THE FOUNDING POPULATIONS AND THE CRITICAL FACTORS REQUIRED FOR THE ESTABLISHMENT OF ENDOTHELIAL PROGENITOR CELLS

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THE FOUNDING POPULATION AND THE CRITICAL FACTOR REQUIRED FOR THE ESTABLISHMENT OF ENDOTHELIAL PROGENITOR CELLS

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ABSTRACT

Recent studies revealed that EPCs have been successfully isolated from the MNCs of both peripheral blood (PB) and umbilical cord blood (UCB). However, UCB-MNC population -which is the founding population of EPCs - and the critical factors which are required for the establishment of EPC colonies from UCBs had still not been identified.

In this study, the founding population of EPCs was identified. Four fractions of cell subpopulations including $CD14^+$, $CD14^-$, $CD14^-/CD34^-$ and $CD14^-/CD34^+$ were immuno-magnetically separated and their abilities investigated in forming EPC colonies. In addition, the soluble factors, that play the critical role in EPC generation, were also determined using ELISA and functional assays.

Consequently, the EPC colonies could only be observed in $CD14^-$ and $CD14^-/34^+$ subpopulations which were co-cultured with $CD14^+$ cells in both transwell and direct co-cultures. The result of cytokine screening has demonstrated that the angiogenin (ANG) might be required for the generation of EPC colonies. The functional assays of ANG revealed that it was the critical factor in EPC generation in $CD14^-$ subpopulation cultures and exhibited increased colony numbers of EPCs in $CD14^-/34^+$ subpopulation cultures, while the neutralizing of ANG could not completely inhibit the generation of EPC colonies. Taken together, this study demonstrates that the $CD14^-/CD34^+$ subpopulation is the founding population of EPCs while $CD14^+$ subpopulation is the source of cytokine “ANG” which is one of the critical factors that is required for the establishment of EPCs in UCB samples.

KEY WORDS: ENDOTHELIAL PROGENITOR CELLS/ ORIGIN/ ANGIOGENIN

65 pages

การค้นพบกลุ่มประชากรเซลล์และปัจจัยสำคัญที่จำเป็นสำหรับการเกิดขึ้นของเซลล์ต้นกำเนิดหลอดเลือด THE FOUNDING POPULATION AND THE CRITICAL FACTOR REQUIRED FOR THE ESTABLISHMENT OF ENDOTHELIAL PROGENITOR CELLS

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บทคัดย่อ

จากการศึกษาที่ผ่านมาประสบผลสำเร็จในการแยก EPCs จากเซลล์โมโนนิวเคลียร์จากเลือดและเลือดจากสายสะดือทารก ถึงอย่างไรก็ตามในตัวอย่างของเลือดจากสายสะดือทารกยังไม่มีการศึกษาแน่ชัดว่า กลุ่มประชากรเซลล์และไซโตไคน์ กลุ่มใดที่เป็นต้นกำเนิดและมีความสำคัญต่อการเกิดขึ้นของโคโลนี EPCs ที่ผ่านมามีรายงานสำคัญที่ว่า เมื่อมีการแยกเลี้ยงกลุ่มประชากรเซลล์ $CD14^+$ และ $CD14^-$ จะไม่สามารถตรวจพบโคโลนีของ EPCs บนจานเลี้ยงเซลล์ได้

ดังนั้น ในการศึกษาครั้งนี้จึงมุ่งไปที่การค้นหากลุ่มประชากรเซลล์และปัจจัยสำคัญต่อการเกิดขึ้นของ EPCs วิธีการทดลองคือ นำเซลล์ทั้งสี่กลุ่มคือ $CD14^+$, $CD14^-$, $CD14^-/CD34^-$ และ $CD14^-/CD34^+$ ที่แยกได้โดยอาศัยหลักการทางด้านวิทยาภูมิคุ้มกัน (immune-magnetic cell sorting) มาเลี้ยงทั้งแบบร่วมกันและแยกกัน บนจานเลี้ยงเซลล์แบบ transwell และแบบปกติ ร่วมกับการศึกษาไซโตไคน์ที่หลั่งออกจากกลุ่มประชากรเซลล์ต่างๆ

จากการทดลองพบว่ามีเฉพาะกลุ่มเซลล์ $CD14^-$ และ $CD14^-/34^+$ ที่ทำการเลี้ยงร่วมกับกลุ่มประชากรเซลล์ $CD14^+$ แบบ transwell และ direct co-cultures ที่สามารถตรวจพบโคโลนีของ EPCs บนจานเลี้ยงเซลล์ ขณะที่การตรวจสอบชนิดของไซโตไคน์พบว่า angiogenin อาจจะมีบทบาทสำคัญต่อการเกิดโคโลนีของ EPCs จากการศึกษาบทบาทของไซโตไคน์ชนิดนี้พบว่ามีบทบาทในการช่วยสร้างโคโลนีของ EPCs จากการเลี้ยงกลุ่มประชากรเซล์ $CD14^-$ และยังมีบทบาทในการเพิ่มจำนวนโคโลนีของ EPCs ในการเลี้ยงกลุ่มประชากรเซลล์ $CD14^-/34^+$ ขณะที่เมื่อบดบังการทำงานของไซโตไคน์ชนิดนี้โดยการบล็อคจะไม่มีผลยับยั้งการสร้างโคโลนีของ EPCs ระหว่างการเลี้ยง $CD14^-$ ร่วมกับ $CD14^+$ แบบ transwell co-culture จากทั้งหมดสรุปได้ว่า $CD14^-/34^+$ เป็นกลุ่มประชากรเซลล์ที่เป็นแหล่งของ EPCs และ angiogenin คือหนึ่งในปัจจัยสำคัญต่อการเกิดขึ้นของ EPCs ในตัวอย่างเลือดที่ได้จากสายสะดือทารก

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
BM	bone marrow
DM	diabetes mellitus
EBM-2	endothelial basal medium-2
EC	endothelial cell
EGM-2	endothelial growth medium-2
EPC	endothelial progenitor cell
Et al	et alii
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGF-β	fibroblast growth factor beta
FITC	flourescein isotiocyanate
HGF	hepatocyte growth factor
HSC	hematopoietic stem cell
IGF	insulin growth factor
MCP-1	monocyte chemotactic protein-1
min	minute
ml	milliliter
mmol	millimole
MNCs	mononuclear cells
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PE	phycoerytin
SDF-1	stromal cell-derived factor-1
TNF-α	tomor necrosis factor alpha
UCB	umbilical cord blood

LIST OF ABBREVIATIONS (cont.)

VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
vWF	von Willebrand factor

CHAPTER I
INTRODUCTION

Bone marrows (BMs) have various stem/progenitor cells, comprised of, not only hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) but also endothelial progenitor cell (EPCs). There were evidences suggest that both HSCs and EPCs derived from the same progenitor called hemangioblast that resides in BM. This hemangioblast might be received certain signals which are involving in the determination of cell fate toward hematopoietic and endothelial cell lineages (1). EPCs, which were initially reported by Asahara *et al*. in 1997 were isolated from human peripheral blood (hPB) using the combination of cell surface molecules specified for hematopoietic and endothelial cell lineages (2). Those EPCs are demonstrated to be the progenitors of endothelial cells (ECs) and perform the essential roles in vessel homeostasis through the mechanism called postnatal vasculogenesis (3-7).

Due to their properties, EPCs are considered as a renewable source of mature endothelial cells which can be used to treat patients with vascular diseases. However, the main obstacle which limits the use of EPCs in medical application is the low number of EPCs in circulation. Thus, the EPC enrichment procedure is required. These procedures might include cell sorting and *ex vivo* expansion of EPCs (5). Hence, most EPCs were generated by an ex *vivo* cultivation from either PB or UCB-MNCs on fibronectin- coated tissue culture plate (8). To enrich EPCs from those MNC populations, the cell surface molecules which are associated with the founding population of EPCs in MNCs must be identified. However, the cell surface markers that are associated with the EPC population are still controversial. Some studies have indicated that the EPC population is enriched in $CD14^-$ population whereas the other studies suggest that EPCs reside in the $CD14^+$ population (8-13). Nevertheless, it was shown that the separated $CD14^+$ or $CD14^-$ population alone could not generated EPC colonies, so the interaction between $CD14^+$ and $CD14^-$ subpopulation through soluble factors and/or cell surface molecule might be essential (10). The aim of this study is to

identify the founding population of EPCs in UCB-MNCs by systematic cell sorting using the combination of several cell surface molecules including CD14 and CD34. In addition, the soluble factors that play the critical role in EPC generation were also identified using ELISA and functional assays.

CHAPTER II
OBJECTIVES

The overall objective of this study is to determine the founding population of EPCs and the cytokines which are critical for the establishment of EPC colonies from UCB-MNC population.

To accomplish the overall objective, three specific objectives must be performed as follows.

1) To separate several UCB-MNC subpopulation including $CD14^+$, $CD14^-$, $CD14^-/34^+$ and $CD14^-/34^-$ and determine their capacities to generate EPC colonies.

2) To study the phenotypic and functional characteristic of EPCs generated from each subpopulation in comparison to those generated from MNCs.

3) To identify the critical factors which play critical roles on the establishment of EPC colonies from UCB-MNCs.

CHAPPER III
LITERATURE REVIEW

3.1 Endothelial Progenitor Cells

Bone marrows (BMs) have various stem/progenitor cell population including not only hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) but also endothelial progenitor cell (EPCs). There were evidences suggest that HSCs and EPCs are derived from a common precursor called hemangioblast (1). EPCs were reported to co-express several cell surface molecules associated with hematopoietic cells (CD133, and CD34) and mature endothelial cells (VEGFR2 and Tie-2) (2, 12, 14-17). EPCs are the precursor of mature endothelial cells (ECs) and play essential roles in vascular homeostasis through the mechanism called postnatal vasculogenesis. They also have the ability to extensively migrate, proliferate and form primitive vascular structure. (4-6). Several *in vivo* studies demonstrated that EPCs have contributed to the neovascularization and reendothelialization of vascular grafts in animal models suffered from high limb ischemia and myocardial infarction (11, 19, 21-23,). In addition, several *in vitro* studies also demonstrated that EPCs could differentiate to mature ECs and form capillary like structure under appropriated condition (18-23).

3.2 Postnatal Neovascularization

During the postnatal period, the neovascularization was previously believed to be generated by angiogenesis which involves the proliferation and migration of mature ECs from the nearby pre-existing vessels. This angiogenetic process plays roles in both physiological and several pathological conditions including wound healing, organogenesis, and tumor metastasis (5-7). The mechanism is regulated by various cytokines which can be categorized into angiogenic and anti-angiogenic factors (3, 5). Firstly, the pre-existing ECs proliferate and migrate from the

pre-existing vessel to generate new collateral branches. The important angiogenic factors involved in the process are acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), angiogenin, vascular endothelial growth factor (VEGF) and their receptors (3). In addition to angiogenesis, the vasculogenesis has been recently demonstrated to be involved in the neovascularization during postnatal period (2-5). The vasculogenesis involved BM-derived endothelial cell precursors called EPCs which have abilities to extensively proliferate, migrate and incorporate into new vessels (4-6, 24-26). Apart from bone marrow, EPCs had been recently isolated from other tissues such as skeletal muscle and waton's jelly tissues, so the tissue specific stem/progenitor cells might provide the alternative sources of EPCs other during postnatal vasculogenesis (27, 28). During vasculogenesis, the EPCs have egressed from BMs into circulation. When these EPCs receive the angiogenic signal, they proliferate, migrate, penetrate and differentiate to form new blood vessel *de novo* (1, 4, 5).

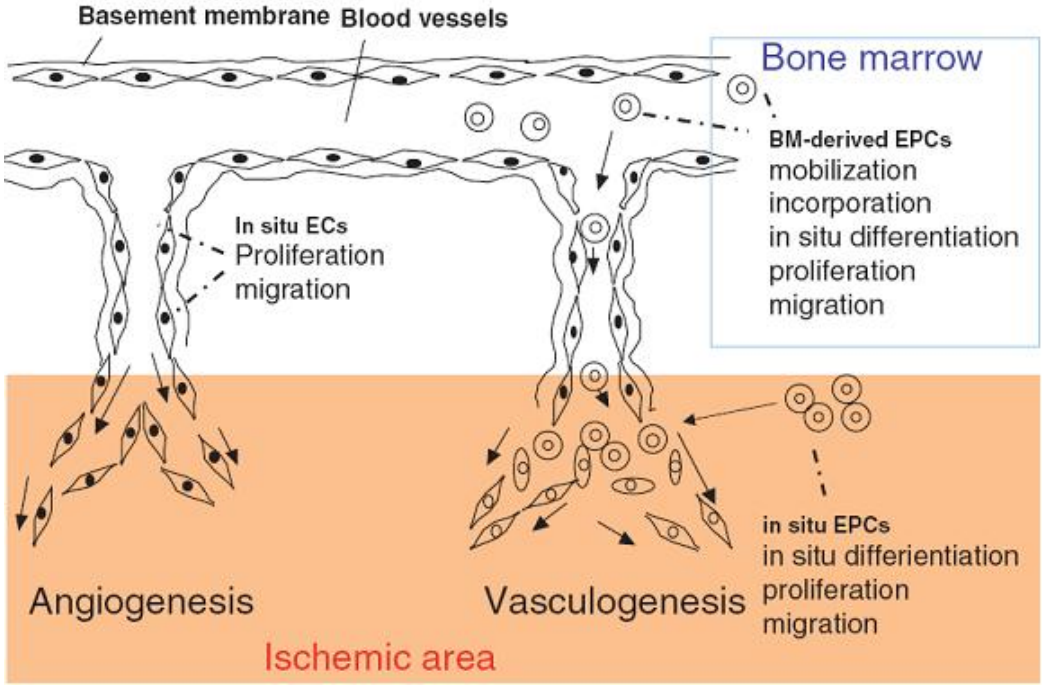


Figure 3.1 Schematic diagram illustrated the postnatal neovascularization (Egushi *et al*., 2008)

3.3 Endothelial Progenitor Cell Biology

EPCs were isolated for at the first time from human peripheral bloods (hPBs) using the hematopoietic and endothelial cell surface markers, CD34 and Flk-1. The isolated $CD34^+$ and $Flk\text{-}1^+$ populations exhibited the properties of ECs by engulfing Dil-acetylic low density lipoprotein (Dil-acLDL) and incorporating with the ECs to reendothelialize the damaged vessels (2). EPCs could be categorized into two types, early EPCs and late EPCs which could be generated under different culture condition (figure 3.2). The early EPCs co-express monocytic marker CD14, pan-leukocytic (CD45), and endothelial marker VEGFR-2 and could be maintain in culture for a short period of time due to their limit proliferative capacity. In contrast to early EPCs, late EPCs which exhibit homogenous cobblestone morphologies and co-express hematopoietic stem cell marker CD34 and mature ECs VEGFR2 have height proliferative capacity and could be extensively propagated in culture (8). The general definition of EPCs was proposed by which the EPCs are circulating, BM-derived cells that are functionally and phenotypically distinct from mature ECs. They must be able to differentiate to ECs in vitro and contributed to *vivo* vasculogenesis and/or vascular homeostasis (14, 29). According to these definitions, the only type of true EPCs is late EPCs (9, 30, 31).

