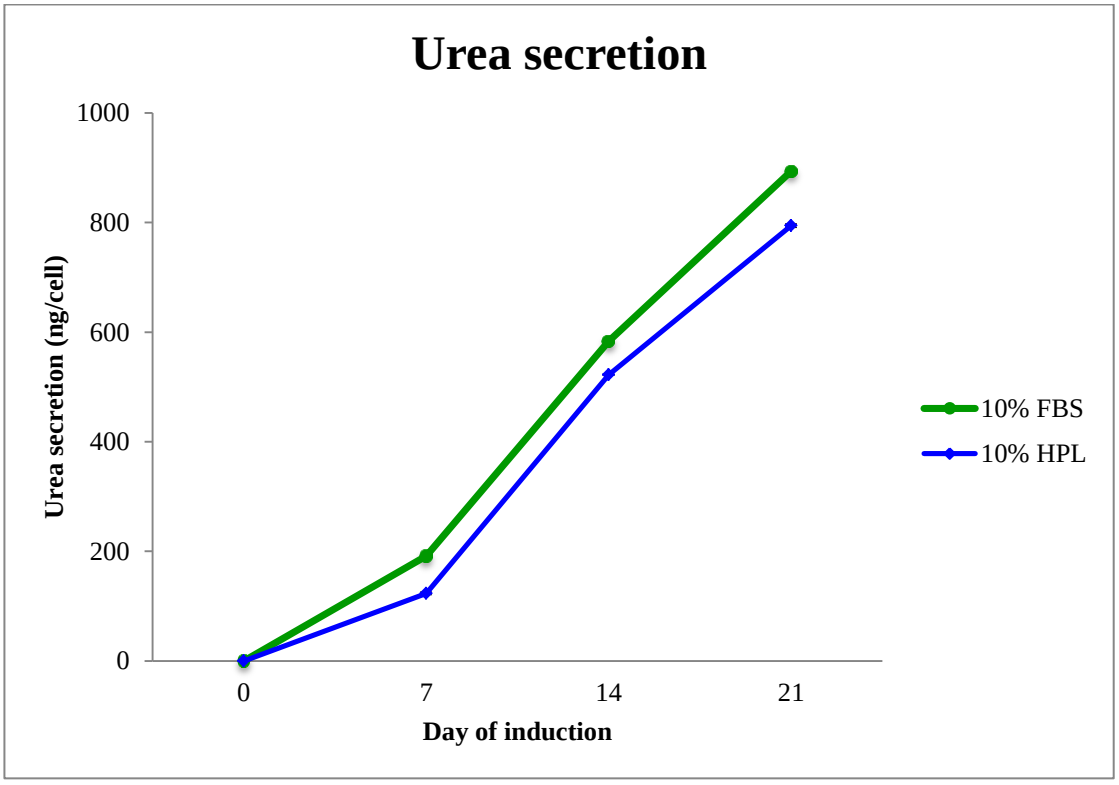

Figure 4.51: Urea secretion of SCD hepatocyte-like cells expanded in FBS and HPL.

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

This study was carried out with the objective to evaluate HPL as an alternative culture media to FBS for the cultivation of SCD in the aspect of proliferation rate, endodermal differentiation potential, immunophenotypic and immunocytochemistry analysis together with the gene expression. Human dental pulp stem cells from deciduous teeth (SCD) were successfully isolated (n=4). Dental pulp from deciduous teeth was chosen with the purpose of obtaining a very proliferative cell population (Miura et al., 2003) and considered to be strongly prepared for differentiation. Mohd Hilmi et al. (2008) suggested that since deciduous teeth were developed in intra-uterine and thus its pulp may contain very young stem cells which are capable to undergo multilineage differentiation. In addition, the potential of stem cells to proliferate and differentiate decline with age (D'Ippolito et al., 1999), thus justifying the selection of deciduous teeth as the source of stem cells for this study.

