ผลของลิโพพอลิแซ็กคาไรด์ต่อการเพิ่มจำนวนและการแปรสภาพไปเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดจากปุ่มเนื้อปลายรากฟันที่มีปริมาณการแสดงออกของซีดี 24 ที่แตกต่างกัน

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EFFECT OF LIPOPOLYSACCHARIDE ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF STEM CELLS FROM APICAL PAPILLA WITH DIFFERENT AMOUNT OF CD24 EXPRESSING CELLS

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ภาณุรุจ อากิลาร์ : ผลของลิโพพอลิแซ็กคาไรด์ต่อการเพิ่มจำนวนและการแปรสภาพไปเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดจากปุ่มเนื้อปลายรากฟันที่มีปริมาณการแสดงออกของซีดี 24 ที่แตกต่างกัน (EFFECT OF LIPOPOLYSACCHARIDE ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF STEM CELLS FROM APICAL PAPILLA WITH DIFFERENT AMOUNT OF CD24 EXPRESSING CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพ. ดร.วีระ เลิศจิราการ, 159 หน้า.

มีความเชื่อว่าเซลล์ต้นกำเนิดปุ่มเนื้อปลายรากฟันเป็นเซลล์หลักที่ทำหน้าที่ในการรักษาด้วยวิธีการคืนสภาพทางวิทยาเอ็นโดดอนต์ อย่างไรก็ตามพบว่าเนื้อเยื่อแข็งที่สร้างใหม่ในการรักษาดังกล่าวมีลักษณะคล้ายกระดูกหรือเคลือบรากฟัน ดังนั้นจึงเป็นไปได้ว่าการติดเชื้อภายในคลองรากฟันอาจมีผลต่อการเปลี่ยนสภาพของเซลล์ดังกล่าวไปเป็นเซลล์ที่สร้างเนื้อเยื่อคล้ายกระดูกหรือเคลือบรากฟันแทน ลิโพพอลิแซ็กคาไรด์เป็นสารที่ออกมาจากจุลชีพภายในคลองรากฟันที่ติดเชื้อและสามารถแพร่ออกมาสู่เนื้อเยื่อรอบปลายรากได้ ดังนั้นลิโพพอลิแซ็กคาไรด์อาจมีผลในการปลี่ยนสภาพของเซลล์ดังกล่าว อนึ่ง ซีดี24 ซึ่งพบการแสดงออกบนเซลล์ดังกล่าวอาจมีความเกี่ยวข้องกับการควบคุมพฤติกรรมในทางชีววิทยาของเซลล์ต้นกำเนิด เช่น การเพิ่มจำนวนเซลล์ การจำลองตนเอง และการเปลี่ยนสภาพ เนื่องจากปริมาณร้อยละของซีดี24ที่แสดงออกมีช่วงห่างระหว่างการศึกษามาก ดังนั้น ซีดี24ก็อาจมีบทบาทในวิธีการคืนสภาพทางวิทยาเอ็นโดดอนต์ด้วยได้นอกเหนือไปจากลิโพพอลิแซ็กคาไรด์ การศึกษานี้ได้ยืนยันความแปรผันของจำนวนร้อยละของซีดี 24 ในเซลล์ต้นกำเนิดปุ่มปลายรากฟัน โดยได้แบ่งกลุ่มเซลล์ที่คัดแยกได้จากตัวอย่างออกเป็นกลุ่มที่มีการแสดงออกซีดี24สูงและต่ำ พบว่าทั้งกลุ่มที่มีการแสดงออกของซีดี24สูงและต่ำนั้นมีการแสดงออกของตัวบ่งชี้ความเป็นเซลล์ต้นกำเนิดมีเซนไคม์และความสามารถในการเพิ่มจำนวนไม่แตกต่างกัน แต่กลุ่มที่มีการแสดงออกของซีดี24สูงมีคุณสมบัติการเปลี่ยนสภาพของเซลล์ได้ดีกว่า ในทางตรงข้ามกลุ่มที่มีการแสดงออกของซีดี24ต่ำ กลับมีคุณสมบัติในการจำลองตนเองดีกว่า นอกจากนี้ยังพบว่าระดับการพัฒนาของรากฟันในช่วงต้นนั้นเซลล์มีการแสดงออกร้อยละของซีดี24สูงกว่าเซลล์ที่ได้มาจากตัวอย่างที่มีการพัฒนารากในช่วงปลาย คุณสมบัติของเซลล์ต้นกำเนิดในการเพิ่มจำนวนเซลล์และการเปลี่ยนสภาพไปเป็นเซลล์สร้างเนื้อเยื่อแข็งมีความสำคัญในกระบวนการคืนสภาพทางวิทยาเอ็นโดดอนต์ ซึ่งเซลล์เหล่านี้ภายหลังการเผยลิโพพอลิแซ็กคาไรด์ความเข้มข้น 0.001 ถึง 5 ไมโครกรัม/มิลลิลิตร พบว่าไม่มีการเปลี่ยนแปลงต่อการเพิ่มจำนวนเซลล์ทั้งในกลุ่มที่มีการแสดงออกของซีดี24สูงและต่ำ แต่มีผลในการตกตะกอนของเนื้อเยื่อแข็งของกลุ่มที่มีการแสดงออกของซีดี24สูง โดยลิโพพอลิแซ็กคาไรด์ 1 และ 5 ไมโครกรัม/มิลลิลิตร มีการตกตะกอนได้มากกว่ากลุ่มควบคุม นอกจากนี้ในกลุ่มของเซลล์ที่มีการแสดงออกของซีดี24สูงมีการแสดงออกของยีนโบนไซอะโลโปรตีนที่พบมากในกระดูกและเคลือบรากฟัน มีการแสดงออกมากกว่าอย่างมีนัยสำคัญที่ความเข้มข้น 1 และ 5 ไมโครกรัม/มิลลิลิตร เมื่อเทียบกับกลุ่มควบคุม ในขณะเดียวกันลิโพพอลิแซ็กคาไรด์ไม่มีผลต่อการตกตะกอนของกลุ่มที่มีการแสดงออกของซีดี24ต่ำเมื่อเทียบกับกลุ่มควบคุม แต่พบว่าลิโพพอลิแซ็กคาไรด์ความเข้มข้น 5 ไมโครกรัม/มิลลิลิตร สามารถเพิ่มการแสดงออกของยีนโบนไซอะโลโปรตีนได้มากกว่ากลุ่มควบคุม การแสดงออกของเดนทีนไซอะโลฟอสโฟโปรตีนซึ่งเป็นตัวบ่งชี้สำหรับเนื้อฟันหรือเซลล์สร้างเนื้อฟันนั้นไม่มีความแตกต่างกันระหว่างกลุ่มควบคุมและกลุ่มที่เผยต่อลิโพพอลิแซ็กคาไรด์ทั้งเซลล์ที่มีการแสดงออกของซีดี24สูงและต่ำ ดังนั้นการศึกษานี้จึงใช้อธิบายผลการศึกษาทั้งในสัตว์ทดลองและผู้ป่วยจริงที่พบว่าการสร้างเนื้อเยื่อใหม่ด้วยวิธีการคืนสภาพทางวิทยาเอ็นโดดอนต์ได้เนื้อเยื่อแข็งที่มีลักษณะคล้ายกระดูกหรือเคลือบรากฟัน

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5476054232 : MAJOR ORAL BIOLOGY
KEYWORDS: SCAPS / CD24 / LIPOPOLYSACCHARIDE / CELL PROLIFERATION / OSTEOGENIC DIFFERENTIATION

PANUROOT AGUILAR: EFFECT OF LIPOPOLYSACCHARIDE ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF STEM CELLS FROM APICAL PAPILLA WITH DIFFERENT AMOUNT OF CD24 EXPRESSING CELLS. ADVISOR: ASSOC. PROF. VEERA LERTCHIRAKARN, Ph.D., 159 pp.

Stem cells from apical papilla (SCAPs) were believed the source of regenerative endodontic procedure (REP) that is performed in infected immature tooth. However, the new hard tissues were reported to be bone or cementum-like structure. It was possible that root canal infection might alter SCAPs to generate bone or cementum-like structure rather than dentin. This might due to the effects of lipopolysaccharide (LPS) in infected root canal. In addition, the percentages of CD24 expressing SCAPs varied among the studies. CD24 involved many cellular activities such as cell proliferation, differentiation and self-renewal of stem cells. Therefore, CD24 might affect to REP, as well. In this thesis, the variation of CD24 expression was confirmed. To facilitate the investigation of amount of CD24, SCAPs were classified into two groups, High and Low-CD24. The mesenchymal stem cell markers and cell proliferation were not different between two groups. However, High-CD24 demonstrated lower self-renewal than Low-CD24 but higher multi-differentiation capacity than Low-CD24. High-CD24 associated with early root formation while Low-CD24 related to later stage of root formation. After exposure with 0.001-5μg/ml of LPS, cell proliferation and migration of all experimental groups were not different from their control groups of both High and Low-CD24. However, High-CD24 significantly increased mineralization activity when exposed to 1 and 5 μg/ml of LPS. Moreover, bone sialopreotein (BSP) gene expression that extensively expressed in bone and cementum, significantly increased when exposed to 1 and 5 μg/ml of LPS. Low-CD24 did not alter mineralization activity when compared with non-exposure LPS group. However, the BSP gene expression increased only when compared with 5 μg/ml exposure. Dentin sialophosphoprotein (DSPP) gene expression was not different between LPS pre-exposure and control group of both High and Low-CD24. The results from this study may explain why the bone or cementum-like structure was formed by SCAPs after performing REP.

Field of Study: Oral Biology Student's Signature .....

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ALP	alkaline phosphatase
Alpha-MEM	alpha minimum essential medium
AP	apical papilla
bFGF	basic fibroblast growth factor
BMSCs	bone marrow stem cells
BSP	bone sialoprotein
CAP	cementum attachment protein
CD	cluster of differentiation
CFU	colony forming unit
CO_2	carbon dioxide
CXCR4	C-X-C chemokine receptor 4
DAB	3,3’-diaminobenzinidine
DFSCs	dental follicle stem cells
DMP1	dentin matrix protein 1
DPP	dentin phosphoprotein
DSP	dentin sialoprotein
DSPP	dentin sialophosphoprotein
E.coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
ESCs	embryonic stem cells
FA	fluocinolone acetonide
GAPDH	glyceraldehyde phosphate dehydrogenase

GLA-protein	gamma-carboxyglutamic protein
GPI	glycosylphosphatidylinositol
HERS	Hertwig's epithelial root sheath
HLA-DR	human leukocyte antigen D related
HRP	horseradish peroxidase
IBMX	isobutylmethylxanthine
IgG	immunoglobulin
IL	interleukin
LAL	limulus ameobocyte lysate
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MTA	mineral trioxide aggregate
MSCs	mesenchymal stem cells
NANOG	nanog homeobox
NFIC	nuclear factor I C
OCN	osteocalcin
OCT4	octamer-binding transcription factor 4
OPN	osteopontin
p38	protein 38
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDLSCs	periodontal ligament stem cells
PG	*Porphyromonas gingivalis*

RNA	ribonucleic acid
REP	regenerative endodontic procedure
RUNX2	runt-related transcription factor 2
SCAPs	stem cells from apical papilla
SDF-1	stromal cell-derived factor 1
SHEDs	shedding human exfoliated deciduous teeth
SOX2	sex determine region Y-box 2
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
Wnt	wingless-type MMTV integration site

CHAPTER 1
INTRODUCTION

Regenerative endodontic procedure or REP is the biological based procedure that can re-establish the continuing tooth development both increasing root dentin wall thickness and root length of necrotic immature tooth (Hargreaves *et al*., 2013; Murray *et al*., 2007). Stem cells from apical papilla or SCAPs were believed as a source of stem cells for this treatment (Ding *et al*., 2009; Huang *et al*., 2008; Sonoyama *et al*., 2006). After homing SCAPs into the root canal spaces, cells must proliferate into proper numbers before undergoing to differentiation (Smith *et al*., 2016). Therefore, dentin is expected to re-establish and continue the root development process until reaching its full length. However, human teeth (Lei *et al*., 2015; Shimizu *et al*., 2013) and animal studies (Thibodeau *et al*., 2007; Yamauchi *et al*., 2011) demonstrated bone or cementum-liked tissue in the root canal space instead of dentin. Although these structures occurred, the clinical outcome was claimed as a successful treatment if periapical periodontitis had been resolved (Bose *et al*., 2009; Jeeruphan *et al*., 2012; Kahler *et al*., 2014; Nagata *et al*., 2014).

The occurrence of these regenerative structures was suggested as the results of the stem cells recruitment from the other sources of stem cells such as dental follicle, periodontal ligament and osteoblasts into the root canal (Huang and Garcia-Godoy, 2014). The other possibility is SCAPs may alter their phenotype from dentin to bone or cementum after challenging with the infection. The recently animal studies demonstrated viability of SCAPs after experimental pulpal space infection (Tobias Duarte *et al*., 2014; Yoo *et al*., 2016). Interestingly, these cells enhanced mineralization

capacity and cell proliferation when compared to unaffected teeth (Yoo *et al*., 2016). The *DSPP*, the dentin specific marker, was increased but the other gene or protein expressions that specific to cementum or bone were not investigated. Thus, the consequence of long-term root canal infection on SCAPs's differentiation phenotype is still unknown.

Gram-negative bacteria species were the most common microorganisms in necrotic pulp (Siqueira *et al*., 2002). The continuous influx of the cell wall component of this bacteria, lipopolysaccharide or LPS, can cause the apical periodontitis. LPS has been demonstrated its effects on biological responses of SCAPs by releasing various cytokines such as IL-6, IL-8 and TNF-α (Zhang *et al*., 2013). The alteration of mineralization due to LPS exposure in other dental stem cells such as dental pulp, periodontal ligament and dental follicle were reported (Kato *et al*., 2014; Morsczeck *et al*., 2012; Yamagishi *et al*., 2011). In addition, some of these studies also showed the effect of LPS to cell proliferation (He *et al*., 2015; Kato *et al*., 2014). However, the effects of LPS to SCAPs proliferation and osteogenic differentiation have never been investigated.

Cluster of differentiation 24 or CD24 was demonstrated to express in SCAPs while stem cells from other origin such as dental pulp stem cells did not express (Osathanon *et al*., 2014; Sonoyama *et al*., 2006). Therefore, CD24 was suggested to use as a specific marker for SCAPs more than a decade (Sonoyama *et al*., 2006). CD24 involves in many cellular activities such as cell migration/invasion, cell apoptosis, cell proliferation and differentiation (Baumann *et al*., 2005; Fang *et al*., 2010; Kim *et al*., 2008). The function of CD24 in SCAPs is still unclear. It might involve in the

osteogenic differentiation process (Sonoyama *et al*., 2006). However, CD24 has been demonstrated lower number of positive cell and shown the wide range percentage of positive cells among the studies (3% - 20%). In contrast, the other surface mesenchymal stem cell markers such as CD90, CD44, CD105 and CD73 extensively expressed constantly between 60%-99% of positive cells (Bakopoulou *et al*., 2013; Schneider *et al*., 2014; Sonoyama *et al*., 2006; Zhang *et al*., 2014). Recent studies suggested that CD24 might indicate the committed progenitor cells or advance stage of differentiation (Chang *et al*., 2015; Rodeheffer *et al*., 2008). Therefore, the different amount of CD24 expressing cells may influence to SCAPs behavior especially, cell differentiation, self-renewal and proliferation. Moreover, the cause of different percentages of CD24 expressing cells among the studies is still unknown.

Both CD24 and LPS may have the interaction effects on SCAPs behaviors especially, cell proliferation and osteogenic differentiation that are crucial for REP. Thus, the objective of this study was to investigate the effects of LPS and amount of indigenous CD24 expressing SCAPs on SCAPs proliferation and osteogenic differentiation. This knowledge might explain the repairing or results of REP.

CHAPTER 2
REVIEW LITERATURE

2.1 Root dentinogenesis

Root dentin formation begins when inner and outer epithelial cells fuse together (Bosshardt *et al*., 2015; Sakano *et al*., 2013). This double layer of epithelium structure calls Hertwig's epithelial root sheath (HERS). Generally, the root dentin formation uses the same interaction between epithelial cells and mesenchymal cells underneath HERS (Huang and Chai, 2012; Thesleff, 2003; Tummers and Thesleff, 2009). HERS releases growth factors to mesenchymal cells. Then, the signals between HERS and mesenchymal cells are reciprocately released (Huang and Chai, 2012). The growth factors and their receptors strictly function in concentration and time dependent (Thesleff, 2003) otherwise malformation of root will be occurred (Luder, 2015; Wright, 2007). HERS will detach after odontoblasts differentiate. Some dental follicles cells are recruited to outer root dentin and differentiate to be cementoblasts to generate cementum and periodontal ligament cell (Nanci, 2008).

2.1.1 Odontoblast differentiation and markers

Mesenchymal cells, underlying the inner enamel epithelium and HERS, develop to be odontoblasts and dental pulp cells. These mature odontoblasts response to generate dentin (Thomas, 1995). Recent studies showed the mesenchymal cells that became to odontoblasts were heterogeneous origins which derived from different cell sources (Kaukua *et al*., 2014; Ono *et al*., 2016). Regardless to their origins, odontoblast differentiation process is the same. The odontoblast progenitors will reorganize the

intracellular organelles and nucleus for extracellular matrix secretion after receiving the proper signals between HERS and mesenchymal cells interaction. The cell morphology also changes into cuboidal shape (Lesot, 2000; Ruch *et al*., 1995). The most abandon dentin matrix is collagen type I which serves as the template for dentin mineralization. Then, the other dentin matrix proteins such as phosphoproteins, glycoproteins and other type of collagens are synthesized for regulation of proper root dentin mineralization (Boskey *et al*., 2000; Butler *et al*., 2002; Kawashima and Okiji, 2016).

Dentin shows unique structure that the tubular calcified structure covers only the process of odontoblast while bone and cementum contains the whole cell's body inside calcified matrix (Nanci, 2008). Even though the extracellular matrixes from calcified tissues are the same among dentin, bone and cementum, the level or amount of expression and matrix orientation are different (Butler *et al*., 2003). Extracellular matrix proteins are different between dentin and bone or cementum. Dentin phosprotein (DSP) and dentin phosphorin (DPP) widely express in dentin more than in bone or cementum (Baba *et al*., 2004; Qin *et al*., 2003). Thus, they are used as the dentin specific markers for several decades. These two proteins are translated from dentin sialophosphoprotein or *DSPP* gene (Prasad *et al*., 2010). To identify odontoblast differentiation, the *DSPP* gene expression is also used as the dentin specific marker. However, DSPP gene expression was demonstrated in bone, as well, but it presented only one in 400 folds of dentin (Qin *et al*., 2002).

DSP and DPP are the members of phosphorylated protein. They are negative charge molecules because there are numerous phosphate groups in their molecules. The role of DSP and DPP are believed to involve in regulation of the volume of dentin

mineralization (Suzuki *et al*., 2009). Dentin matrix protein1 or DMP1, another phosphoprotein, also is proposed as a specific marker for dentin (Ching *et al*., 2017). However, DMP1 is also highly expressed in bone and cementum. *DMP1* is required for *DSPP* expression, then the *DMP1* expression decreases in mature odontoblast (Balic and Mina, 2011; Hao *et al*., 2004). Thus, DMP1 may not strong enough to be a specific marker for dentin like DSP/DPP or *DSPP*. The other non-collagenous dentin matrix proteins, such as osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP) also were investigated both *in vivo* and *in vitro* to determine along the odontoblastic differentiation. (Boskey *et al*., 2000; Butler *et al*., 2003; Ruch *et al*., 1995). However, OPN and BSP, which are phosphoprotein like DSP and DPP, also express in bone. Both OPN and BSP highly express in bone and cementum than dentin (Bosshardt *et al*., 1998; Foster, 2012)

Interestingly, tertiary dentin, the pathologic response of dentin, expressed both OPN and BSP higher than primary dentin while DSP was lower when compared to the adjacent unaffected (Aguiar and Arana-Chavez, 2007; Xie *et al*., 2014; Yuan *et al*., 2012). The role of OPN is to regulate the size of hydroxyapatite by attaching to the apatite crystal and inhibiting phosphate ion to form hydroxyapatite (Huang *et al*., 2004; Rittling *et al*., 1998). The function of BSP is believed to be the nucleator for mineralization (Harris *et al*., 2000; Qin *et al*., 2004; Sinha and Zhou, 2013). The other dentin matrix protein which is frequently used as marker is osteocalcin (OCN). OCN is one of the gamma-carboxyglutamic-containing protein or GLA-protein unlike to most non-collagenous proteins which is phosphoprotein. In osteoblast differentiation, OCN expresses in the late stage of differentiation and mineralization (Lian and Stein, 1995). The function of OCN may relate to bone remodeling and regulation of the

mineralization process. The function of OCN in odontoblast is still unknown although it expresses in dentin matrix (Gorter de Vries *et al*., 1987). OCN expression can be detected only in the early stage of odontoblast differentiation, the polarizing stage in dentin (Gorter de Vries *et al*., 1987). This detection suggests the role of OCN may involve in the early phase of odontoblast differentiation that is different from osteoblasts. The other protein that is widely used for odontogenic/osteogenic differentiation is alkaline phosphatase or ALP, the cell surface enzyme. The function of ALP in calcified tissue is breaking the pyrophosphate into free phosphate group that can bind to calcium ion.