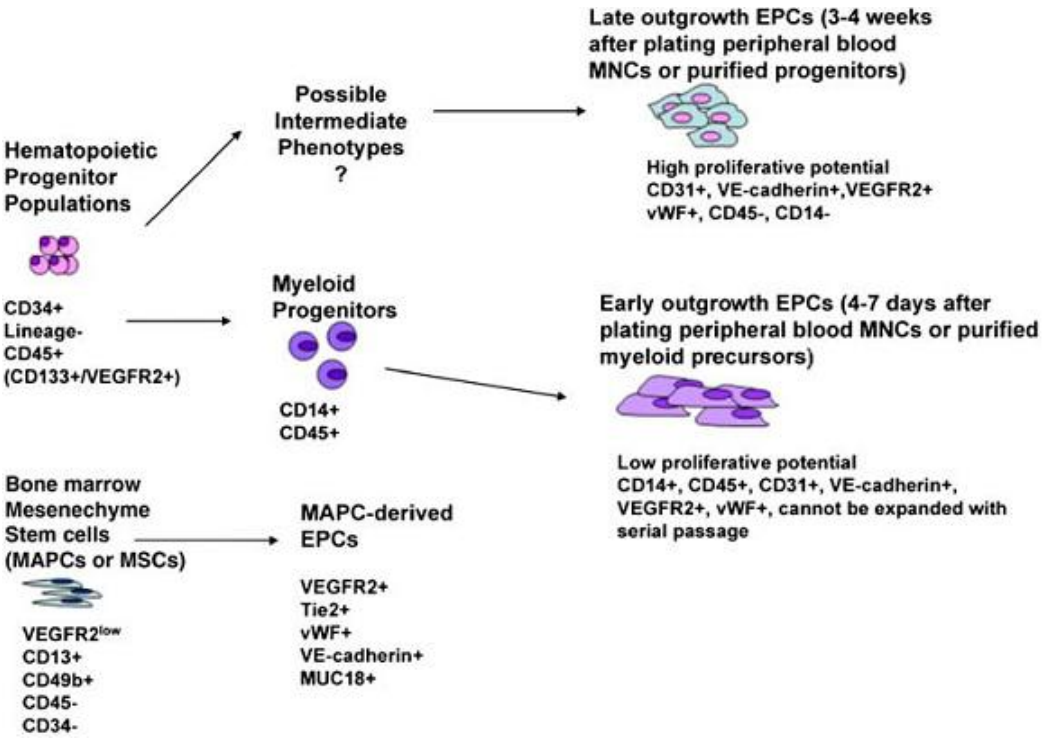
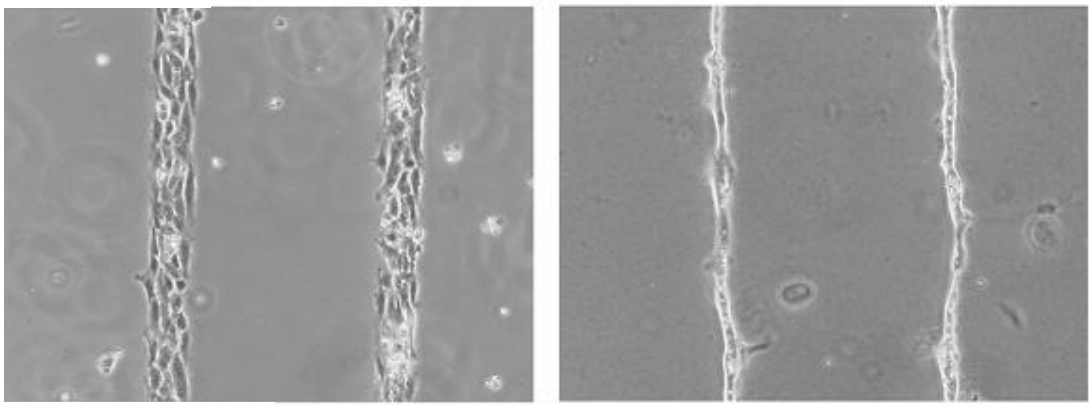


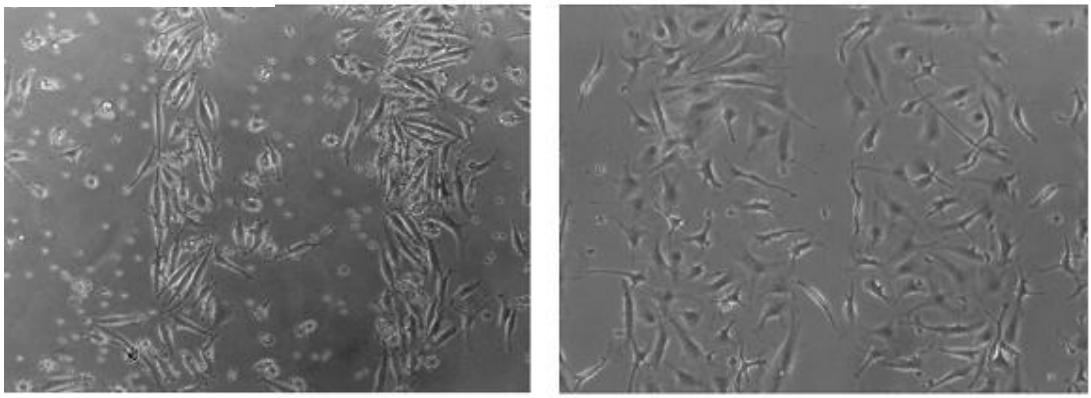
Figure 3.2 Schematic diagram illustrated the model of characterized culture-expanded EPC populations (Young *et al.*, 2007)

There are several surface molecules that have been used to isolated EPC population including hematopoietic stem cell markers (CD34, CD133), endothelial cell marker (VEGFR2) and monocyte specifi peripheral blood (CD14). EPCs have been firstly isolated from umbilical cord bloods since 2000 (32). These UCB-derived EPCs exhibited the same characteristic as those derived from PBs, being cobblestone shape cells, co-expressing CD34 and VEGFR2 and generating capillary like structure both in vitro and in vivo (13, 18-21, 32-34, 52). Moreover, these UCB-derived EPCs showed higher proliferative capacity and telomerase activity than PB-EPCs (19, 21, 32-34). However, similar to PB-derived EPCs, the identification of founding EPC population in UCBs using a combination of cell surface molecules is still not investigated. Mukai *et al.* have demonstrated the new technology for assess the capillary like structure comparison between CB and PB derived EPCs *in vitro*. They found that late EPCs derived from CB could form capillary like structure on glass slide while PB derived EPCs could not which has shown in figure 3.3 (18). Recently, the founding populations of EPCs in CB sample have studied in $AC133^{-}/CD14^{+}$ subpopulation only which is still controversial (20).

HUVECs



PB derived EPCs



CB derived EPCs

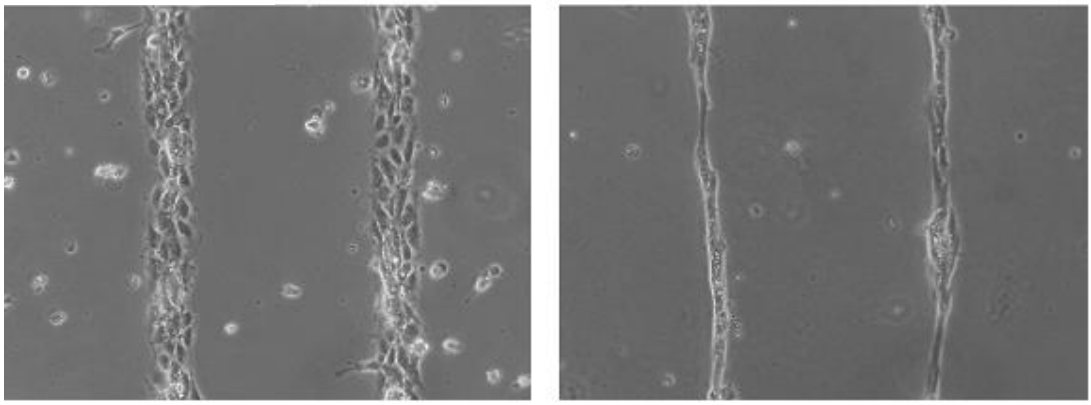


Figure 3.3 Schematic diagram illustrated the vessel formation comparison between CB and PB derived EPCs

This figure was adapted from Mukai *et. al.*, 2008

3.4 The critical Factors Involved in the Establishment of Endothelial Progenitor Cell Colonies

The critical factors which play important roles in the proliferation and migration of mature ECs have been identified. These factors included interleukin 6 (IL-6), which increase in the proliferation migration and vessel forming capacity of ECs, interleukin 8 (IL-8) which induces the differentiation of mature ECs and VEGR which regulate EC mobilization, EC differentiation and postnatal vasculogenesis through several signaling molecules such as BAkt, ERK1/2, PI3 and JNK/SAPK (35-38). In addition IL-6, IL-8 and VEGF, the additional factor which plays an important role in the EC proliferation and angiogenesis is angiogenin (ANG). However, the roles of these factors in the establishment of EPC colonies are still poorly understood. Angiogenin is the member of RNase A super family which has the molecular weight is about 14,400 Da (39). Angiogenin could stimulate basement membrane degradation, enhancing rRNA transcription and increasing angiogenic activity by ECs. ANG bind to it's a 170-kDa receptor, translocate into nucleus and exert its effect through ERK1/2, PKB/Akt, JNK/SAPK and eNOS signaling pathways resulting in the promotion of EC proliferation and angiogenesis (40-46). Moreover, angiogenin have been found to collaborating with aFGF, bFGF and VEGF in regulating angiogenesis (46).

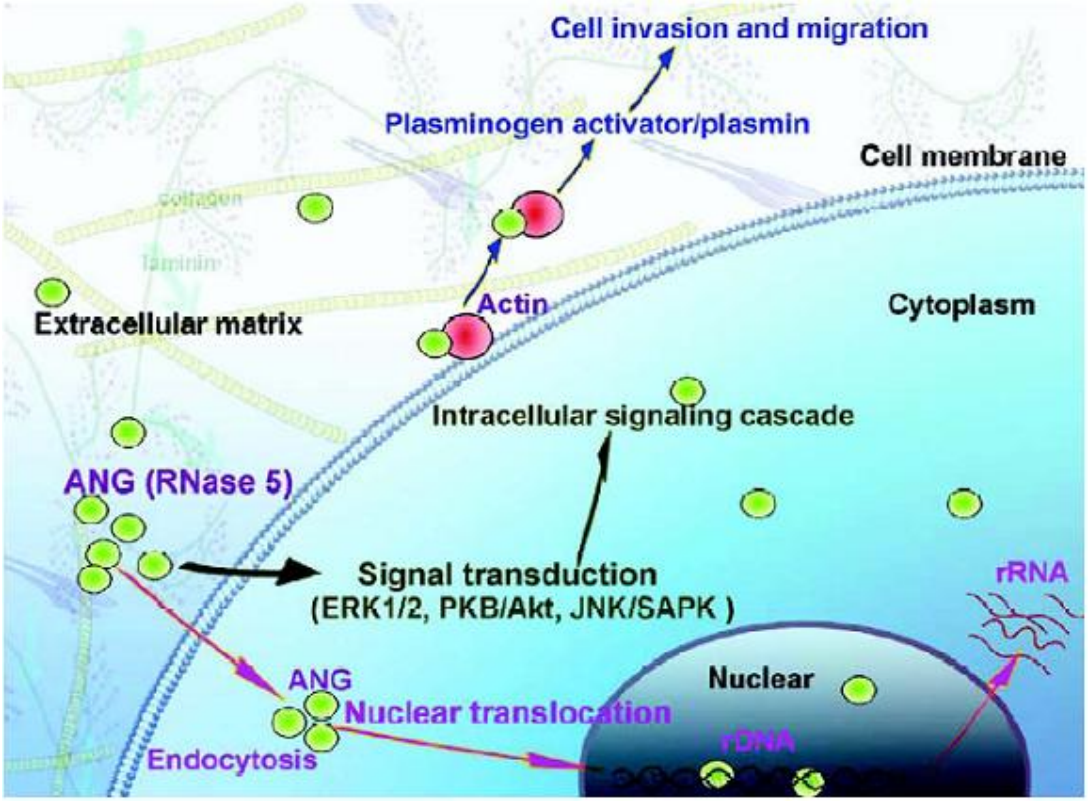


Figure 3.4 Schematic diagram illustrated the mechanism of angiogenin (Goa *et. al.*, 2008)

3.5 Therapeutic Application of **Endothelial Progenitor Cells**

According to their properties, EPC transplantation has been considered as a potential treatment for several vascular diseases such as diabetes mellitus (DM), cerebrovascular diseases and cardiovascular diseases. Several studies in animal model demonstrated that transplantation and mobilization of EPCs could improve the vaiability and function of ischemic tissue by increasing the vessel density in the infract area (19, 22, 32-34, 47-51). The overall possible therapeutic application of EPCs and their secreted factors have shown in figure 3.6 (5). Despite their potential, a critical limitation of EPC transplantation is their low number in the circulation and slow proliferation. Therefore, the procedures which can enrich EPC population from PBs or UCBs have to be developed. Those enrichment procedures might involve cell sorting, mobilization, *ex vivo* expansion of EPCs (4-6). However, the candidate marker that cloud be used to enrich EPCs from both PBs and UCBs is still not yet identify.

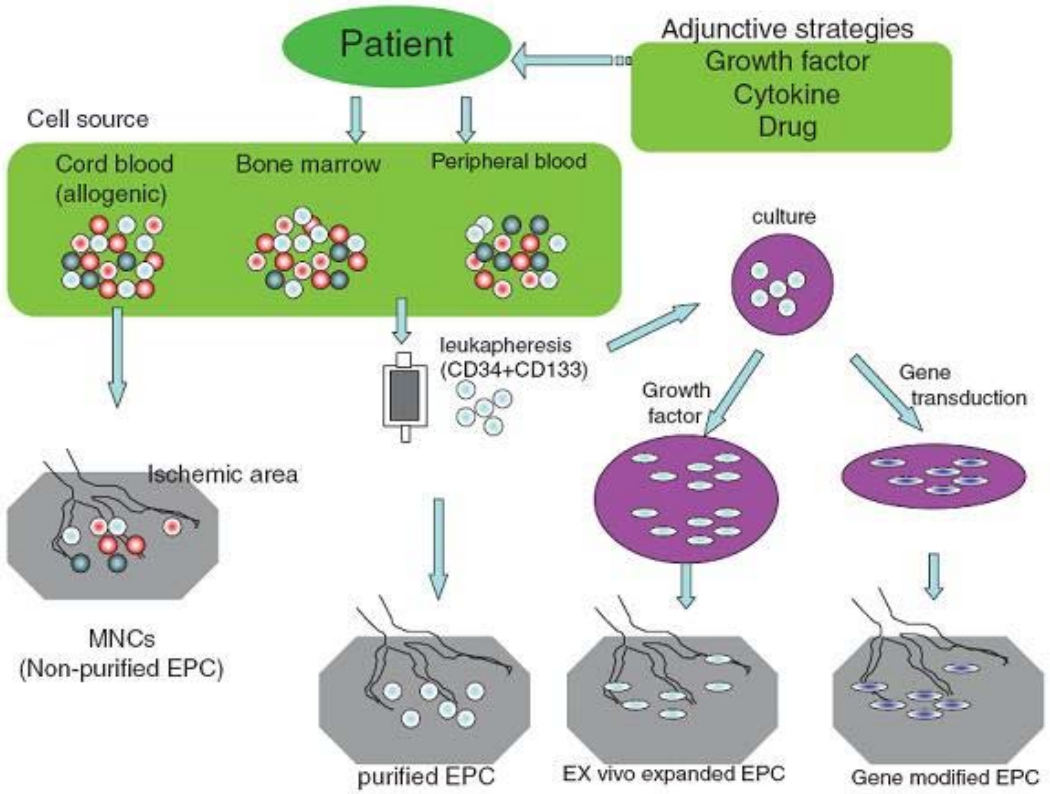


Figure 3.5 Schematic diagram illustrated the therapeutic applications of EPCs for neovasculogenesis (Eguchi *et. al*., 2007)

CHAPTER IV
MATERIALS AND METHODS

4.1 Subjects

This study was approved by the Ethical Committee of Faculty of Medicine Siriraj Hospital, Mahidol University (No. Si586/2008). All of donors which were included in this study had enrolled in Siriraj Hospital. After informed consent, 18 cord blood (CB) samples were collected from umbilical vein while the placentas were still in utero using Heparin Leo 20 international units (i.u.) per ml of blood.

