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Background and significance: Echogenic liposomes can be used as drug and cell delivery vehicles that reduce atheroma progression. Vascular endothelial growth factor (VEGF) is a signal protein that induces vasculogenesis and angiogenesis. VEGF functionally induces migration and proliferation of endothelial cells and increases intracellular vascular permeability. VEGF activates angiogenic transduction factors through VEGF tyrosine kinase domains in high-affinity receptors of endothelial cells. Bevacizumab is a humanized monoclonal antibody specific for VEGF-A which was developed as an anti-tumor agent. Often, anti-VEGF agents result in regression of existing microvessels, inhibiting tumor growth and possibly causing tumor shrinkage with time. During atheroma progression neovasculation in the arterial adventitia is mediated by VEGF. Therefore, bevacizumab may be effective in inhibiting atheroma progression. Stem cells show an ability to inhibit atheroma progression. We have previously demonstrated that monocyte derived CD-34+ stem cells that can be delivered to atheroma by bifunctional-ELIP (BF-ELIP) targeted to Intercellular Adhesion Molecule-1 (ICAM-1) and CD-34. Adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) are expressed by endothelial cells under inflammatory conditions. Ultrasound enhanced liposomal targeting provides a method for stem cell delivery into atheroma and encapsulated drug release. This project is designed to examine the ability of echogenic liposomes to deliver bevacizumab and stem cells to inhibit atheroma progression and neovasculation with and without ultrasound in vitro and optimize the ultrasound parameters for delivery of bevacizumab and stem cells to atheroma.

Hypotheses: Previous studies showed that endothelial cell VEGF expression may relate to atherosclerosis progression and atheroma formation in the cardiovascular system. Bevacizumab-loaded ELIP will inhibit endothelial cell VEGF expression in vitro. Bevacizumab activity can be enhanced by pulsed Doppler ultrasound treatment of BEV-ELIP. I will also test the hypothesis that the transwell culture system can serve as an in vitro model for study of US-enhanced targeted delivery of stem cells to atheroma. Monocyte preparations will serve as a source of CD34+ stem cells.

Specific Aims: Induce VEGF expression using PKA and PKC activation factors to endothelial cell cultures and use western blot and ELISA techniques to detect the expressed VEGF.

- Characterize the relationship between endothelial cell proliferation and VEGF expression to develop a specific EC culture based system to demonstrate BEV-ELIP activity as an anti-VEGF agent. Design a cell-based assay for in vitro assessment of ultrasound-enhanced bevacizumab release from echogenic liposomes.
- Demonstrate ultrasound delivery enhancement of stem cells by applying different types of liposomes on transwell EC culture using fluorescently labeled monocytes and detect the effect on migration and attachment rate of these echogenic liposomes with and without ultrasound in vitro.

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I. INTRODUCTION

Cardiovascular diseases are wide spread diseases that cause the highest rates of death among humans. Atherosclerosis, also known as arteriosclerotic vascular disease (ASVD), is a chronic arterial disease that is considered one of the most common cardiovascular diseases (Thomas et al., 1988). Atherosclerosis is a chronic disease that is caused by various cellular and immunological factors, which lead to fat and cholesterol accumulation on the arterial wall. The fat and cholesterol accumulated in the arterial wall obstruct the blood flow and cause loses of arterial flexibility.

Atherosclerosis is a term derived from two combined Greek words: “athera” (gruel) and “sclerosis” (stiffness). Historically, atherosclerosis was first described by Leonardo da Vinci (1452-1519), who conducted an autopsy on an elderly man to ascertain the cause of sudden death. He stated that “vessels in the elderly restrict the transit of blood through thickening of the tunics”(Davies and Eollman, 1996) "In 1768, a British doctor, William Heberden, was the first doctor who described Angina Pectoris symptoms. He indicated that patients afflicted with it are seized, but when they walk they have the most painful and disagreeable sensation in the breast, which seems as if it will extinguish their life if it were to increase or continue, but the moment they stand still, all uneasiness vanishes" (Khan and Metha, 2002).

In 2006, more than 81 million Americans suffered from cardiovascular of diseases, atherosclerosis is most prevalent (Lloyd-Jones, 2010). In the United States

atherosclerosis have higher mortality rates than cancer. The latest statistics indicate that men have a higher chance of developing atherosclerosis than women in the United States (Virmani et al, 2000). Untreated atherosclerosis can ultimately lead to heart attacks often fatal.

A distinguishing feature of atherosclerosis is the stiffness and inflexibility of the damaged artery due to impairment of the internal elastic lamina layer of the arterial wall. The internal lamina which responds to blood pressure changes is responsible for the arterial elasticity. The internal elastic lamina starts degenerating during middle ages. However, there are many other factors that degrade the elastic lamina such as life style, diet, genetics and stress. The arterial endothelial layer contributes to many physiological and pathological processes. It is involved in the regulation of vascular tone by producing a number of vasodilator and vasoconstrictor substances that control the circulation pressure (Furchgott and Zawadski, 1980). The factors that contribute to the development of atherosclerosis are called risk factors. These factors contribute in initiation development of atherosclerosis via various cellular and molecular mechanisms. (Blankenhorn and Hodis, 1993).

Atherosclerosis is generated because of the deposition of low density lipoprotein (LDL) in the arterial wall. In atherosclerotic conditions, LDL accumulates in the sub-endothelial matrix of the arteries (Lusis, 2000). High circulation levels of LDL increase atheroma progression by optimizing the retention of LDL at the sites of lesion formation. The atherosclerotic lesion can be described as a series of changes in the histological and histochemical structure of the prone

region, along with the cellular matrix changes (Stary et al., 1992). This pathologic process is mediated by enzymes such as sphingomyelinase. Atheroma is defined as a stage after lesion formation in the affected region, in which fat and cholesterol are accumulated on the arterial wall causing arterial swelling and stiffness (Aldons and Lusis 2000).