2.1.2 Root dentin formation

Root dentin formation is the reciprocation signaling between epithelial cells and mesenchymes as the same principle of crown dentin. However, there are some details are different between root and crown dentin formation. The nuclear factor I-C (NFIC) demonstrated as the first essential protein for root dentin formation (Steele-Perkins *et al*., 2003). NFIC knockout mice showed rootless phenotype, whereas, this deletion did not affect to crown dentin structure (Steele-Perkins *et al*., 2003). The later studies revealed that NFIC of peripheral mesencymes, that was adjacent to HERS, was induced by HERS to regulate odontoblast differentiation (Huang *et al*., 2010; Liu *et al*., 2015b). However, *NFIC* gene and protein expression locate only at odontoblast layer and fully developed dental pulp cells while the apical papilla did not express NFIC (Gao *et al*., 2014). NFIC regulates *osterix*, *DSPP* and DMP1 in the differentiation process during root formation (Zhang *et al*., 2015). Interestingly, osterix knockout mice demonstrated rootless phenotype like NFIC knockout mice that crown dentin was unaffected (Kim *et*

al., 2015; Zhang *et al*., 2015). The target gene of osterix, *DSPP* and *DMP1*, were decreased and root odontoblasts were impaired to differentiation (Zhang *et al*., 2015). Osterix expressing cells could be observed throughout the apical papilla tissue (Ono *et al*., 2016). These osterix expressing cells could differentiate to root odontoblasts and generated root dentin (Ono *et al*., 2016). This evidences suggested that were not only mesenchymes which adjacent to HERS gave rise to odontoblast differentiation but also the inner mesechymes. Hence, osterix expressing cells in apical papilla may suitable for indication of odontoblast progenitor cells. The mechanism of osterix expression in these osterix expressing cells is unclear. TGF-β signaling played as significant role for osterix expressing cells in apical papilla (Wang *et al*., 2013b). TGF-β receptor 2 or Tgfbr2 knockout mice that under controlled with osterix gene expression ($Tgfbr2^{CKO}$; Cre-Osterix) showed the rootless phenotype while tooth crown development was unaffected. Moreover, these knockout mice showed decreasing *DSPP* gene expression but *NFIC* expression did not change in developing root (Wang *et al*., 2013b). The other osterix deletion by using OCN or collagen type I promoter also showed the similar results that was shorten root without any effects to a crown dentin (Kim *et al*., 2015).The osterix expressing cells may be the suitable candidate for odontoblast progenitor cells. However, the further study is needed to elucidate the role and association between TGF-β or other signal pathways to regulate odontoblast progenitors for root dentin formation.

The root formation should be accomplished with normal development process. In some situations, the root formation is disturbed by root canal infection. Root canal infection which frequently occur by dental caries, trauma or crown development defect can arrest root formation process. The REP becomes the treatment of choice for these cases.

The knowledge of tooth development process is important for dental tissue regeneration. The newly regenerated tissue should be imitated or adapted from natural process of root development, especially dentin.

2.2 The treatment of necrotic immature teeth

The objectives of necrotic immature tooth treatment are the same as fully root development cases which are prevention and/or treatment of apical periodontitis (Orstavik and Pitt Ford, 2007). Due to a wide apical foramen and the short root with thin dentinal wall, the root canal procedures, especially mechanical instrumentation and obturation, are difficult to control within ideal treatment. Moreover, thin dentin root canal wall is easy to crack or fracture. The treatment procedure that provides the apical barrier and increases dentin wall thickness is the ideal treatment for this case.

2.2.1 Apexification

Apexification is an endodontic treatment procedure that can induce apical hard tissue barrier formation to limit the level of root canal filling material and contribute to the quality of root canal obturation. This apical hard tissue barrier can be established by long-term root canal medication with calcium hydroxide (Frank, 1966). The irritation of high pH from calcium hydroxide is believed to be the key mechanism to induce hard tissue formation (Javelet *et al*., 1985). Apexification can preserve the tooth and give a favorable outcome. (Cvek, 1992; Kerekes *et al*., 1980; Mackie *et al*., 1988). However, the main disadvantage of this treatment is a thin root dentin wall that is prone to a root fracture (Cvek, 1992; Hecova *et al*., 2010). Multiple appointments for replacement of medication every 3 or 6 months until hard tissue barrier existing were also claimed as the disadvantage (Caliskan and Sen, 1996; Finucane and Kinirons, 1999). In addition, the porous structure of hard tissue barrier may compromise the apical seal (Steiner and Van Hassel, 1971; Walia *et al*., 2000). Recently, MTA is suggested as a material to use in the apexification procedure. MTA apexification

showed the favorable outcome. Some studies demonstrated new cementum deposition on the material surface (Mente *et al*., 2009; Simon *et al*., 2007). However, the weak root structure is still the main problem.

2.2.2 Regenerative endodontic procedure or REP

In the past two decades, the knowledge of regenerative medicine has been extensively studied in many fields of health research including endodontics. The first evidence of regenerative medicine in endodontics was reported 40 years ago (Ostby, 1961). The pulp tissue could regenerate into pulpal space by initiating blood clot into the root canal. The regenerated pulp contained fibroblastic cells, blood vessels and nerve fibers (Ostby, 1961). The result from this study implied that blood from peripaical tissue might contain the potential cells for pulp regeneration. This hypothesis was supported later. The stem cells that obtain by blood clot from inflamed periapical tissue of immature teeth express mesenchymal stem cell markers (Lovelace *et al*., 2011). Interestingly, in some cases of conventional apexification showed continuing root development (Cvek, 1972; Torneck and Smith, 1970; Torneck *et al*., 1973a, b, c). These evidences suggested that these stem cells survived in chronic inflamed periapical area during root canal infection and responded for a root development. However, the outcome of continuing root development by conventional apexification is unpredictable.

The regenerative endodontics has been interested again when Banchs and Trope (2004) implemented the new treatment protocol that could continue the root and pulp development in necrotic immature tooth with chronic apical abscess. The treatment was called revascularization. The blood clot is initiated into the root canal by extruding

endodontic file beyond the root apex without instrumentation after the proper disinfection of root canal systems (Banchs and Trope, 2004). The following clinical studies showed the favorable successful outcomes of this protocol (Bose *et al*., 2009; Jeeruphan *et al*., 2012; Kahler *et al*., 2014; Nagata *et al*., 2014). The radiographic findings showed increasing root length and dentin wall thickness with the resolution of periapical lesion. Then, the term "regenerative endodontics procedure or REP" is widely used for such the treatment procedures due to regain and continue pulpo-dentinal complex development that previously disturbed by infection.

2.2.3 Treatment protocol for current REP

The current protocol for REP comprises of two main steps of treatment (Bansal *et al*., 2014; Kontakiotis *et al*., 2015). The objective of the first appointment is to disinfect the root canal. After tooth isolation, the access opening is established. Vigorous irrigation is performed by 1.5% of sodium hypochlorite without any instrumentation. Then, root canal is flushed with normal saline or EDTA. All chemical irrigants are gently flushed to avoid periapical tissue damage. The root length is estimated and confirmed by a radiograph. Then, the proper medication is introduced into the root canal at least for one to four weeks. Temporary filling is placed into the access opening. The objective of the second appointment is homing stem cells into the root canal space. Any clinical signs and symptoms should diminish. The anesthesia without vasoconstrictor is administration. Temporary filling is removed under tooth isolation with rubber dam. The medicament is removed by irrigation with 17% EDTA. Blood clot initiation is performed by insertion endodontic file beyond the root apex 2

mm. After blood is completely clotted, the suitable matrix is placed at cemento-enamel junction. Then, the coronal pulp space is filled with proper materials.

The important key to achieve the success of REP is the stem cells recruitment into the root canal space (Hargreaves *et al*., 2013). The proper disinfection of the root canal environment allows these stem cells to attach (Trevino *et al*., 2011), proliferate, differentiate, and develop the pulpo-dentinal structure. In addition, blood clot acts as a scaffold that enriches with various growth factors (Nagy *et al*., 2014).

Stem cells are the actual key factor for the successful in REP. SCAPs were the most potential candidate due to their roles in root dentin development process (Sonoyama *et al*., 2006; Sonoyama *et al*., 2008). Therefore, dentin and dental pulp tissue are expected from regenerative endodontic outcome.

2.3 The histologic outcome of REP

Repair by REP

After the successful treatment outcome of REP case report by Banch and Trope in 2004 (Banchs and Trope, 2004), the first animal study was performed and demonstrated the bone liked-structure instead of dentin in the root canal space (Thibodeau *et al*., 2007). The later animal study showed the same results (Yamauchi *et al*., 2011). The cementum-liked hard tissue was also observed. Another animal study found that periodontal tissue extended into the root canal (Wang et al., 2010). The human fractured teeth after this treatment also showed bone-liked structure (Lei *et al*., 2015; Shimizu *et al*., 2013). The newly form hard tissue both animal studies and human samples were calcified matrix embedded with round or spindle shaped cells without a tubular structure (Thibodeau *et al*., 2007; Wang *et al*., 2010).

Interestingly, the progenitor cells that differentiated and expressed bone sialoprotein (BSP) (Shimizu *et al*., 2013). This BSP extensively expressed in bone and cementum but lesser expression in dentin (Foster, 2012). The regenerative calcified structure appeared outside root canal at the apex (Shimizu *et al*., 2013). Although, the generated hard tissue was not dentin-liked structure, the outcome was acceptable because apical periodontitis was resolved.

The histological appearance of the bone or cementum-liked structure embarks on the question what the exact source of the stem cells are recruited. Due to the repaired tissue was generated, cementoblast, periodontal ligament cells or bone progenitors are suggested to survive during infection and recruited inside the root canal after creating

the blood clot (Huang and Garcia-Godoy, 2014). SCAPs were also suggested that they could not survive under root canal infection (Huang and Garcia-Godoy, 2014). Recent study of human extracted incomplete tooth root development with apical periodontitis failed to demonstrated SCAPs or apical papilla structure (Ricucci *et al*., 2017). On the other hand, some researchers believed that SCAPs were survived and were the main source for REP (Hargreaves *et al*., 2013; Sonoyama *et al*., 2008). There are animal studies demonstrated the availability of SCAPs after the experimental pulp necrosis in immature teeth (Tobias Duarte *et al*., 2014; Yoo *et al*., 2016). SCAPs from experimental necrotic teeth still proliferated and could be induced to osteogenic differentiation (Yoo *et al*., 2016). SCAPs from infected teeth also presented better osteogenic differentiation ability than uninfected teeth (Yoo *et al*., 2016). However, this study did not observe which type of calcified tissue was generated. Thus, SCAPs could survive under root canal infection. Infection from root canal might change or alter the SCAPs behaviors especially osteogenic differentiation.

2.4 Role of lipopolysaccharide to apical periodontitis

More than 400 species of bacteria reside in necrotic pulp space (Siqueira and Rocas, 2009). Due to low or absent of oxygen tension of infected necrotic pulp microenverionment, the polymicrobial infection mostly comprises of strictly anaerobe or facultative anaerobe bacteria such as *Fusobacterium, Porphyromonas, Prevotella* which are gram-negative bacteria and other microorganisms (Narayanan and Vaishnavi, 2010). To prevent apical bone marrow infection, body defense mechanism creates the space around the apical foramen to accumulate the immune cells for bacteria invasion prevention (Lopez-Marcos, 2004).

Even bacterial infection from root canal cause apical periodontitis (Kakehashi *et al*., 1965; Moller *et al*., 1981), inflamed periapical tissue is claimed as the bacteria-free zone (Ricucci *et al*., 2006). Only the bacteria by product can release from infected root canal to periapical tissue and initiate periapical periodontitis (Siqueira and Rocas, 2007). One of the most important of bacteria byproducts that plays a major role to cause the pulp and periapical pathology is lipopolysaccharide or LPS (Hong *et al*., 2004; Siqueira and Rocas, 2007).

LPS is the component of gram-negative bacteria cell wall. LPS is released from dividing bacteria or dead bacteria (Rietschel and Cavaillon, 2003). LPS could induce apical periodontitis in animal studies (da Silva *et al*., 2008; Nelson-Filho *et al*., 2002; Ricucci *et al*., 2011). The amount of LPS has been demonstrated to relate with the clinical symptoms severity (Jacinto *et al*., 2005). The high concentration of LPS was found from the root canal that associated with painful on biting or abscess formation and vis versa (Jacinto *et al*., 2005). However, the exact concentration that could produce

clinical signs and symptoms was inconsistent among the studies although they used the same methods of sampling and laboratory measurement that were used (Martinho *et al*., 2011; Sousa *et al*., 2014). Regardless to clinical symptoms, the concentrations of LPS were in the range of 0.001μg/ml to 0.1μg/ml by using of limulus amoebocyte lysate assay or LAL assay (Jacinto *et al*., 2005; Marinho *et al*., 2012; Marinho *et al*., 2015; Martinho *et al*., 2011). Because of the disadvantage of this method that all LPS molecules are not obtained from root canal, so the concentration of LPS in the root canal may be higher than reports. Unfortunately, the amount of LPS in periapical lesion has not been investigated. However, the existence of LPS in human periapical lesion (Dahlen and Hofstad, 1977) and animal study (Yamasaki *et al*., 1992) were demonstrated.

The effects of LPS to SCAPs were reported only on immune response of SCAPs, such as cytokines releasing or related signaling pathway of immunological responses (Wang *et al*., 2013a; Zhang *et al*., 2013). However, the exposure time of LPS in these studies was not more than 48 hours (Wang *et al*., 2013a; Zhang *et al*., 2013) which did not reflect the chronic or long-term infection in clinical situation. The effects of LPS that relate to regenerative endodontic treatment of SCAPs such as proliferation and osteogenic differentiation have not been examined. These effects were studied on the other types of mesenchymal stem cells including dental tissue origin stem cells.

2.4.1 Effect of LPS on mesenchymal cell proliferation

Proliferation of stem cells is an important biological activity for development and differentiation. The proliferation is also crucial for maintaining tissue homeostasis and replacing the dead cells by the local stem cells that reside in each organ.

Cell proliferation in apical papilla is required for root development process. The evidence showed that cell proliferation was active at the root forming region but not at furcation area (Sohn *et al*., 2014). Inhibition of cell proliferation by chemical administration, cytocalasin-D, could impair root development and dentin structure (Sohn *et al*., 2014). Cell proliferation for regenerative medicine is more important in cell free-based approach, such as REP, by using only blood clot for homing stem cells into the root canal space. The recruited stem cells have to proliferate to proper cell numbers before differentiation.

The effects of LPS on stem cells proliferation were studied in various cell types (Chatzivasileiou *et al*., 2013; He *et al*., 2015; Kato *et al*., 2014; Mo *et al*., 2008). Bone marrow mesenchymal stem cells (BMSCs), the prototype of mesenchymal stem cell, showed the exposure to LPS could increase their proliferation (Giuliani *et al*., 2014). In contrast, the other studies showed LPS had no effect on cell proliferation of BMSCs (Chatzivasileiou *et al*., 2013; Mo *et al*., 2008). The possibility of this difference would be discussed later.

The influence of LPS on cell proliferation was also investigated in dental origin stem cells. Dental pulp stem cells (DPSCs) showed increase in cell proliferation when exposing to LPS (Liu *et al*., 2014b). However, the other studies showed decreasing cell

proliferation in DPSCs (He *et al*., 2015; Liu *et al*., 2014b). Periodontal ligament stem cells (PDLSCs) increased cell proliferation when exposed to LPS (Kato *et al*., 2014). In contrast, the other study of PDLSCs demonstrated LPS exposure had no effect to cell proliferation (Albiero *et al*., 2015). On the other hand, dental follicle stem cells (DFSCs) demonstrated that LPS had no effect to cell proliferation (Chatzivasileiou *et al*., 2013; Morsczeck *et al*., 2012).

The current evidences of the effect of LPS on mesenchymal stem cell proliferation are controversial among the cell types and even in the same cell type. The different cell types showed different response to LPS. LPS increased cell proliferation in BMSCs (Giuliani *et al*., 2014) but decreased in DPSCs (He *et al*., 2015) and showed no effect on DFSCs (Chatzivasileiou *et al*., 2013) at the same concentration of 1 μg/ml. Concentration of LPS also affected the proliferation of the same cell type. DPSCs showed cell proliferation increased at 0.1 μg/ml of LPS, but no effect to cell proliferation at 1 μg/ml. In addition, LPS at 10 μg/ml decreased cell proliferation in the same study (He *et al*., 2015). However, there are some studies showed the opposite results in the same cells with the same concentration of LPS. DPSCs with 0.1 μg/ml of LPS increased cell proliferation in He and his colleague's study (He *et al*., 2015) but decreased DPSCs cell proliferation in Liu and co-worker's study (Liu *et al*., 2014b). The different results may be possibility of different starting cell numbers, medium culture system and the time points of data collection.

LPS from the different bacteria also affects to cell proliferation. LPS from *Porphyromonas gingivalis* showed increasing cell proliferation with concentration dependent manner in PDLSCs (Kato *et al*., 2014) while LPS from *E.coli* showed

decreasing cell proliferation (Albiero *et al*., 2015). The effect of source of LPS to cell proliferation may explain by the different structure of LPS that has different bioactivity to LPS receptor, Toll-like receptor (TLR) (Ogawa and Yagi, 2010). These controversial results may also cause by the different culture system between the studies (Zhang *et al*., 2015). The summary of these studies is presented in Table 2.1. Unfortunately, the effects of LPS, especially from endodontic pathogen, to SCAPs on either cell's survival or proliferation after exposure to different concentrations of LPS have not been investigated.

Authors	LPS	concentration	result	Time (day)
BMSCs				
Mo *et al*., 2008	*E.coli*	0.01μg/ml	no effect	7
		0.1μg/ml	no effect	
		1μg/ml	no effect	
Chatzivasileiou *et al*., 2013	*E.coli*	1 μg/ml	no effect	3
		10 μg/ml	no effect	
		50 μg/ml	no effect	
Giuliani *et al*., 2014	*E.coli*	1 μg/ml	increase	2
Tang *et al*., 2015	*PG*	0.1 μg/ml	increase	3
		1 μg/ml	no effect	
		10 μg/ml	decrease	
DPSCs				
Liu *et al*., 2014	*E.coli*	0.1μg/ml	decrease	7
		1μg/ml	decrease	
		10 μg/ml	decrease	
He *et al*., 2015	*E.coli*	0.1 μg/ml	increase	14
		1 μg/ml	no effect	
		10 μg/ml	decrease	
PDLSCs				
Albieno *et al*., 2014	*E.coli*	0.1 μg/ml	no effect	10
		1 μg/ml	no effect	
		10 μg/ml	decrease	
Kato *et al*., 2014	*PG*	1 μg/ml	increase	7
		10 μg/ml	increase	
DFSCs				
Chatzivasileiou *et al*., 2013	*PG*	1 μg/ml	no effect	3
		10 μg/ml	no effect	
		50 μg/ml	no effect	
Morsczeck *et al*., 2012	*E.coli*	1 μg/ml	no effect	3
	PG	1 μg/ml	no effect	

Table 2.1 Effect of LPS on mesenchymal stem cell proliferation.

BMSCs=bone marrow stem cells; DPSCs=dental pulp stem cells; PDLSCs=periodontal ligament stem cells; DFSCs=dental follicle stem cells; PG=Porphyromonas gingivalis.

2.4.2 Effect of LPS on osteogenic differentiation

The effects of LPS on osteogenic differentiation of various types of stem cells were demonstrated in many *in vitro* studies (Cho *et al*., 2010; Fiedler *et al*., 2013; He *et al*., 2015; Kato *et al*., 2014; Waterman *et al*., 2010; Yamagishi *et al*., 2011). BMSCs were extensively studied (Croes *et al*., 2015; Liotta *et al*., 2008; Mo *et al*., 2008; Raicevic *et al*., 2012; Tang *et al*., 2015; Waterman *et al*., 2010). Some studies showed LPS exposure decreased osteogenic differentiation on BMSCs (Croes *et al*., 2015; Tang *et al*., 2015). However several studies demonstrated LPS exposure increased BMSCs cell differentiation (Mo *et al*., 2008; Waterman *et al*., 2010). Moreover, some studies showed LPS had no effect on BMSCs osteogenic differentiation (Liotta *et al*., 2008; Raicevic *et al*., 2012). However, adipose tissue derived-stem cells (ADSCs), another type of mesenchymal stem cell, consistently increased osteogenic differentiation when exposed to LPS (Cho *et al*., 2006; Cho *et al*., 2010; Fiedler *et al*., 2013).

The influence of LPS on osteogenic lineage differentiation also investigated in many dental origin stem cells like cell proliferation. LPS was reported to inhibit osteogenic differentiation of DPSCs (Yamagishi *et al*., 2011). In contrast, LPS increased osteogenic differentiation of DPSCs in the other study (He *et al*., 2015). This different result might be the effect of concentration of LPS. Higher concentration, 5-20 μg/ml, demonstrated inhibitory effect (Yamagishi *et al*., 2012) but lower concentration, 1 μg/ml, showed increasing osteogenic differentiation (He *et al*., 2015). PDLSCs also exhibited the similar results to DPSCs. LPS decreased osteogenic differentiation with higher concentration, 10 μg/ml (Kato *et al*., 2014; Li *et al*., 2014a). However, the lower concentration, 1 μg/ml, increased osteogenic differentiation (Albiero *et al*., 2015). In

contrast, LPS decreased osteogenic differentiation of DFSCs even in lower concentration, 1 μg/ml (Morsczeck *et al*., 2012). These results suggested that the high concentration of LPS could decrease osteogenic differentiation whereas lower concentration increased osteogenic differentiation. In addition, different cell types may response to LPS in different results during osteogenic differentiation. The source of LPS also could affect the osteogenic differentiation. LPS from *Porphyromonas gingivalis* decreased osteogenic differentiation at concentration of 1 μg/ml (Kato *et al*., 2014) but LPS from *E.coli* increased osteogenic differentiation with the same concentration (Albiero *et al*., 2015). However, the source of LPS had no effect to DFSCs (Morsczeck *et al*., 2012). The different effects of LPS on osteogenic differentiation among studies were summarized in Table 2.2. The effects of LPS from *Porphyromonas gingivalis* on osteogenic lineage differentiation of SCAPs are still unknown.