4.2 Mononuclear Cell Isolation

The MNCs were isolated using Ficoll-Paque density gradient centrifugation (47). Briefly, 50 ml of cord blood were diluted 1:1 with phosphate buffered saline (PBS) and then were overlaid onto the equivalent volume of Ficoll-Plaque (Amersham Bioscience, USA). The blood was centrifuged at 980×g (BECKMAN, USA) at room temperature (RT) for 20 min. MNCs were collected and washed twice with PBS supplemented with 1% penicillin/streptomycin (Invitrogen, UK).

4.3 Cell Separation

4.3.1 CD 14 Cell Separation

EasySep® Phycoerythrin (PE) selection Kit, (StemCell Technologies, USA) which base on PE labeled immuno-magnetic bead positive selection was used for CD14 separation. One million of MNCs were resuspended in 1 ml of PBS containing 2% fetal bovine serum (Lonza, USA) and 1 mM of ethylene diamine tetra-acetic acid (BHD Chemicals Ltd, U.K.) (2% FBS in PBS/EDTA) at a concentration of 1×10^8 cells/ml. The suspension was incubated with 100 µl of anti-CD32 ($F_c\gamma R$ blocking)

together with 200 µl of anti-CD14 PE labeled for 15 min at RT in the dark. One hundred microliters of PE-selection cocktail was added and incubated for further 15 min at RT in the dark. This cocktail comprises of anti-PE and anti-dextran, are called tetrameric antibody complex (TAC). Afterward, 50 µl of nano-particle was added to the cell suspension and was incubated for 10 min RT in the dark. The cell suspension was added upto 2.5 ml with 2% FBS in PBS/EDTA. The selection was started by placing the tube into *EasySep*® Magnet and incubated for 5 min at RT in the dark. The *EasySep*® Magnet and tube were picked up and inverted with in one continuous motion, the supernatant which was $CD14^-$ fraction was then poured off into another tube. The magnetically labeled cells, which were $CD14^+$ fraction, were remained inside the tube and held by the magnetic field of the *EasySep*® Magnet. The selected cells were washed 4 times with 2% FBS PBS/EDTA and resuspended in Endothelial Growth media-2 (EGM-2) (Clonetics; Walkersville, USA) for further studies.

4.3.2 CD 34 Cell Separation

CD34 selection Kit, (StemCell Technologies, USA) which base on CD34 labeled immuno-magnetic bead positive selection was used for CD34 separation. One million of MNCs were resuspended in 1 ml of 2% FBS in PBS/EDTA at a concentration of 1×10^8 cells/ml. The cells were incubated with 100 µl of anti-CD32 for $F_c\gamma R$ blockling. One hundred microliters of CD34 selection cocktail was added to the cells and incubated for 15 min at RT in darkness. The CD34 cocktail comprises of anti-CD34 and anti-dextran are called tetrameric antibody complex (TAC). Then 50 µl of nano-particle was added and incubated for 10 min at RT in the dark. The cell suspension was added upto 2.5 ml with 2% FBS in PBS/EDTA. Thereafter, the tube was placed in *EasySep*® Magnet and incubated for 5 min at RT in the dark. The *EasySep*® Magnet and tube were picked up and inverted with in one continuous motion, the supernatant which was $CD34^-$ fraction was then poured off into another tube. The magnetically labeled cells which were $CD34^+$ fraction were remained inside the tube, held by the magnetic field of the *EasySep*® Magnet. The selected cells were washed 4 times with 2% FBS PBS/EDTA and resuspended in EGM-2 for further studies.

4.3.3 CD14/34 Cell Separation

EasySep® fluorescein isothiocyanate (FITC) selection Kit was used in CD14 separation. Two millions of CB-MNCs were resuspended in 1 ml of 2% FBS PBS/EDTA at a concentration of 2×10^8 cells/ml. The suspension was incubated with 200 µl of anti-CD32 ($F_c\gamma R$ blocking) accompanied with 200 µl of anti-CD14 FITC labeled for 15 min at RT in the dark. Two hundred microliters of FITC-selection cocktail was added and incubated for further 15 min at RT in the dark. This cocktail comprises of anti-FITC and anti-dextran, are called tetrameric antibody complex (TAC). Afterward, 100 µl of nano-particles were added to the cell suspension and was incubated for 10 min at RT in the dark. The cell suspension was added upto 2.5 ml with 2% FBS in PBS/EDTA. Thereafter, tube was placed in *EasySep*® Magnet and incubated for 5 min at RT in the dark. The *EasySep*® Magnet and tube were picked up and inverted with in one continuous motion, the supernatant which was $CD14^-$ fraction was then poured off into another tube. The magnetically labeled cells which were $CD14^+$ fraction were remained inside the tube, held by the magnetic field of the *EasySep*® Magnet. The selected cells were washed 4 times with 2% FBS PBS/EDTA. After finish CD14 cell separation, the cell fractions comprise of CD14 negative and positive subpopulations. CD14 negative fraction was subsequently separated by CD34 marker. 1.2 to 1.6 × 10^8 of CD14 cells were resuspended in 1 ml of 2% FBS in PBS/EDTA. The cells were incubated with 100 µl of anti-CD32 ($F_c\gamma R$ blockling). One hundred microliters of CD34 selection cocktail was added to the cells and incubated for 15 min at RT in the dark. The CD34 cocktail comprises of anti-CD34 and anti-dextran are called tetrameric antibody complex (TAC). Then, 50 µl of nano-particle was added and incubated for 10 min at RT in the dark. The cell suspension was added upto 2.5 ml with 2% FBS in PBS/EDTA. Thereafter, the tube was placed in *EasySep*® Magnet and incubated for 5 min at RT in the dark. The *EasySep*® Magnet and tube were picked up and inverted with in one continuous motion, the supernatant which was $CD14^-/CD34^-$ fraction was then poured off into another tube. The magnetically labeled cells which were $CD14^-/CD34^+$ fraction were remained inside the tube, held by the magnetic field of the *EasySep*® Magnet. The selected cells were washed 4 times with 2% FBS PBS/EDTA and resuspended in EGM-2 for further studies.

4.4 Culture of Endothelial Progenitor Cells

The culture of EPC colonies from CB cell subpopulation were performed as with some modification (47). The experiments were described as following;

4.4.1 Culture of CB-MNC and CD14 Subpopulations

After cell separation, 5 x 10^4 cells/cm^2 of CB-MNC, 1 x 10^4 cell/cm^2 of $CD14^+$ subpopulation, 4 x 10^4 cells/cm^2 of $CD14^-$ subpopulation, and 1 x 10^1 cells/cm^2 of $CD14^+$ subpopulation together with 4 x 10^4 cell/cm^2 of $CD14^-$ subpopulation (co-culture) were cultured in 2 ml of EGM-2 (Clonetics; Walkersville, USA). The plates were coated with 10 μg/ml human fibronectin (Amersham Biosciences, USA) before use and incubated at 37 °C, in 5% CO_2 incubator. (SANYO, Japan). After culture for 3 days, non-adherent cells were removed and fresh medium was added. Thereafter, the medium was replaced every 3 days for the entire culture period. The colonies of EPC were observed under inverted microscope on day 14.

4.4.2 Transwell Culture of CB derived $CD14^+$ and $CD14^-$ fractions

Transwell plate contains six wells and each well contained the transwell polycarbonate membrane which has pore size of 0.3 μm. Each well and the transwell inserts were pre-coated with 10 ng/ml fibronectin (Invitrogen, USA) for 30 min at RT before cell plating. Each wells and its transwell inserts were incubated with 2.4 ml and 1.6 ml of EGM-2 (Clonetics; Walkersville, USA), respectively. 1 × 10^5 cells/cm^2 of $CD14^+$ cells and 4 × 10^5 cells/cm^2 of $CD14^-$ cells were cultured in EGM2 as shown in figure 4.1A. The culture of MNCs and co-culture were performed as the positive control for EPC colonies. The cultures of $CD14^+$ cell or $CD14^-$ cell alone was used as negative control. The cultures were incubated at 37 °C in 5% CO_2 incubator. (SANYO, Japan). After culture for 3 days, non-adherent cells were removed and fresh medium was added. Thereafter, the medium was replaced every 3 days for the entire culture period. The colonies of EPCs were observed under inverted microscope on day 14.

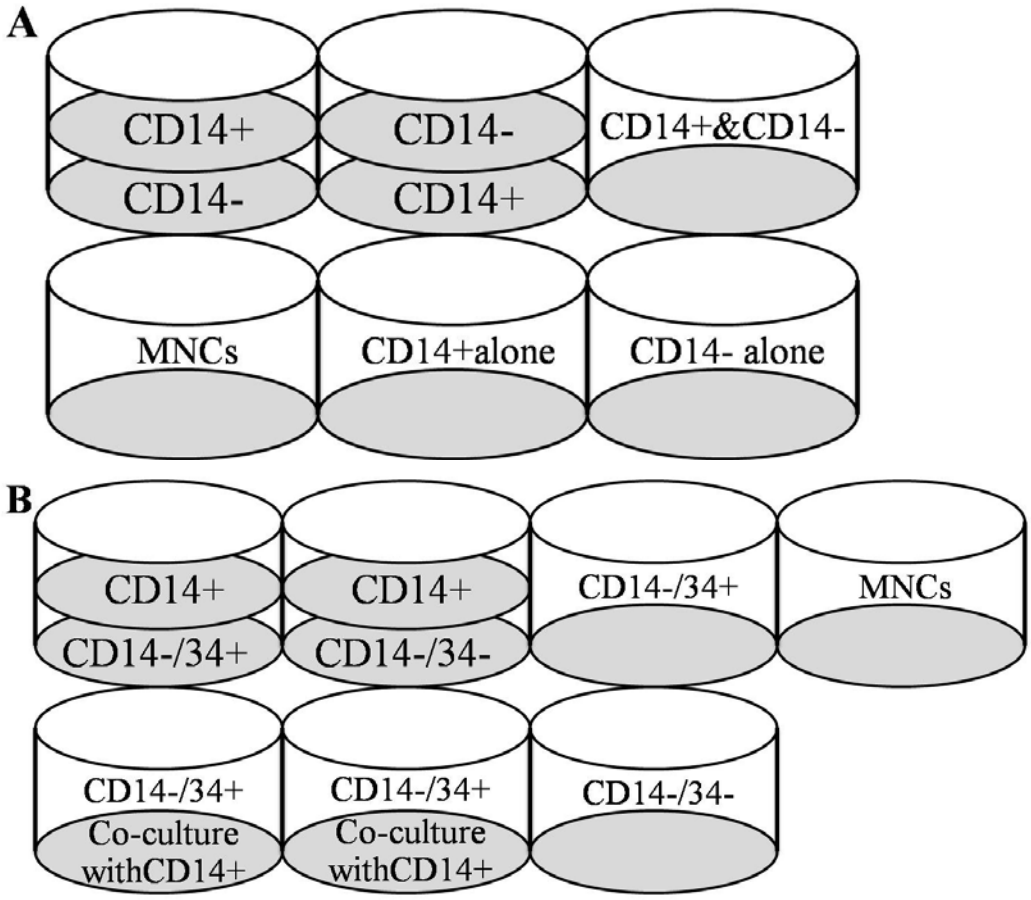


Figure 4.1 Schematic diagram illustrated the cultures of $CD14^+$, $CD14^-$, $CD14^-/34^+$ and $CD14^-/34^-$ cell subpopulations.

4.4.3 Transwell Culture of CB derived $CD14^-/34^+$, $CD14^-/34^-$ and $CD14^+$ fractions

Three fractions of $CD14^-/34^+$, $CD14^-/34^-$ and $CD14^+$ were colleted from CD34 and CD14 cell separations as described above (4.3.3). Transwell plate contains six wells and each well contained the transwell polycarbonate membrane which has pore size of 0.3 μm. The plates including each well and the transwell inserts were pre-coated with 10 ng/ml fibronectin (Invitrogen, USA) for 30 min at RT before cell plating. Each wells and its transwell inserts were incubated with 2.4 ml and 1.6 ml of EGM-2 (Clonetics; Walkersville, USA), respectively. 1×10^5 cells/cm^2 of $CD14^+$ cells, 3×10^5 cells/cm^2 of $CD14^-/CD34^-$ cells and 3×10^4 cells/cm^2 of $CD14^-/CD34^+$ cells were cultured in EGM2 as shown in figure 4.1B. The culture of MNC derived

EPCs was performed as the positive control for EPC colonies. The cultures were incubated at 37 °C in 5% CO_2 incubator. (SANYO, Japan). After culture for 3 days, non-adherent cells were removed and fresh medium was added. Thereafter, the medium was replaced every 3 days for the entire culture period. The colonies of EPC were observed under inverted microscope on day 14.

4.5 Immunophenotyping of Endothelial Progenitor Cells

5×10^5 of EPCs (from the third or the fourth passage) derived from MNC, $CD14^-$ or $CD14^-/34^+$ cells were incubated with anti-CD146 FITC conjugated (BD bioscience, USA), anti- von willebrand factor (vWF) FITC conjugated (AbDserotec, UK) , anti-CD34 PE conjugated (BD bioscience, USA), anti-vascular endothelial growth factor receptor-2 (VEGFR-2) PE conjugated (R&D System, USA) or isotype controls which are anti-mouse IgG1 FITC conjugated and anti-mouse IgG2 PE conjugated (both from BD bioscience, USA). All anti-bodies were added to EPCs 3 µl each and incubated at 4 ºC for 30 min in the dark. Afterward, the cells were washed twice with PBS and resuspended in 500 µl of 1% paraformaldehyde in PBS. The cells were analyzed by flow cytometer using CellQuest software (FACSCalibur, Becton Dickinson, USA) (9, 10, 30, 47, 52).

4.6 *In vitro* Tube Formation

This experiment was modified from Glulati, *et al.,* 2003 (30). Briefly, 48 well culture plates (Corning, USA) were coated with 60 µl of matrigel™ (Sigma Aldrich, USA) and allowed to polymerize for 30 mins at RT. 4×10^4 EPCs which derived from MNC, $CD14^-$ or $CD14^-/34^+$ cells were seeded into matrigel™ coated plates and incubated for 24 hr at 37°C in 5% CO_2 incubator (SANYO, Japan). Capillary like structures were observed by inverted microscope. Experiments were performed in triplicate.

4.7 Cytokine Array

RayBio® Human cytokine antibody array Kits 3 were purchased from Ray Biotech, Inc. (RayBiotech, USA). This kit contains the membrane that has been labeled with antibodies against 42 cytokines. The mechanism for cytokine detection is base on chemiluminescence imaging system. The procedure was performed according to the manufacturer's instruction. Briefly, the membrane was incubated with 2 ml of blocking buffer which provided from the kit for 30 min with shaking. Thereafter, the membrane was incubated with 1 ml of certain condition media (condition media of $CD14^-$ or $CD14^+$ cell culture) for 2 hr with continuously shaking. The membrane was incubated with 2 ml of biotinylated Ab (cocktail of Biotin-Ab) with shaking for 2 hr and then incubated with 2 ml of HRP-labeled streptavidin solution for 2 hr with continuously shaking. Afterward, 500 µl of the substrate (reagent C and D) which contain in the detection was added and incubated for 30 sec in the dark. Finally, the membrane was exposed to the x-ray film for detection of the signals and then further analyzed the data by densitometry (BioRad, USA). All steps are shown in figure 4.2. The membrane must be washed with wash buffer I 3 times and wash buffer II 2 times in every step and allow 5 min per wash with shaking.