Atherosclerosis is initiated due to high activities of risk factors that cause endothelial dysfunction. Later, atheroma is generated by the development of atherosclerotic lesions. Lesion formation is a complicated process which many factors involves such as immunological, physiological, and cellular factors that suppress the elastic lamina of the arterial wall. Atheroma is developed by the high activity of the inflammation and stress signals. Atheroma progression causes many symptoms such as hypertension and myocardial morphological changes (Assmann et al., 2002).

High levels of LDL and other risk factors form lesions by activating cellular and molecular factors that induce adhesion molecule expression and cellular migration (Luscher and Barton 1997; Kinlay et al., 2001). LDL carries endogenous cholesterol to peripheral tissues. LDL's major components are cholesterol and cholesteryl esters. LDL is synthesized in response to the cellular demand of chlesterol. It is formed in liver, transported and deposited in peripheral tissues (Assmann et al., 2002).

Apo-B 100 is the identical protein particle on the surface of LDL, which is bound to LDL receptors on cell membranes for further digesting and restoring. The

LDL plays a crucial role in atheroma progression due to the involvement of macrophages which respond to LDL accumulation in the plaque area (Kumar et al., 2011). The digestions of LDL by macrophages cause accumulation the LDL inside these cells and transform them to foam cells in the atheroma. Foam cell accumulation builds the plaques lipid core plaque (Wiley, 2013).

Macrophages play a central role in the atherogenic process as modulators of both lipid metabolism and the immune response (Kruth et al., 2002). Macrophages accumulate cholesterol by a specific process called fluid phase endocytosis. This process produces foam cells that depend not only on the modified LDL, but also on macrophage activity (Kruth, 2002).

Foam cells are lipid-loaded macrophages which can be found in blood vessel walls filled with fatty material. Macrophages are produced by monocytes differentiation (Wiley, 2013). In atherosclerosis, foam cells are formed when the macrophages target the fatty deposit location on the blood vessel wall (Manning, 2013). Macrophages function to digest and destroy the lipid accumulated on the arterial wall. After targeting the fatty materials, macrophages become filled with lipids giving them a "foamy" appearance (Kumar et al., 2010). The lipids and lipoproteins aggregated within the tunica intima layer of arteries are oxidized by macrophages or endothelial cell free oxygen radicals. The macrophages digest the oxidized LDL by endocytosis via scavenger receptors, which are LDL-specific. The oxidized LDL accumulated inside the macrophages and other phagocytes cause foam cell formation (Berkerman, 2013). Foam cells formation represents a defense

mechanism to exclude cholesterol from the arterial wall. Foam cells which are small cells that lack characteristic cell-surface markers do not cause clinical dysfunction directly, but contribute to progression by creating the necrotic core progression atheroma. If the fibrous cap of the atheroma eroded by the necrotic core, the lumen of vessel ruptures, forming thrombotic embolus occluding the artery.

Endothelial function can be characterized as a balance between vascular cell protection mechanisms and risk factors. In response to the physiological condition, a vascular endothelial cell has the potential to be as an anti-thrombogenic. Anti-thrombogenic endothelial cells produce pro-inflammatory cytokines or extracellular matrix as defensive tools. Endothelial dysfunction results from cell damage produced by inflammatory response. The cellular dysfunction will cause an imbalance between the protective endothelial activities and the anticoagulant properties that become pro-thrombotic.

Nitric oxide plays a significant role in managing the dysfunctional progression. Endothelium dysfunction increases when nitric oxide levels drop. Reducing nitric oxide levels will increase the oxidative stress, which is an important promoter of the inflammatory process (Bonetti et al., 2003; Sela et al., 2002). As the endothelial dysfunction process is initiated, it may be considered an early attribute of atherosclerosis (Luscher, 1997).

Risk factors

Risk factors can be defined as factors that contribute to initiate and develop atherosclerosis and the associated symptoms, which lead to often fatal heart attacks and chronic strokes. These factors are hypercholesterolemia, high plasma LDL levels, hypertension, cigarette smoking (Nicotine), age and gender (higher in male than female), diabetes mellitus (high plasma glucose), high-fat diet, physical inactivity, family history of premature coronary heart disease (Cayatte et al., 1994).

In the presence of cardiovascular risk factors, not all individuals develop an arterial thrombotic process at the same level, but several additional factors can be involved in coronary events. These additional factors can be, for instance, levels and function of coagulation factors, local blood flow conditions (shear stress), circulating progenitor cells and genetic factors. Inappropriate generation of thrombin may lead to vascular occlusion (Mann et al., 2003).

Under conditions of atheroma, coagulant and anti-coagulant forces together with pro / anti fibrinolytic substances to determine a delicate balance. Hereditary or 4acquired defects of blood clotting factors, impairment of the anti-coagulant system or fibrinolytic mechanism, and inflammation can promote plasmatic and local hyper coagulation state. Local thrombin generation is not only resulted in a mixed of fibrin platelets clot, but also thrombin itself has pro-inflammatory activity. Plaque in unstable angina possesses elevated levels of tissue factors that can be released during inflammation, precipitating acute clinical syndromes (Matthay, 2001).

During early stages of atherosclerotic lesion formation, leukocytes adherent to the endothelium at particular sites in the arterial wall *(*Gerrity, 1981*;* Faggiotto et al., 1984; Davies et al., 1988). Under inflammatory conditions, the lymphocytes migrate across the endothelial layer and accumulate in the sub-endothelial space, where some of the macrophages ingest LDL and form foam cells. However, atherosclerosis appears to be a specialized inflammatory response in which leukocyte recruitment occurs in lesion-prone areas of the arterial wall and causes lymphocyte accumulation in the sub-endothelial matrix. This recruitment of specialized leukocytes continues for a period of time as long as there is hypercholesterolemia. During that time, the vascular endothelial layer remains intact and active in recruitment of leukocytes by expression of specific leukocyte adhesion molecules.