Authors	LPS	concentration	result	Time (day)
BMSCs				
Mo *et al*., 2008	*E.coli*	0.01μg/ml	no effect	14
		0.1μg/ml	increase	
		1μg/ml	increase	
Raicevic *et al*., 2012	N/A	10 μg/ml	no effect	21
Croes *et al*., 2015	*E.coli*	0.5 μg/ml	decrease	22
Li *et al*., 2014	*E.coli*	10 μg/ml	no effect	28
Tang *et al*., 2015	*PG*	0.1 μg/ml	increase	14
		1 μg/ml	decrease	
		10 μg/ml	decrease	
DPSCs				
Yamagishi *et al*., 2012	*PG*	5 μg/ml	decrease	14
		10 μg/ml	decrease	
		20 μg/ml	decrease	
He *et al*., 2015	*E.coli*	0.01μg/ml	no effect	14
		0.1μg/ml	increase	
		1 μg/ml	increase	
		10 μg/ml	increase	
PDLSCs				
Albieno *et al*., 2014	*E.coli*	1 μg/ml	increase	21
Kato *et al*., 2014	*PG*	1 μg/ml	decrease	21
		10 μg/ml	decrease	
DFSCs				
Morsczeck *et al*., 2012	*E.coli*	1 μg/ml	decrease	21
	PG	1 μg/ml	decrease	

Table 2.2 Effect of LPS on mesenchymal stem cell osteogenic differentiation.

BMSCs=bone marrow stem cells; DPSCs=dental pulp stem cells; PDLSCs=periodontal ligament stem cells; DFSCs=dental follicle stem cells; N/A=not available; PG=Porphyromonas gingivalis.

The most current studies regarding to the effects of LPS on SCAPs related to immediate cell responses such as cytokine releasing (Zhang *et al*., 2013) and the signaling pathway (Wnt/β-catenin and p38 MAPK) that regulated the inflammatory response (Wang *et al*., 2013a). The exposure time of LPS to SCAPs in these studies were only 24 to 48 hours (Wang *et al*., 2013a; Zhang *et al*., 2013). The periapical tissue and cells always relate to long-term inflammation by root canal microorganisms in the most of the periapical periodontitis, so the effects of LPS should be investigated as the long-term exposure. To elucidate the effects of LPS on SCAPS that relate to REP, especially cell proliferation and osteogenic lineage differentiation, it is necessary to design the experiment that resemble to the clinical situation. In addition, the source of LPS should be awareness. Even LPS from *E.coli* is a gold standard for observing general endotoxin responses in many studies, this species is not found during root canal infection. The LPS from infected root canal flora such as LPS from *Porphyromonas gingivalis* is more suitable. After proper root canal disinfection, the long-term LPS exposed SCAPs are recruited into the clean root canal by blood clot initiation. Thus, cell proliferation and osteogenic lineage differentiation should be investigated after long-term treated with LPS.

2.5 SCAPs markers for mesenchymal stem cell

2.5.1 Mesenchymal stem cells markers

Mesenchymal stem cells (MSCs) refer to stem cells that reside tissue organ stromal. In general, these cells function as repairing cells for the tissue organ damage. Thus, mesenchymal stem cells are limited the multi-differentiation to their lineages. Cells from apical papilla tissue of root developing tooth were firstly isolated by Sonoyama and co-workers in 2006 (Sonoyama *et al*., 2006). To identify these cells whether they are mesenchymal stem cells, several laboratory methods are utilized. Like other discovered dental stem cells such as dental pulp stem cells, periodontal ligament stem cells and dental follicle stem cells, the mesenchymal stem cells characteristics have to be tested (Huang *et al*., 2009). Bone marrow stem cells (BMSCs) were frequently used as a gold standard for mesenchymal stem cells identification because they were isolated and have been studied for several decades.

BMSCs were firstly isolated from mouse bone marrows. At the first glance of bone marrow MSCs from culture disc, they were fibroblastic shape that attached to culture disc surfaces (Friedenstein *et al*., 1970). These cells revealed the colony forming unit capacity and could be induced to osteogenic, adpogenic and chondrogenic lineage of differentiation (Friedenstein *et al*., 1987; Pittenger *et al*., 1999). The other cells from bone marrow also demonstrated the heterogeneity population. Thus, the markers that could distinguish MSCs from others were used. According to the different protein expressions on cell surface of these two stem cell types, these cell surface markers were utilized by antigen-antibody reaction assay, flow cytometry. In addition, the hematopoietic stem cells might be isolated along with BMSCs. Thus, surface markers

that abundantly expressed on the hematopoietic cell surface such as CD45, CD34 and HLA-DR were used as negative selection of MSCs from bone marrow (Boxall and Jones, 2012; Pittenger *et al*., 1999; Rasini *et al*., 2013). The other markers, STRO-1, CD105, CD90, CD73, CD44, CD29 and CD34 were used as a positive selection for BMSCs (Barry *et al*., 2001; Barry *et al*., 1999; Pittenger *et al*., 1999; Simmons and Torok-Storb, 1991a, b). The other positive markers such as CD271 (Jones *et al*., 2002), CD146 (Delorme *et al*., 2008), CD106 (Gronthos *et al*., 2001) were also used. CD90 or Thy-1, a part of TGF-β receptor and CD44, hyaluronan receptor that normally relate to stem cell signal especially for stem cell differentiation allow stem cells to interact with cell matrix and stimuli. All these proteins could send the specific signals into stem cells and regulate the stem cells behaviors (Boxall and Jones, 2012).

After discovery of BMSCs, other types of mesenchymal stem cells from other organs were discovered and investigated, including mesenchymal stem cells from oral tissue (Huang *et al*., 2009). Thus, the international society for cellular therapy or ISCT has declared the minimum requirements for extracted mesenchymal stem cells characteristics as the following. Firstly, the cells can adhere to the culture disc. Secondly, mesenchymal stem cell candidcates should express CD105, CD73 and CD90 more than 95% of total population and express CD45, CD31, CD14, CD11b, CD19, CD79α and HLA-DR less than 2%. Thirdly, cells could be derived at least into three lineages namely osteogenic, adipogenic and chondrogenic differerntiation (Dominici *et al*., 2006). These criteria are set as a standard for mesenchymal stem cell studies regardless the source of cells. However, the specific marker of MSCs from each source is used in some MSCs to specify the origin of the obtained stem cells, for example, CD133 for renal and liver stem cells (Bruno *et al*., 2006; Kordes *et al*., 2007), CD24

for adipose and neural stem cells (Berry and Rodeheffer, 2013; Rodeheffer *et al*., 2008). Hence, some surface mesenchymal stem cell markers should appropriately selected regard to tissue origin, as well.

There are many MSCs from dental tissue origins such as dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs), shedding human exfoliated dental stem cells (SHEDs) and stem cells from apical papilla (SCAPs). These stem cells were proved as MSCs following the ISCT's criteria. However, the differentiation capacity of some lineages and the rate of proliferation or colony forming unit or CFU were not the same. The osteogenic differentiation capacity between dental stem cells has been reported. SCAPs showed better osteogenic differentiation than DPSCs (Gosau *et al*., 2013). However, SCAPs demonstrated the same osteogenic differentiation capacity as SHEDs (Bakopoulou *et al*., 2011). Proliferation rate of SCAPs was faster than DPSCs even though they showed the same amount of surface MSCs markers (Huang *et al*., 2008; Sonoyama *et al*., 2006; Sonoyama *et al*., 2008). Thus, the surface markers may be sufficient for MSCs identification and distinction from HSCs but they cannot reflect stem cell stage, self-renewal and the capacity of stem cell differentiation.

However, these ISCT's surface markers do not mean the stem cell property, self-renewal and differentiation capacity. CD73, CD105 and CD90 expressed the same percentage between early and late passage although the later passage showed decreasing colony forming capacity and differentiation (Digirolamo *et al*., 1999). In addition, CD73 and CD90 still expressed after differentiation process (Wagner *et al*., 2008). Each clone from MSCs demonstrated the different potency of differentiation

(Wagner *et al*., 2008). This suggested that the explanted MSCs were heterogeneous population although the surface MSCs markers expressed beyond 95% (Pittenger *et al*., 1999). In contrast, CD105 and CD73 can express in the other somatic cells that are not MSCs for example, skin fibroblast (Ishii *et al*., 2005).

2.5.2 Using pluripotent marker of embryonic stem cells to identify stem cell characteristics of MSCs

Stem cells that obtained from inner cell mass of embryo can derive into all cell types of three germ layers. According to this ability, these stem cells are also called "embryonic" stem cells or ESCs. ESCs use various proteins such as growth factors and cytokines to maintain the stem cell characteristics and regulate lineage the differentiations by acting as cell-cell communication or regulation the cell development process (Goldberg *et al*., 2011). These regulators transmit the signals through cytoplasm proteins into nucleus. There are regulatory networks that comprise of some proteins are essential for controlling the proper signals by regulating the essential gene expression to maintain stem cell characteristics and initiate the differentiation (Murry and Keller, 2008; Rodda *et al*., 2005).

These proteins of ESCs are often used to determine mesenchymal stem cell characteristics and stage of differerntiation. There are several pluripotent markers that are widely used such as Oct4, Nanog and Sox2. These three proteins are transcription factors that respond to gene transcription into messenger RNA. These transcription factors also regulate themselves by binding to the promoter sequences of their genes (Niwa, 2007). These transcription proteins control their functions by their interactions to each other. Knockdown the gene expression of these proteins demonstrated alteration

of ESCs's behaviors for examples, the reduction of proliferation and self-renewal. In contrast, knockdown these proteins increased the related differentiation genes (Masui *et al*., 2008; Torres and Watt, 2008). These studies confirmed the roles of these transcription factors for stemness and differerntiation in ESCs. Thus, Oct4, Nanog and Sox2 are used as pluripotent marker that could identify stemness and differentiation stage of ESCs.

The "core" pluripotent markers also express in adult stem cell including various types of MSCs (Tsai and Hung, 2012). However, the level of gene and protein expression was less than ESCs (Esteves *et al*., 2014). The current evidences suggested that pluripotent markers might function as shown in ESCs (Tsai and Hung, 2012). Overexpression of Oct4 or Nanog could increase cell proliferation by regulation the cell cycle proteins which was important to maintain stemness and related to self-renewal. In contrast, Knockdown Oct4 and Nanog could decrease cell proliferation (Tsai and Hung, 2012; Tsai *et al*., 2012). However, there was a study demonstrated that Sox2 regulated the MSCs stem cell characteristics but Oct4 and Nanog did not (Pierantozzi *et al*., 2011). The another pluripotent transcription factor that frequent used to determine the pluripotncy in ESCs is Rex1. Rex1 also played the significant roles in MSCs (Bhandari *et al*., 2010). The gene expressions of four pluripotent markers, Oct4, Nanog, Sox2 and Rex1 were general used for stem cell characteristics identification of MSCs.

The pluripotent markers also express in various types of dental stem cells and are used along with the expression of surface stem cell markers and multi-lineage differentiation to examine stem cell characteristics. DPSCs, SHEDs and PDLSCs expressed these markers and some studies demonstrated the same function of these

markers in the other sources of MSCs as to maintain stem cell characteristics (Huang *et al*., 2009; Wu *et al*., 2012). SCAPs also expressed these markers and genes (Wu et al., 2012). Oct4, Nanog, Sox2 and Rex1 could be increased by treating with basic fibroblast growth factors (bFGF) which was regulated the stemness, self-renewal and increased cell proliferation, as well (Wu *et al*., 2012).

To confirm the mesenchymal stem cell characteristics of obtained SCAPs from apical papilla *in vitro*, surface mesenchymal stem cell markers, multi-lineage differentiation and expression of pluripotent markers are sufficient. Moreover, cell proliferation and CFU should be investigated. SCAPs are accepted as mesenchymal stem cell following the minimum criteria by ISCT and also express the core pluripotent markers. However, there were some studies found that SCAPs expressed CD24 marker (Bakopoulou *et al*., 2013; Schneider *et al*., 2014; Sonoyama *et al*., 2006; Zhang *et al*., 2014). The function of this marker in SCAPs has not been investigated

2.6 CD24 as a marker to SCAPs

2.6.1 The structure of CD24

CD24 was first discovered as a cell surface marker for leukocyte differentiation in mice 40 years ago (Springer *et al*., 1978). The human and mouse precursor of CD24 composed of 76 and 80 amino acids, respectively (Ayre *et al*., 2016). CD24 precursor was modified by cleaving the N-terminal region that was important for signal transduction. Then, CD24 was translocated to cell membrane by using glycophosphophatidylinositol or GPI part (Kay *et al*., 1991). The mature of CD24 protein core is contain 32 residues in human and 27 residues in mouse (Kay *et al*., 1991). Like other GPI-anchorage proteins, CD24 was modified by N or O-linked glycosylation to increase the molecular mass (Fang *et al*., 2010). The different cell type showed the different glycosylation modification form. It was suggested that CD24 could interact more than one molecule with different functions.

The function of CD24 is diversity. The early studies of CD24's function found that it involved in B cell maturation. CD24 expressed at the early stage of B cell development in bone marrow but it did not express at the later stage of B cell in peripheral lymphoid organs or blood circulation (Hardy *et al*., 1991; Wenger *et al*., 1995). Recent study showed that CD24 was important to cell-cell communication between B cells via the vesicle releasing (Ayre *et al*., 2015). CD24 also functioned in T cell and dendritic cell that related to cell proliferation and acted as co-stimulator, respectively (Crispe and Bevan, 1987; Hubbe and Altevogt, 1994). CD24 also expressed in myeloid cells such as granulocytes as an adhesion molecule (Aigner *et al*., 1995).

CD24 expressed in the other somatic cells or tissue such as brain and nervous system, fat tissue, skin, kidney, liver, hair follicle, intestine including tooth bud (Shirasawa *et al*., 1993). Similar to B cell, CD24 in most somatic cells transiently expresses and its function mostly relates to cell maturation. CD24, then, it decreases when these cells reach the fully maturation stage (Berry and Rodeheffer, 2013; Shirasawa *et al*., 1993). Some organs, such as kidney and liver, contained a few CD24 expressing cells for repairing the organ damages (Angelotti *et al*., 2012; Fomin *et al*., 2011). The relation between CD24 and cell differentiation during organ development are mostly unknown. It is expected that CD24 may interact with growth factors and appropriately modulate cell signals. In addition, CD24 has been extensively studied in cancer research. CD24 associated with poor prognosis or invasiveness in many cancer types for example, breast, pancreatic, liver, lung cancer and squamous cell carcinoma (Jaggupilli and Elkord, 2012). These cancer tissues or cells highly expressed CD24. The higher CD24 expression always correlates with the poor prognostic cancers (Jaggupilli and Elkord, 2012) that are more cell migration, proliferation rate and metastasis. Thus, CD24 is used for the poor prognostic marker of cancer. CD24 also involves in fibrosis lesion (Kim *et al*., 2009). High migration capacity usually relates to high CXCR4 expression that involves in cell chemoattractant and regulates cell cytoskeleton (Leelawat *et al*., 2013). Highly proliferation rate also related to increase of cell cycle protein regulation and involved in stem cell characteristics in some cancer stem cell types (Yang *et al*., 2014; Yeung *et al*., 2010). In spite of strong evidences about the role of CD24 in cancer cell behaviors, the insight mechanism of CD24 is not clear.

2.6.2 Roles of CD24 and SCAPs

During adipogenic differentiation of adipocyte progenitors, CD24 expression was increased (Smith *et al*., 2015), and then could not detect after adipogenic differentiation process (Berry and Rodeheffer, 2013; Rodeheffer *et al*., 2008). Moreover, CD24 expressing stem cells from adipose tissue demonstrated better adipogenic differentiation than non-expressing cells (Rodeheffer *et al*., 2008). CD24 was also examined in mouse BMSCs. CD24 expressing BMSCs demonstrated the osteoprogenitor cell phenotype. They expressed osterix, alkaline phosphatase gene expression more than non-CD24 expressing cells (Chang *et al*., 2015).

CD24 was suggested that this marker might involve or associate with SCAPs behaviors. Sonoyama suggested that CD24 might involve in osteogenic differentiation process because the expression of CD24 was undetected after osteogenic differentiation (Sonoyama *et al*., 2006), like adipogenic differentiation of adipocyte progenitor cells (Rodeheffer *et al*., 2008; Smith *et al*., 2015). Sonoyama and his colleagues (2006) suggested that CD24 might be used as a specific marker for SCAPs because it expressed in SCAPs but did not express in DPSCs. However, unlike other surface mesenchymal stem cell markers that expressed more than 90%, CD24 expressed only 7.56% from total of population (Sonoyama *et al*., 2006). In addition, the percentage of CD24 positive SCAPs was demonstrated in a wide range among the studies between 3% and 20% (Bakopoulou *et al*., 2013; Schneider *et al*., 2014; Sonoyama *et al*., 2006; Zhang *et al*., 2014). Their suggestion is still doubted. On the other hand, the cause of this variation is still unknown.

The relationship of CD24 and stem cells behaviors, especially SCAPs, has not been investigated. However, the variation of percentage of CD24 expressing SCAPs may influence these behaviors.

2.7 Gap in knowledge and research question

The number of CD24 expressing SCAPs might be benefit for REP. Factors or biological pathways that participated or regulated CD24 of SCAPs should be also investigated for better understanding the insight mechanisms. This knowledge would be benefit for root dentin regeneration.

LPS has been shown that it affected to cell proliferation and osteogenic differentiation in other mesenchymal stem cells. These cell behaviors are crucial for REP. The effects of LPS on SCAPs proliferation and osteogenic differentiation have not been investigated. In additions, the different CD24 expressing SCAPs might also influence to cell behaviors especially cell proliferation and differentiation of mesenchymal stem cells after LPS exposure. The question of this study was LPS exposure whether affected to cell proliferation and osteogenic differentiation of different percentage of CD24 expressing SCAPs.

2.8 Objectives of the study

1. To determine the variation of percentage of CD24 expressing cells among the SCAPs cell lines.

2. To characterize the mesenchymal stem cell characteristics of SCAPs between different percentage of CD24 expressing SCAPs.

3. To determine the effect of lipopolysaccharide to SCAPs survival of different percentage of CD24 expressing SCAPs.

4. To determine the effects of lipopolysaccharide pre-exposure to cell proliferation and osteogenic differentiation of SCAPs with different percentage of CD24 expressing SCAPs.

Objective 1 and 2 were performed in Chapter 3.

Objective 3 and 4 were performed in Chapter 4.

CHAPTER 3

COMPARISON OF STEM CELL BEHAVIORS BETWEEN INDIGENOUS HIGH AND LOW-CD24 PERCENTAGE EXPRESSING CELLS OF STEM CELLS FROM APICAL PAPILLA [1]

3.1 Abstract

CD24 was suggested as a marker to SCAPs and has been reported for a decade. CD24 has been shown to involve stem cell activities such as self-renewal, proliferation and differentiation. However, the percentage variations of CD24 positive cells were reported among the studies. It is possible that this variation may affect these SCAPs behaviors. In this study, the variation was confirmed. To elucidate the influence of CD24 positive cells quantity on SCAPs stem cell behaviors, the 3 cell lines with the most maximum and the least numbers of CD24 positive cells (High-CD24 and Low-CD24 group) were selected to study. Both groups expressed the same mesenchymal stem cell markers and negative to hematopoietic marker. High-CD24 group demonstrated less self-renewal capacity by lower colony-forming-unit count and pluripotency marker gene expressions. However, cell proliferation was not different. In contrast, osteogenic and adipogenic differentiation were better than Low-CD24 group. The early stage of root development demonstrated higher CD24 expressing cells

[1] Based on manuscript, submitted and published to Tissue and Cell; volume 8 issue5; page 397-406. Copyright with permission, © 2016 Elseveir Ltd, license number 4105211269849.

than later stage. In conclusion, quantity of CD24 expressing cellsself-renewal and multi-lineage differentiation but did not influence on cell proliferation. Stage of root development influenced to CD24 expressing cell numbers.

3.2 Introduction

Stem cells from apical papilla or SCAPs are one of the interesting postnatal mesenchymal stem cells. SCAPs were shown multi-lineage differentiation capacity such as osteogenic, adipogenic, chondrogenic and neurogenic lineage like other dental stem cells origins (Dong *et al*., 2013; Gervois *et al*., 2015; Huang *et al*., 2009; Prateeptongkum *et al*., 2015; Vanacker *et al*., 2014). Recently, SCAPs were used as a stem cells source to reconstruct the spinal cord injury in animal model (De Berdt *et al*., 2015). Therefore, SCAPs are interesting in the regenerative medicine.

SCAPs were first isolated and studied by Sonoyama and co-workers in 2006 (Sonoyama *et al*., 2006). SCAPs expressed many surface mesenchymal stem cell markers such as CD90, CD105, CD73, CD44, STRO-1 and negative to hematopoietic cell lineage marker, CD45 (Huang *et al*., 2009; Prateeptongkum *et al*., 2015; Sonoyama *et al*., 2006; Sonoyama *et al*., 2008). The other pluripotent stem markers such as Rex-1, Nanog, Sox2, and Oct4, that frequently were used to identify mesenchymal stem cell, were also expressed in SCAPs (Bakopoulou *et al*., 2013; Wu *et al*., 2012). These markers regulated self-renewal and proliferation in mesenchymal stem cells (Kashyap *et al*., 2009; Niwa, 2007; Son *et al*., 2013). However, these markers are not specific to SCAPs. They generally expressed in most types of postnatal mesenchymal stem cell (He *et al*., 2009; Huang *et al*., 2009). Sonoyama and his colleagues proposed CD24 and Survivin as the specific markers for SCAPs (Sonoyama *et al*., 2006).