Most liver diseases can lead to the development of hepatocyte dysfunction with the possibility of the liver failure. Liver transplantation is an attractive therapeutic approach for patients suffering from liver conditions such as cirrhosis and hepatocellular carcinoma. However, liver transplantation has limitations such as shortage of suitable donor, possibility of rejection and maintenance of immunosuppressant (Ohashi et al. 2001; Najimi & Sokal, 2005). Furthermore, liver transplantation could cause considerable consequence to both the donor and the recipient (Kakinuma et al., 2009). Alternative treatments are actively searched and stem cells have become one of the promising sources for tissue regeneration and regenerative medicine.

The successful culture of mesenchymal stem cell (MSCs) is dependent on many factors and one of them is the culture condition. Culture condition is important especially during the initial derivation of MSCs. A major concern is the application of animal-based components which has the potential cross-species contamination including virus or protein transmission that may affect the culture of MSCs (Shahdadfar et al., 2005). One of the most commonly used culture medium is fetal bovine serum (FBS), a serum derived from calf fetus. The usage of FBS may potentially contribute to unknown pathogen into cell cultures, as it has high level of batch variation according to its nature (Gruen & Grabel, 2006). Furthermore, the FBS-cultured cells may contribute to immune-reactions problems when they are applied clinically (Horwitz et al., 2002). It has been suggested that MSCs culture and expansion requires suitable alternatives supplement to replace FBS in order to achieve good manufacturing practice (Preti, 2001). Therefore, this study aimed at evaluating human platelet lysate (HPL) as an alternative medium for stem cells culture followed by induction to hepatocyte-like cells.

The HPL was recently proposed as a suitable substitute for FBS in human MSCs expansion (Doucet et al., 2005; Capelli et al., 2007; Govindasamy et al., 2011). The application of HPL as a culture medium may provide promising features in the clinical situations due to the several reasons. According to Sugiyama et al. (2011), HPL is safe and easily-obtained source of serum since it can be harvested from the thrombocyte concentrates which are continuously produced and regularly applied in clinical treatment. Moreover, the utilization of HPL for cell culture is highly beneficial in terms of production cost compared to the other exogenous growth factors. It can also serve as a natural source of growth factors. Johansson et al. (2003) also suggested that HPL can offer substantial benefits for various applications in cell culture from a standpoint of performance, economy and product safety. They reported that the cost-benefit would be

favorable as HPL can be produced at a much lower cost which is 20-50% less than of FBS and yet it is able to retain similar performance as FBS.

In this study, the characterization of SCD cultured in FBS and HPL were examined through several ways including proliferation rate, multilineages differentiation and cell phenotype analysis. SCD in both media showed rapid initial growth, indicating easy adaptation to the new environment in the culture flask as they started to grow around the tissues after two days of isolation. It is important to note dissimilarities in morphology of SCD cultured in both media. SCD grown in HPL adopted a more spindled-shaped cells. Several studies had reported that the use of human serum triggered the production of smaller spindle shaped cells (Schallmoser et al., 2007; Mirabet et al., 2008). As cellular therapy become more established, the ability to expand large numbers of cells in HPL may become very useful. Therefore, this study was carried out to evaluate SCD proliferation rate in HPL compared to FBS. Overall, the results displayed a significantly higher proliferation rate of SCD expanded in HPL compared to FBS for population doubling and population doubling time ($p<0.05$). The same scenario was reported by Doucet et al. (2005) in the expansion of BMSCs. It has been already demonstrated that cultures using HPL displayed a significantly higher cell-proliferative rate compared to FBS in the culture of MSCs (Stute et al., 2004; Kocaomer et al., 2007). The reason for this different growth pattern can be explained through the abundance growth factors in HPL compared to FBS (Doucet et al., 2005; Lange et al., 2007). Concerns could be raised as high rate of proliferation could give rise to tumor formation. However, several studies had reported that MSCs cultured in HPL did not trigger tumor formation in nude mice under *in vivo* condition (Schallmoser et al., 2007). Moreover, the preliminary data from various researches also reported to have no predominant gross karyotyping lesion in MSCs cultured in HPL (Wang et al., 2005; Govindasamy et al., 2011). Chevallier et al. (2010) also testified that the higher

proliferation rate and the differences in cell morphology of MSCs cultured in HPL were not related to the abnormality in the c-myc and htert expressions which are the primary indicators used for the safety assessments of MSCs preparation. Thus, the use of HPL can be claimed to be a safe component for SCD expansion.