As a result of leukocyte recruitment, a special adhesion molecule is activated to regulate different stages of monocyte and lymphocyte migration to inflammatory sites in a long term process (Springer and Cybulsky, 1996).

Vascular cell adhesion molecule -1 (VCAM-1) and Intercellular adhesion molecule-1 (ICAM-1) are membrane glycoprotein that mediate the recruitment of leukocytes in a given pathological condition. These proteins are highly expressed by inflammatory endothelial cells. Tumor necrosis factor alpha (TNF-α) is another risk factor that is produced by the T-cells, and it activates ICAM-1 and VCAM-1 expression in endothelial cells (Myers et al., 1992; Henseleit et al., 1994). TNF-α along with interleukin-1 (IL-1) are inflammatory factors that trigger both ICAM-1 and VCAM-1 expressions (Wong and Dorovini-Zis, 1995).

VCAM-1 is an immunoglobulin-like adhesion molecule located on vascular endothelial cells surface as membrane marker (Osborn et al., 1989). VCAM-1 binds to alpha and beta subunits of integrins and is involved in lymphocyte and monocyte migration. At the beginning of lesion formation, VCAM-1 is highly expressed in atherogenesis as a stress response molecule (Chen et al., 1999).

Nitric Oxide is another remarkable factor that affects atherosclerosis formation, which is activated by stress signals. Although NO has a variety of functions, its most important one is to maintain vascular homeostasis. NO is a highly diffusible inorganic radical gas with a very short half-life 3 - 6 seconds that is synthesized by the conversion of L-arginine to L-citrulline, mediated by enzymatic activity (nitric oxide synthase) (Moncada et al., 1991). NO mediates many biological activities such as monocyte and leukocyte adhesion and migration through the endothelium *(*De Caterina et al., 1995; Davenpeck et al., 1994).

NO also increases endothelial permeability and reduces vascular pressure, which causes a reduction of lipoprotein flow in the vessels (Hinder et al., 1997).Finally, NO is involved in vascular smooth muscle cell proliferation. It causes an inhibition in smooth muscle cell proliferation and also inhibits monocyte migration (Draijer et al., 1995; Cardona-Sanclemente and Born 1995). Reduced endothelial nitric oxide synthase (eNOS)-derived NO bioactivity is a critical step of atherogenesis (Palmer et al., 1987). Previous studies showed that the deficient of NO as vasodilator in hypercholesterolemia conditions is contributed in atherosclerosis initiation (Cooke et al., 1992). Endothelial cell types that are responsible for NO

production can be dysfunctional in atherosclerosis. These cells serve as an anti-inflammatory factor under normal conditions (Moroi et al., 1998). Nitric oxide synthesis is induced by the Ca^{2+}/calmodulin-dependent activation pathway that is mediated by NO synthase.

Nitric oxide is also produced by vascular smooth muscles cells that surround the endothelial cells in the artery. The action of NO not only decreases the muscular contraction of smooth muscle cells, but also activates guanylyl cyclase which increases cellular permeability (Furchgott,F.R. Vanhoutte, 1989). NO synthesis is activated by a high level of cAMP that associates with cellular permeability reduction (Bredt et al., 1990; Lowenstein and Snyder, 1992). Thus Nitric Oxide plays central role in cellular permeability regulation. All the mentioned afore risk factors altogether participate at atherosclerosis formation and atheroma progression in different levels and through different mechanisms.

Adhesion Molecules in Atherosclerosis:

The immune system plays a crucial role in proatherosclerosis levels and atherosclerosis progression. The molecular mechanism of atherosclerosis can be described as an inflammatory response by the immune system to the risk factors. The excessive immune activities contribute to plaque formation, necrosis, and lipid accumulation later. Different cell types are involved in this immune process by expressing various extracellular matrixes and membrane molecules. These cells

include smooth muscle cells, T-cells, endothelial cells, and platelets (Binder et al., 2002; Hansson et al., 2002).

The most important anti-inflammatory molecules associated with atherosclerosis initiation and developments are ICAM-1, P-Selectin and VCAM-1. ICAM-1 and P-Selectin are both expressed by inflammatory endothelial cells and they mediate neutrophil recruitment and adherence (Johnson-Tidey et al., 1994). Smooth muscle cells also have the potential to express both ICAM-1 and VCAM-1 in the arterial wall (Jang et al., 1994). VCAM-1 is not directly involved in atherosclerotic lesion formation, but it significantly contributes to lesion development and lipid core formation later (Risau and Flamme, 1995).

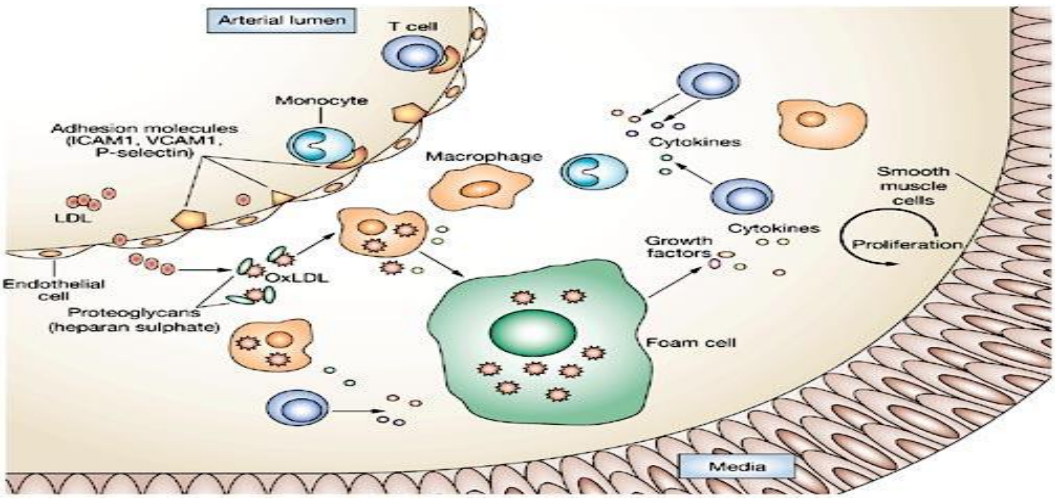


Figure 1. **Adhesion Molecules Functions.** Adhesion molecules roles in atherosclerotic lesion formation and cell that expression of molecules (Sherer and Shoenfeld , 2006. License No. 3199560393174 LWW publication of NCPR volume 2, No.2).