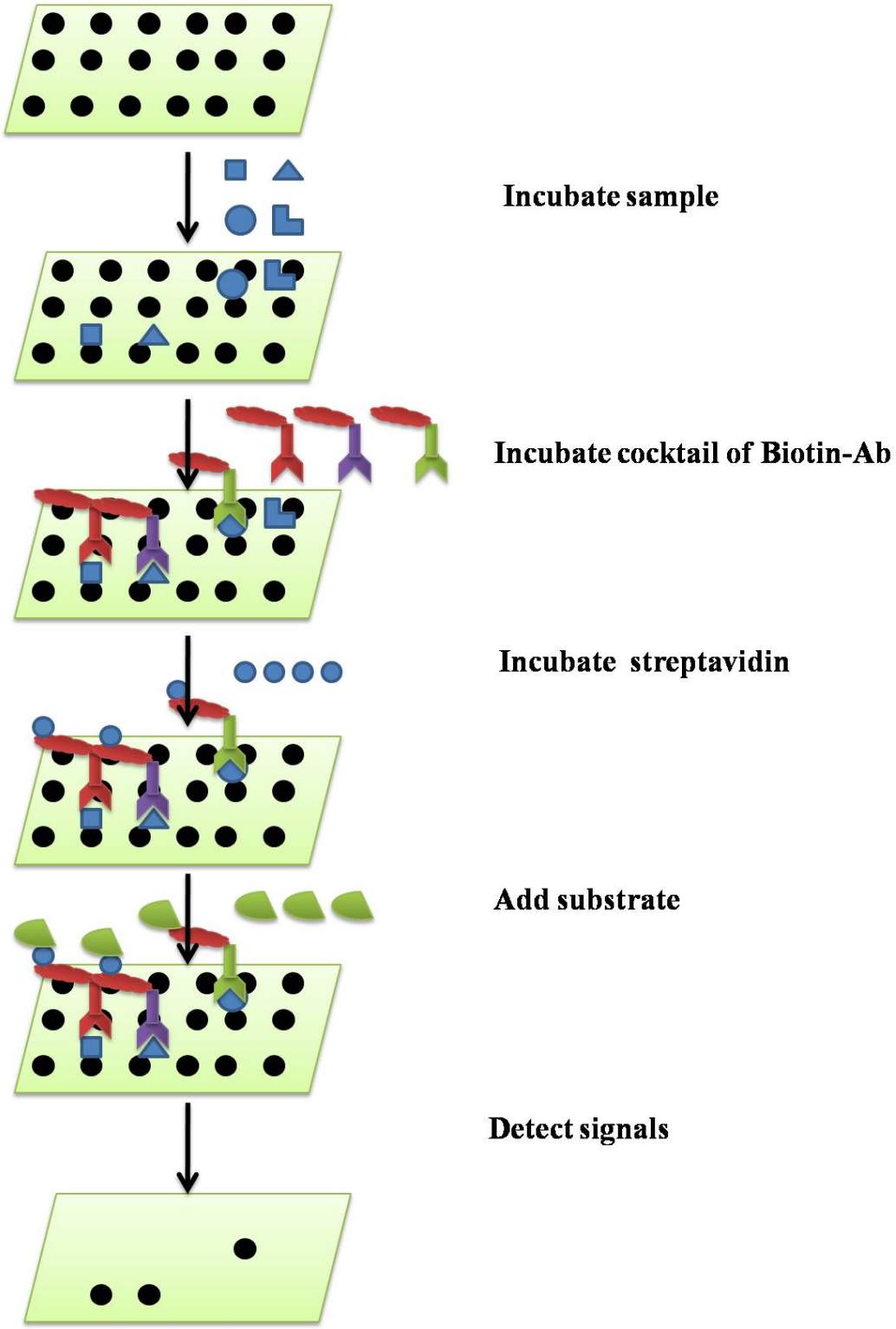


Figure 4.2 Schematic diagram illustrated the principle of human cytokine antibody array

4.8 Neutralization and Stimulation of EPCs Using Angiogenin

4.8.1 Angiogenin Blocking

The neutralizing of angiogenin (ANG) was performed as shown in figure 4.3A. The transwell culture between $CD14^+$ and $CD14^-$ subpopulations was performed as described in section 4.4.1, 1×10^5 cells of $CD14^+$ cells and 4×10^5 cells of $CD14^-$ were seeded in upper transwell insert and lower chamber, respectively. Goat anti-human ANG antibody (R&D System, USA) was added at a final concentration of 5 and 50 μg/ml. The cells were cultured in EGM-2 with 6 well plates [coated with 10 μg/ml human fibronectin (Amersham Biosciences, USA)] at 37 °C in 5% CO_2 incubator (SANYO, Japan). After culture for 3 days, non-adherent cells were removed and fresh medium was added. Thereafter, the medium was replaced every 3 days for the entire culture period. The colonies of EPC were observed under inverted microscope on day 14.

4.8.2 Angiogenin Stimulation

3×10^5 of $CD14^-$ cells or 1×10^5 of $CD34^+$ cells were cultured in EGM-2 in the present of recombinant human ANG (rhANG) (R&D System, USA) at a final concentration of 10, 20, 100 ng/ml, respectively (41, 42, 46) in fibronectin coated plates as shown in figure 4.3B. $CD14^-$ or $CD34^+$ alone was used as the negative control. The culture was incubated at 37 °C in 5% CO_2 incubator (SANYO, Japan). After culture for 3 days, non-adherent cells were removed and fresh medium was added. Thereafter, the medium was replaced every 3 days for the entire culture period. The colonies of EPC were counted under inverted microscope on day 14.

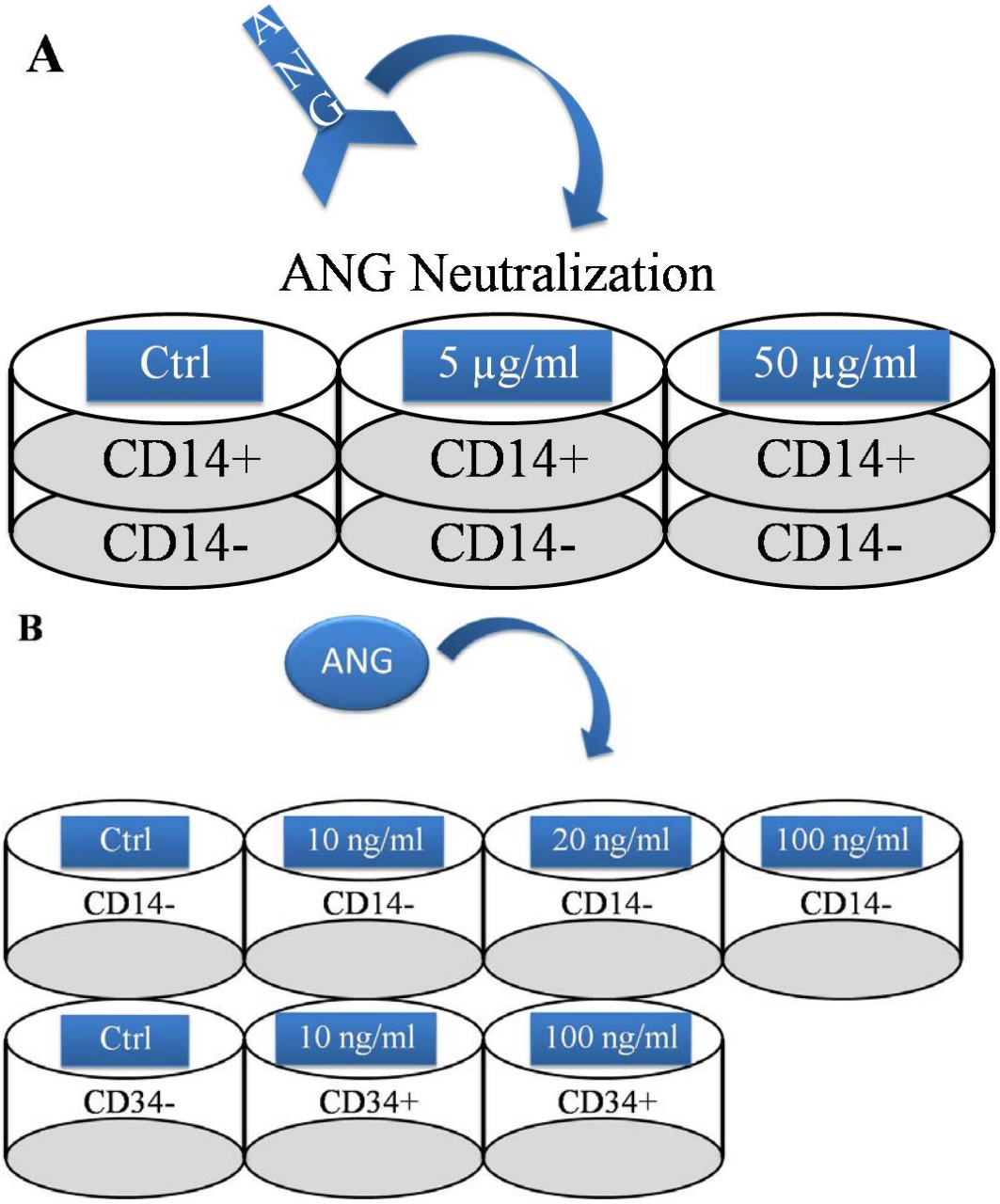


Figure 4.3 Schematic diagram illustrated the neutralization and stimulation of EPCs by an angiogenin

4.9 Data analysis

The data was presented as mean ± SD or mean ± SEM. The statistical significance of differences in the data was evaluated by using the analysis of the dependent student t-test. A value of $P<0.05$ was accepted as statistically significant.

* mean P value < 0.05 and ** mean P value < 0.01.

CHAPTER V
RESULTS

5.1 Culture of CD14 Fractions

To identify the founding subpopulation of EPCs in UCBs, the UCB-MNCs were separated to CD14 expressing cell population ($CD14^+$ cells) and CD14 negative cell population ($CD14^-$ cells) by magnetic cell sorting as described in method section 4.3.1. The enriched $CD14^+$ (the percentage in this population was 95.56 ± 4.12 %) and $CD14^-$ (the percentage in this population was 96.10 ± 2.37 %) were culture either separately or in combination to assess their capacity to generated EPC colonies. The purity of each cell subpopulation ($CD14^+$, $CD14^-$) has shown in figure 5.1. This result has indicated that the enriched $CD14^+$ or $CD14^-$ subpopulation alone could not generate any observable EPC colonies. The results have indicated that the separated culture of $CD14^+$ or $CD14^-$ subpopulation could not be observed EPC colonies. In contrast the direct co-culture of $CD14^+$ and $CD14^-$ subpopulations yield several EPC colonies in a number similar to those observed in MNC culture. In addition, the interaction between $CD14^+$ and CD14- subpopulation through the transwell culture also yield several EPC colonies. However, the numbers of EPC colonies observed in transwell culture were lower than those observed in MNC and direct $CD14^+/CD14^-$ culture (figure 5.2, 5.3). In addition, the EPC colonies from transwell co-culture system could be observed only when $CD14^-$ subpopulation received the soluble factors from $CD14^+$ subpopulation in transwell while the $CD14^+$ subpopulation which receives the soluble factors from $CD14^-$ subpopulation could not observe EPC colonies (figure 5.2, 5.3). The EPCs generated from MNCs, $CD14^+/CD14^-$ direct co-culture and $CD14^+/CD14^-$ transwell culture exhibited the typical EPC characteristics, being cobblestone shaped cells, expressing CD34, CD146, VEGFR2 and vWF (figure 5.4A) and form capillary like structur on Matrigel™ (figure 5.4B).

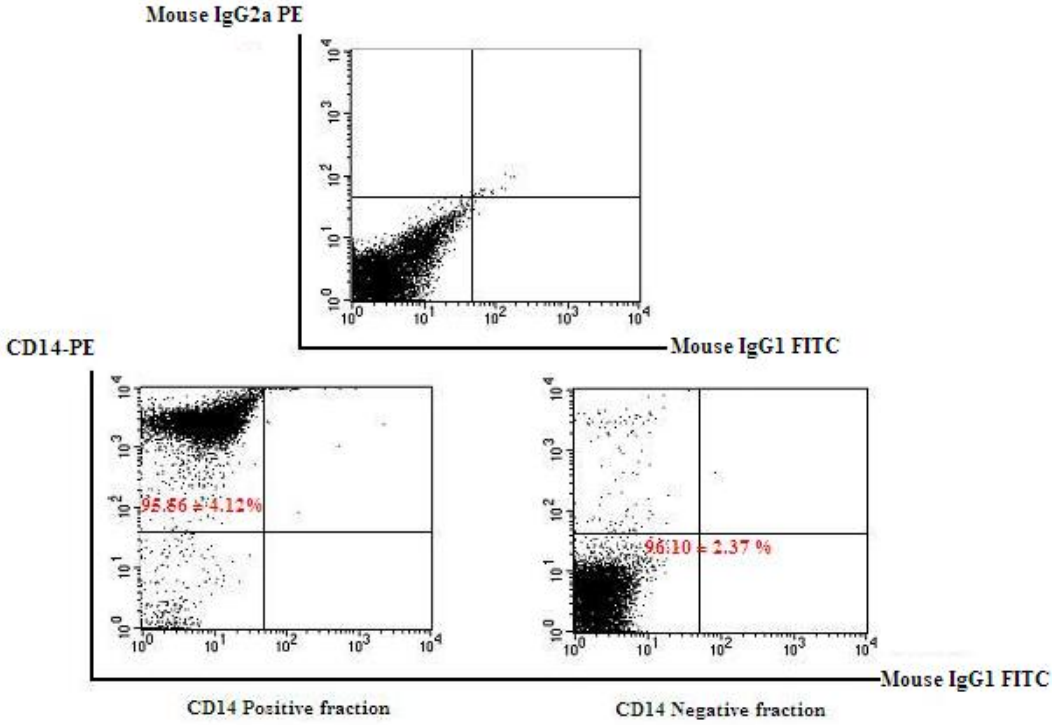


Figure 5.1 The purity of $CD14^+$ and $CD14^-$ subpopulations

$CD14^+$ and $CD14^-$ fractions were assesed by flowcytometer. Upper middle was the isotype control which stained with mouse IgG1 FITC and mouse IgG2a, respectively. Lower left and lower right showed the purity of $CD14^+$ and $CD14^-$ fractions, respectively. The experiment was perfromed in 6 cases (n=6).