The expansion of SCD also demonstrated a higher number of CFU activities for SCD cultured in HPL medium, indicated by the presence of larger colonies compared to the SCD expanded in FBS. In the CFU experiment, the expansion capabilities of SCD cultured in HPL were higher and reaching twice as many cells compared to FBS. This distinct result may be due to the differences of cell morphology of SCD cultured in FBS compared to the HPL. The SCD expanded in HPL displayed elongated spindle-shaped cell and thus resulting in higher cell number per growth area compared to the FBS where the cells were thicker flattened in morphology.

Generally, MSCs will proliferate for a limited number of populations doubling depending on the type of cells and eventually enter into an arrested state, where cells remained alive however do not proliferate in response to the presence of mitogen. This Hayflick's limit phenomena was first described by Leonard Hayflick in 1960, where it reflected the aging process of the whole organism. Senescence cells can be characterized by their enlarged and irregular shape of cells (Wagner et al., 2008). In addition, the senescence MSCs may exibited lower potential of differentiation upon prolonged *in vitro* culture as described by previous studies (Banfi et al., 2000; Baxter et al., 2004; Bonab et al., 2006). The SCD cultured in HPL displayed lower level of senescence activity at passage 4 compared to FBS. This suggests the usage of HPL in SCD expansion media may lower the senescence activity in cells and therefore maintain the differentiation potential of SCD.

As recommended earlier by the International Society for Cell Therapy, a population of human MSCs must possess a specific phenotype profile (Dominici et al., 2006). The evaluation of surface antigens namely CD 34, CD 44, CD 45, CD 73, CD 90, CD 166, 7-AAD and HLA-DR by flow cytometry revealed that the phenotype of SCD expanded in FBS and HPL expressed > 92% of MSCs marker. Moreover, the SCD cultured in FBS and HPL expressed < 0.3% of the hematopoietic markers indicating that the SCD population in both media were not hematopoietic cells. This part of our study is in accordance with the data recently reported by others (Doucet et al., 2005; Capelli et al., 2007; Schallmoser et al., 2007).

The SCD expanded in HPL displayed similar potency to differentiate into adipogenic, chondrogenic and osteogenic. Positive staining results for trilineage differentiation proved that the usage of HPL does not affect the SCD potential to differentiate. Earlier studies also reported that MSCs expanded in HPL can maintain their capacity of multineages differentiation (Doucet et al., 2005; Bernardo et al., 2007). HPL not only contributed to elongated spindle-shaped cell but also encourage rapid self-renewal and higher clonogenicity with higher potential towards other lineages (Colter et al., 2000; Sekiya et al., 2002). Sankaranarayan et al. (2011) also stated that MSCs expanded in media supplemented with HPL presented a higher proliferative rate and maintained the capacity to differentiate while in their immuno-silent state.

The result of this study also revealed that there were no distinct differences in the RT-PCR and immunocytochemistry profile of SCD cultured in HPL and FBS. The SCD showed positive expression for the pluripotent stem cells markers (Oct 4, Sox 2, Nanog and SSEA4), ectoderm markers (Nestin, βIII Tubulin, MAP 2 and Vimentin), mesoderm markers (GATA 2, MSX 1, Hand 1 and BMP 4) and endoderm markers (HNF3β and AFP) . The expressions of Oct 4 and Nanog are crucial in most of the stem