Stem Cells and Regenerative Medicine

Stem cells undifferentiated or ‘blank’ cells that are capable of developing many different other cells, which are later specialized to perform specific physiological functions in organs and tissues. The majorities of the human somatic cells are differentiated and specialized to perform defined functions in tissue, which provides organs characteristic functions. Differentiation consists of cellular developmental processes resulting from undifferentiated cell division in response to appropriate conditions and signals. Furthermore, the differentiation process provides the differentiated cell with a particular function and morphological appearance (Little et al., 2006). The most significant properties of stem cells are a potential for self-renewal, unspecialized cell types, and the differentiation to other types of cells that give a specific tissue function (Rafii and Lyden 2003).

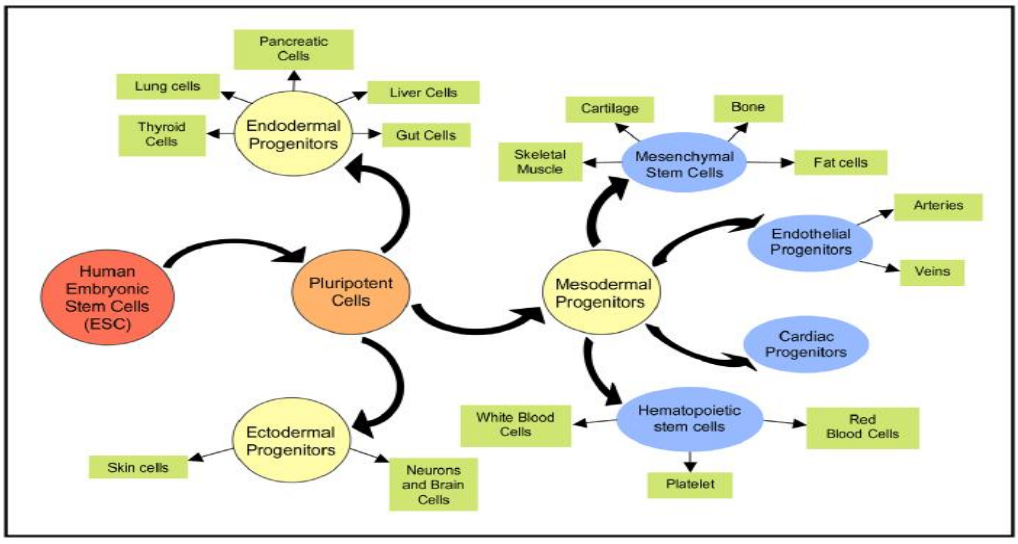


Figure 2. **Stem Cell Source.** Cells that are derived from differentiation of these cells.

Stem cells can be classified based on the stages of organismal developmental. Pluripotent stem cells that exist in embryonic stages have the potential to differentiate to all cells types. Furthermore, they can form the progenitor cells that are considered a source for specific types of differentiated cells in tissue such as brain, bone, heart, and skin.

Multipotent stem cells are type of cells that exist in adults, as well as in umbilical cords, and have less ability or strict potential to differentiate to a specific type of cells. For instance, Multipotent stem cells found in the bone marrow that have the potential to differentiate to red blood cells, platelets, or white blood cells, but not into skin or brain cells (O Choi, 2006).

Stem cells hold a lot of promise in the field of regenerative medicine providing a therapeutic approach for tissue repair and functional regeneration, in many diseases and disorders. Stem cells offer a new look at old problems and diseases such as burns, neurological and cardiovascular disorders, and diabetes. Although this field is considered recent, the impact of new discoveries can dramatically change clinical research and therapeutic approaches (Matthews, 2009). However, stem cells in regenerative medicine face many limitations and disadvantages, which can be summarized by:

1. Difficulty to obtain efficient and reproducible derivation stem cells that has the ability to divide indefinitely and give rise to the specific progenitor cells type without production of cancer cells (Lee et al., 2012).

2. Ethical issues. Stem cells now face a many limitations, especially embryonic stem cell research due to ethical codes and regulation policies that are considered a big dilemma for stem cell researches progression especially embryonic stem cells research (Borstlap et al., 2010).

3. Immunological rejection. In regenerative medicine research, immune resistance for stem cell grafting could be another obstacle of stem cell researches.

Although recent scientific researches resolved some of these problems mentioned above, some of these limitations are still unsolvable because of the current policies and regulations that ban embryonic stem cells researches (Kishigami et al., 2006).

In order to progress in stem cell research and avoid the strict policies, new methods have been invented with the use of somatic cell and nuclear transfers (cells cloning technology) to generate specific tissues that could only be derived previously from embryonic stem cells. This method has potential to overcome stem cell research challenges (Kishigami et al., 2006).

Stem Cells in Atherosclerosis

Atherosclerotic pathogenesis can be defined as endothelial dysfunction and loss of vascular tissue function. This damage happens due to risk factors and inflammatory activity (Ross., 1999, Blann et al., 1998). Preliminary studies showed that transplanted stem cells give rise to neointima smooth muscle cells and functional improvement in recipient animal vessels (Tsai et al., 2009; Hristov et al., 2008).