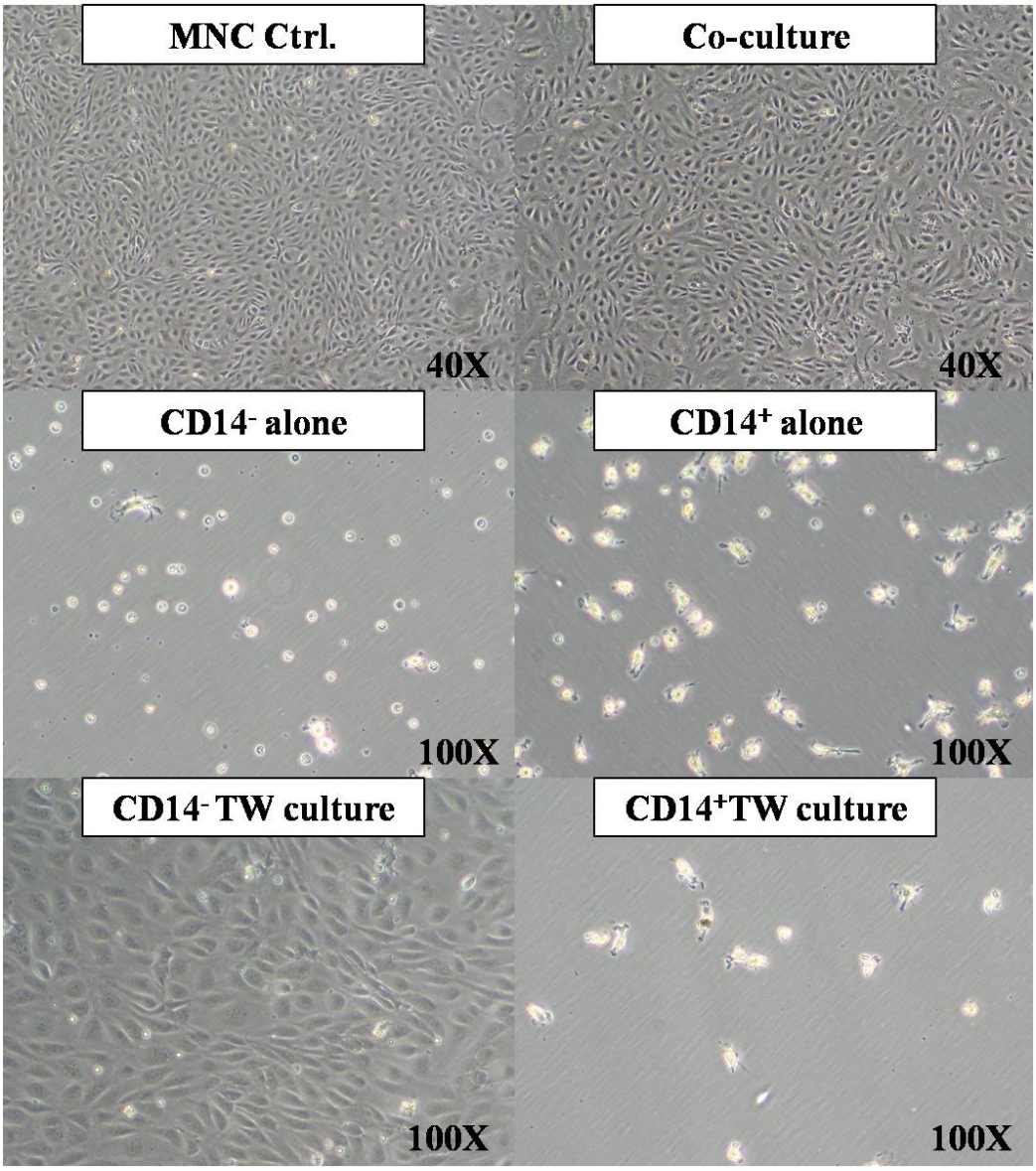


Figure 5.2 Represent figure of CD14 each subpopulation culture

The founding population of EPCs were investigated by the culture of CD14 each fractions. MNCs means culture of MNCs, co-culture means direct co-culture between $CD14^+$ and $CD14^-$ subpopulations, $CD14^-$ alone means culture of $CD14^-$ subpopulation alone, $CD14^+$ alone means culture of $CD14^+$ subpopulation alone, and CD14- TW culture means transwell co-culture between $CD14^-$ and $CD14^+$ subpopulation that observed EPC colonies in the $CD14^-$ fraction while CD^+ TW culture, EPC colonies were observed in the $CD14^+$ fraction. The experiment was performed in 6 cases (n=6).

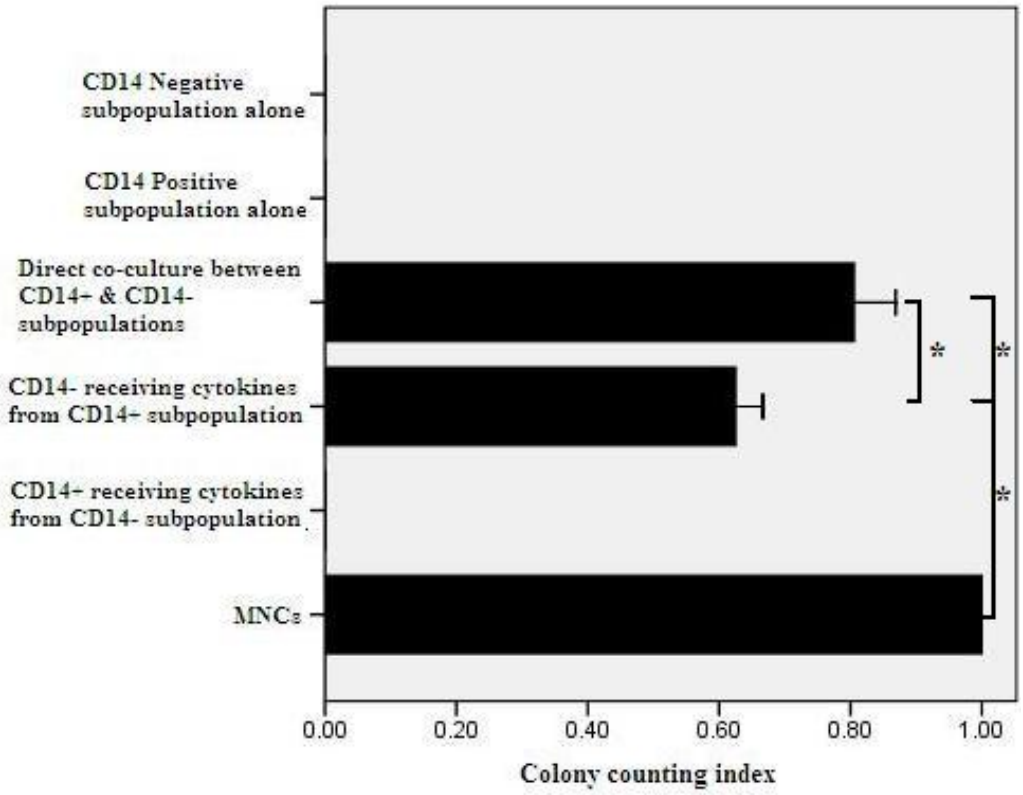


Figure 5.3 The colony counting index of CD14 each subpopulation culture

Colony counting index was performed by the total colony counts of each condition divided by the colony count of MNCs. This graph showed the various culture condition of CD14 each subpopulation which was described above in figure 5.2. The experiment was performed in 6 cases (n=6).

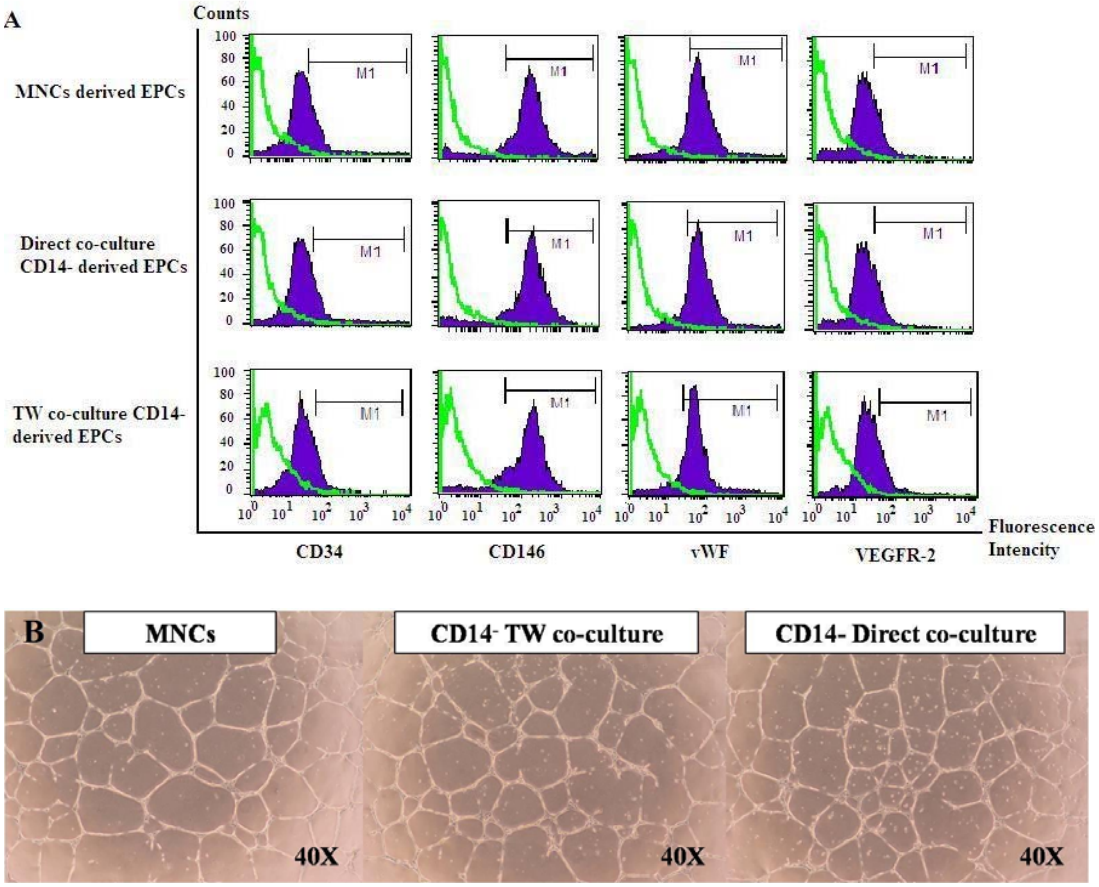


Figure 5.4 CD14 each subpopulation derived EPC characterization

Molecular marker expression and capillary like structure were performed in MNC, $CD14^-$ TW co-culture and $CD14^-$ direct co-culture which were performed by flow cytometer and in vitro tube formation, respectively. The experiments were performed in 3 cases (n=3).

5.2 Determination of the Critical Factors Involving in the Establishment EPC Colonies

The results described in section 5.1 suggest that there are $CD14^+$ derived soluble factors which play critical roles in the establishment of EPC colonies. To determine those factors, we compared the amount of several 42 pro-angiogenic cytokines secreted from $CD14^+$ and $CD14^-$ subpopulation using kit as described in section 4.7. Using this method, we cloud indentify factors that are selectively release

from $CD14^+$ fraction. The result indicates that angiogenin was exclusively expressed in $CD14^+$ fraction and might play an important role in the establishment of EPC colonies. In addition to angiogenin, the amount of secreted IL-8, EGF and VEGF was also significantly higher in $CD14^+$ subpopulation compared with the amount secreted from $CD14^-$ subpopulation (figure 5.5). Taken together, these results suggest a critical role of angiogenin, IL-8, EGF and VEGF in the establishment of EPC colonies from UCBs.

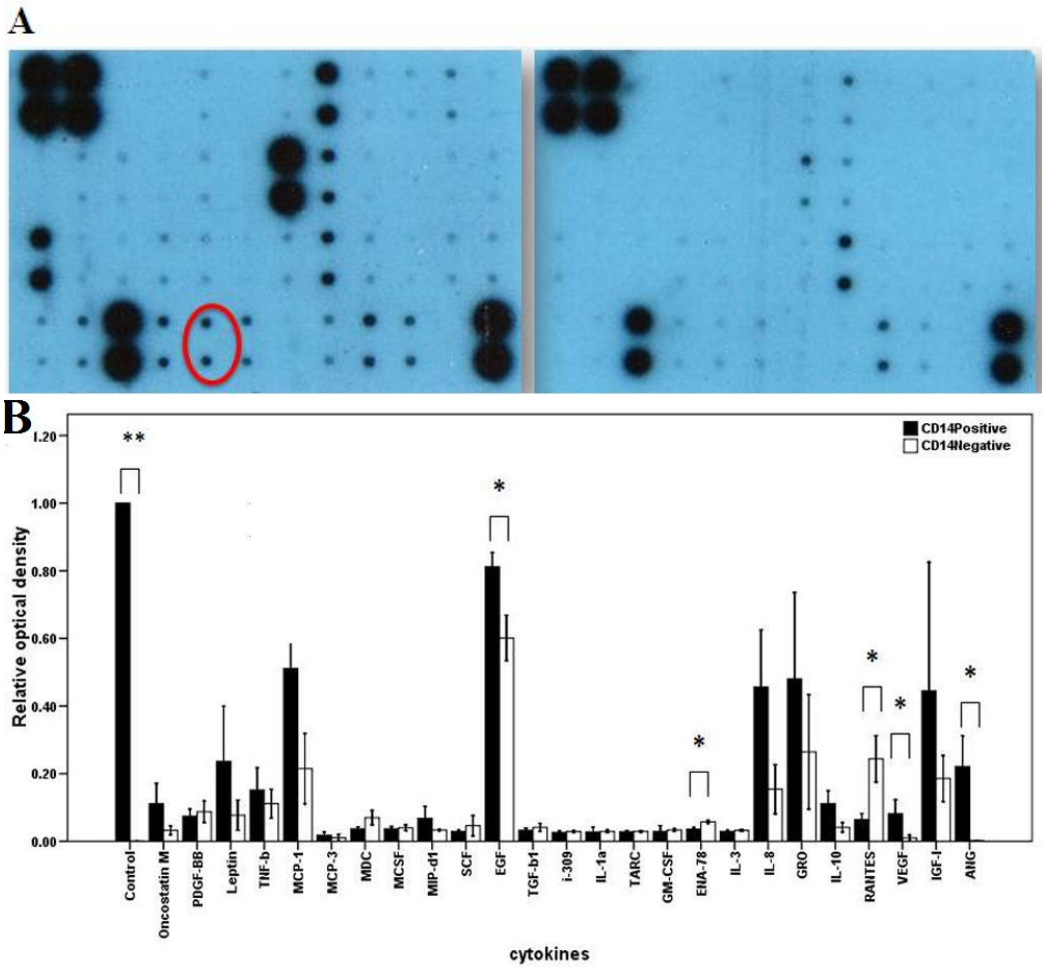


Figure 5.5 The determination of cytokines involving in EPC establishment

The cytokines were determined by cytokine antibody array. The direction of this kit was described in section 4.7. Figure 5A illustrated the array membranes between $CD14^+$ condition media derived (right) and $CD14^-$ condition media derived (left) membranes. Figure 5B represented the cytokine graph which compared between $CD14^+$ and $CD14^-$ derived cytokines. The experiment was performed in 4 cases (n=4).

5.3 Culture of CD14/34 Fractions

The previous results described in section 5.1 demonstrated that the founding populations of EPCs were enriched in $CD14^-$ subpopulation. To further characterize the EPC founding population in $CD14^-$ subpopulation, the $CD14^-$ subpopulation were separated into CD14 negative CD34 expressing cells ($CD14^-/34^+$) and CD14 negative CD34 negative cells ($CD14^-/34^-$) subpopulation of EPCs using magnetic cell sorting as described in section (3.3.3). The purity of each cell subpopulation ($CD14^+$, $CD14^-/34^+$, $CD14^-/34^-$) was shown in figure 5.6. The result indicated that the direct co-culture between $CD14^-/34^+$ (the percentage in this population was 93.08 ± 9.20 %) and $CD14^+$ subpopulation yield several EPC colonies while $CD14^-/34^-$ (the percentage in this population was 96.01 ± 2.67 %) subpopulation alone, direct co-culture between $CD14^-/34^-$ and $CD14^+$ subpopulation, or transwell co-culture between $CD14^-/34^-$ and $CD14^+$ subpopulation cloud not yield any EPC colonies (figure 5.7, 5.8). In addition, the result also demonstrated that $CD14^-/34^+$ subpopulation alone cloud generated EPC colonies in a number similar to those observed in MNC and co-culture between $CD14^-/34^+$ and $CD14^+$ subpopulation (figure 5.7, 5.8). This result suggested that the founding population of EPCs were enriched in $CD14^-/34^+$ subpopulation. In addition, The EPCs generated from CD14-/34+ culture alone, $CD14^-$/34+ direct co-culture and $CD14^-$/34+ transwell culture with CD14+ exhibited the typical EPC characteristics, being cobblestone shaped cells, expressing CD34, CD146, VEGFR2 and vWF (figure 5.9A) and form capillary like structur on Matrigel™ (figure 5.9B).