cells as these marker are necessary to sustain the pluripotency and self-renewal of stem cells (Niwa et al., 2000; Carpenter et al., 2003). Sox 2 also play similar role in sustaining the stemness of the cells (Lengerke et al., 2011). The expression of SSEA4 is used to identify the MSCs population and mark human embryonic specifically (Gang et al., 2007). Nestin and Vimentin are classified as one of the intermediate filament that can be detect in stem cells in central nervous system (CNS) of human and rat (Messam et al., 2000). Moreover, βIII Tubulin and MAP 2 have been widely used as the neuronal markers for mouse and human stem cells (Moe et al., 2005). The expression of mesoderm markers indicated that SCD cultured in both media have potential to form mesoderm lineages. GATA 2 and MSX 1 are early markers for hematopoietic mesoderm and they play important roles during the vertebrate development (Kelly et al., 1994; Zhang et al., 2003). In addition, several studies have revealed critical roles of Hand 1 in the formation and development of placenta, heart and neural crest-derivative (Riley et al., 1998; Morikawa & Cserjesi, 2004).

HPL as an alternative medium proved to have a good effect on MSCs growth rate, differentiation and cellular phenotype. Thus, the data for characterization of SCD expanded in FBS and HPL suggested that SCD expanded in both media displayed a very similar phenotype and characteristics. Although there may be some dissimilarity in the other properties, this has yet to be investigated. This study only focused on the characterization of SCD cultured in FBS and HPL. However, further investigation is needed to gain better understanding on the effect of HPL to the SCD cultivation especially from the immunoregulatory aspect. Immunoregulatory property is a major aspect for clinical application and the exploration in this area may propose a good model for human MSCs expansion using HPL.

The study of SCD cultured in HPL and FBS was further continued with the differentiation of SCD into hepatocyte lineage. This approach was used to evaluate the capacity of SCD to support hepatocyte differentiation following expansion in HPL compared to FBS. According to Behbahan et al. (2011), a consensus has not yet been reached for compulsory characterization protocol of hepatocyte-like cells derived from stem cells. In general, hepatocyte-like cells could be identified from their morphological appearance, expression of hepatocyte specific markers and functional abilities (Synkers et al., 2009).

The entire components used in the hepatocyte induction media were selected carefully as it may affect the outcome of this study. According to Snykers et al. (2009), there were several methods that can be used to induce hepatocyte differentiation and the addition of hepatocyte growth factor (HGF) proved to be effective in developing expression of specific hepatocyte markers. Furthermore, HGF also has been listed as a regulator of hepatoblast bipotency (Suzuki et al., 2002; 2003). Dexamethasone is one of the crucial component in the hepatocyte induction media as it is based on a non-proteinaceous chemical compounds that is active in the induction of gluconeogenesis enzyme in the liver (Michalopoulos et al., 2003). Previous studies reported that dexamethasone significantly influenced hepatocyte differentiation of mouse embryonic stem cells (mES) (Yin et al., 2008). Moreover, the use of dexamethasone may increase the expression of specific hepatocyte markers and may affect the cell morphology (Arterburn et al., 1995; Yin et al., 2008). Oncostatin M (OsM) was also added into the hepatocyte induction media to promote the *in vitro* maturation process of SCD as reported by others (Kamiya & Gonzalez, 2004; Istikivies et al., 2010). According to Ochiya et al. (2010), the OsM was needed to synegize with HNF4α for hepatocyte maturation.

In this study, SCD cultured in FBS and HPL showed morphological transformation from fibroblastic-like shape to polygonal shape which confirms to one of the hepatocyte characteristics. Binucleation was also observed in the polygonal shape of SCD hepatocyte-like cells cultured in both media. Behbahan et al. (2011) stated that the appearance of polygonal shaped cells with multiple nuclei is indicative of hepatocyte cells. Previous studies reported that the round nuclei can be observed under transmission electron microscopy with evenly distributed chromatin and Golgi complex as well as fine developed bile canaliculi are considered to be the characteristics of mature hepatocytes (Bahanvard et al., 2008; Basma et al., 2009). Although the cellular mechanism that governs the passage from mononucleated MSCs to binucleated hepatocyte remains obscure, recent studies suggest that the hepatocyte binucleation could be a result of the cell exit from mitosis due to the absence or abortion of cytokinesis (Guidotti et al., 2003; Alison et al., 2004).