Atheroma progression depends on endothelial inflammatory mechanisms that cause aggregation of LDL, lipids, and macrophages in the sub-endothelial layer (Bosco et al., 2008). Many approaches to cardiovascular therapy such as revascularization techniques, bypass and graft surgery, and coronary angioplasty, are of limited benefits in the long term (Seiler, 2003; Barner, 2008).

Stem cells and progenitor cells have unique abilities for self-renewal and differentiation capacity to various types of cells, providing methods for tissue regeneration and restoral of function in many diseased organs and tissues disorders (Rafii and Lyden, 2003). Since atherosclerosis is considered disease of endothelial dysfunction, regenerative cell-based therapy can be an effective approach to ameliorating atherosclerotic pathogenesis. This approach has been supported by data obtained from animal studies. Currently, there is an uncertainty regarding characterization of specific cell types to achieve significant therapeutic results (Dotsenko, 2010).

Stem Cells Types Used in Atherosclerosis

Embryonic stem cells can differentiate and give rise to all cell types in the body, however the application of these cells are often not permissible due to ethical codes and government regulations except for some cases that have been approved by the U.S. Food and Drug Administration (Lui et al., 2009). Adult stromal cell reprogramming methods have recently been reported as a stem cell source for clinical use. Fibroblasts have been commonly used in the reprogramming method to generate induced pluripotent stem cells (IPSC). Generally, multipotent stem cells are

responsible for tissue repair and regeneration under normal conditions (Jaenish and Young, 2008).

Hematopoietic and mesenchymal stem cells can be efficient types of multipotent stem cells that have the ability to regenerate and repair cardiovascular tissues. Hematopoietic stem cells can be obtained from many sources in the body such as bone marrow and umbilical cords (Gallacher et al., 2000). However, mesenchymal stem cells are mainly located in bone marrow. Also, mesenchymal stem cells have the potential to differentiate into adipocytes, osteoblasts, chondrocytes and myoblasts (Chamberlain et al., 2007).

Clinical experiments successfully demonstrated cardiac and vascular regeneration using mesenchymal stem cells (Psaltis et al., 2008). Mesenchymal stem cells have unique advantages in cardiovascular repair, such as ease of recovery, high yield from cultures required for transplantation, low immune resistance to the cells, and a potential to mediate vascular repair for both endothelial and smooth muscle cells damaged by the atherosclerotic process (Toma et al., 2002). Previous studies showed that the tunica media in the arterial wall is the final differentiation site for transplanted mesenchymal stem cells, giving rise to endothelial cells and smooth muscle cells in the injured area (Babaev et al., 1990).

Mesenchymal stem cells also can differentiate to peripheral blood mononuclear cells, which give rise to endothelial progenitor cells under the right conditions in response to appropriate signals. Among the factors controlling endothelial progenitor cell proliferation in vivo are hyperlipidemia, hormone levels

and risk factors. Low number of endothelial progenitor cells associate with atherosclerotic lesion formations. The endothelial progenitor cells number drop in circulation because of risk factors activity predisposing toward atheroma progression (Xu et al., 2003; Werner et al., 2002). Therefore, a most worthwhile approach for atheroma progression inhibition and arterial repair is to provide the circulation with infusion appears to be endothelial progenitor cells (Xu et al., 2003).

Stem Cells Markers

Each cell type has a characteristic surface molecule that gives it special properties and differentiates it from other cell types. Stem cells are considered undifferentiated cells that have a special surface molecule. Stem cells have special surface molecules called clusters of differentiation (CD). Cluster of differentiation is a glycoprotein located on the cell surface (Lie et al., 1999). Cluster of differentiation is considered a trans-membrane protein that is produced as a glycoprotein on the cell membrane and plays a crucial role as signal protein on the cell surface. Also, it acts as adhesion molecules that lead to alternative behavior or changes in the cells function after it is activated by an external signal (Zola et al., 2006).

Cluster of differentiation can provide a feasible method to identify cells populations and cell membrane molecules. There are more than 320 CD molecules. Thus, cells can be classified as positive or negative for particular CD depending on the presence or absence of each CD on the cell surface. CD has a potential of high specific binding to specific monoclonal antibody, since it is considered a surface

antigen. These properties can be used to detect the cells CD population (Tekstra et al., 1996).

Generally, stem cell populations have specific CD molecules on their surfaces that are used as identification cell markers for stem cells populations. CD34 is a special surface molecule that exists on many stem cell type surfaces and is considered an identifying molecule of stem cell populations. CD34 plays an important role as an adhesion of stem cells to stromal cells that lead to further tissue regeneration by stem cells differentiation (Satterthwaite et al., 1992). CD34 molecules existence on the cell surface can provide a practical method isolating those cells by flow cytometery to obtain a pure CD34+ stem cell population that can be used in stem cell transplant therapy (Gulati et al., 2003).

Mesenchymal stem cells and CD34+ endothelial progenitor cells play an important role in arterial injury therapy and it provide a reliable method for progenitor cells numeration (Abedin et al., 2004). Clinical studies showed that early stage atherosclerosis can be detected by decreased levels of CD34 as indication of low vascular stem cells in circulation that essential for arterial repair (Fadini et al., 2006). As afore mentioned, CD34 mediated stem cells adherence to stromal cells, thus CD34 is very important particles that characterize the targeting of stem cells to the desired injured tissues. Strong stem cell adhesion molecule binding to the endothelial cell ligand triggers the differentiation process. Endothelial cells secrete angiogenic growth factors that induce migration of surrounding mature endothelial

cells to the damaged tissue, leading to activation of tissue regeneration and functional restoration process (Urbich et al., 2005).