CD24 is a short glycophosphatidylinositol membrane protein with heavily glycosylation (Lim, 2005; Pierres *et al*., 1987). CD24 first used for B cell identification (Fischer *et al*., 1990) and involved in B cell maturation (Wenger *et al*., 1995). The

somatic cells such as neuroblasts, epithelial cells, keratinocytes and hair follicle cells expressed CD24 as well (Fang *et al*., 2010). CD24 participated in various cellular activities such as, proliferation, migration, apoptosis (Fang *et al*., 2010; Jaggupilli and Elkord, 2012; Kristiansen *et al*., 2004) including stem cell self-renewal and differentiation (Lee *et al*., 2011; Liu *et al*., 2014a; Petkova *et al*., 2013; Pruszak *et al*., 2009). The role of CD24 in SCAPs was supposed to involve in osteogenic differentiation (Sonoyama *et al*., 2006). Up to date, the exact function of CD24 remains unclear.

Interestingly, CD24 was shown wide range of the percentage expressing cells between 3% and more than 20% (Abuarqoub *et al*., 2015; Bakopoulou *et al*., 2013; Schneider *et al*., 2014; Zhang *et al*., 2014). This indigenous variation may influence to SCAPs behaviors especially mesenchymal stem cell characteristics. The objective of this study was to compare the influences of the indigenous high and low percentage of CD24 expressing SCAPs on the mesenchymal stem cell characteristics, such as the surface stem cell markers, self-renewal, cell proliferation and multi-lineage differentiation.

3.3 Materials and methods

3.3.1 Sample collection and cell culture

SCAPs were obtained from human permanent immature third molar teeth from 17–20 year-old patients at Department of Oral and Maxillofacial Surgery, Faculty of Dentistry Chulalongkorn University with approved protocol by the Ethics Committee (Study code: HREC-DCU-P 2015-001). The cells were collected using an outgrowth technique. Briefly, the apical papilla tissue was minced by using surgical blades and placed on culture dishes to allow for cell expansion in growth medium, and then incubated at 37°C in 5% CO_2. The growth medium was α-MEM (GIBCO/Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, TX, USA), 2 mmol/L glutamine (GIBCO), 100 U/ml penicillin with 100 µg/ml streptomycin (GIBCO). Ten donor cell lines were used to characterize the SCAPs.

The percentage of CD24 and other surface mesenchymal stem cell markers expressions were measured by flow cytometry at passage 2. Cell lines at passage 4 were used in further experiments. Experiments were performed in triplicate. The three cell lines with the most and the least of CD24 expressing cells percentage (High-CD24 and Low-CD24) were selected for further experiments. The surface mesenchymal stem cell markers and functional assay for self-renewal were measured by flow cytometry and CFU respectively. The pluripotent marker genes, *Rex-1*, *Nanog*, *Sox2* and *Oct4* and *Survivin*, another marker for SCAPs, were determined by PCR. Due to the major function of apical papilla was to develop root dentin (Sonoyama *et al*., 2006), the baseline differentiated odontoblast gene markers, *Runx2*, *Osterix* and *DSPP* (Chen *et al*., 2009), were also examined by PCR.

3.3.2 Flow cytometry analysis of surface mesenchymal stem cell markers and CD24

Cells were grown in 10 cm culture dish until confluence. Cells were subcultured and counted for flow cytometry. Each experiment used SCAPs at passage 2 with 100,000 cells per test performed on a BD FACsCalibur (BD Biosciences Pharmingen, CA, USA), following previous protocol (Mahanonda *et al*., 2008).

Mesenchymal stem cell surface markers were measured by using fluorescent tagged-antibodies, Alex Fluor®647 conjugated mouse IgM anti-human STRO-1 antibody (BioLegend, CA, USA), PE conjugated mouse IgG1 anti-human CD90 (BioLegend), FITC conjugated mouse IgG1 anti-human CD44 (BD Bioscience), FITC conjugated mouse IgG1 mouse anti-human CD105 (ImmunoTools, Friesoythe, Germany), PE conjugated mouse IgG2a anti-human CD24 (BioLegend). FITC conjugated mouse IgG1 anti-human CD45 (BioLegend) was used to confirm that the cells were not the hematopoietic lineage. Immunoglobulin isotypes were served as a negative control.

3.3.3 Self-renewal assays

3.3.3.1 Colony-forming unit assay

The SCAPs were seeded on 6 cm diameter culture dish at 100 cells and cultured for 10 days in growth medium. The cells were fixed with 10% formalin, washed with deionized water and stained with 10% (g/v) crystal violet in methanol. The cells were then washed and dried. Cell aggregations of more than 50 cells were counted as one colony. The colony counting was performed under a microscope.

3.3.3.2 Pluripotent gene markers expression

Total RNA from 500,000 SCAPs was extracted using the RNEasy kit (QIAGEN, CA, USA) following the manufacturer's instructions. The 1 μg of total RNA was converted to cDNA by using iScript™ cDNA synthesis kit (Bio-rad, CA, USA). The quantitative real-time polymerase chain reactions were performed by using iTaq Universal SYBR Green Supermix (Bio-Rad) with 40 cycles in a LightCycler®480 machine (Roche diagnostic, IN, USA) with 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. The primer sequences of *Rex1*, *Nanog*, *Oct4* and *Sox2* were shown in Table 3.1.

Gene Name	Sequence ID	Primer Sequences (5'-3')	Product Size
Rex-1	NM_174900.4	GCTGACCACCAGCACACTAGGC TTTCTGGTGTCTTGTCTTTGCCCG	298
Nanog	NM_024865.3	ATGCCTCACACGGAGACTGT AGGTGGGTTGTTTGCCTTTG	101
Oct4	NM_002701.5	AGACCCAGCAGCCTCAAAATC GCAACCTGGAGAATTTGTTCCT	181
Sox2	NM_003106.3	ACCAGCTCGCAGACCTACAT ATGTGTGAGAGGGGCAGTGT	319
Runx2	NM_001024630.2	CCCCACGACAACCGCACCAT CACTCCGGCCCACAAATC	289
Survivin	NM_001012270.1	CATGCAAAGGAAACCAACAA GGTGGCACCAGGGAATAAAC	161
Osterix	NM_001173467.1	GCCAGAAGCTGTGAAACCTC GCTGCAAGCTCTCCATAACC	161
DSPP	NM_014208.3	ATATTGAGGGCTGGAATGGGG A TTTGTGGCTCCAGCATTGTCA	136
GAPDH	NM_002046.4	GAAGGCTGGGGCTCATTT CAGGAGGCATTGCTGATGAT	137

Table 3.1 Primer sequences for real-time PCR.

3.3.4 Proliferation assay

SCAPs were seeded in 24-well plates (Thermo Scientific, MA, USA) at 5,000 cells per well. After incubation for 24, 72, 120, and 168 hours, the growth media was replaced with growth medium without phenol red with 1:10 AlarmarBlue® solution (GIBCO/Invitrogen) and incubated for 180 minutes. The absorbance was measured by spectrophotometry with an Anthos Zenyth rt200 microplate reader at 570 nm (Biochrom, Cambridge, UK).

3.3.5 Multi-lineage differentiation

3.3.5.1 Osteogenic differentiation

The SCAPs were seeded on 24 well-plates with 50,000 cells per well (Thermo Scientific). The confluent SCAPs were induced to osteogenic differentiation by replacing with induction medium. The osteogenic induction medium (Nowwarote *et al*., 2015) was growth medium supplemented with 100 nmol/L dexamethasone (Sigma-Aldrich), 50 µg/ml ascorbic acid (Sigma-Aldrich) and 10 mmol/L β-glycerophosphate (Sigma-Aldrich). The duration of the osteogenic induction was 14 days. To determine the extent of osteogenic differentiation, cells were washed with PBS and fixed with cold methanol. The cells were washed with deionized water and stained with 1% (g/v) Alizarin Red S solution at pH 4.2 (Sigma-Aldrich). The cells were then washed and dried. The stained precipitation was dissolved with 10% (g/v) cetylpyridinium chloride monohydrate (Sigma-Aldrich) and the spectrophotometry was measured at 570 nm by microplate reader (Biochrom).

The baseline odontogenic lineage differentiation markers, *Runx2*, *Osterix* and *DSPP* of both High-CD24 and Low-CD24 group were determined by PCR with the same protocol in 2.3.2. The primer sequences were shown in Table 3.1.

3.3.5.2 Adipogenic differentiation

The cell numbers and culture plates in this experiment were the same as in the osteogenic differentiation assay. The adipogenic induction medium (Nowwarote *et al*., 2015) was growth medium supplemented with 1 μmol/L dexamethasone (Millipore, MA, USA) and 0.5 mmol/L 3-isobutyl-1-methyl-xanthine (IBMX) (Millipore). The confluent cells were treated with adipogenic induction medium for 48 hours then replaced with maintenance medium to allow for lipid accumulation for 48 hours. Maintenance medium was growth medium with 10 μg/ml insulin (Millipore). This induction process was repeated for 3 cycles. The cells were washed with PBS and stained with Oil Red O solution (Millipore), then washed with washing buffer (Millipore). The staining was quantified by dissolving the stain in eluting solution (Millipore) and the spectrophotometry was measured at 520 nm by microplate reader (Biochrom).

3.3.6 Tissue preparation and immunohistochemistry observation

The stage of root development of samples were recorded and categorized by modification of Demirjian and co-workers's tooth development stage criteria (Demirjian *et al*., 1973) into the root formation less than 1/2 group (≤1/2) and the root formation between 1/2 and 3/4 group (>1/2-3/4) (Figure. 3.6A). Tooth was cut along the long axis by diamond disc with running water both mesial and distal sites. Tooth

was split and soft tissue both dental pulp and apical papilla were removed. The specimens were fixed with 10% formalin solution at room temperature for 24 hours. Tissues were embedded in paraffin block and kept in room temperature. Serial section was performed by tissue microtome with 4 μm thickness then mounted on glass slides. Each section was de-paraffin in xylene and rehydrated with serial ethanol solution (100%, 95% and 80%) and washed with distilled water. CD24 positive cells were identified with Polymer/HRP and DAB chromogen technique. Primary mouse anti-human CD24 IgG2a antibody (BioLegend) was diluted with 1:200 and incubated overnight. Isotype antibody (IgG2a) (BioLegend) was used as negative control with the same concentration. Antigen retrieval was performed by using citrate buffer with pH 6.0 and then heated by microwave for 10 minutes with 700 watts. Secondary goat anti mouse antibody was further incubated for 60 minutes. DAB substrate for peroxidase enzymatic reaction (Dako EnVision™, Glostrup, Denmark) was then performed. Counterstaining was done with haematoxylin. The sections were investigated under light microscope (Olympus CX41, Olympus America Inc, PA, USA) for observing CD24 positive cells.

3.3.7 Statistical analysis

The data were shown as a mean and standard deviation. The Mann-Whitney U test was performed to compare the dependent variables between two groups. The statistical significance was set at $p < 0.05$.

3.4. Results

3.4.1 Percentage of CD24 expressing cells of SCAPs

The percentage of CD24 positive cells varied among the SCAPs lines, ranging from 2.21%–47.94% (n=10) with a mean of 15.82±16.37% and median of 7.55% (Figure 3.1). Three cell lines from the most (cell line 1-3) and the least (cell line 8-10) percentage of CD24 expressing cells were selected to examine the influence of CD24 expressing cells (High-CD24 and Low-CD24 group) in the next experiments. The mean percentages of selected 3 cell lines from High-CD24 and Low-CD24 were 36.81±13.9% (47.94%, 41.26% and 21.23%) and 3.49±1.61% (5.29%, 2.96% and 2.21%), respectively (Figure 3.2A). *Survivin*, another proposed SCAPs marker, was detected in both groups but it was not statistically different ($p > 0.05$) between groups (Figure 3.2B).

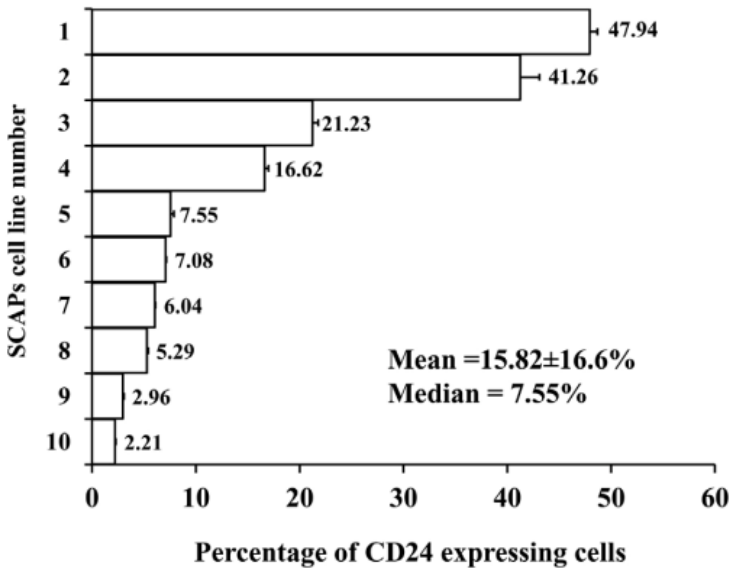


Figure 3.1 Percentage variation of CD24 expressing cells at passage 2**.**

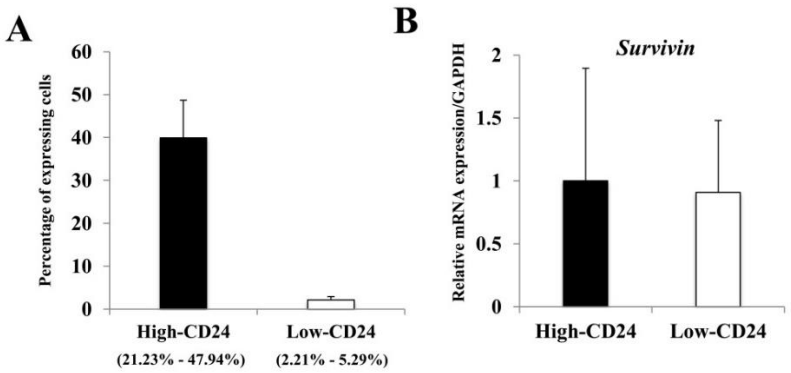


Figure 3.2 Percentage of CD24 expressing cells and Survivin expression between High-CD24 and Low-CD24.

(A) The mean of percentages of the 3 highest and lowest CD24 expressing cell lines were 36.81±13.9% and 3.49±1.61%, respectively. (B) Baseline mRNA expression of Survivin at passage 4 between High-CD24 and Low-CD24 (not significant, $p > 0.05$).

3.4.2 Surface mesenchymal stem cell markers of High and Low-CD24 group

The results from flow cytometry showed more than 95% of the SCAPs in both groups were positive to CD44 and CD90, approximately 65% positive to CD105 and were negative to CD45, a hematopoietic cell surface marker (Figure 3.3A). Furthermore, the High-CD24 group showed a significant higher STRO-1 expression level than the Low-CD24 group at $p < 0.05$ (Figure 3.3A). However, these STRO-1 positive cells in High-CD24 group totally vanished at passage 4, while CD24 positive cells were not different between passage 2 and passage 4 (Figure 3.3B).

A

Markers\Types	High-CD24	Low-CD24
STRO-1	8.35%±1.76*	0.62%±0.37
CD90	98.61%±1.03	99.51%±0.37
CD44	97.8%±1.45	94.93%±5.58
CD105	64.66%±3.72	64.56%±3.47
CD45	1.07%±1.34	0.25%±0.2

B

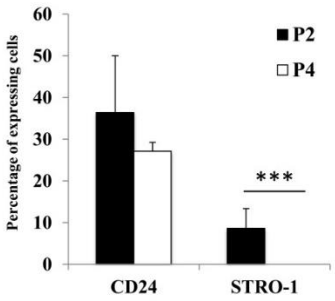


Figure 3.3 Surface mesenchymal stem cell markers between High-CD24 and Low-CD24 of SCAPs at passage 2.

(A) The percentage surface mesenchymal stem cell markers (*significant, $p < 0.05$). (B) The percentage of CD24 expressing cells in the High-CD24 group when compared between passage 2 and passage 4 in the same cell line. The STRO-1 expressing cells were significantly reduced at passage 4 (***$p < 0.001$).

3.4.3 Percentage of CD24 expressing cells of SCAPs on self-renewal

Low-CD24 group exhibited a significant greater number of CFU when compared to High-CD24 group (Figure 3.4A-left). The mean colony counting numbers of Low-CD24 (each cell line was 46, 30 and 33) and High-CD24 (each cell line was 18, 23 and 16) were shown in bar graph (Figure 3.4A-right). The gene expression of pluripotent markers, *Rex-1*, *Nanog*, *Sox2* and *Oct4* were also significant higher in the Low-CD24 group than in High-CD24 group (Figure 3.4B). However, cell proliferation was not different between groups (Figure 3.4C).

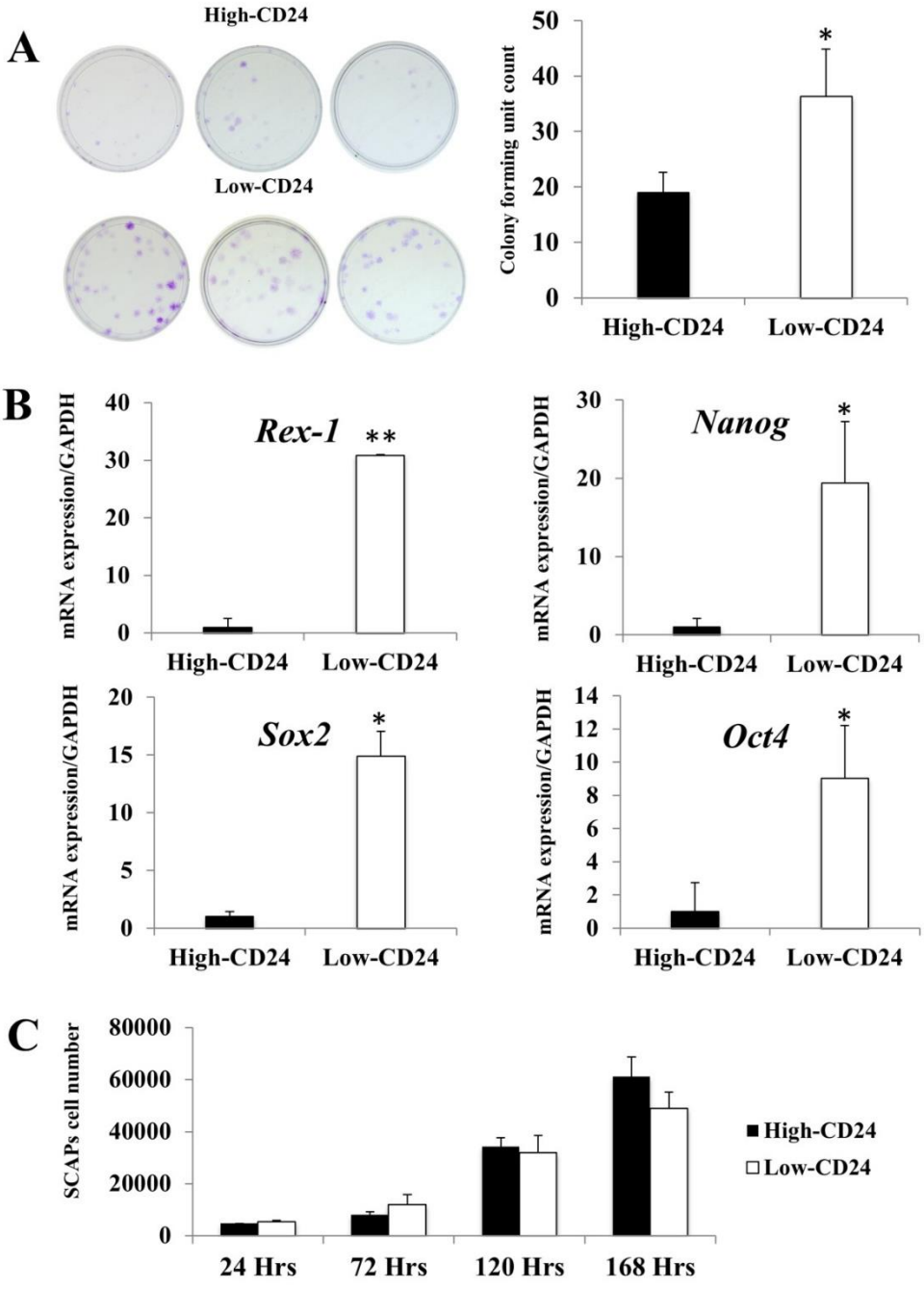

A
High-CD24
Low-CD24
Colony forming unit count
50
40
30
20
10
0
*
High-CD24
Low-CD24
B
Rex-1
**
mRNA expression/GAPDH
40
30
20
10
0
High-CD24
Low-CD24
Nanog
*
30
20
10
0
Sox2
*
20
15
10
5
0
Oct4
*
14
12
10
8
6
4
2
0
C
SCAPs cell number
80000
60000
40000
20000
0
24 Hrs
72 Hrs
120 Hrs
168 Hrs
High-CD24
Low-CD24

Figure 3.4 Comparison of self-renewal and cell proliferation between High-CD24 and Low-CD24 of SCAPs.

(A) The colony formation from each cell line of High and Low-CD24 group were shown in the left. Colony counting analysis between High-CD24 group and Low-CD24 group were shown in the right as bar graphs (*$p < 0.05$). (B) The baseline mRNA expression pluripotent gene markers between High-CD24 and Low-CD24 group. All gene expressions were normalized to GAPDH and then mRNA expression of the High-CD24 normalized to the Low-CD24 (*$p < 0.05$, **$p < 0.01$). (C) Cell proliferation between Low and High-CD24 group (not significant in each time point, $p > 0.05$).