Hepatocellular function of SCD hepatocyte-like cells cultured in FBS and HPL were detected by using periodic acid Schiff (PAS) staining. The SCD hepatocyte-like cells in both media showed increased amount of purple stained cell from day 7 to day 21. This qualitative result suggests that the glycogen storage in the SCD hepatocyte-like cells increasing proportionally with the day of hepatocyte induction. The amount of purple stained cells was higher at day 21 as there were more mature SCD hepatocyte-like cells present with higher glycogen storage. SCD in FBS and HPL demonstrated similar positive results in PAS staining at day 21. The assessment of glycogen storage by PAS staining is necessary to confirm mature hepatocyte in hepatocyte differentiated (Baharvan et al., 2008; Duan et al., 2010).

The morphological observations of SCD hepatocyte-like cells cultured in HPL and FBS were further validated using hepatocyte specific markers expression. All tests

to detect the expression of hepatocyte specific marker were done using pooled SCD hepatocyte-like cells. This step has been proposed to be able to overcome the donor-to-donor variability and to offer consistent population of MSCs (Govindasamy et al., 2011). Although pooled cells could provide a reliable average cell population, in long term, cells from one donor with higher proliferation rate may become predominant and thus gave more optimal results (Chase et al., 2010).

The result showed high expression of Sox 17 at the early phase of hepatocyte induction. Sox 17 is recognized as a marker for endoderm lineages (D'Amour et al., 2005; Synkers et al., 2009). Early hepatocyte differentiation has also been commonly evaluated by the expression of HNF3β and GATA 4. Hepatocyte nuclear factors (HNF) have been revealed to be a fundamental transcription factor for liver development during mouse embryogenesis (Watt et al., 2003). Among this group, HNF3β is the key marker expressed during endoderm differentiation (Levinson & Benvenisty, 1997; Ishizaka et al., 2002; Yamamoto et al., 2005) and this was observed on day 7. The expression of HNF4α increased from day 7 to day 21 as expected for hepatocyte differention. HNF4α is expressed in the hepatic diverticulum (Duncan et al., 1994) and it plays an important role in hepatocyte differentiation and liver gene expression (Khurana et al., 2009). GATA 4 has been localized in the developing mammalian liver and gastrointestinal tract (Divine et al., 2004) thus high expressions of this markers in the early phase of hepatocyte induction and later decrease at the maturation phase was observed in this study. The expression of alpha-fetoprotein (AFP) also showed gradual decrease from day 7 to day 21 in the immunocytochemistry analysis and real-time PCR quantification. This result is in agreement with previous reported studies (Kazemnejad et al., 2008; Zemel et al., 2009; Ochiya et al., 2010). AFP is the earliest hepatocyte specific marker that can be detect at early phase of hepatocyte differentiation (Agarwal et al., 2008; Zemel et al., 2009). According to Ochiya et al. (2010), although AFP

expressed in the early stage of hepatocyte differentiation, it decreases immediately after birth and the total amount of mRNA expressed is identified to be less compared to the fetal period.

Cytokeratin 19 (CK 19) is a cell marker for bile duct epithelial cells and hepatic progenitor cell (Petersen et al., 1998; Theise et al., 1999; Funakoshi et al., 2011). In this study, the expression of CK 19 showed gradual increase from the early induction until the maturation phase in immunocytochemistry analysis, immunophenotype analysis and real-time PCR quantification. The similar pattern of expression was observed with the albumin and Cytokeratin 18 (CK 18). Albumin is a specific hepatocyte marker and can be categorized as the major protein in plasma and manufactured by hepatocyte cells (Cai et al., 2007; Yin et al., 2008). Albumin plays a crucial role in promoting the development and formation of complexes and improves their transportation in plasma (Yin et al., 2008). The expression of albumin in the immunocytochemistry, immunophenotype profile and in mRNA level indicated the maturation of SCD hepatocyte-like cells cultured in FBS and HPL.