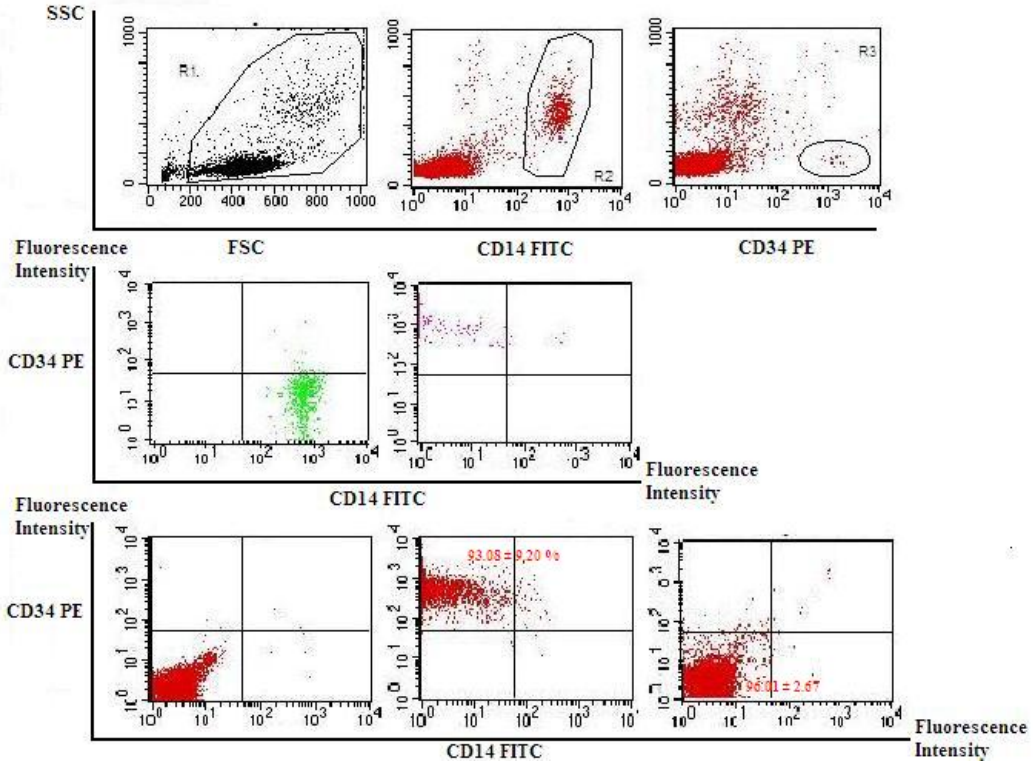


Figure 5.6 The purity of $CD14^{-}/34^{+}$ and $CD14^{-}/34^{-}$ subpopulations

The purity of $CD14^{-}/34^{+}$ and $CD14^{-}/34^{-}$ subpopulations were assessed by flow cytometer. The upper middle and right showed the $CD14^{+}$ and $CD34^{+}$ which contained in MNCs. Region 2 (R2) was CD14 positive cells which were subsequently opened in CD34 (middle left). Region 3 (R3) was CD34 positive cells which were subsequently opened in CD14 (middle right). $CD14^{-}/34^{+}$ and $CD14^{-}/34^{-}$ purities were shown in the lower middle and right, respectively. The experiments were performed in 3 cases (n=3).

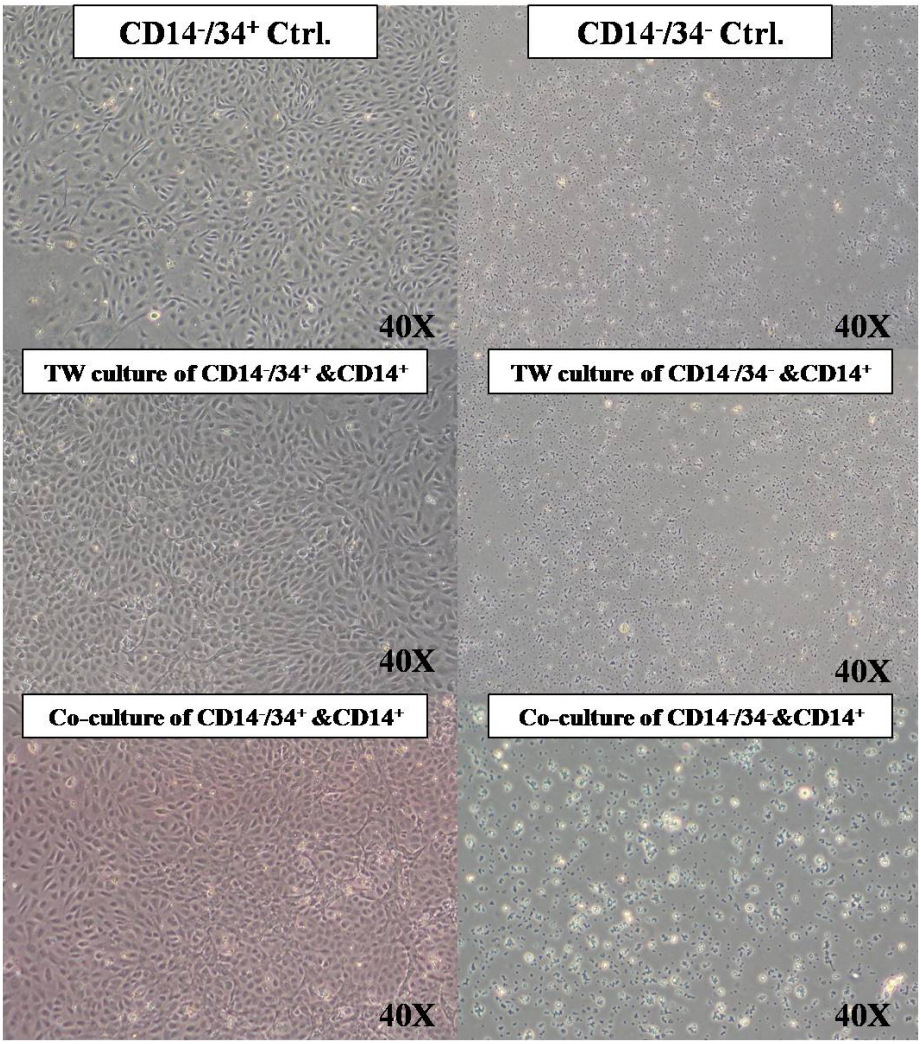


Figure 5.7 Represent figures of culture conditions of $CD14^+$, $CD14^-/34^+$ and $CD14^-/34^-$ each subpopulation

The founding population of EPCs was subsequently investigated by culture of each subpopulation which selected by CD14 and CD34 markers. $CD14^-/34^+$ Ctrl means culture of $CD14^-/34^+$ subpopulation alone, $CD14^-/34^-$ Ctrl means culture of $CD14^-/34^-$ alone, TW culture of CD14-/34+ & CD14+ means the transwell co-culture between $CD14^-/34^+$ and $CD14^+$ subpopulations, TW culture of $CD14^-/34^-$ & $CD14^+$ means the transwell co-culture between $CD14^-/34^-$ and $CD14^+$ subpopulations, co-culture of $CD14^-/34^+$ & $CD14^+$ means the direct co-culture between $CD14^-/34^+$ and $CD14^+$ subpopulations, and co-culture of $CD14^-/34^-$ & CD14+ means the direct co-culture between $CD14^-/34^-$ and $CD14^+$ subpopulations. The experiment was performed in 3 case (n=3).

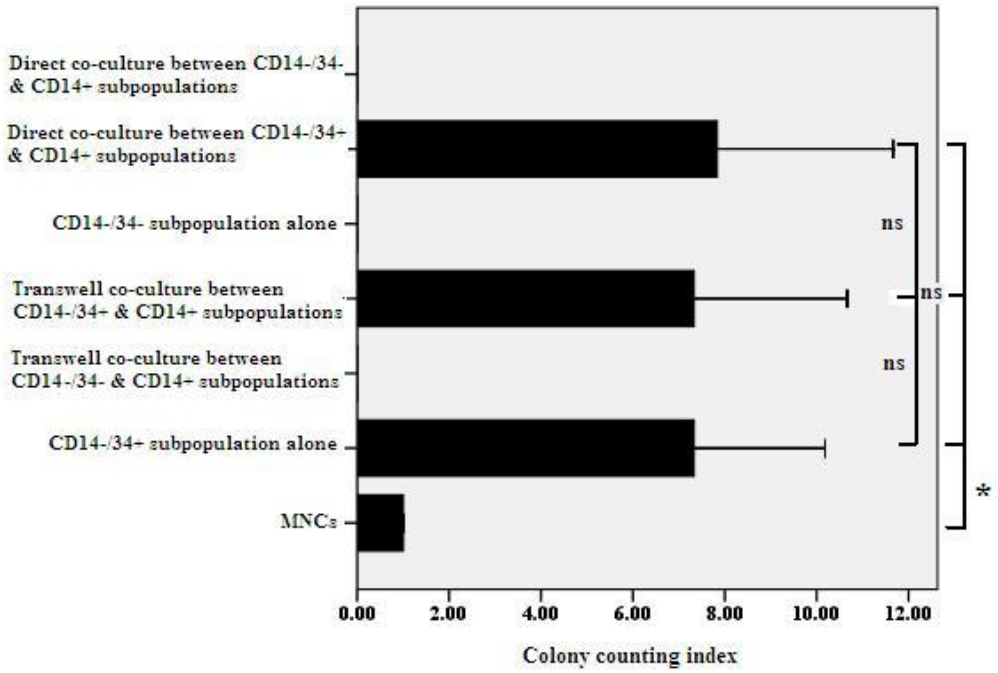


Figure 5.8 The colony counting index of $CD14^+$, $CD14^-/34^+$ and $CD14^-/34^-$ each subpopulation culture

Colony counting index was performed by the total colony counts of each condition divided by the colony count of MNCs. This graph showed the various culture condition of CD14/34 each subpopulation which was described above in figure 5.7. The experiment was performed in 3 cases (n=3).

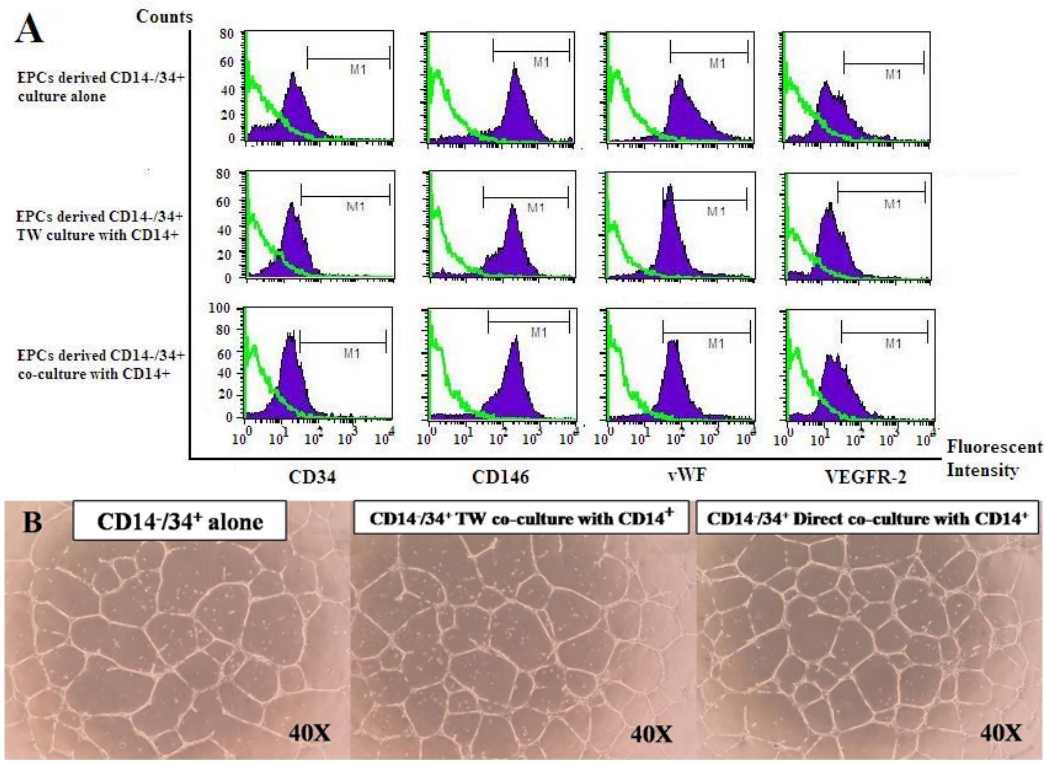


Figure 5.9 CD14/34 each subpopulation derived EPC characterization

Molecular marker expression and capillary like structure were performed in $CD14^{-}/34^{+}$ alone, $CD14^{-}/34^{+}$ TW co-culture with CD14+ and $CD14^{-}/34^{+}$ direct co-culture with $CD14^{+}$ which were performed by flow cytometer and in vitro tube formation, respectively. The experiments were performed in 3 cases (n=3).

5.4 Role of Angiogenin in the Establishment of EPC Colonies

The result described in section 5.2 suggests that angiogenin secreted from $CD14^{+}$ subpopulation might play an important role in the establishment of EPC colonies from $CD14^{-}$ subpopulation. To verify this hypothesis, the function of angiogenin was determined using an in vitro culture system. The effects of angiogenin in the establishment of EPC colonies was assessed by the addition of exogenous angiogenin into $CD14^{-}$ subpopulation which by itself could not generated any EPC colonies. The result showed that angiogenin can significantly increase the number of EPC colonies generated from $CD14^{-}$ subpopualation compared with control in a dose

dependent manner (figure 5.10). In addition, angiogenin could also significantly increase the number of EPC colonies generated from $CD14^-/34^+$ subpopulation compared with control (figure 5.11). However, the inhibition of angiogenin activity by angiogenin neutralizing antibody was not shown any negative effects on the number of EPC colonies generated from $CD14^+/CD14^-$ transwell culture (figure 5.12).

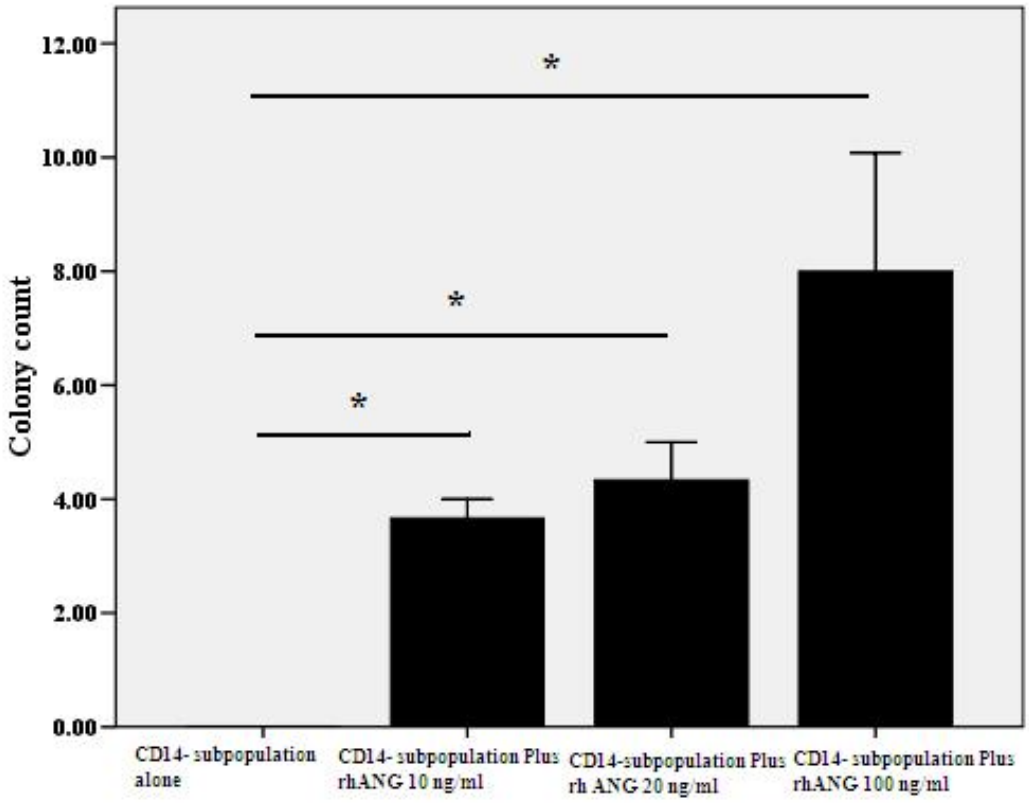


Figure 5.10 The colony count of $CD14^-$ stimulation with rhANG

The role of ANG was claimed by adding rhANG to the culture of $CD14^-$ subpopulation. The molecular rhANG was serially added (10, 20, 100 ng/ml). The experiment was performed in 3 cases (n=3).