3.4.4 Percentage of CD24 expressing cells of SCAPs on multi-lineage differentiation

High-CD24 group showed significant higher osteogenic differentiation than Low-CD24 group by calcium deposition assay (Figure 3.5A). The High-CD24 group expressed the baseline *Osterix* and *DSPP* gene expressions more than the Low-CD24 group (Figure 3.5B). In contrast, *Runx2* expressed in the High-CD24 lower than in the Low-CD24 group. Adipogenic differentiation also demonstrated higher oil droplet accumulation in the High-CD24 group (Figure 3.5C).

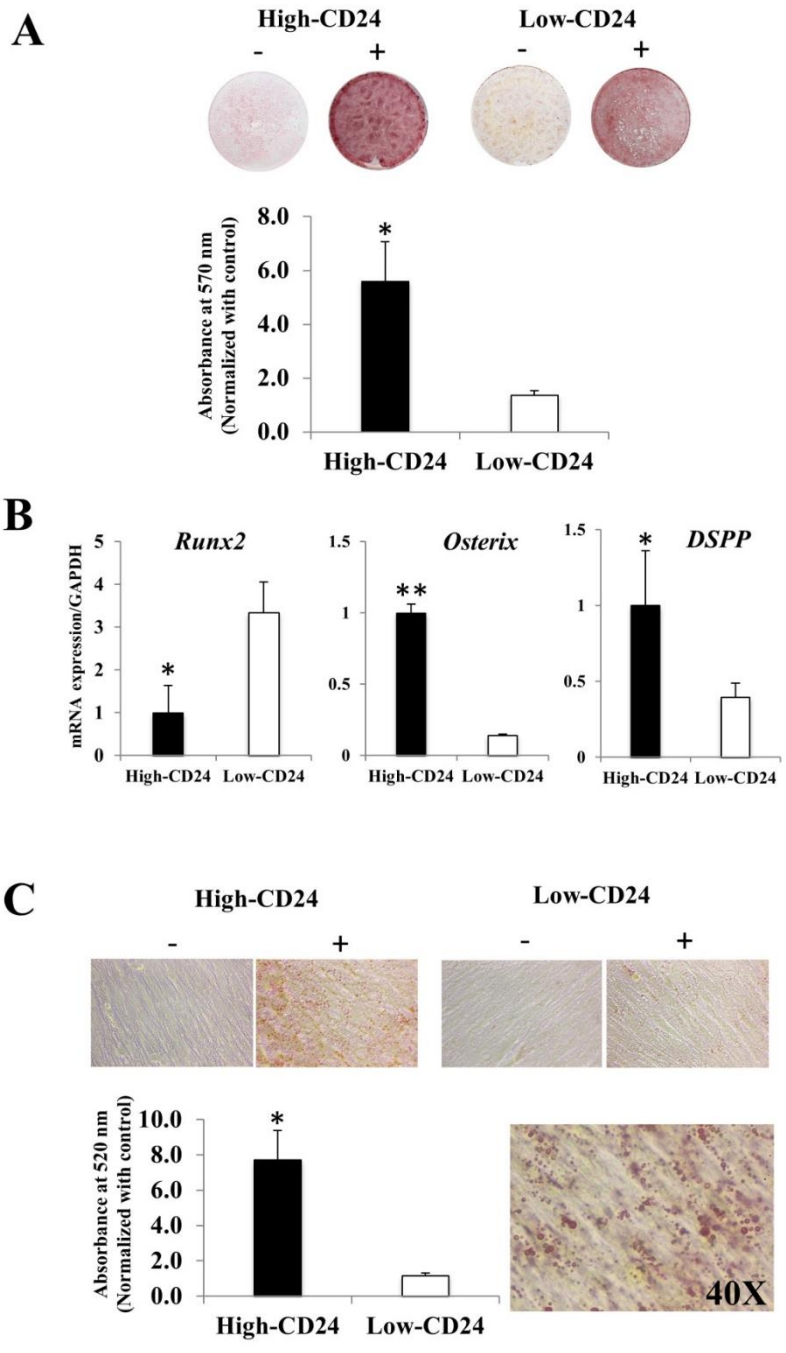

A
High-CD24
Low-CD24
-
+
-
+
Absorbance at 570 nm
(Normalized with control)
8.0
6.0
4.0
2.0
0.0
*
High-CD24
Low-CD24
B
mRNA expression/GAPDH
Runx2
Osterix
DSPP
*
**
*
High-CD24
Low-CD24
C
High-CD24
Low-CD24
Absorbance at 520 nm
(Normalized with control)
10.0
8.0
6.0
4.0
2.0
0.0
*
High-CD24
Low-CD24
40X

Figure 3.5 Multi-differentiation capacity between High-CD24 and Low-CD24 expressing cells of SCAPs.

(A) Osteogenic differentiation; upper figures showed osteogenic differentiation induction capacity in High-CD24 and Low-CD24 group at 1X magnification, **-** Complete growth medium, **+** Osteogenic induction medium. The lower figures presented the quantification of the mineral content using dye elution (*$p < 0.05$). (B) The baseline gene expression of differentiated odontoblast markers, Runx2, Osterix and DSPP between High-CD24 and Low-CD24 group (*$p < 0.05$, **$p < 0.01$). All gene expressions were normalized to GAPDH and then mRNA expression of the High-CD24 normalized to the Low-CD24. (C) Adipogenic differentiation; upper figures showed the oil drop accumulation of High-CD24 and Low-CD24 group at 20X magnification, **-** Complete growth medium, **+** Adipogenic induction medium. The lower figures showed lipid droplet quantification by using dye elution (*$p < 0.05$) and the image at 40X showed lipid drop accumulation in the High-CD24 group.

3.4.5 Stage of root development and percentage of CD24 expressing cells

Ten immature third molar teeth with various stage of root development were collected and categorized into the group of root formation, less than 1/2 group and between 1/2 and 3/4 group as shown in figure 3. 6A (≤1/2 group-left, >1/2-3/4 group-right). The results from H&E staining section demonstrated the different architecture between root dental pulp (DP) and apical papilla (AP) in Figure 3.6B. Root dental pulp contained more cell density and number of blood vessels than in apical papilla (Figure 3.6B). Immunohistochemical observation revealed that ≤1/2 group (Figure 3.6C-left) exhibited more CD24 positive cells than >1/2-3/4 group (Figure 3.6C-right). Either odontoblasts or root dental pulp cells were not positive to CD24 according to observation by the location of pulp tissue (data not shown). The ≤1/2 group showed significant by higher percentage of CD24 expressing cells and MFI than >1/2-3/4 group (Figure 3.6D).

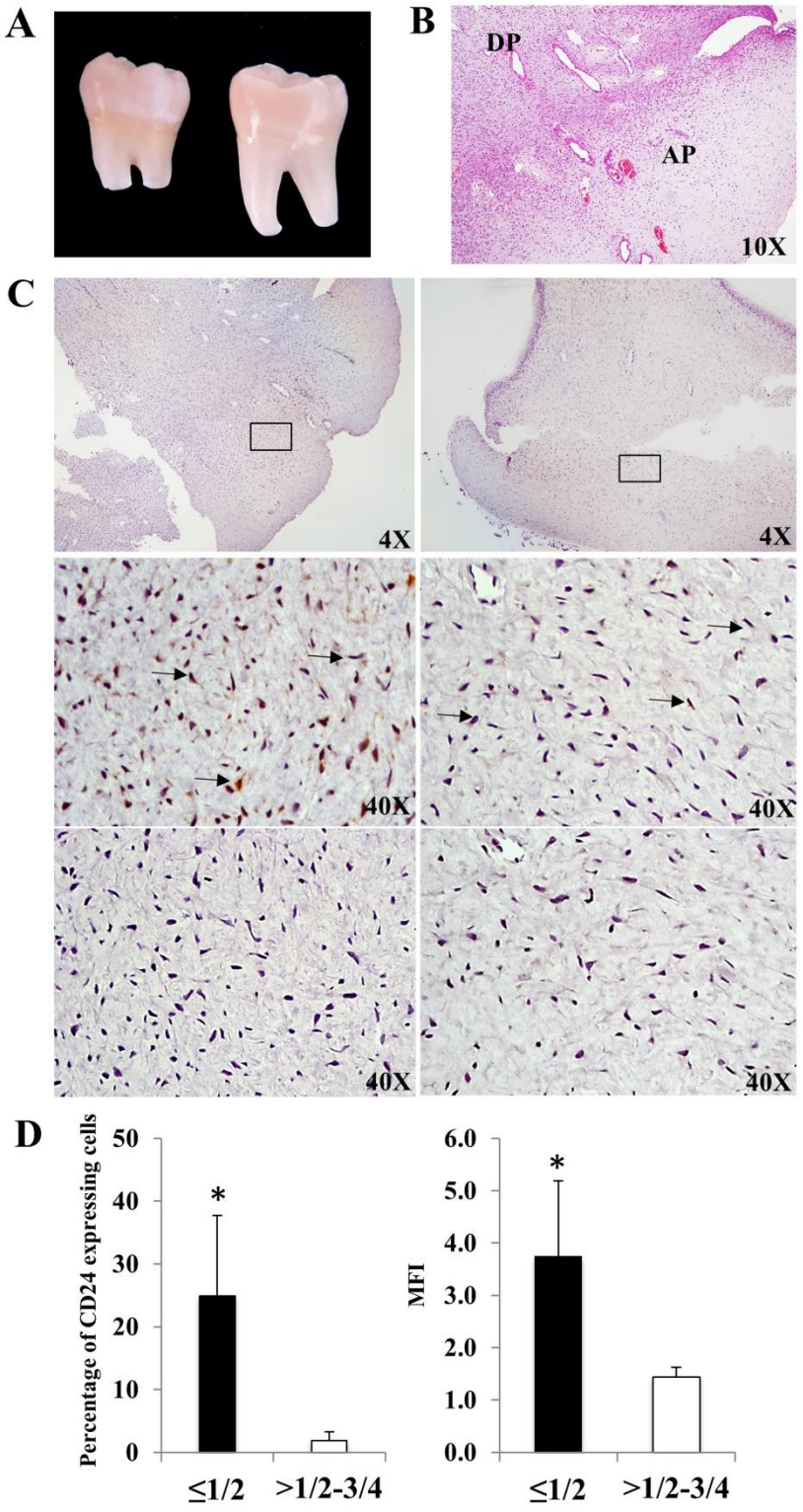

A
B
DP
AP
10X
C
4X
4X
40X
40X
40X
40X
D
Percentage of CD24 expressing cells
50
40
30
20
10
0
*
≤1/2
>1/2-3/4
MFI
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
≤1/2
>1/2-3/4

Figure 3.6 Stage of root development and percentage of CD24 expressing cells.

(A) Extracted third molar tooth with the root formation less than 1/2 group (≤1/2, left) and the root formation between 1/2 to 3/4 group (>1/2-3/4, right). (B) The H&E staining showed histological architecture between root dental pulp (DP) and dental papilla (AP) at 10X. (C) Upper figures showed immunohistochemistry sections stained with antibody against to human CD24 of root dental pulp and apical papilla of ≤1/2 group, left, and >1/2-3/4 group, right, at 4X. Middle panel showed CD24 expressing cells (black arrow) from box area of upper figures at 40X. Lower panel showed isotype control at the same area of ≤1/2 group, left and >1/2-3/4 group, right, at 40X. (D) Showed flow cytometry analysis of SCAPs at passage 2, left, and mean fluorescent intensity (MFI) normalized with isotype antibody staining, right, between ≤1/2 group and >1/2-3/4 group (>1/2-3/4) (*$p < 0.05$). Scale bars, 200 μm.

3.5 Discussion

CD24 was proposed to use as a specific marker for SCAPs (Sonoyama *et al*., 2006). However, CD24 also expressed in many cells types. During embryogenesis, CD24 expressing cells were observed in various cell types such as cells from nervous system, salivary gland cells, kidney cells, hair follicular cells including differentiating odontoblasts (Shirasawa *et al*., 1993). Some CD24 expressing cells were absent in some cell types such as dental pulp cells (Sonoyama *et al*., 2006), odontoblasts and adipocytes (Rodeheffer *et al*., 2008), when they reached the maturation stage. In contrast, differentiated neural cells constantly expressed CD24 (Pruszak *et al*., 2009;

Yuan *et al*., 2011). Nevertheless, CD24 positive cells were found in matured organ in very few numbers (Qiu *et al*., 2011; Romagnani and Remuzzi, 2014). These positive cells were claimed to be progenitor cells for repairing the injured tissue (Qiu *et al*., 2011; Romagnani and Remuzzi, 2014). These evidences suggest that temporary or permanent CD24 expression may depend on cell type or tissue organ. The wide range percentage of CD24 expressing cells of SCAPs was also reported in this study. Thus, CD24 may not be the suitable specific marker to SCAPs regarding to its inconsistent expression. However, CD24 may be used to distinguish SCAPs from dental pulp cells. The other purposed SCAPs marker, Survivin, was also investigated in our study.

Survivin was suggested to function as an anti-apoptotic protein and cell proliferation regulator which was necessary for developmental process (Fukuda and Pelus, 2006). In this study, *Survivin* expression was the same in both groups of SCAPs. This result suggested that *Survivin* expression did not depend on the percentage of CD24 expressing cell or stage of root development. The same *Survivin* expression level might explain the same cell proliferation rate between high and low CD24 expressing SCAPs because of its proliferation regulation.

The different percentage of CD24 expressing cells may affect the SCAPs behaviors especially the mesenchymal stem cell characteristics. However, High and Low-CD24 groups were not different in expression of surface mesenchymal stem cell markers (CD44, CD90 and CD105). Interestingly, High-CD24 group demonstrated higher STRO-1 expressing cells than Low-CD24 group. The STRO-1-sorted SCAPs were shown a higher potential to osteogenic differentiation than the non-sorted cells in previous study (Bakopoulou *et al*., 2013). To exclude the effects of STRO-1 on

osteogenic differentiation of SCAPs in our study, the further subculture was continued until STRO-1 was not detected which was at passage 4. Therefore, the SCAPs at passage 4 were used to investigate the effects of CD24 on SCAPs behaviors. Although STRO-1 expressing SCAPs were not detected on the cell surface at passage 4, the effects of STRO-1 might still have an impact to High-CD24 SCAPs behavior especially osteogenic differentiation. $CD146^{+}/STRO\text{-}1^{+}$ sorted SCAPs at passage 2 demonstrated more mineralization capacity than non-sorted SCAPs when induced to osteogenic differentiation at passage 4-6 (Bakopoulou *et al*., 2013). However, the number of $STRO\text{-}1^{+}$ SCAPs of $CD146^{+}/STRO\text{-}1^{+}$ sorted cells did not measure before induction. Interestingly, the same study also showed that $CD146^{+}/STRO\text{-}1^{+}$ sorted SCAPs contained CD24 expressing cells more than non-sorted SCAPs. It was possible that some cells of High-CD24 groups of this study also positive to both CD24 and STRO-1, only positive to CD24 and only positive to STRO-1. To confirm the effect of higher osteogenic differentiation of High-CD24 group was the result from CD24 or STRO-1, the cell sorting at passage 2 of each group, $CD24^{+}/STRO\text{-}1^{+}$, $CD24^{+}/STRO\text{-}1^{-}$, $CD24^{-}/STRO\text{-}1^{+}$ and non-sorted cells of High-CD24 group, should be performed in the passage 4.

Self-renewal is an ability to give rise the two progenies from one mother cell that one daughter cell will undergo differentiation while the other still be the stem cell after mitosis. However, self-renewal is difficult to examine *in vitro* study by this definition. It was usually determined by CFU assay *in vitro* study (Becker *et al*., 1963; Ogawa, 1993). Self-renewal assay also determined by the clonality of single cell that increased along pluripotent gene markers expression as in the previous studies (Nowwarote *et al*., 2015; Sukarawan *et al*., 2014). The High-CD24 group demonstrated

the lower numbers of CFU than the Low-CD24 group. The genes that regulated stem cell self-renewal, *Rex-1, Nanog*, *Sox2* and *Oct4*, were also lower than Low-CD24 group. These results indicated that Low-CD24 group showed more self-renewal capacity than High-CD24 group. The less self-renewal of higher CD24 expressing cells in this study was similar to previous reports that some cell types such as keratinocytes and luminal cells of prostate gland were demonstrated as a differentiated or committed progenitor cells with less self-renewal (Bergoglio *et al*., 2007; Petkova *et al*., 2013). However, cell proliferation was not different between both groups in our results. Cell proliferation could determine only the capacity of the whole cell population to grow but did not determine the self-renewal ability.

In the osteogenic differentiation assay, the High-CD24 group of SCAPs demonstrated a higher capacity of this differentiation than the Low-CD24 group by calcium deposition assay. The baseline gene expressions that related to odontoblast differentiation without osteogenic induction, *Dentin sialophosphoprotein* (*DSPP*), *Osterix* and *Runx2* were also investigated. The late stage of odontogenic differentiation markers, *DSPP* and *Osterix* of High-CD24 were higher than Low-CD24 group. In contrast, the early stage marker, *Runx2*, was lower than Low-CD24 group. These results indicated that High-CD24 group exhibited more advance stage of differentiation and more susceptible to induce. These results were the same as the other cell type reports. The higher CD24 expressing cells demonstrated the features of committed progenitor cells or more advance stage of cell differentiation of keratinocytes (Bergoglio *et al*., 2007), the luminal cells of prostate gland (Petkova *et al*., 2013), progenitor cells of adipocyte (Berry and Rodeheffer, 2013; Rodeheffer *et al*., 2008) , neural progenitor cells (Pruszak *et al*., 2009). Recently, bone marrow stem cells that expressed CD24

were also demonstrated to committed progenitor cell to osteoblast (Chang *et al*., 2015). Thus, higher CD24 expressing cells of SCAPs may contain more the higher committed progenitor cells as well.

Furthermore, High-CD24 group showed a higher differentiated capacity to adipogenic differentiation. SCAPs could be forced into adipogenic differentiation by the specific induction medium, although adipogenic differentiation of SCAPs did not participate in root development. Others dental mesenchymal stem cells, dental pulp stem cells (Gronthos *et al*., 2002) and periodontal ligament stem cells (Seo *et al*., 2004) could also induce to the adipogenic differentiation. The results from this study suggested that indigenous higher number of CD24 expressing cells in early stage of root development was more susceptible to adipogenic differentiation. The high CD24 expression was proposed as the committed or more advance stage of progenitor cells of adipocyte and osteoblast (Chang *et al*., 2015; Rodeheffer *et al*., 2008). Our study demonstrated that the multi-lineage differentiation, both osteogenic and adipogenic differentiation, was better in higher CD24 expressing cell numbers than lower CD24 expressing cell numbers. Thus, the proper induction of high CD24 expressing SCAPs can induce them to other cell lineages and may benefit to regenerative medicine in further study.

The factors that may affect the percentage of CD24 expressing cells of SCAPs have not been investigated. The previous studies reported that the density of dentinal tubules in cervical root dentin was more than in apical root dentin (Harran Ponce *et al*., 2001; Mannocci *et al*., 2004). Each dentinal tubule was normally occupied by only one odontoblast (Mjor *et al*., 2001). This implies that the progenitor cell number for

odontoblast may decrease in later stage of root development. Thus, the stage of root development may influence the number of CD24 expressing cells of SCAPs. According to root development stage criteria in this study, the samples with root formation less than 1/2 group and the root formation between 1/2 and 3/4 were categorized in figure 3.6A (≤1/2 group-left, >1/2-3/4 group-right). The first group represented the early root development stage and the latter represented the later stage of root development. The percentage of CD24 expressing SCAPs and mean fluorescence intensity (MFI) were higher in early stage of root formation in this report. Moreover, apical papilla tissue from the early root formation contained more density of CD24 positive cells. These results suggested that SCAPs obtained from early stage of root development teeth might contain more committed progenitor cell numbers to osteogenic differentiation or to be odontoblasts. These findings may explain the higher density of odontoblast in cervical root dentin than apical root dentin.

Dental mesenchyme migration is crucial for mesenchyme condensation and these events are pre-requisite for odontoblast differentiation during tooth development (Mammoto *et al*., 2011). The high percentage of CD24 expressing cells demonstrated the higher cell migration ability in cancer cell line (Mierke *et al*., 2011) by cooperating with integrin and focal adhesion kinase (FAK) pathway (Baumann *et al*., 2012; Bretz *et al*., 2012; Runz *et al*., 2008). Thus, CD24 may regulate cell migration of SCAPs during root development and should be further investigated, as well.

The results from this study showed that the CD24 expression transiently expressed in SCAPs from early stage of root development and might contain more committed progenitor cells than the late stage of root development. However, this study

reported only the roles of indigenous or native CD24 expressing cells on stem cell behaviors. The further investigation need to confirm about the effects of CD24 expressing cell numbers on self-renewal and differentiation capacity of SCAPs by silencing of CD24 in High-CD24 expression and overexpression of CD24 in Low-CD24 expression. These further studies are very useful to recapitulate results and confirm the results from indigenous expression of CD24.

In conclusion, the percentage of CD24 expressing cells was related to root development stage. SCAPs from early stage of root development contained more CD24 expressing cells and demonstrated higher differentiation capacity. However, both High-CD24 and Low-CD24 group showed the mesenchymal stem cell characteristics of SCAPs. The amount of CD24 may refer to the number of committed progenitor cells of the SCAPs in apical papilla tissue.