The expression of hepatocyte marker namely HNF4α (FBS: 98.06 ± 0.37; HPL: 97.76 ± 0.04), CK 19 (FBS: 87.07 ± 1.62; HPL: 89.92 ± 3.52), albumin (FBS: 90.71 ± 0.83; HPL: 92.82 ± 0.81) and CK 18 (FBS: 96.07 ± 0.51; HPL: 97.93 ± 1.17) at day 21 of the immunophenotype analysis appear to be highly expressed compared to hepatocyte differentiated from ESCs (Si-Tayeb et al., 2010). It would have been expected that SCD hepatocyte-like cells to have lower expression of these markers as they are of ectoderm in origin. At this junction, no explaination could be offered and further studies should be conducted. However, one limitation that has been identified in the immunophenotype analysis is the absent of control group, which is liver cells. Within the time frame of this study, human hepatocyte cells were unattainable.

SCD hepatocyte-like cells in FBS and HPL were also defined by the expression of specific hepatocyte late marker such as tryptophan-oxygenase (TO), tyrosine amino-transferase (TAT), tryptophan 2,3-dioxygenase (TDO) and specific cytochrome (CYP) family (Synkers et al., 2009). CYPs family has mixed monooxygenases function and has been categorized as a major phase I enzyme. CYP3a4 is a marker involved in the metabolism of xenobiotics (Song et al., 2009). CYP7a1 is not expressed in fetal liver thus it is considered as a hepatocyte maturation marker (Funakoshi et al., 2011). Mature hepatocytes also have a role in the amino acid metabolism and plasma protein secretion. The presence of TO and TAT expression in the SCD hepatocyte-like cells cultured in FBS and HPL showed that they have potential to undergo amino acid metabolism while expression of AAT represent the ability to secrete plasma protein.

The successful differentiation into hepatic lineages of SCD cultured in FBS and HPL was also confirmed using urea secretion assay. The urea production assay was carried out to ascertain cellular function of the SCD hepatocyte-like cells. The result revealed that SCD differentiated in presence of HPL produced lower levels of urea when compared to those obtained in FBS medium. This may be due to lack of matured SCD hepatocyte-like cells when cultured in HPL. This *in vitro* hepatocyte differentiation was performed according to the condition optimized for SCD cultured in FBS. Therefore, this aspect may contribute to the lack of matured SCD hepatocyte-like cells in HPL and thus resulting lower level of urea production. Total number of matured hepatocyte cells present in the tested population is important for urea secretion assay as only matured hepatocyte cell are able to produce urea (Kazemnajad et al., 2008; Shiota & Yasui, 2012). Further study has to be conducted to optimized the induction media for hepatocyte differentiation in HPL as Nimura et al. (2008) also reported that MSCs expanded in human serum may need different condition for optimal *in vitro* differentiation.

In general, the results of this study presented that the HPL is a potentially effective medium to increase growth rate of SCD. Interestingly, HPL stimulated high proliferation of MSCs and at the same time able to maintain their trilineage differentiation potential and immunophenotypic characteristics, and the data concurred with that reported by Doucet et al. (2005). Moreover, the results in this study also demonstrated the potential of SCD to differentiate into hepatocyte lineage in HPL, an animal-free serum. Further investigation is needed to explore and understand the significant components in HPL when used for hepatocyte differentiation of SCD. Furthermore, *in vivo* study is also essential to ascertain if SCD hepatocyte-like cells can be functional and have a positive effect to liver when applied clinically. Knowledge in molecular mechanisms of endodermal differentiation for dental stem cells are also lacking and has not been fully explored. A more detailed insight into the molecular pathway for endodermal differentiation in dental stem cells is a very crucial aspect and needs to be explored to ensure that any differentiated cells can be applied clinically.

CHAPTER 6

CONCLUSION

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Within the limitation of this study, the following conclusions can be drawn;

1. The proliferation rate of SCD cultured in HPL is statistically higher compared to FBS.
2. SCD cultured in HPL also exhibited similar characteristics to those in FBS.
3. SCD cultured in both media displayed ability to differentiate into hepatocyte-like cells when induced in hepatocyte induction media.
4. SCD hepatocyte-like cells from both media were able to expressed hepatocyte specific markers.