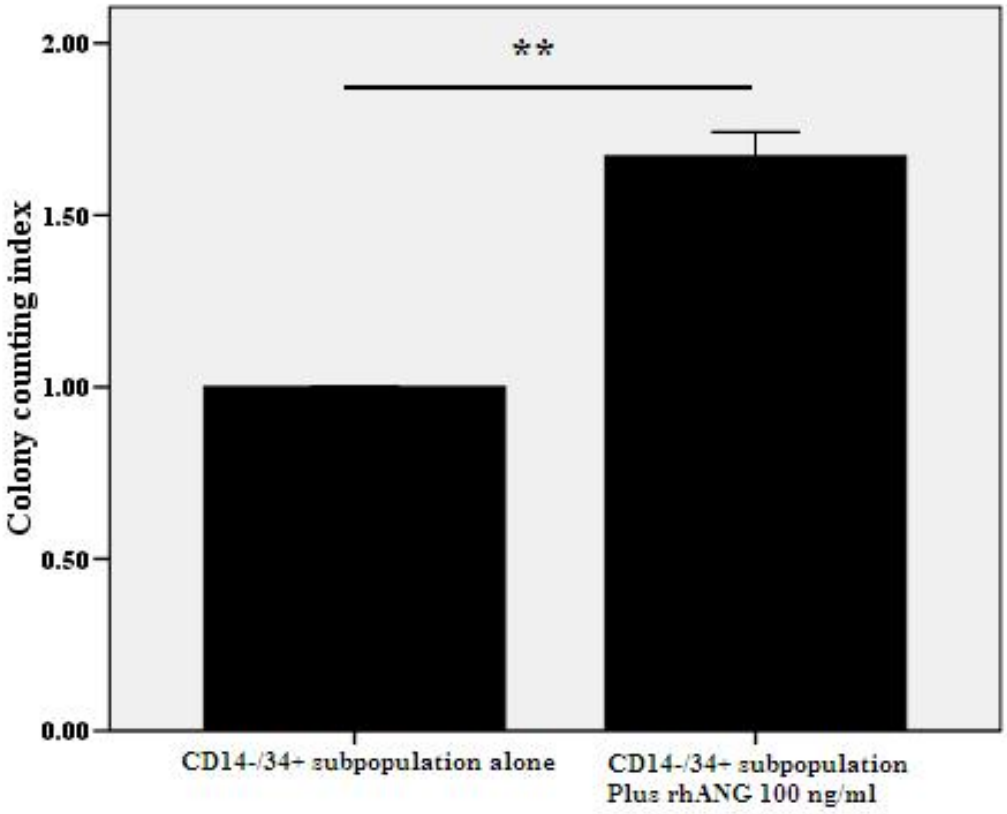


Figure 5.11 The colony count index of $CD14^{-}/34^{+}$ stimulation

The role of ANG was claimed by adding rhANG to the culture of $CD14^{-}/34^{+}$ subpopulation. One hundred nanogram per milliliter of rhANG was added. Colony counting index was performed by the total colony counts of each condition divided by the colony count of $Cd14^{-}/34^{+}$ culture alone. The experiment was performed in 3 cases (n=3).

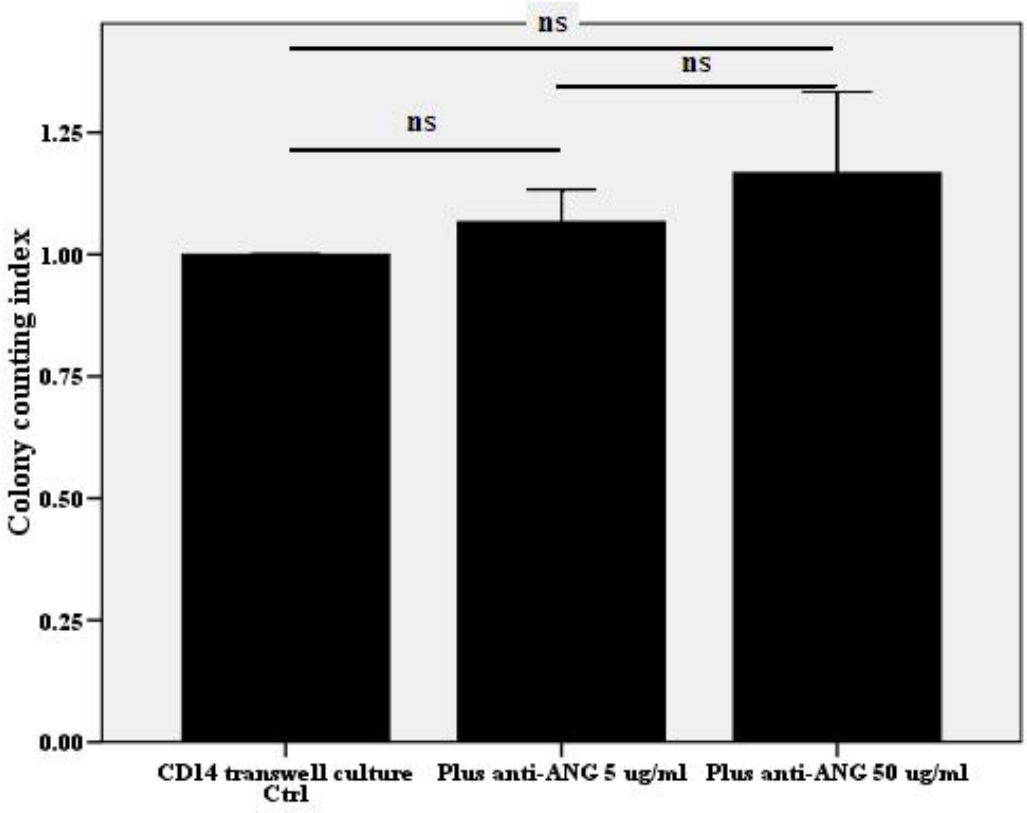


Figure 5.12 Angiogenin blocking of $CD14^{+}/CD14^{-}$ transwell culture

The blocking of ANG in $CD14^{+}/14^{-}$ transwell culture was performed for study the role of ANG. $CD14^{+}/14^{-}$ transwell culture conditions were added neutralizing antibody to ANG at concentration 5 and 50 µg/ml, respectively. Colony counting index was performed by the total colony counts of each condition divided by the colony count of $CD14^{+}/14^{-}$ transwell culture. The experiment was performed in 3 cases (n=3).

CHAPTER V
DISCUSSION

The founding population of EPCs derived from cord bloods was investigated in this study. Previous report showed that culture of either $CD14^+$ or $CD14^-$ subpopulation gave no EPC colonies, however, there were EPC colonies when co-culture of $CD14^+$ and $CD14^-$ subpopulation. Furthermore, $CD34^+$ subpopulation can also give EPC colonies in culture. We further explored the founding population of EPCs derived from cord bloods. EPCs were characterized by the presence of CD34, VEFFR, vWF and CD146 and the ability to form capillary like structure. By using antibody to CD14, MNCs from cord blood were separated into $CD14^+$ and $CD14^-$ subpopulations. Culture of either $CD14^+$ or $CD14^-$ subpopulation gave no EPC colonies. However, EPC colonies were presented in both direct and transwell co-culture of those two subpopulations. The transwell co-culture was the first experimental platform for exploring the effect of cytokine effect on EPCs. Our results indicated that EPCs derived from cord blood are confined in the $CD14^-$ subpopulation, however the colony growth required the cytokines secreted from $CD14^+$ subpopulation. We further screened the cytokine involving the generation of EPCs cytokine antibody array. The cytokine tested were anti-inflammatory cytokine (IL-8), chemokines (ENA-78), and growth factors (VEGF, angiogenin). It was found that the cytokine produced from $CD14^+$ subpopulation was angiogenin (ANG). ANG is an important angiogenic factor which plays an important role in blood vessel growth. ANG can bind to its receptor, or bind to ANG binding protein, and undergo translocation into the nuclease. ANG is one of the critical factor for EPCs as the addition of the recombinant human angiogenin into the culture of $CD14^-$ subpopulation yielded EPC colonies. However, the addition of neutralization antibody of ANG into the upper wells of the transwell co-culture between $CD14^+$ and $CD14^-$ failed to reduce the EPC colony. This may be due to the presence of cytokines other than we tested that can stimulate EPCs. Previous reports indicated that culture of the

$CD34^+$ subpopulation yielded the EPC colonies. We then separated the $CD14^-$ subpopulation into $CD14^-/34^+$ and $CD14^-/34^-$ subpopulations. Based on direct and transwell co-culture of these subpopulations with CD14+ subpopulation, the EPC colonies were observed only with $CD14^-/34^+$ subpopulation. However, culture of $CD14^-/34^+$ subpopulation alone also yielded EPC colonies. It may possible that the numbers of EPCs in the $CD14^-/34^+$ subpopulation are higher than that in $CD14^-$ subpopulation. ANG also had stimulating effect on $CD14^-/34^+$ subpopulation. Taken together, this study provided the evidence that $CD14^-/34^+$ subpopulation is the founding population of EPCs. Our report is the first study in the founding population by systemic cell sorting and thus provides the new experimental platform using cord blood sample.

CHAPTER VII
CONCLUSION

In summary, the founding population of EPC in umbilical cord blood mononuclear cells (UCB-MNCs) is enriched in $CD14^-/34^+$ subpopulation. The result demonstrates that $CD14^-/34^+$ cells had an ability to generate EPCs which express typical EPC markers (CD34, vWF, CD146 and VEGFR2) and are able to form capillary-liked structure *in vitro*. However, the generation of EPCs from $CD14^-$ population required some critical soluble factors from $CD14^+$ subpopulation. Several critical factors secreted from $CD14^+$ population were detected by our experimental system and the result indicates that one of those critical factors is an angiogenic cytokine “angiogenin”. This study provides the experimental platform that could be use to identify the founding population of EPCs as well as the critical factors involving in the generation of EPC colonies. The valuable information generated by this study will greatly improve our knowledge on the origin and biology of EPCs.

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APPENDICES

APPENDIX A
PATIENT INFORMATION SHEET

เอกสารชี้แจงข้อมูลคำแนะนำแก่ผู้เข้าร่วมโครงการ

(Patient Information Sheet)

ชื่อโครงการวิจัย การค้นพบกลุ่มประชากรเซลล์และปัจจัยสำคัญที่จำเป็นสำหรับการเกิดขึ้นของเซลล์ต้นกำเนิดหลอดเลือด

ชื่อผู้วิจัย นายศักดิ์ชัย สุดชะดา คณะแพทยศาสตร์ศิริราชพยาบาล ม. มหิดล
ศ.นพ. สุรพล อิสรไกรศีล คณะแพทยศาสตร์ศิริราชพยาบาล ม. มหิดล
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โทรศัพท์ 02-4199465-6

สถานที่ทำการวิจัยและ/หรือเก็บข้อมูล

โรงพยาบาลศิริราช คณะแพทยศาสตร์ศิริราช ม. มหิดล

ความเป็นมาของโครงการวิจัย

โครงการวิจัยนี้เป็นศึกษาแหล่งกำเนิดของเซลล์ต้นกำเนิดหลอดเลือดจากตัวอย่างเลือดจากสายสะดือทารก จากรายงานที่ผ่านมาในการศึกษากลุ่มเซลล์ที่เป็นแหล่งของเซลล์ต้นกำเนิดหลอดเลือดจากเลือดคนปกติมีรายงานค่อนข้างแน่ชัดแล้ว แต่เซลล์ต้นกำเนิดหลอดเลือดที่ได้จากเลือดมีประสิทธิภาพที่ด้อยกว่าจากเลือดสายสะดือทารกในด้าน การแบ่งตัว ความสามารถในการสร้างหลอดเลือดทั้งในหลอดทดลองและสัตว์ทดลอง และปัจจุบันยังไม่มีรายงานแน่ชัดว่ากลุ่มเซลล์ใดเป็นเซลล์ต้นกำเนิดหลอดเลือด เพื่อให้ทราบแน่ชัดว่ากลุ่มเซลล์ชนิดใดในเซลล์โมโนนิวเคลียร์ที่เจริญไปเป็นเซลล์ต้นกำเนิดหลอดเลือดต่อไป ทางคณะผู้วิจัยจึงได้สนใจที่จะศึกษาหากลุ่มเซลล์และปัจจัยสำคัญต่างๆ ที่มีผลต่อการเกิดขึ้นของเซลล์ต้นกำเนิดหลอดเลือด จากตัวอย่างเลือดจากสายสะดือทารก

วัตถุประสงค์และประโยชน์ของการศึกษาที่จะได้รับ

๑) เพื่อให้ทราบแน่ชัดว่ากลุ่มเซลล์ใดเป็นแหล่งของเซลล์ต้นกำเนิดหลอดเลือด

๒) เพื่อเป็นองค์ความรู้พื้นฐานที่จะใช้ในการประยุกต์ในการรักษาโรคระดับเซลล์ที่เกี่ยวกับการบกพร่องของเซลล์ต้นกำเนิดหลอดเลือดต่อไป

ความเสี่ยงในการเก็บตัวอย่าง

อาสาสมัครไม่มีความเสี่ยงเพิ่มเติมจากการเก็บตัวอย่างเพื่อใช้ในงานวิจัย หากมีข้อมูลเพิ่มเติมทั้งด้านประโยชน์และโทษเกี่ยวข้องกับการวิจัยนี้ ผู้วิจัยจะแจ้งให้ทราบโดยไม่ปิดบังอย่างรวดเร็ว

การเก็บข้อมูลของอาสาสมัคร

ข้อมูลส่วนตัวของอาสาสมัครจะถูกเก็บรักษาไว้ ไม่เปิดเผยต่อสาธารณะเป็นรายบุคคล แต่จะรายงานผลการวิจัยเป็นข้อมูลส่วนรวม ข้อมูลของอาสาสมัครเป็นรายบุคคลอาจมีคณะบุคคลบางกลุ่มเข้ามาตรวจสอบได้ เช่น ผู้ให้ทุนวิจัยม สถาบัน หรือองค์กรของรัฐที่มีหน้าที่ตรวจสอบ, คณะกรรมการจริยธรรมเป็นต้น

สิทธิอาสาสมัครในการถอนตัวออกจากโครงการ

อาสาสมัครที่เข้าร่วมการวิจัยมีสิทธิ์ถอนตัวออกจากโครงการวิจัยเมื่อใดก็ได้ โดยไม่ต้องแจ้งให้ทราบล่วงหน้า และการไม่เข้าร่วมการวิจัยหรือถอนตัวออกจากโครงการวิจัยนี้จะไม่มีผลกระทบต่อการบริการและการรักษาที่สมควรจะได้รับประการใด

โครงการวิจัยนี้ได้รับความเห็นชอบจากคณะกรรมการจริยธรรมการวิจัยในมนุษย์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล เลขที่ Si 586/2008

APPENDIX B
INFORMED CONSENT FORM

หนังสือยินยอมโดยรับการบอกกล่าวและเต็มใจ

(Informed Consent Form)

ชื่อโครงการวิจัย การค้นพบกลุ่มประชากรเซลล์และปัจจัยสำคัญที่จำเป็นสำหรับการเกิดขึ้น ของเซลล์ต้นกำเนิดหลอดเลือด

ชื่ออาสาสมัคร.....อายุ.....ปี

คำยินยอมของอาสาสมัคร

ก่อนจะลงนามในหนังสือยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจาการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว ซึ่งผู้วิจัยได้ตอบคำถามต่างๆ ที่ข้าพเจ้สงสัยด้วยความเต็มใจ ไม่ปิดบัง ซ่อนเร้น จนข้าพเจ้าพอใจ และเข้าร่วมโครงการนี้โดยสมัครใจ

ข้าพเจ้ามีสิทธิ์ที่จะบอกเลิกการเข้าร่วมการวิจัยนี้เมื่อใดก้ได้ ถ้าข้าพเจ้าปรารถนาโดยไม่เสียสิทธิในการรักษาพยาบาลที่จะเกิดขึ้นตามมาในโอกาสต่อไป

ผู้วิจัยรับรองว่า จะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็ผลการวิจัยโดยรวม (หรือข้าพเจ้าอนุญาตให้ผู้วิจัยเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องได้ตามที่ผู้วิจัยเห็นสมควร) การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ ที่มีสาเหตุจากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษา พยาบาลโดยไม่คิดมูลค่าเพิ่ม และจะได้รับการชดเชย ตลอดจนเงินทดแทนความพิการที่อาจเกิดขึ้นและตามความเหมาะสมดังกล่าว

ข้าพเจ้ายินยอมให้ผู้กำกับดูแลการวิจัย ผู้ตรวจสอบ คณะกรรมการจริยธรรมการวิจัยในมนุษย์ และคณะกรรมการที่เกี่ยวข้อง สามารถเข้าไปตรวจสอบบันทึกข้อมูลทางการแพทย์ของข้าพเจ้า เพื่อเป็นการยืนยันถึงขั้นตอนโครงการวิจัยทางคลินิกโดยไม่ล่วงละเมิดเอกสิทธิ์ ในการปิดบังข้อมูลของการสมัครตามกรอบที่กฎหมายและกฎระเบียบได้อนุญาตไว้

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว และข้าพเจ้าจึงลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

ลงนาม.....พยาน

(.....)