CHAPTER 4

THE EFFECTS OF LIPOPOLYSACCHARIDE ON STEM CELLS FROM APICAL PAPILLA PROLIFERATION, MIGRATION AND OSTEOGENIC DIFFERENTIATION WITH DIFFERENT INDEGENOUS CD24 EXPRESSING CELLS[2]

4.1 Abstract

Dentin regenerative process after root canal infection requires SCAPs proliferation, migration and osteogenic differentiation for regenerative endodontic procedure. However, the effects of root canal infection especially LPS to these capacities have never been studied. LPS with 0.001, 0.01, 0.1, 1 and 5 μg/ml did not affect on SCAPs viability of both High and Low-CD24 SCAPs. After challenging with various LPS concentrations to High and Low-CD24 SCAPs 168 hours, these SCAPs were investigated for cell proliferation and migration and osteogenic differentiation. LPS did not affect cell proliferation and cell migration numbers of both High and Low-CD24 SCAPs. High-CD24 SCAPs showed higher mineralization ability than Low-CD24 SCAPs by Alizarin Red staining. Interestingly, 1 and 5 μg/ml of LPS significantly increased Alizarin Red staining and *BSP* gene expression of High-CD24 SCAPs when compared to non-LPS exposure SCAPs (control). In contrast, LPS did not affect to Alizarin Red staining in Low-CD24 SCAPs. However, only 5 μg/ml LPS

[2] This chapter based on manuscript, submitted to Tissue and Cell.

increased *BSP* gene expression when compared to Non-LPS exposure of Low-CD24 SCAPs. LPS pre-exposure at any concentration to both groups of SCAPs did not affect the *DSPP*, *OCN*, and *OPN* gene expressions.

4.2 Introduction

Tooth root development could be interrupted by root canal infection following dental caries or traumatic injuries. Regenerative endodontic procedure could regain root development by increasing the root canal wall thickness and the root length (Bose *et al*., 2009; Jeeruphan *et al*., 2012; Kontakiotis *et al*., 2015). The principle of REP was recruitment of stem cells around tooth apex into root canal space (Hargreaves *et al*., 2013). Stem cells from apical papilla or SCAPs were presumed as the stem cells for this regeneration (Huang *et al*., 2008; Sonoyama *et al*., 2008) because these cells responded to physiological root dentin development (Sonoyama *et al*., 2006). SCAPs were demonstrated as the mesenchymal stem cells by using various methods such as, positive to some surface mesenchymal stem cell markers could be induced to multi-lineage of differentiation (Aguilar and Lertchirakarn, 2016; Bakopoulou *et al*., 2013; Ding *et al*., 2009; Prateeptongkum *et al*., 2015; Sonoyama *et al*., 2006). SCAPs also showed cell proliferation, migration and osteogenic differentiation capacity (Huang *et al*., 2008; Sonoyama *et al*., 2006; Sonoyama *et al*., 2008).

In REP, the recruited SCAPs were usually exposed to LPS via root canal infection. Other dental stem cells such as dental pulp, periodontal ligament and dental follicle stem cells have been shown that LPS could increase, decrease or not alter these cell proliferation and osteogenic differentiation. The results depended on type of stem cells, source of LPS and concentrations (Albiero *et al*., 2015; Chatzivasileiou *et al*., 2013; He *et al*., 2015; Kato *et al*., 2014; Liu *et al*., 2014b; Morsczeck *et al*., 2012; Yamagishi *et al*., 2011). In addition, LPS could increase dental pulp cells migration to injury site of carious lesion for repairing dentin formation (Chmilewsky *et al*., 2015; Li

et al., 2014a; Liu *et al*., 2014b). However, there is no evidence to show the effects of LPS on SCAPs behaviors. Thus, LPS was suggested to influence the SCAPs behaviors, cell proliferation, migration and osteogenic differentiation, which required for root dentin regeneration (Huang and Garcia-Godoy, 2014; Smith *et al*., 2016). The effects of LPS on SCAPs were demonstrated only the immunological responses such as cytokines and inflammatory mediator releases (Wang *et al*., 2013a; Zhang *et al*., 2013).

In previous study, we presented the various percentages of CD24 expressing SCAPs and showed high percentage of CD24 expressing SCAPs were better osteogenic differentiation than low percentage of CD24 expressing. In contrast, the percentage of CD24 expressing cells did not affect to SCAPs proliferation (Aguilar and Lertchirakarn, 2016). These SCAPs might expose to LPS before the regenerative procedure initiation. The pre-exposure of LPS might affect the stem cells behaviors of SCAPs with different CD24 expressing cells and might impact to root dentin regeneration.

The objective of this study was to investigate the influence of LPS pre-exposure to different CD24 expressing SCAPs (High-CD24 or Low-CD24 SCAPs) on cell proliferation, migration and osteogenic differentiation that were crucial for the dentin regeneration.

4.3 Materials and Methods

4.3.1 Sample collection and cell culture

SCAPs were obtained from human permanent immature third molar teeth from 17–20 year-old patients at Department of Oral and Maxillofacial Surgery, Faculty of Dentistry Chulalongkorn University with approved protocol by the Ethics Committee (Study code: HREC-DCU-P 2015-001). The amount of CD24 expressing cells in apical papilla tissue was determined by immunohistochemistry analysis as previously described (Aguilar and Lertchirakarn, 2016). The mesenchymal stem cell markers and CD24 of these cells were determined before using in further experiments. Three cell lines with High-CD24 and Low-CD24 SCAPs were used in the following experiments. The experiments were performed in triplicate.

4.3.2 Effect of LPS exposure on SCAPs viability

Three cell lines of both high and low percentage of CD24 expressing SCAPs at passage 4 (High-CD24 and Low-CD24 SCAPs) were seeded at 5,000 cells/well in 24-well plates. These cells were incubated in growth medium (Aguilar and Lertchirakarn, 2016) with 0.001, 0.01, 0.1, 1 and 5 μg/ml of *Porphyromonas gingivalis* LPS (Invivogen, San Diego, CA, USA). Growth medium without LPS was used as a control. The same medium with or without LPS were replaced every 48 hours. At the end of exposure time at 24, 72, 120 and 168 hours, all SCAPs aviability in every groups were determined by AlarmarBlue® solution (GIBCO/Invitrogen, CA, USA) following the previous methods (Aguilar and Lertchirakarn, 2016).

4.3.3 Effect of LPS exposure on percentage of CD24 expressing SCAPs

Three cell lines of both high and low percentage of CD24 expressing SCAPs at passage 4 (High-CD24 and Low-CD24 SCAPs) were seeded at 120,000 cells/well in 6-well plates. These cells were incubated in growth medium (Aguilar and Lertchirakarn, 2016) with 0.001, 0.01, 0.1, 1 and 5 μg/ml of *Porphyromonas gingivalis* LPS. Growth medium without LPS was used as a control. The same medium with or without LPS were replaced every 48 hours. All experimental groups were incubated for 168 hours At the end of exposure time, all SCAPs in every groups were detached and 100,000 cells from all conditions of each cell line were determined the percentage of CD24 expressing cells by flow cytometry.

4.3.4 SCAPs proliferation after LPS exposure

All experimental and control group of High and Low-CD24 SCAPs at 168 hours were detached and seeded at 5,000 cells in 24-well plates in growth medium without LPS. All conditions were replaced with growth medium without LPS every 48 hours including control group. Cell proliferation was measured as the percentage of control at 24, 72, 120 and 168 hour by AlarmarBlue® solution following the previous methods (Aguilar and Lertchirakarn, 2016).

4.3.5 SCAPs migration after LPS exposure

After incubation with and without LPS for 168 hours, both High and Low-CD24 SCAPs were seeded at 20,000 cells on the upper chamber of 8-micron pore sized polycarbonate membrane, Transwell®insert system (Corning, MA, USA) for 6 hours to allow cells to attach on the membrane of upper chamber. Then, upper chamber of

Transwell®insert system with attached cells was transferred to 24-well plates with 600 µl of growth medium. After 24 hours, each membrane was fixed with 10% formalin for 15 minutes. Non-migrated cells on upper surface membrane were swabbed with moist cotton bud. Then, migrated cells were stained with 10% (g/v) crystal violet in methanol. To quantify the migrated cells, five pictures of five random areas of each experimental group were taken (Li *et al*., 2014a) at 10X magnification (Olympus CK21, Olympus America Inc, PA, USA). Cell counting was performed at this magnification. The migrated cells of five areas were averaged and represented as migrated cell numbers of each condition (Li *et al*., 2014a). Previous studies demonstrated that the C-X-C chemokine receptor type 4 (CXCR4) participated in SCAPs migration (Chen *et al*., 2016; Liu *et al*., 2015a). Thus, the *CXCR4* gene expression of both High and Low-CD24 SCAPs in all conditions was examined. Total RNA of all conditions were converted to cDNA and determined *CXCR4* gene expression by PCR. *CXCR4* primer sequences were shown in Table 4.1.

4.3.6 SCAPs osteogenic differentiation after LPS exposure

SCAPs at the same conditions of both High and Low-CD24 SCAPs were seeded at 50,000 cells per well in 24-well plates. Osteogenic induction medium without LPS and osteogenic differentiation methods were followed as the previous study (Aguilar and Lertchirakarn, 2016). After 14 days of induction, the mineralization was determined by Alizarin Red staining and quantified as previously described (Aguilar and Lertchirakarn, 2016). The total RNA after osteogenic induction of each group was harvested. Osteogenic differentiation markers, *DSPP*, *BSP*, *OCN*, *OPN* and *CAP* were

determined by PCR with the same methods of 2.4. Primer sequences of *DSPP*, *BSP*, *OCN, OPN* and *CAP* were shown in Table 4.1.

4.3.7 Statistical Analyses

The data were shown as mean and standard deviation. The Kruskal-Wallis test was performed to investigate the effects of LPS and followed by Dunn-Sidak's test for both High and Low-CD24 SCAPs. Mann-Whitney U test was performed to compare the migrated cell numbers between High and Low-CD24 SCAPs in each LPS concentration and control. Mann-Whitney U test was also performed to compare the difference of *CXCR4* gene expression between control groups of High and Low-CD24 SCAPs. The significance level was set at $p < .05$.

Gene Name	Sequence ID	Primer Sequences (5'-3')	Product Size
CXCR4	NM_001008540.1	TACACCGAGGAAATGGGCTCA AGATGATGGAGTAGATGGTGGG	110
DSPP	NM_014208.3	ATATTGAGGGCTGGAATGGGGA TTTGTGGCTCCAGCATTGTCA	136
BSP	NM_004967.3	ATGGCCTGTGCTTTCTCAATG AGGATAAAAGTAGGCATGCTT	123
OCN	NM_199173.2	CTTTGTGTCCAAGCAGGAGG CTGAAAGCCGATGTGGTCAG	166
OPN	NM_001040060.1	AGGAGGAGGCAGAGCACA CTGGTATGGCACAGGTGATG	150
CAP	NM_014241.3	CTGCGCGCTGCACATGG GCGATGTCGTAGAAGGTGAGCC	256
GAPDH	NM_002046.4	GAAGGCTGGGGCTCATTT CAGGAGGCATTGCTGATGAT	137

Table 4.1 Primer sequences for real-time PCR.

4.4 Results

4.4.1 SCAPs viability during LPS exposure.

All concentrations of LPS did not affect to SCAPs viability of both High-CD24 SCAPs (Figure 4.1A) and Low-CD24 SCAPs (Figure 4.1B) at 24, 72, 120 and 168 hours.

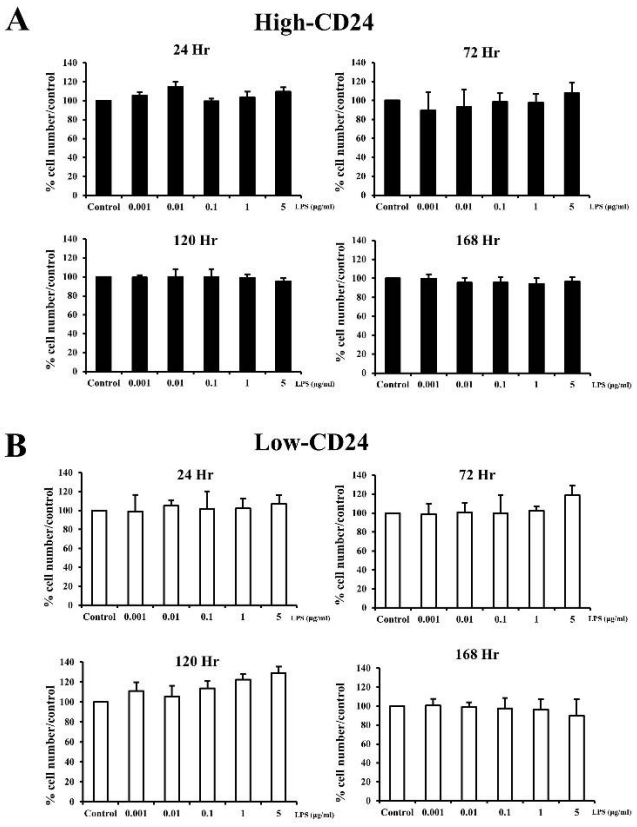
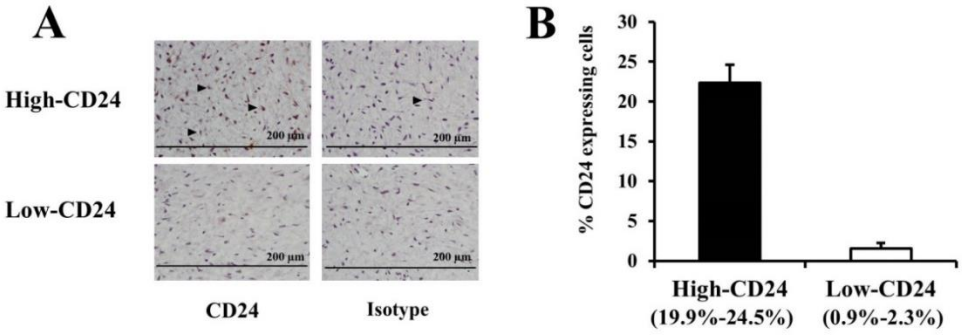


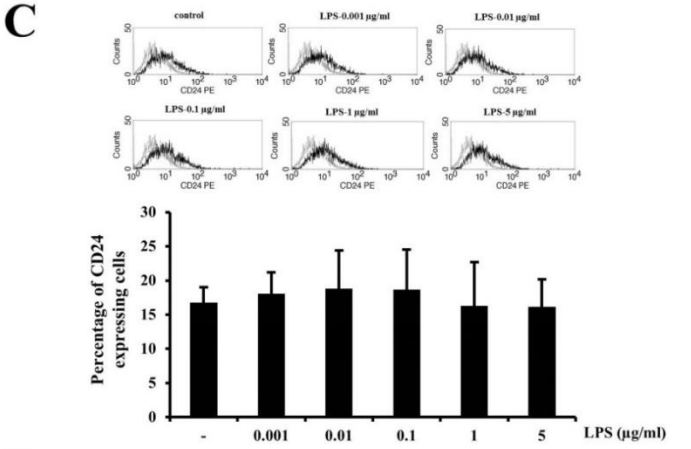
Figure 4.1 Cell viability during LPS exposure of High and Low-CD24 SCAPs.

(A) Cell viability during LPS exposure at 24, 72, 120 and 168 hours of High-CD24 SCAPs. (not significant in each time point, $p > 0.05$). (B) Cell viability during LPS exposure at 24, 72, 120 and 168 hours of Low-CD24 SCAPs (not significant in each time point, $p > 0.05$).

4.4.2 The percentage of CD24 expressing cells after LPS exposure

The immunohistochemistry analysis of CD24 confirmed the numerous CD24 positive cells in apical papilla tissue from ≤1/2 root formation stage group and lower number of CD24 positive cells in >1/2 – 3/4 root formation stage group (Figure 4.2A). Three cell lines from High-CD24 SCAPs (22.32% ± 2.29) and Low-CD24 SCAPs (1.54% ± 0.69) were selected for the following experiments (Figure 4.2B). After exposure to LPS for 168 hours, the percentage of CD24 expressing cells of all LPS treated groups and control were not significantly different from before exposure in both High-CD24 (Fig. 4.2C) and Low-CD24 SCAPs (Figure. 4.2D).


A
High-CD24
Low-CD24
200 µm
CD24
Isotype
B
% CD24 expressing cells
30
25
20
15
10
5
0
High-CD24
(19.9%-24.5%)
Low-CD24
(0.9%-2.3%)


C
control
LPS-0.001 µg/ml
LPS-0.01 µg/ml
LPS-0.1 µg/ml
LPS-1 µg/ml
LPS-5 µg/ml
Counts
CD24 PE
Percentage of CD24 expressing cells
30
25
20
15
10
5
0
-
0.001
0.01
0.1
1
5
LPS (µg/ml)

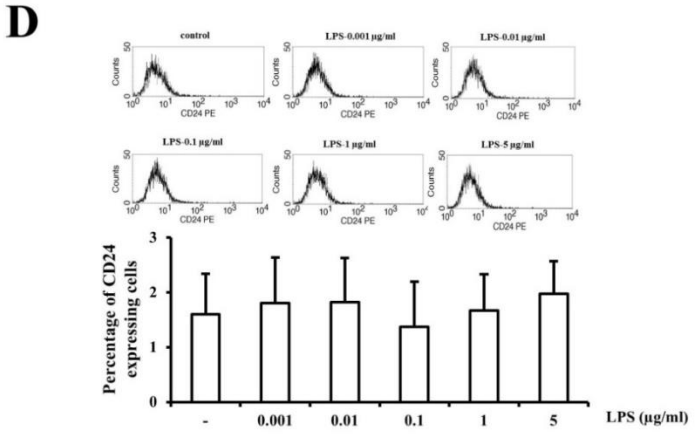

D
control
LPS-0.001 µg/ml
LPS-0.01 µg/ml
LPS-0.1 µg/ml
LPS-1 µg/ml
LPS-5 µg/ml
Counts
CD24 PE
Percentage of CD24 expressing cells
3
2
1
0
-
0.001
0.01
0.1
1
5
LPS (µg/ml)

Figure 4.2 Percentage of CD24 expressing cells after LPS exposure.

(A) CD24 expressing cells from apical papilla tissue. Upper left and right figures were stained with CD24 antibody of <1/2 and ≥1/2 - 3/4 root formation stage groups (<1/2 and ≥1/2 - 3/4 groups), respectively. Black arrow head pointed to CD24 expressing cells. Lower left and right figures were stained with isotype antibody. (B) The average percentage of CD24 expressing cells of the High-CD24 and Low-CD24 SCAPs, used in this study, at passage two. (C) Illustrated High-CD24 expressing cells after LPS exposure. Upper figures represented the CD24 positive cells (black line) and background fluorescence of High-CD24 SCAPs (grey line). Lower bar graph showed percentages of CD24 of High-CD24 SCAPs of three cell lines after LPS exposure ($p > 0.05$). (D). Illustrated Low-CD24 expressing cells after LPS exposure. Upper figures represented the CD24 positive cells (black line) and background fluorescence of Low-CD24 SCAPs (grey line). Lower bar graph showed percentages of CD24 of Low-CD24 SCAPs of three cell lines after LPS exposure ($p > 0.05$).

4.4.3 The effect of LPS on SCAPs proliferation

SCAPs proliferation after exposure to LPS at any concentrations and control groups were not different in both High-CD24 (Figure 4.3A) and Low-CD24 SCAPs (Figure 4.3B) at 24, 72, 120 and 168 hours.

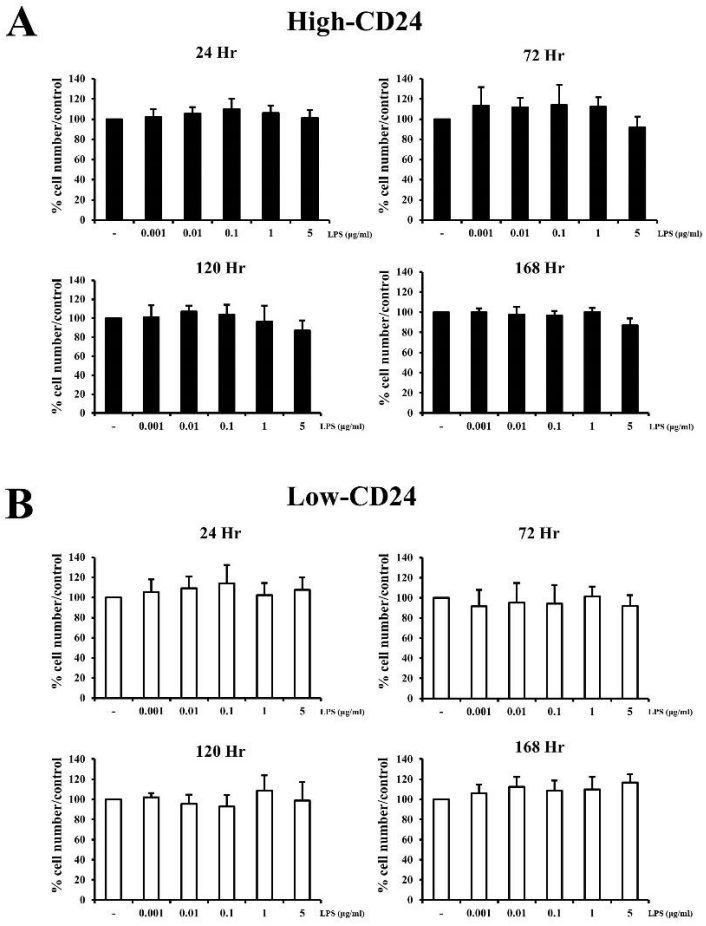
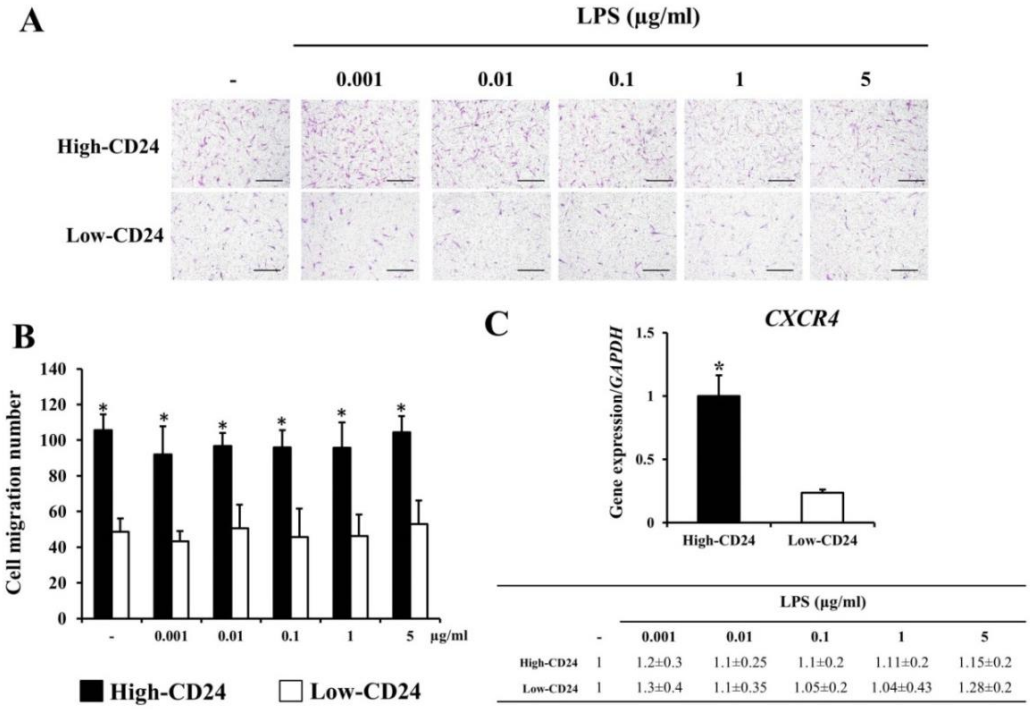


Figure 4.3 Cell proliferation after LPS exposure of High and Low-CD24 SCAPs. (A) Cell proliferation after LPS exposure at 24, 72, 120 and 168 hours of High-CD24 SCAPs. (not significant in each time point, $p > 0.05$). (B) Cell proliferation after LPS exposure at 24, 72, 120 and 168 hours of Low-CD24 SCAPs (not significant in each time point, $p > 0.05$).