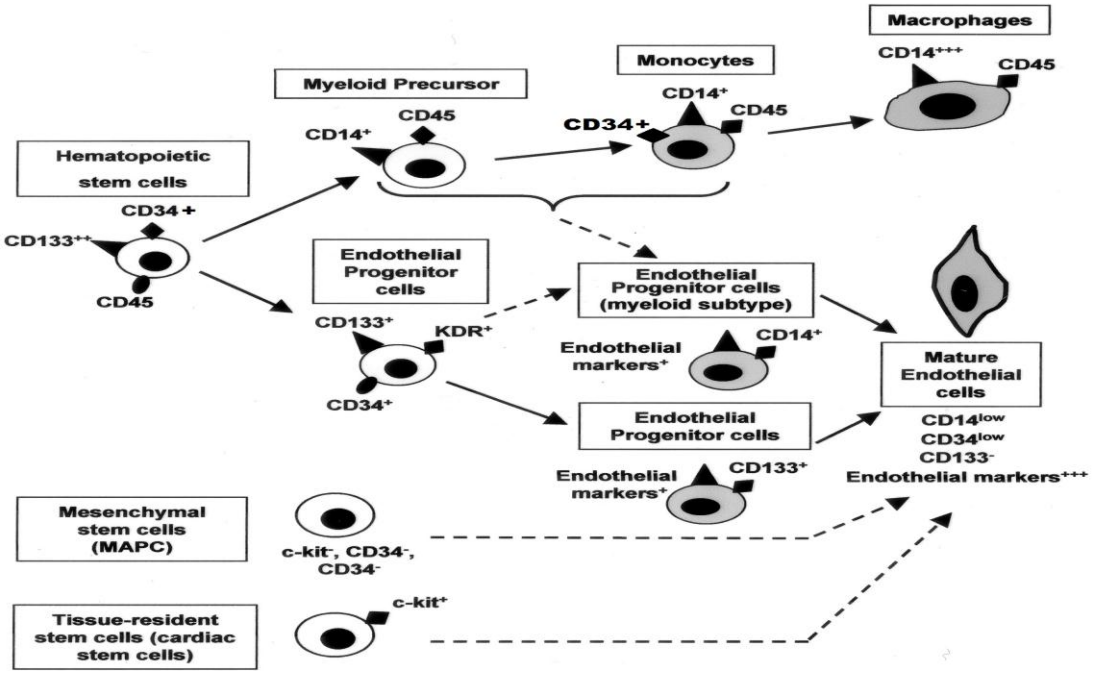


Figure 3. Ste**m Cell Types and Cluster of Differentiation**. Endothelial stem cell sources associated with surface markers and the derivative cells (Urbich and Dimmeler, 2004. License no 3200341076041 LWW publications J Mol Cell Cardiol. 2005;39:733–742).

Therefore, this targeting and adherence of endothelial progenitor cells and mesenchymal stem cells mediated by CD provide methods for arterial repair and endothelial cells regenerations.

Vascular Endothelial Growth Factor

The cardiovascular system begins to develop and become functional in very early embryonic stages (Breier et al., 1992). The initial step is called vasculogenesis, which generally can be described as new blood vessel formation from the differentiation of progenitor cells (Risau and Flamme, 1995). The newly formed blood micro-vessels develop and enlarge by recruiting more endothelial cells to increase the vessels' diameter in a process called "angiogenesis." Angiogenesis is defined as new vessels development to a mature phase from pre- existing micro vessels (Risau, 1997).

A very vital process in body growth and development angiogenesis is responsible for blood supplies for newly grown organs and tissue repair (Folkman, 1995). Although angiogenesis is an essential process of embryonic development under normal conditions, it may have a pathological role. Angiogenesis can be an essential factor in many pathological conditions such as excessive cell proliferation (hyperplasia), retinopathies, muscular degeneration, tumors and psoriasis (Flokman, 1995; Garner, 1994). The initiation signal of angiogenesis is triggered by family signal proteins of collectively called vascular endothelial growth factor (VEGF) which is considered the main inducer of new blood vessel formation and growth (Clark et al., 1998).

VEGF belongs to a family of growth factors that are synthesized as heparin-binding homodimeric glycoproteins. There are many types of VEGF that belong to

the same family and have a similar course of action but they perform in different growth stages (Dvorak et al., 1995).

VEGF family members	Receptors	Functions
VEGF (VEGF-A)	VEGFR-1, VEGFR-2, neuropilin-1	Angiogenesis Vascular maintenance
VEGF-B	VEGFR-1	Angiogenesis (embryonic)
VEGF-C	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-D	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-E (viral factor)	VEGFR-2	Angiogenesis
PlGF	VEGFR-1, neuropilin-1	Angiogenesis Inflammation

Table 1. **VEGF Types and Functions.** Types of VEGF, their functions and their receptors (Ferrara, 2004).

Each VEGF type has various isoforms that appear to react differently with tyrosine kinase receptors. Generally, there are three main types of VEGF receptors: VEGFR1, VEGF2 and VEGFR3, which exist as membrane binding receptors or soluble receptors (Fujita et al., 2008). VEGF isoforms are produced by differential mRNA splicing (Ferrara et al., 1996). VEGF family molecules bind to tyrosine kinase receptors on the cell surface that leads to activation of transphosphorylation pathways. Each VEGF type has a high specificity of binding to a certain receptor, but a common pathway (Holmes et al., 2003).

VEGF type A is the most abundant and important member of this growth factors family. VEGF-A doe not only affects endothelial cells and angiogenesis, but also many other cells functions such as macrophage migration, neurons, cancer cells,

epithelial cells, and inflammatory processes (Leung et al., 2013). VEGF A is activated by many factors that ultimately induce angiogenesis. Previous studies showed that VEGF expression is activated in response to parathyroid and growth hormones (Rashid et al., 2008). Many studies suggested that VEGF expression can be activated via protein kinase pathways by different mechanisms. Angiogenesis activated by norepinephrine is mediated by the PKC signaling pathway that induces VEGF expression. Angiogenesis also can be activated in responce to neurotransmitters via the PKA signaling pathway that leads to activate VEGF expression as well (Fredriksson et al., 2000). Endogenous NO appears to enhance vascular smooth muscle cells VEGF expression (Dulak et al., 2000).