ลงนาม.....พยาน

(.....)

คำอธิบายของแพทย์หรือคณะผู้วิจัย

ข้าพเจ้าได้อธิบายรายละเอียดของโครงการ ตลอดจนประโยชน์และข้อเสี่ยงที่อาจจะเกิดขึ้นแก่ผู้ยินยอมตนให้ทำวิจัยทราบแล้วอย่างชัดเจนโดยไม่มีสิ่งใดปิดบังซ่อนเร้น

ลงนาม.....(แพทย์หรือคณะผู้วิจัย

(.....)

APPENDIX C

DOCUMENT FROM HUMAN RESEARCH ETHICS COMMITTEE

2 PRANNOK Rd. BANGKOKNOI
BANGKOK 10700

Tel. (662) 4196405-6
FAX (662) 4196405

MAHIDOL UNIVERSITY
Since 1888
Siriraj Ethics Committee

Certificate of Approval COA no.Si 586/2008

Protocol Title : A study of endothelial progenitor cells in normal healthy subjects and diabetes.

SiEC number : 550/2551(EC1)

Principal Investigator/Affiliation: Prof. Surapol Issaragrisil, M.D. / Department of Medicine
Faculty of Medicine Siriraj Hospital, Mahidol University

Research site : Faculty of Medicine Siriraj Hospital

Approval includes :

1. EC Submission Form
2. Participant Information Sheet
3. Informed Consent Form
4. Executive Summary

Approval date : October 30, 2008
Expired date : October 29, 2009

This is to certify that Siriraj Ethics Committee is in full Compliance with International Guidelines For Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Prof. Jariya Lertakyamanee, M.D.
Chairperson

November 3, 2008
date

(Clin. Prof. Teerawat Kulthanan, M.D.)
Dean of Faculty of Medicine Siriraj Hospital

November 4,2008
date

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APPENDIX D
REAGENTS AND INSTRUMENTS

Reagents

10 mM EDTA	
2.5% Trypsin-EDTA	(GibcoBRL, UK)
FAC lysing solution	(BD Biosciences, USA)
Fetal bovine serum	(Lonza, USA)
Fibronectin	(Sigma Aldrich, USA)
Ficoll-Hypaque solution	(GE Helthcare Bio-science AB, Sweden)
Heparin	(Leo, Thailand)
Human recombinant angiogenin	(R&D System, USA)
IsoPrep	(Robbins Sciencetific Corporation, USA)
KH_2PO_4	(Sigma Aldrich, USA)
NaCl	(Sigma Aldrich, USA)
Na_2EDTA	(Sigma Aldrich, USA)
Na_2HPO_4	(Sigma Aldrich, USA)
Paraformaldehyde	(Fisher Scientific Company, USA)
Pennicillin/Streptomycin	(GibcoBRL, UK)
Tryphan blue	(GibcoBRL, UK)

Monoclonal antibody

FITC-conjugated anti-mouse IgG1 antibody	(BD bioscience, USA)
FITC-conjugated anti-human CD146 antibody	(BD bioscience, USA)
FITC-conjugated anti-human vWF antibody	(AbDserotec, UK)
FITC-conjugated anti-human CD14	(BD bioscience, USA)
FITC-conjugated anti-human CD34	(BD bioscience, USA)
PE-conjugated anti-mouse IgG2a antibody	(BD bioscience, USA)
PE-conjugated anti-human CD34 antibody	(BD bioscience, USA)
PE-conjugated anti-human VEGFR2 antibody	(R&D System, USA)
PE-conjugated anti-human CD14	(Biolegend, USA)

Neutralizing polyclonal antibody

Goat anti- human angiogenin (R&D system, USA)

Media

Endothelial basal media-2 (EBM-2)	(Clonetics; Walkersville, USA)
Endothelial growth media (EGM-2)	(Clonetics; Walkersville, USA)

Instrumentation

24 well cell culture plate	(Corning Incorporation, USA)
50 pH meter	(Bacman, USA)
6 well cell culture plate	(Corning Incorporation, USA)
90 mm cell culture Disc	(Corning Incorporation, USA)
Allega™ 6R centrifuge	(Bacman, USA)
BD FACScalibur™	(Becton Dickinson, USA)
CellQuest® solfware	(Becton Dickinson, USA)
CO_2 incubator	(Sunyo. Japan)
Inverted microscopy CKX41	(Olympus, Japan)
Light microscopy CX31	(Olympus, Japan)
Model J-6B centrifuge	(Bacman, USA)
Sunyo Biomedical Freezer	(Sanyo. Japan)
Transwell insert products	(Corning Incorporation, USA)
Water bath	(Memment, USA)

Kit

Human cytokine antibody array kit 3	(Raybiotech.Inc, USA)
Human CD34 selection kit	(Stem cell Technologies, USA)
FITC selection kit	(Stem cell Technologies, USA)
PE selection kit	(Stem cell Technologies, USA)

Preparation of Reagents

10X Phosphate buffer saline (PBS)

NaCl	38.25 g.	
Na_2HPO_4	4.97 g.	
KH_2PO_4	2.04 g.	
Distilled water to	500 ml	Adjust pH 7.4

The solution was steriled by autoclaving for 15 min at 121 °C, 15lb/square inch and stored at 4 °C.

1X Phosphate buffer saline (1X PBS)

10X PBS	50 ml.
Distilled water to	500 ml.

The solution was steriled by autoclaving for 15 min at 121 °C, 15lb/square inch and stored at 4 °C.

10 mM EDTA

Na_2EDTA	1.861 g.
Distilled water to	500 ml.

The solution was steriled by autoclaving for 15 min at 121 °C, 15lb/square inch and stored at 4 °C.

0.5% Trypsin/EDTA

5.0% trypsin	10 ml
10 mM EDTA	10 ml
PBS	80 ml

The solution was mixed using heater and stored at 4 °C.

1% paraformaldehyde

Paraformaldehyde	1 g
PBS	100 ml

The solution was mixed on heater and stored at 4 °C.

1X Penicillin/Streptomycin

10X Penicillin/Streptomycin	5 ml
1X PBS	45 ml

The solution was mixed and stored at 4 °C.

Endothelial Growth Medium-2

Endothelial basal medium	500 ml
Fetal bovine serum	10 ml
Hydrocortisone	0.2 ml
Human fibroblast growth factor	2 ml
Vascular endothelial growth factor	2 ml
Recomb. Long R insulin-like growth factor-1	0.5 ml
Ascorbic acid	0.5 ml
Human epidermal growth factor	0.5 ml
Gentamycin sulfate amphotericin B	0.5 ml
Heparin	0.5 ml

Antibody Array Protocol

1. Place the membrane into the provided eight-well tray ("-" means the antibody printed side).

2. Add 2 ml 1X Blocking buffer and incubate at room temperature for 30 min to block membranes. Make sure there are no bubbles between the membranes.

3. Decant Blocking buffer from each container, and incubate membranes with 1ml of condition media which comes from $CD14^+$ or $CD14^-$ cell culture alone at RT for 2 h.

4. Decant the condition media from the container, and wash 3 times with 2 ml of 1X Wash buffer I at RT with shaking (5 min per wash).

5. Wash 2 times with 2 ml of 1X Wash buffer II at RT with shaking (5 min per wash).

6. Prepare working solution for primary antibody. Add 100 µl of 1X blocking buffer to the biotin-conjugated anti-cytokine tube. Mix gently and transfer all mixture to a tube containing 2 ml of 1X blocking buffer. Adjust volume to 2 ml. (one mixture can be used for 2 membrane)

7. Add 1 ml of diluted biotin-conjugated antibodies to the membrane. Incubate at RT for 1-2 h with shaking.

8. Repeated steps 4 and 5 1 time.

9. Prepare 1,000 fold diluted HRP conjugated streptavidin by add 2µl of HRP-conjugated streptavidin to 1998 µl of 1X Blocking buffer

10. Add 2 ml of 1,000 fold diluted HRP-conjugated streptavidin to the membrane. Incubate at RT for 2 h with shaking.

11. Repeated steps 4 and 5 1 time.

12. Prepare Detection buffer by adding 250 µl of 1X Detection buffer C and 250 µl of 1X Detection buffer D and mix together.

13. Drain off excess wash buffer by holding the membrane vertically with forceps. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Pipette the mixed Detection buffer on to the membrane and cover with another clean plastic sheet.

14. Incubated at RT about 2 min.

15. Drain off any excess detection reagent by holding the membrane vertically with forceps and touching the edge against a tissue.

16. Gently place the membrane, protein side up, on a piece of plastic sheet ("-" mark is on the protein side top left corner). Cover another piece of plastic sheet on the array. Gently smooth out any air bubble. Avoid using pressure on the membrane.

17. Expose the array to x-ray film for 30 sec, 1 min, 2min and 5 min.

18. Save membranes in –20 °C to –80 °C for future references.

19. Read the density of cytokine by densitometry.

20. Interpret the result.

Human Cytokine Antibody Array Map

	a	b	c	d	e	f	g	h	I	j	k	l
1	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1 β
2	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1 β
3	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40p70	IL-13	IL-15	IFN-γ
4	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40p70	IL-13	IL-15	IFN-γ
5	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 δ	RANTES	SCF	SDF-1	TARC	TGF- β1
6	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 δ	RANTES	SCF	SDF-1	TARC	TGF- β1
7	TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos
8	TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos

CD34 Selection Protocol

1. Resuspended 10^8 cells of MNCs in Falcon® 5 ml Polystyrene tube containing 1 ml of 2% FBS PBS/EDTA.

2. Add *EasySep*® Positive Selection Cocktail at 100 µl/ml cells. Mix well and incubate at RT for 15 minutes.

3. Mix *EasySep*® Magnetic Nanoparticles to ensure that they are in a uniform suspension by vigorously pipetting up and down more than 5 times.

4. Add the nano-particles at 50 µl. Mix well and incubate at RT for 10 min.

5. Bring the cell suspension to a total volume of 2.5 ml by adding 2% FBS PBS/EDTA. Mix the cells in the tube by gently pipetting up and down 2-3 times. Place the tube (without cap) into the magnet. Tube was incubated in magnetic field for 5 min.

6. Pick up the magnet in one continuous motion for pouring off the supernatant fraction (CD34 negative cell). The magnetically labeled cells (CD34 positive cell) will remain inside the tube, held by the magnetic field of the EasySep® Magnet.

7. Remove the tube from the magnet and add 2.5 ml % FBS PBS/EDTA. Mix the cell suspension by gently pipetting up and down 2-3 times. Place the tube back in the magnet and tube was incubated in magnetic flied for 5 minutes.

8. Repeat Steps 6 and 7 four times.

9. Remove tube from magnet and resuspend cells in EGM-2. The positively and negatively selected cells are now ready for use.

FITC Selection Protocol

1. Resuspended 10^8 cells of MNCs in Falcon® 5 ml Polystyrene tube containing 1 ml of 2% FBS PBS/EDTA.

2. Add species-specific FcR blocking antibody at 100 µl for human cells

3. Add FITC-conjugated antibody 100 µl. Mix well and incubate at RT for 15 min.

4. Add *EasySep*® FITC Selection Cocktail at 100 µl. Mix well and incubate at RT for 15 min.

5. Mix *EasySep*® Magnetic Nanoparticles to ensure that they are in a uniform suspension by vigorously pipetting up and down more than 5 times. 4. Add the nano-particles at 50 µl. Mix well and incubate at RT for 10 min.

6. Bring the cell suspension to a total volume of 2.5 ml by adding 2% FBS PBS/EDTA. Mix the cells in the tube by gently pipetting up and down 2-3 times. Place the tube (without cap) into the magnet. Tube was incubated in magnetic flied for 5 min.

7. Pick up the magnet in one continuous motion for pouring off the supernatant fraction (CD14 negative cell). The magnetically labeled cells (CD14 positive cell) will remain inside the tube, held by the magnetic field of the *EasySep*® Magnet.

8. Remove the tube from the magnet and add 2.5 ml % FBS PBS/EDTA. Mix the cell suspension by gently pipetting up and down 2-3 times. Place the tube back in the magnet and tube was incubated in magnetic flied for 5 min.

9. Repeat Steps 7 and 8 four times.

10. Remove tube from magnet and resuspend cells in EGM-2. The positively and negatively selected cells are now ready for use.

PE Selection Protocol

1. Resuspended 10^8 cells of MNCs in Falcon® 5 ml Polystyrene tube containing 1 ml of 2% FBS PBS/EDTA.

2. Add species-specific FcR blocking antibody at 100 µl for human cells

3. Add FITC-conjugated antibody 100 µl. Mix well and incubate at RT for 15 min.

4. Add *EasySep*® PE Selection Cocktail at 100 µl. Mix well and incubate at RT for 15 minutes.

5. Mix EasySep® Magnetic Nanoparticles to ensure that they are in a uniform suspension by vigorously pipetting up and down more than 5 times. 4. Add the nano-particles at 50 µl. Mix well and incubate at RT for 10 min.

6. Bring the cell suspension to a total volume of 2.5 ml by adding 2% FBS PBS/EDTA. Mix the cells in the tube by gently pipetting up and down 2-3 times. Place the tube (without cap) into the magnet. Tube was incubated in magnetic flied for 5 minutes.

7. Pick up the magnet in one continuous motion for pouring off the supernatant fraction (CD14 negative cell). The magnetically labeled cells (CD14 positive cell) will remain inside the tube, held by the magnetic field of the EasySep® Magnet.

8. Remove the tube from the magnet and add 2.5 ml of 2% FBS PBS/EDTA. Mix the cell suspension by gently pipetting up and down 2-3 times. Place the tube back in the magnet and tube was incubated in magnetic flied for 5 min.

9. Repeat steps 7 and 8 four times.

10. Remove tube from magnet and resuspend cells in EGM-2. The positively and negatively selected cells are now ready for use.

BIOGRAPHY

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