4.4.4 The effect of LPS on SCAPs migration.

High-CD24 SCAPs showed significant more migrated cell numbers than Low-CD24 SCAPs in control and all LPS pre-exposure groups (Figure 4.4A, 4.4B). However, all concentration of LPS and control in the same percentage of CD24 expressing cells showed no effect of LPS to SCAPs migration. The control group of High-CD24 SCAPs demonstrated more CXCR4 gene expression than Low-CD24 SCAPs (Figure 4.4C-upper). In addition, exposure to LPS at any concentrations did not affect CXCR4 gene expression in both High and Low-CD24 SCAPs from control group (Figure 4.4C-lower).



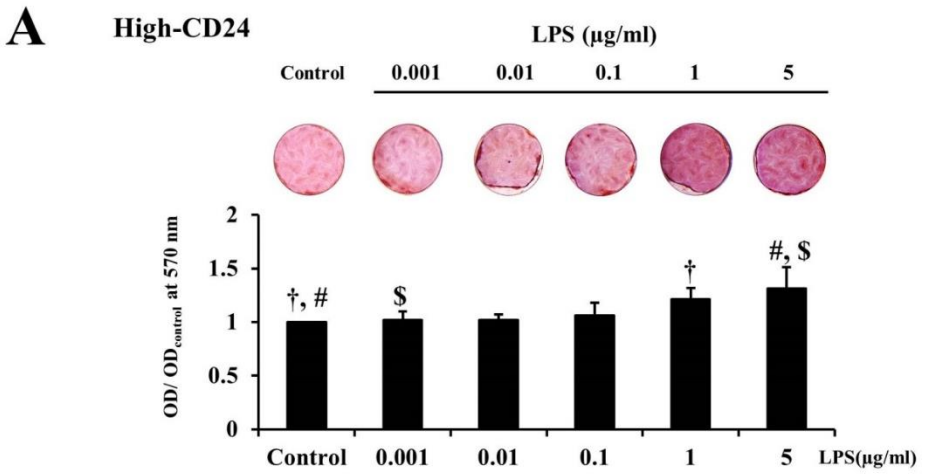
		LPS (µg/ml)				
	-	0.001	0.01	0.1	1	5
High-CD24	1	1.2±0.3	1.1±0.25	1.1±0.2	1.11±0.2	1.15±0.2
Low-CD24	1	1.3±0.4	1.1±0.35	1.05±0.2	1.04±0.43	1.28±0.2

Figure 4.4 Cell migration after LPS exposure of High and Low-CD24 SCAPs.

(A) Showed migrated cells from High-CD24 SCAPs (upper panel) and Low-CD24 SCAPs (lower panel) after exposure to LPS. Bar line in each figure was 25 µm. (B) Numbers of migrated cell of High and Low-CD24 SCAPs after LPS exposure. The numbers of migrated cell of High-CD24 SCAPs after LPS exposure and control were significant more cells when compared with Low-CD24 SCAPs in the same condition (*$p < 0.05$). (C) Upper figure showed CXCR4 gene expression between High and Low-CD24 SCAPs of control groups (*$p < 0.05$). Lower figure showed CXCR4 gene expression of High and Low-CD24 SCAPs, compared with control, after LPS exposure ($p > 0.05$).

4.4.5 The effect LPS on SCAPs osteogenic differentiation.

In High-CD24 SCAPs, exposure with 1 and 5 µg/ml of LPS significantly increased Alizarin Red staining when compared to control and 0.001 µg/ml LPS groups (Fig. 4.5A). In contrast, the Alizarin Red staining of all LPS concentrations and control groups were not different in Low-CD24 SCAPs (Figure 4.5B). *DSPP*, *OCN*, *OPN* and *CAP* gene expression of all LPS concentrations and control groups of both High and Low-CD24 SCAPs were not different (Figure 4.6A, 4.6B). However, 5 µg/ml LPS exposure of both High-CD24 and Low-CD24 SCAPs demonstrated significantly more *BSP* gene expression than control group (Figure 4.6A, 4.6B) but 1 µg/ml LPS increased *BSP* gene expression only in High-CD24 SCAPs when compared to control (Figure 4.6A).


A
High-CD24
LPS (µg/ml)
Control
0.001
0.01
0.1
1
5
OD/ OD control at 570 nm
2
1.5
1
0.5
0
†, #
$
†
#, $
Control
0.001
0.01
0.1
1
5
LPS(µg/ml)

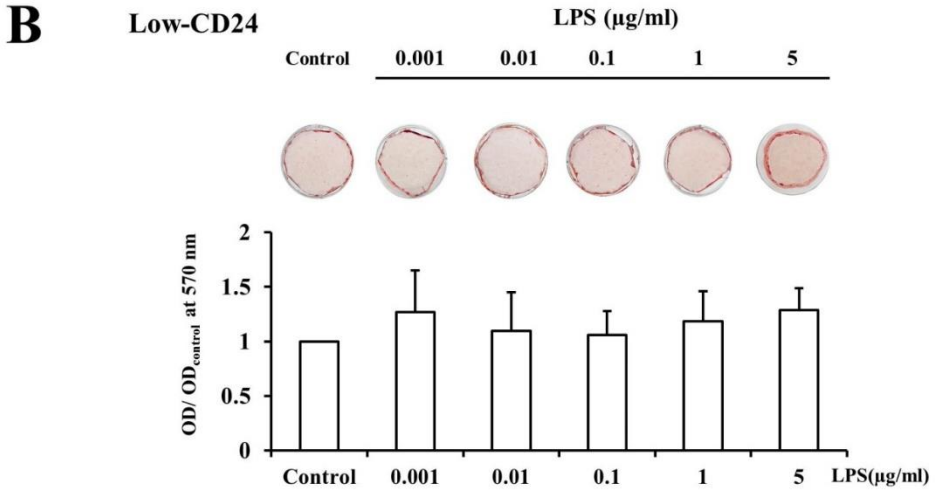

B
Low-CD24
LPS (µg/ml)
Control
0.001
0.01
0.1
1
5
OD/ OD control at 570 nm
2
1.5
1
0.5
0
Control
0.001
0.01
0.1
1
5
LPS(µg/ml)

Figure 4.5 Effects of LPS pre-exposure to mineralization of High and Low-CD24 SCAPs.

(A) Upper figures showed Alizarin Red staining after LPS exposure and further osteogenic differentiation induction of High-CD24 SCAPs. Lower graph presented the quantification of the mineral content using dye elution between the groups ($p < 0.05$). Bar graphs with the same symbols (†, # and $) were significantly different by pair-wise comparison test ($p < 0.05$). (B) Upper figures showed Alizarin Red staining after LPS exposure and further osteogenic differentiation induction of Low-CD24 SCAPs. Lower graph presented the quantification of the mineral content using dye elution between the groups ($p > 0.05$).

A

High-CD24

		LPS (µg/ml)				
	Control	**0.001**	**0.01**	**0.1**	**1**	**5**
DSPP	1	1.13±0.3	1.31±0.5	1.32±0.54	0.3±0.64	1.69±0.58
BSP	$1^{\dagger,\#}$	$1.5\pm0.2^{\P,\$}$	1.7±0.3	2.9±0.8	$3.3\pm1^{\dagger,\P}$	$4\pm1.1^{\#,\$}$
OCN	1	1.08±0.22	0.94±0.23	0.85±0.21	1.09±0.26	1.18±0.55
OPN	1	1.22±0.60	1.23±0.79	1.3±0.6	1.32±0.98	1.83±1.38
CAP	1	1.01±0.11	0.86±0.28	1±0.26	0.91±0.2	1.15±0.18

B

Low-CD24

		LPS (µg/ml)				
	Control	**0.001**	**0.01**	**0.1**	**1**	**5**
DSPP	1	1.03±0.18	1.19±0.28	1.40±0.17	1.5±0.87	3.08±2.34
BSP	$1^{\dagger}$	1.51±0.27	1.42±0.3	1.91±0.78	2.14±0.71	$5\pm1.72^{\dagger}$
OCN	1	1.08±0.51	0.95±0.36	0.97±0.21	0.97±0.21	1.12±0.42
OPN	1	1.64±0.24	1.24±0.39	1.46±0.12	1.78±0.9	2.22±1.45
CAP	1	0.7±0.08	0.87±0.21	0.97±0.14	0.78±0.28	0.75±0.09

Figure 4.6 Effects of LPS pre-exposure to osteogenic gene markers of High and Low-CD24 SCAPs.

(A) Gene expression of High-CD24 SCAPs after LPS exposure and further osteogenic differentiation induction. Only BSP gene expression was different among the groups ($p < 0.05$). The groups with the same symbols (†, #, ¶ and $) were significantly different by pair-wise comparison test in Dunn-Sidek's method ($p < 0.05$). (B) Gene expression of Low-CD24 SCAPs after LPS exposure and further osteogenic differentiation induction. BSP gene expressions were different among the groups ($p < 0.05$) and the groups with the same symbols (†) were significantly different by pair-wise comparison test in Dunn-Sidek's method. ($p < 0.05$).

4.5 Discussion

Tissue around infected root canal tooth was continuously challenged by the microorganism byproducts such as enzymes and bacterial endotoxin (Aw, 2016; Sundqvist, 1994). LPS, belonged to gram-negative bacteria cell wall was extensively studied and demonstrated that it could induce inflammatory reaction and bone resorption around root apex (da Silva *et al*., 2008; Hong *et al*., 2004; Tang *et al*., 2014). *Porphyromonas gingivalis*, gram-negative bacteria, was frequently detected in the root canal (Gomes *et al*., 2005; Siqueira *et al*., 2008), so LPS from *Porphyromonas gingivalis* was used in this study. The amounts of LPS from infected root canals in previous reports were range between 0.001 and 0.1 μg/ml by limulus ameobocyte lysate or LAL assay (Marinho *et al*., 2012; Martinho *et al*., 2011). However, the higher concentration of LPS in root canal was found and associated with severe pain and abscess formation around the root apex (Jacinto *et al*., 2005). In addition, 1 and 5 μg/ml of LPS demonstrated the biological responses of SCAPs such as IL-6, IL-8 and TNF-α releasing, activated Wnt-β catenin pathway (Wang *et al*., 2013a; Yamagishi *et al*., 2011; Zhang *et al*., 2013). Thus, these concentrations were included in this study.

Due to anatomical landscape of apical tissue, SCAPs located at the most apical part of immature tooth root that constantly challenged to LPS from infected root canal during infected period before regenerative procedure initiation. To simulate this situation, SCAPs were exposed to LPS in those concentrations before investigation of cell proliferation, migration and osteogenic differentiation. Because of the limitation of cell culture technique, the pre-exposure time of LPS in this study was only 168 hours that SCAPs could maintain the monolayer condition (data not shown). During

incubation with LPS, all concentrations of LPS in this study did not affect on cell viability at 24, 72,120 and 168 hours both High and Low-CD24 SCAPs. After LPS incubation for 168 hour, SCAPs were incubated in fresh growth medium without LPS and replaced this medium every 48 hours to eradicate the remained effects of LPS and inflammatory mediators from SCAPs releasing. These results coincided with animal studies that SCAPs could survive after experimental pulp necrosis in immature teeth (Tobias Duarte *et al*., 2014; Yoo *et al*., 2016). Then, the cell proliferation, migration and osteogenic differentiation were investigated.

Cell proliferation is an important circumstance to increase the proper cell numbers before odontoblast progenitor differentiation and contribute to the root formation process during tooth development (Sohn *et al*., 2014). The results of pre-exposure with LPS did not affect cell proliferation in both High and Low-CD24 SCAPs when compared to control groups. Thus, LPS from root canal infection may not influence to the SCAPs proliferation in REP. The cell numbers of recruited SCAPs in this procedure may be more significant.

Cell migration is also crucial for the regenerative process by stem cells or wound healing. These cells were recruited by various chemokines into the injured site (Rennert *et al*., 2012). In REP, SCAPs around the root apex were recruited into the root canal space for dentin regenerative process. The source of stem cells that contained high numbers of migrated stem cells could increase the possibility of tissue regeneration (Ikebe and Suzuki, 2014). Many cancer cells such as breast cancer, ovarian cancer or bile duct cancer demonstrated higher numbers of migrated cells and CD24 expressing cells, and expressed *CXCR4* gene expression (Kang *et al*., 2013; Leelawat *et al*., 2013;

Schabath *et al*., 2006). The results of our study the were same as the cancer cells reports that High-CD24 SCAPs also showed higher numbers of migrated cell than Low-CD24 SCAPs. In addition, High-CD24 SCAPs showed more *CXCR4* gene expression than Low-CD24 SCAPs. However, LPS did not alter numbers of migrated cell of both High and Low-CD24 SCAPs from their control groups. Thus, the modulation of CXCR4 of SCAPs might increase the SCAPs migration without concerning about the effects of LPS.

Stem cells from other sources of oral tissue, such as dental pulp, periodontal ligament and dental follicle, that could differentiate into osteogenic lineage, demonstrated the alteration of osteogenic differentiation during LPS exposures and responded to LPS in different manners. These responses depended on concentration of LPS, source of LPS and stem cells. The osteogenic differentiation of dental pulp cells decreased when they were exposed to LPS at 0.1 μg/ml, but did not affect by 0.01 μg/ml of the *E.coli* LPS (He *et al*., 2015). LPS from *Porphyromonas gingivalis* increased osteogenic differentiation (Kato *et al*., 2014) but LPS from *E.coli* decreased osteogenic differentiation in periodontal ligament cells (Albiero *et al*., 2015). The source of stem cells also showed the different effects of the same LPS. LPS from *E.coli* at 1 μg/ml increased osteogenic differentiation in periodontal ligament cells (Albiero *et al*., 2015) but decreased osteogenic differentiation in dental follicle cells (Morsczeck *et al*., 2012). The results of our study showed that only the high concentration of LPS at 1 and 5 μg/ml pre-exposure could increase mineralization capacity in High-CD24 SCAPs while LPS did not affect Low-CD24 SCAPs mineralization capacity by Alizarin Red staining study. This suggested that High-CD24 SCAPs were more sensitive to high concentration of *Porphyromonas gingivalis* LPS than Low-CD24 SCAPs. Our results

coincided with the previous studies that LPS could increase osteogenic differentiation in periodontal ligament and dental pulp cells by Alizarin Red staining (Albiero *et al*., 2015; He *et al*., 2015). However, LPS demonstrated to decrease Alizarin Red staining in some studies (Kato *et al*., 2014; Morsczeck *et al*., 2012; Yamagishi *et al*., 2011). These different results might depend on experiment design, source of LPS and cell type as the earlier discussion.

Unfortunately, *in vitro* mineralization assay cannot distinguish types of calcified tissues. Calcified tissues in osteogenic lineage of oral tissue stem cells can be bone, dentin or cementum. Each lineage demonstrates the different organic and inorganic parts that some of the matrix proteins can be used for calcified tissue identification (Foster, 2012; Goldberg *et al*., 2011; Yoo *et al*., 2016). Thus, BSP, the general bone and cementum marker, that expressed in bone and cementum more than dentin (Foster, 2012) was examined by PCR in this study. The *BSP* gene expression increased after exposure to the high concentration of LPS, 1 and 5 μg/ml, and 5μg/ml of High and Low-CD24 SCAPs, respectively. *BSP* gene expression after LPS exposure at 1 and 5 μg/ml significantly increased and corresponded to Alizarin red staining in High-CD24 SCAPs. In contrast, *BSP* gene expression of Low-CD24 SCAPs significantly increased only the exposure to LPS at 5 μg/ml but Alizarin red staining in the same condition was not different when compare to control. Our previous report suggested that High-CD24 SCAPs contained more committed odontoblast progenitor cells than Low-CD24 SCAPs (Aguilar and Lertchirakarn, 2016). The committed odontoblast progenitor cell numbers of Low-CD24 SCAPs expressed *BSP* enough to be detected by PCR. However, the mineralization capacity of these progenitor cells might not be sufficient to detect by Alizarin red staining. Unlike High-CD24 SCAPs, the great committed

odontoblast progenitor cell numbers were adequate for detection *BSP* gene expression of both PCR and mineralization capacity by Alizarin red staining. Osteocalcin (*OCN*) and osteopontin (*OPN*), the other bone markers (D'Errico *et al*., 1997), did not alter after LPS exposures. In addition, dentin specific marker, *DSPP*, was not change by any concentration LPS exposures. All our results were the same as the previous study of dental pulp stem cells that demonstrated LPS increased *BSP* gene expression and did not alter *DSPP* and *OCN* gene expression (Abe *et al*., 2010). We further investigated the effect of LPS to the specific marker of cementum, cementum attachment protein (CAP), to confirm the cementum phenotype (Arzate *et al*., 2015). *CAP* was not altered by LPS, as well. Therefore, SCAPs tend to generate other calcified tissue either bone or cementum rather than dentin if they expose to high concentration of LPS, especially SCAPs with high percentage of CD24 expressing cells. The results from this study could explain and support the phenomenon of clinical case reports (Lei *et al*., 2015; Shimizu *et al*., 2013) and animal studies (Thibodeau *et al*., 2007; Wang *et al*., 2010) that the regenerative calcified tissue in these reports and studies were bone and cementum rather than dentin.

In conclusions, SCAPs from early stage of root development or High-CD24 SCAPs provided better cell migration numbers and osteogenic differentiation than late stage of root development associated with more CD24 expressing SCAPs. LPS pre-exposure did not affect to cell proliferation and migration of both High and Low-CD24 SCAPs. In contrast, high concentration of LPS pre-exposure, 1 or/and 5 μg/ml, significantly increased *BSP* gene expression and tended to generate bone or cementum rather than dentin.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

The current REP protocols (Kontakiotis *et al*., 2015) were used and demonstrated the favorable clinical success. The infected incomplete root formation teeth showed continuing the root development along with resolving the periapical periodontitis (Bose *et al*., 2009; Jeeruphan *et al*., 2012; Kahler *et al*., 2014; Kontakiotis *et al*., 2015). After proper root canal disinfection, SCAPs were recruited into the root canal via bleeding initiation from periapical tissue (Hargreaves *et al*., 2013; Kontakiotis *et al*., 2015). Blood clot was served as a scaffold and also being the source of growth factor such as PDGF and SDF-1 (Yang *et al*., 2016). Root canal dentin wall also released of growth factors such as TGF-β1, bFGF or VEGF and dentin matrix proteins which could contribute or involve to cell proliferation, migration and osteogenic differentiation (Hargreaves *et al*., 2013; Huang and Garcia-Godoy, 2014; Smith *et al*., 2016; Zizka and Sedy, 2017). Hence, inside the root canal space environment has already been appropriate for dentin regeneration by SCAPs. The abilities of SCAPs themselves before recruitment via blood clot initiation were hypothesized and suggested to be the important roles for REP, especially root dentin formation.

The variation of percentage of CD24 expressing SCAPs was reported (Bakopoulou *et al*., 2013; Schneider *et al*., 2014; Sonoyama *et al*., 2006; Zhang *et al*., 2014). However, the factor that related to amount of CD24 expressing cells has never been examined. The results of this thesis in chapter III found that the amount of CD24

expressing cells related to stage of root formation. The stage of root formation affected to amount of CD24 expressing cells. The results showed that early root development stage contained higher number of CD24 positive cells than late stage of root development. These results confirmed by immunohistochemistry. Dentinal tubules at the apical third showed less numbers than coronal third and one dentinal tubule was occupied by only one odontoblast. This finding might relate to decreasing number of odontoblast progenitors according to more advance stage of root formation (Mannocci *et al*., 2004). Mouse bone marrow cells with CD24 expression were suggested as the osteoprogenitor cells or committed stem cells osteoblastic lineage (Chang *et al*., 2015). Therefore, CD24 positive SCAPs might be odontoblast progenitor cells, as well. Moreover, previous report demonstrated that osterix expressing cells were also contributed to the odontoblast progenitors (Ono *et al*., 2016). The High-CD24 SCAPs also expressed *osterix* and *DSPP* more than Low-CD24 SCAPs. This result confirmed that early root development provided more CD24 expressing SCAPs. These evidences also suggested the possibility of relation between CD24 and osterix to dontoblast progenitor cells for root dentin formation.