VEGF is over-expressed under hypoxic conditions. During low oxygen conditions, hypoxia-inducible factor 1 (HIF-1) is activated to up regulate the VEGF gene, resulting in a high level of VEGF expression in response to low level of oxygen (Forsythe et al., 1996). HIF-1 plays a crucial role in tumor growth inducing VEGF-A expression, leading to angiogenesis and causing tumor growth providing a well understanding of VEGF-A's pathological role in cancer (Semenza,2003). Also, VEGF receptors VEGFR1 and VEGFR2 are directly affected by hypoxia and seem to be overexpressed under the hypoxic conditions to supply the oxygen needed by activating angiogenesis (Gerber et al., 1997). The central roles of HIF-1 and VEGF in progression are illustrated by the fact that HIF-1 is over expressed by v-src oncogene action leading induction of VEGF mRNA expression (Jiang et al., 1997).

Vascular Endothelial Growth Factor Role in Atherosclerosis

Vascular endothelial growth factor is expressed by many cells types. It was reported that VEGF is produced by smooth muscle cells, macrophages and endothelial cells. VEGF expression level can be varied from normal to pathological conditions. There are many physiological and molecular changes in response to changes VEGF concentrations (Williams et al., 1995; Berse et al., 1992; Namiki et al., 1995).

These cell types, which are important to atheroma progression, share the ability to express VEGF. In atherosclerotic plaques, endothelial cells, macrophages and smooth muscle cells highly express VEGF, along with other growth factors and cytokines that that play a crucial role in atherosclerosis progression. Many studies reported that VEGF expression enhances atherosclerotic plaque progression (Celletti et al., 2001). Recent studies reported that increased neovascularization is associated with atherosclerosis (Heistad and Marcus, 1979). In advanced stages of atherosclerosis increased vasa vasorum can be clearly observed in the arterial adventitia due to the lack of oxygen to the atherosclerotic plaque. Vasa vasorum can be defined as microvessels formation in the adventitial layer of the arteries (Barger and Beeuwkes, 1990). Vasa vasorum proliferation on is activated in response to concentrations of VEGF expressed by the damaged arteries. The neovascularization initiated in the atherosclerotic arteries makes them most likely to be fragile and subject to rupture, explaining how atherosclerosis can become a lethal disease (Kamat et al., 1987).

VEGF not only induces endothelial cell proliferation, but has also been reported predispose the endothelial layer to development of atherosclerosis. Integrins are trans-membrane proteins that mediate cell attachment and migration (Reynolds et al., 2002). Previous studies of the role of α v β3 integrin in atherosclerosis showed that these molecules regulate atherosclerosis formation indirectly by inducing VEGF expression through phosphorylation pathways. Thus, α v β3 integrin is highly expressed in atherosclerotic conditions (Barger et al., 1984).

It has been reported that VEGF mediates angiogenic processes by stimulating endothelial cells to express alpha beta integrins in the atherosclerotic plaque area. Also, it was reported that these molecules mediate smooth muscle cell attachment and accumulation in the intima layer of atherosclerotic arteries and induce macrophage migration and transformation to foam cells in the progression of atheroma (Hoshiga et al., 1995).

There are some aspects of VEGF's role in atherosclerosis that are reveals controversial in regard to mechanism and effect. Although many studies have reported VEGF involvement in atheroma progression through induction of inflammatory molecules, other functions of VEGF have contradictory effects in atherosclerotic arteries. For instance, VEGF has been shown to mediate vascular hypotension by inducing NO synthesis, which appears to be contradictory atherosclerosis, since is characterized by NO decrease and vascular hypertension (Horowitz et al., 1997).

Elevated of VEGF concentration contributes to progress the lesion formation. Since VEGF report to increase the arterial permeability, clinical data showed that

increasing the endothelial permeability by expressed VEGF will lead to increase the arteries stenosis that lead to LDL accumulation in the sub endothelial layer (Sakellarios et al., 2012).

Therapeutic Approach of VEGF Inhibition

While angiogenesis is contributed in organs growth it often plays a harmful role in many diseases. In such cases, VEGF inhibition in cancer, the is key step of tumor progression inhibition. Formation of new blood vessels in tumor increase tumors size because it provides essential supplement to cancer cells (Sitohy et al., 2012).

Inhibition of VEGF expression is an efficient anti-cancer therapeutic strategy. VEGF/ VEGFR inhibitor is a humanized monoclonal antibody that is used to inhibit VEGF expression, leading to general inhibition of angiogenesis. In fact, monoclonal anti VEGF antibody not only inhibits tumor neovascularization, but also inhibits pre-existing vessel (Inai et al., 2004). Bevacizumab (Avastin) is one of the most effective anti- solid tumor drugs that have been prescribed as an anti-cancer drug. It is a humanized antibody to VEGF-A which inhibits the neovascularization causing tumor shrinkage (Hurwitz et al., 2004). Bevacizumab is the first FDA, approved anti-cancer drug that acts through inhibition of angiogenesis (Ferrara, 2004) leading to regression of existing micro vessels of the solid tumor. The US FDA approved bevacizumab for the following clinical cases: metastatic colorectal cancer, nonsquamous cell lung cancer, metastatic renal cell cancer, prostate cancer and

glioblastoma (Mukherji, 2010). For intravenous administration the half-life of this humanized antibody is 20 days (Hicklin and Ellis, 2005).