Regard to the results, in chapter III, the REP should give a higher chance for more calcified tissue in the early stage of root development case than later stage. The recent clinical study supported this suggestion (Chan *et al*., 2017). The early root formation stage demonstrated that the percentage of newly formed calcified tissue was more than late stage of root development.

In spite of SCAPs were presumed as the main population of REP, animal studies and human sample from the fracture teeth (Lei *et al*., 2015; Shimizu *et al*., 2013) revealed bone and/ or cementum-liked structure in the root canal space instead of dentin (Thibodeau *et al*., 2007; Wang *et al*., 2010). The newly formed hard tissue on root canal wall expressed intensively the bone and cementum markers, *BSP* (Shimizu *et al*., 2013). It was that suggested that other stem cells around root apex such as periodontal ligament cells, osteoblast or cementoblast might recruit along with SCAPs by intentional bleeding procedure (Huang and Garcia-Godoy, 2014). The other possibility of this established structure was that SCAPs might change their phenotype into bone or cementum after long-term challenging with infection from root canal. The animal study showed that SCAPs could survive during root canal infection (Tobias Duarte *et al*., 2014; Yoo *et al*., 2016). Recent study also demonstrated that apical papilla tissue was still intact while the coronal and root pulpal tissue were necrosis (Chrepa *et al*., 2017). Interestingly, the animal and human isolated SCAPs from infected root canal increased osteogenic differentiation (Chrepa *et al*., 2017; Yoo *et al*., 2016). SCAPs from infected dog teeth increased *OCN* and *DSPP* gene expression when compared to control (Yoo *et al*., 2016). Thus, root canal infection could alter the osteogenic differentiation of SCAPs both mineralization and gene expression. However, the osteogenic differentiation markers, *OCN*, *DSPP* and *OPN* in this thesis did not change after exposing to LPS. This different result might be the different design or model of the studies. This study was performed in cell culture and investigated only the effect of LPS. In contrast, Yoo and co-workers's study was an animal study. The other biological molecules in periapical lesion that released from SCAPs after exposure to LPS might affect to SCAPs themselves. TNF-α and IL-1β were released by SCAPs after exposure

to LPS (Wang *et al*., 2013a; Zhang *et al*., 2013). TNF- α could increase osteogenic markers such as *OCN*, *OPN*, *BSP*, *DSPP* and *OPN* and mineralization capacity (Li *et al*., 2014b). In contrast, IL-1β could decrease mineralization capacity and osteogenic differentiation markers such as *DSPP* (Liu *et al*., 2016). In addition, periapical lesion environment contains various cytokines, mediators and growth factors from many types of cell, thus, the absolute effects from these inflammatory factors might increase both mineralization and *OCN* and *DSPP* gene expression (Yoo *et al*., 2016). The effects of LPS on SCAPs in this study was the same as in the study of dental pulp cells, the other odontoblast progenitor cell source, that *OCN* and *DSPP* gene expression did not change but BSP expression increased (Abe *et al*., 2010). Only LPS exposure may not alter the gene expression of all osteogenic differentiation markers, excepted *BSP*.

This evidence might be supported by tertiary dentin formation. Tertiary dentin, that was generated by dental pulp cells or obontoblasts, demonstrated bone-liked structure (Xie *et al*., 2014). The tertiary dentin expressed more BSP protein when compared to the adjacent normal dentin. The transplantation of immature tooth also illustrated bone-liked tissue. The hard tissue that generated after transplantation resembled to bone and expressed BSP (Hosoya *et al*., 2003). In contrast, root dentin that was formed before transplantation did not express BSP. Thus, it was possible that the consequences of both trauma and infection might alter odontoblast progenitors to generate the other calcified tissue especially bone liked-structure than dentin. Besides LPS that could effect to osteogenic differentiation, the other biological substances, might also affect. The animal study should be performed to confirm what calcified structure is formed after exposure to LPS.

Because of most of the REP cases related to infected root canal, the effects of LPS to SCAPs with different CD24 expressing cells were studied. The results were reported in chapter IV. The LPS exposure did not affect to cell proliferation and cell migration of both High and Low-CD24 SCAPs. However, LPS at high concentration (1 and 5 μg/ml) increased osteogenic differentiation and activity in High-CD24 SCAPs but did not affect to Low-CD24 SCAPs. In addition, the highest concentration of LPS (5 μg/ml) increased *BSP* gene expression in both High and Low-CD24 SCAPs. High-CD24 SCAPs or early stage of root formation are more sensitive to LPS than Low-CD24 SCAPs or late stage of root formation. Thus, the high concentration of LPS in root canal that relates to tooth with history of severe pain or sinus opening may increases the possibility of bone and/or cementum-liked regenerative calcified tissue especially in short root formation or high CD24 expressing SCAPs. However, the effect of amount of CD24 expressing SCAPs and LPS from this *in vitro* study should be confirmed in animal study.

5.2 Conclusion

SCAPs with high percentage of CD24 expressing cells showed better osteogenic differentiation than low percentage of CD24 SCAPs that obtained from early root development and late root development stage, respectively. Pre-exposure with LPS tended to increase *BSP* gene expression especially in High-CD24 SCAPs. Thus, REP in early root development might give more calcified tissue but tend to generate bone and/or cementum-liked tissue rather than dentin. In addition, LPS did not affect to SCAPs proliferation regardless to amount of percentage of CD24 expressing SCAPs.

5.3 Further study suggestion

The initial numbers of progenitor cells are beneficial for better tissue regeneration or wound healing. Thus, blood clot creation is critical a step that can manipulate to increase initial SCAPs cell numbers. CXCR4 and CD24 are suggested as the targets for increasing the recruited cell numbers especially in late stage of root development.

The medicaments or dentin condition reagents that can promote dentin regeneration by reversing the effect of LPS are suggested to establish. Anti-inflammatory such as fluocinolone acetonide (FA) demonstrated the reversed effect of LPS to dental pulp cells in osteogenic differentiation (Liu *et al*., 2013). Moreover, FA could rescue *DSPP* expression when treated together with LPS in the same study. The application of FA in REP may benefit to dentin regeneration of REP.

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APPENDIX

RAW DATA OF THE STUDY

Table 1 *Survivin* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	1.86	1.56
2	1.05	0.61
3	0.08	0.53
Mean	1	0.91
SD	0.89	0.57

Table 2 Mesenchymal surface stem cell marker of High-CD24 SCAPs.

line	CD24	STRO-1	CD44	CD90	CD45	CD105
1	21.4	6.61	96.51	98.4	2.6	68.44
2	39.83	8.35	99.4	99.72	0.1	64.54
3	47.95	10.08	98	97.7	0.5	61
Mean	36.39	8.35	97.97	98.61	1.07	64.66
SD	13.60	1.74	1.45	1.03	1.34	3.72

Table 3 Mesenchymal surface stem cell marker of Low-CD24 SCAPs.

line	CD24	STRO-1	CD44	CD90	CD45	CD105
1	3.68	1.02	99.26	99.09	0.1	68.57
2	3.93	0.3	88.91	99.64	0.16	62.81
3	3.06	0.55	96.62	99.79	0.5	62.31
Mean	3.55	0.62	94.93	99.51	0.25	64.56
SD	0.44	0.36	5.37	0.36	0.21	3.47

Table 4 Comparison of percentage of CD24 and STRO-1 between passage 2 and passage 4.

	CD24		STRO-1	
line	P2	P4	P2	P4
1	21.4	32.11	6.61	0
2	39.83	26.65	8.35	0
3	47.94	22.6	10.8	0
Mean	36.39	27.12	8.59	0
SD	13.6002978	4.77238934	2.10500198	0

Table 5 Colony forming units of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	18	46
2	23	30
3	16	33
Mean	19	36.33
SD	3.60	8.50

Table 6 *Rex1* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	0.054	14.36
2	0.17	14.70
3	2.77	14.48
Mean	1	14.51
SD	1.53	0.17

Table 7 *Nanog* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	0.41	22.3
2	2.23	10.5
3	0.35	25.39
Mean	1	19.41
SD	1.06	7.86

Table 8 *Sox2* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	0.53	16.44
2	1.37	12.5
3	1.08	15.84
Mean	1	14.92
SD	0.42	2.12

Table 9 *Oct4* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	8.6411E-05	6.44
2	0.000204	0.27
3	2.99	2.04
Mean	1	2.92
SD	1.73	3.17

Table 10 Cell proliferation of High and Low-CD24 SCAPs.

	24 Hour		72 Hour		120 Hour		168 Hour	
line	High	Low	High	Low	High	Low	High	Low
1	4590	5210	7928	11843	34206	31758	61110	48814
2	4750	5101	9228	15854	37777	38508	53410	55129
3	4320	5299	6678	7943	31800	25110	68887	42584
Mean	4553	5203	7945	11880	34594	31792	61136	48842
SD	217.33	99.17	1275.08	3955.63	3007.36	6699.06	7738.53	6272.55

Table 11 Fold change of OD of Alizarin Red staining to control of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	4.71	1.48
2	4.75	1.43
3	7.30	1.18
Mean	5.59	1.36
SD	1.48	0.16

Table 12 *Runx2* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	1.22	3.70
2	1.50	2.52
3	0.29	3.80
Mean	1	3.34
SD	0.63	0.71

Table 13 *Osterix* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	0.96	0.14
2	0.96	0.13
3	1.07	0.15
Mean	1	0.14
SD	0.06	0.01

Table 14 *DSPP* gene expression of High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	1.21	0.29
2	0.58	0.47
3	1.21	0.43
Mean	1	0.40
SD	0.36	0.09

Table 15 Fold change of OD of Oil Red O staining to control of High and Low-CD24 SCAPs.

Line	High-CD24	Low-CD24
1	8.52	1.29
2	8.81	1.20
3	5.80	1.02
Mean	7.71	1.17
SD	1.66	0.14

Table 16 MFI of CD24 of High and Low-CD24 SCAPs by normalization with control isotype.

line	High-CD24	Low-CD24
1	4.1	1.3
2	5.2	1
3	2.6	1.6
Mean	3.86	1.32
SD	1.31	0.31

Table 17 Cell viability during LPS exposure at 24 hour of High-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	103	118	98	100	107
2	100	103	119	100	110	108
3	100	110	109	103	100	115
Mean	100	105.33	115.33	100.33	103.33	110.00
SD	0	4.04	5.51	2.52	5.77	4.36

Table 18 Cell viability during LPS exposure at 72 hour of High-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	96	96	100	97	98
2	100	68	73	88	88	120
3	100	104	110	107	107	104
Mean	100	89.33	93.00	98.33	97.33	107.33
SD	0	18.90	18.68	9.61	9.50	11.37

Table 19 Cell viability during LPS exposure at 120 hour of High-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	102	102	110	96	99
2	100	100	99	94	103	95
3	100	97	99	96	99	92
Mean	100	99.67	100.00	100.00	99.33	95.33
SD	0	2.52	1.73	8.72	3.51	3.51

Table 20 Cell viability during LPS exposure at 168 hour of High-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	103	98	98	97	92
2	100	94	90	89	88	97
3	100	102	99	99	97	101
Mean	100	99.67	95.67	95.33	94.00	96.67
SD	0	4.93	4.93	5.51	5.20	4.51

Table 21 Cell viability during LPS exposure at 24 hour of Low-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	80	101	81	94	105
2	100	115	112	113	114	117
3	100	102	103	112	99	99
Mean	100	99.00	105.33	102.00	102.33	107.00
SD	0	17.69	5.86	18.19	10.41	9.17

Table 22 Cell viability during LPS exposure at 72 hour of Low-CD24 SCAPs

Line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	86	91	86	100	130
2	100	102	110	105	100	110
3	100	108	102	108	108	117
Mean	100	98.67	101.00	99.67	102.67	119.00
SD	0	11.37	9.54	11.93	4.62	10.15

Table 23 Cell viability during LPS exposure at 120 hour of Low-CD24 SCAPs

line		LPS (μg/ml)				
	control	0.001	0.01	0.1	1	5
1	100	99	104	107	106	111
2	100	102	96	100	124	130
3	100	112	117	109	125	129
Mean	100	104.53	105.48	105.33	118.38	123.52
SD	0	6.82	10.82	4.54	10.65	10.71

Table 24 Cell viability during LPS exposure at 168 hour of Low-CD24 SCAPs

line		LPS (μg/ml)				
	Control	0.001	0.01	0.1	1	5
1	100	92	94	86	84	70
2	100	103	99	99	101	102
3	100	106	104	108	104	89
Mean	100	100.33	99.00	97.67	96.33	87.00
SD	0	7.37	5.00	11.06	10.79	16.09

Table 25 Percentage of CD24 expressing cells after LPS exposure of High-CD24 SCAPs.

		LPS (μg/ml)				
Line	control	0.001	0.01	0.1	1	5
1	20.33	23.36	25.4	24.37	20.55	20.42
2	14.87	18.67	14.26	15.86	15.8	16.84
3	15	12.16	16.68	11.8	12.56	11.25
Mean	16.73	18.06	18.78	17.34	16.30	16.17
SD	3.11	5.62	5.85	6.41	4.01	4.62

Table 26 Percentage of CD24 expressing cells after LPS exposure of Low-CD24 SCAPs.

		LPS (μg/ml)				
Line	control	0.001	0.01	0.1	1	5
1	2.39	2.72	2.75	2.32	2.22	2.32
2	0.93	1.1	1.3	0.83	0.94	1.4
3	1.5	1.6	1.43	0.98	1.86	1.2
Mean	1.61	1.81	1.83	1.38	1.67	1.64
SD	0.73	0.82	0.80	0.82	0.66	0.60

Table 27 Cell proliferation after LPS exposure in High-CD24 SCAPs at 24 Hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	96	100	103	101	98
2	100	97	110	110	96	105
3	100	111	111	109	111	100
Mean	100	101.44	105.45	104.95	102.63	97.25
SD	0	8.33	4.40	3.52	7.83	2.41

Table 28 Cell proliferation after LPS exposure in High-CD24 SCAPs at 72 Hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	99	104	99	106	78
2	100	101	101	96	107	83
3	100	136	118	118	111	101
Mean	100	111.86	107.45	104.28	108.16	86.81
SD	0	21.00	9.13	11.82	2.70	5.21

Table 29 Cell proliferation after LPS exposure in High-CD24 SCAPs at 120 Hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	91	103	97	91	79
2	100	110	113	119	121	100
3	100	112	109	102	83	91
Mean	100	104.50	108.43	106.05	98.37	89.73
SD	0	11.59	5.15	11.46	19.98	10.75

Table 30 Cell proliferation after LPS exposure in High-CD24 SCAPs at 168 Hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	101	96	94	101	93
2	100	102	106	103	104	82
3	100	96	92	96	96	80
Mean	100	99.69	98.07	97.64	100.20	85.00
SD	0	3.54	7.13	5.04	4.33	6.78

Table 31 Cell proliferation after LPS exposure of Low-CD24 SCAPs at 24 hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	90	95	99	85	98
2	100	107	103	103	102	103
3	100	104	108	114	110	104
Mean	100	100.46	102.00	105.31	99.20	101.61
SD	0	9.17	6.40	7.79	12.50	3.35

Table 32 Cell proliferation after LPS exposure of Low-CD24 SCAPs at 48 hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	90	104	109	89	93
2	100	107	110	101	112	98
3	100	93	94	96	96	101
Mean	100	96.77	102.77	101.82	98.72	97.58
SD	0	8.78	8.18	6.54	11.73	3.92

Table 33 Cell proliferation after LPS exposure of Low-CD24 SCAPs at 120 hour.

		LPS (μg/ml)				
Line	control	0.001	0.01	0.1	1	5
1	100	100	88	84	108	94
2	100	99	87	84	94	92
3	100	101	102	95	103	100
Mean	100	99.97	92.45	87.59	101.39	89.54
SD	0	0.67	8.05	6.27	7.18	3.93

Table 34 Cell proliferation after LPS exposure of Low-CD24 SCAPs at 168 hour.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	100	110	114	114	119	119
2	100	104	122	102	94	116
3	100	99	99	105	108	114
Mean	100	104.48	111.93	107.04	106.94	116.02
SD	0	5.25	11.70	5.84	12.40	2.57

Table 35 Migrated cell numbers after LPS exposure of High-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	101	80	97	92	99	108
2	115	90	104	90	105	115
3	102	114	90	105	83	94
Mean	106	94.66	97.23	95.66	95.66	105.66
SD	7.81	17.47	7.12	8.14	11.37	10.69

Table 36 Migrated cell numbers after LPS exposure of Low-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	58	50	53	46	46	58
2	49	53	65	66	59	73
3	45	41	33	30	33	45
Mean	50.66667	48	50.33333	47.33333	46	58.66667
SD	6.658328	6.244998	16.16581	18.037	13	14.0119

Table 37 *CXCR4* gene expression between High and Low-CD24 SCAPs.

line	High-CD24	Low-CD24
1	0.81	0.20
2	1.07	0.25
3	1.11	0.25
Mean	1	0.23
SD	0.16	0.02

Table 38 *CXCR4* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.53	1.40	1.39	1.28	1.35
2	1	0.98	1	1	0.98	0.91
3	1	1	0.93	1	1.06	1.18
Mean	1	1.17	1.11	1.13	1.11	1.15
SD	0	0.31	0.25	0.22	0.15	0.22

Table 39 *CXCR4* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.66	1.35	1.05	1.5	1.33
2	1	0.90	0.69	1.08	0.63	1.06
3	1	1.23	1.25	1.04	1	1.45
Mean	1	1.26	1.09	1.05	1.04	1.28
SD	0	0.38	0.35	0.02	0.43	0.19

Table 40 OD of Alizarin Red staining after LPS exposure in High-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	0.4	0.335	0.409	0.454	0.5	0.581
2	0.42	0.461	0.473	0.368	0.56	0.558
3	0.43	0.406	0.435	0.443	0.52	0.55
Mean	0.41	0.41	0.44	0.42	0.526	0.563
SD	0.015	0.06	0.03	0.046	0.03	0.016

Table 41 OD of Alizarin Red staining after LPS exposure in Low-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	0.23	0.243	0.252	0.217	0.239	0.234
2	0.214	0.225	0.202	0.219	0.222	0.226
3	0.225	0.233	0.234	0.225	0.228	0.23
Mean	0.223	0.233	0.22	0.22	0.229	0.23
SD	0.008	0.007	0.021	0.003	0.007	0.003

Table 42 *DSPP* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	2.47	2.29	5.63	6.90	8.09
2	1	0.84	0.73	0.56	0.70	2.37
3	1	2.98	1.31	1.75	1.18	1.5
Mean	1	2.10	1.45	2.65	2.93	3.99
SD	0	1.11	0.78	2.65	3.45	3.58

Table 43 *BSP* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1	1.75	1.40	1.91	1.99
2	1	1.06	1.15	1.36	1.52	1.46
3	1	1.45	1.55	1.70	1.93	1.94
Mean	1	1.18	1.49	1.49	1.80	1.80
SD	0	0.24	0.30	0.18	0.23	0.29

Table 44 *OCN* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (μg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	0.67	0.89	1.12	0.89	1.06
2	1	1.36	1.26	0.65	1.03	1.16
3	1	1.11	1.06	0.82	1.18	1.51
Mean	1	1.05	1.07	0.87	1.04	1.25
SD	0	0.35	0.18	0.23	0.14	0.23

Table 45 *OPN* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	0.72	1.63	1.77	1.02	2.13
2	1	0.96	1.57	1.88	1.50	1.35
3	1	1.02	1.26	0.93	0.97	1.66
Mean	1	0.91	1.49	1.53	1.17	1.72
SD	0	0.15	0.19	0.52	0.29	0.39

Table 46 *CAP* gene expression after LPS exposure of High-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.13	1.19	1.29	0.91	0.95
2	1	1	0.7	0.9	1.1	1.2
3	1	0.9	0.7	0.8	0.7	1.3
Mean	1	1.01	0.86	1.00	0.91	1.15
SD	0	0.11	0.28	0.26	0.20	0.17

Table 47 *DSPP* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.18	1.4	1.5	2.5	3.77
2	1	1.06	0.87	1.2	1	5
3	1	0.82	1.3	1.5	1	0.48
Mean	1	1.02	1.19	1.4	1.5	3.08
SD	0.34	0.18	0.28	0.17	0.86	2.33

Table 48 *BSP* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.7	1.6	2.2	1.9	3.8
2	1	2.2	1.9	3.4	3.3	4.4
3	1	2.14	1.88	1.92	2.36	7.04
Mean	1	2.01	1.79	2.50	2.52	5.08
SD	0	0.27	0.16	0.78	0.71	1.72

Table 49 *OCN* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	0.67	0.89	1.12	0.89	1.06
2	1	1.36	1.26	0.65	1.03	1.16
3	1	1.11	1.06	0.82	1.18	1.51
Mean	1	1.05	1.07	0.87	1.04	1.25
SD	0	0.35	0.18	0.23	0.14	0.23

Table 50 *OPN* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	1.17	0.65	1.05	0.73	0.57
2	1	0.71	1.11	1.04	0.87	1.43
3	1	0.67	0.64	0.72	1.08	1.00
Mean	1	0.85	0.80	0.94	0.89	1.00
SD	0	0.27	0.26	0.19	0.17	0.42

Table 51 *CAP* gene expression after LPS exposure of Low-CD24 SCAPs.

		LPS (µg/ml)				
line	control	0.001	0.01	0.1	1	5
1	1	0.75	0.87	1.12	0.75	0.85
2	1	0.85	1.08	0.86	0.79	0.68
3	1	0.70	0.66	0.93	0.79	0.70
Mean	1	0.76	0.87	0.97	0.78	0.75
SD	0	0.07	0.20	0.13	0.02	0.09

VITA

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