The molecular mechanism of bevacizumab can be explained by the high binding affinity of bevcizumab to the expressed VEGF-A in the circulation. This binding forms a complex that makes VEGF unable to bind to the VEGF receptor on the endothelial cells membrane. Beside the bevaciamab action of trapping the VEGF and inhibiting its activity, it also has the potential to bind to cell surface VEGF receptors preventing unbound VEGF from binding to its receptors (Ferrara et al., 2006; Ziemssen et al., 2009; Holash et al., 2002). (Figure 4)

Since atherosclerosis progression is mediated by VEGF expression, the inhibition of VEGF may reduce plaque neovascularization, eliminating formation of the high-risk atheromatic plaques. There is a suggestion of using bevacizumab as anti VEGF to inhibit plaque formation that mediated by VEGF (Kabbinavar et al., 2003; Hurwitz et al., 2004).

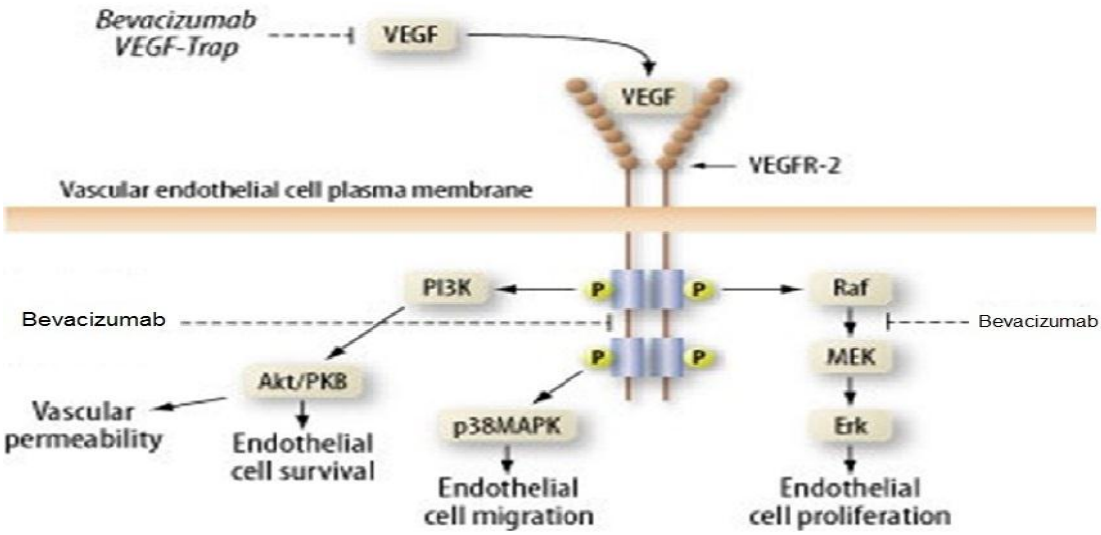


Figure 4. **Bevacizumab Action Sites**: The Bevacizumab molecular mechanism and the inhibition sites of VEGF activity in endothelial cells (Choueiri et al., 2006).

Although bevacizumab shows high efficacy in cancer therapy and other clinical cases as anti VEGF, it has many side effects and devastating activities in the body that limits its use as anti-VEGF therapy. The main side effects that have been reported are hypertension, bleeding, bowel perforation, general growth regression, hair loss and immune suppression (Sliesoriaitis and Tawfik, 2011; Vera et al., 2008).

The regular dosage of bevacizumab prescribed for cancer patients is 5 to 10 mg/kg every 2 to 3 weeks. This high dosage can cause much damage and long term side effects which limit bevacizumab uses (Mishima et al., 2012). An efficient delivery system can be a feasible method to reduce these side effects and optimize drug targeting efficacy (Koukourakis and Sotiropoulou, 2011).

Many studies are necessary before investigating the delivery and targeting mechanisms aimed at developing nanotechnology optimizing bevaciamab efficacy (Rai et al., 2010) while reducing its side effects.

Liposome

Liposomes are nanoparticulate pharmaceutical delivery vehicles used widely to optimize the effectiveness and reduce the side effects of many drugs as a therapeutic approach. Liposomes are spherical structure consisting of a bi-layer membrane with an aqueous core (Pautot et al., 2003). They can vary in size depending on the constituent lipid ratio from 20 nm to several um (Litzingera et al., 1994; Pautot et al., 2003).

Liposomes can be administrated intravenously. Their half-life in the circulation is about 15 to 30 minutes and they are eliminated by the liver (Senior, 1987). Constituent phospholipids consist of hydrophilic heads and hydrophobic tails. In fact, phospholipid properties maintain the stability of the liposome bilayer membrane (Kimballs, 1981) and play the major role in liposome formation.

The hydrophilic heads of the phospholipids that form the liposomal membrane face the aqueous environment inside while the hydrophobic tails of the phospholipids face each other in the intramembranous layer. This structure is similar to the living cellular membrane (Dua et al., 2012).

Proteins can also be conjugated chemically to the phospholipid head groups, especially phosphatidylethanolamine amino functions. Liposome properties of

targeting can be characterized by the molecules attached to their surface, which determine targeting of these molecules in the body. The liposome can be specialized to target specific cells types because of the targeting ligands conjugated to liposomal membranes that bind to specific surface molecules of these cells.

Often, liposomal membrane ligands can be used to enhance tissue penetration, taking advantage of cell surface-attached molecules (Torchilin, 2006; Rolland, 1993).

The liposomal targeting affinity to the targeted organ or tissue can be enhanced in way that increase the effectiveness of liposomal pharmaceutical factors and reduce the liposomal loss in the circulation. There are many methods that have been developed to enhance and characterize the targeting and enhance the delivery efficiency. Liposomes can be classified into many types, based on the molecules conjugated to their membrane that characterize the liposomal targeting.

In Nanomedicine, liposomes are considered composites of molecules that are used to deliver many compounds such as drugs, DNA, nutrient supplements and cells (Blazs and goodbye, 2011; Medina,. 2004). (See figure 5).

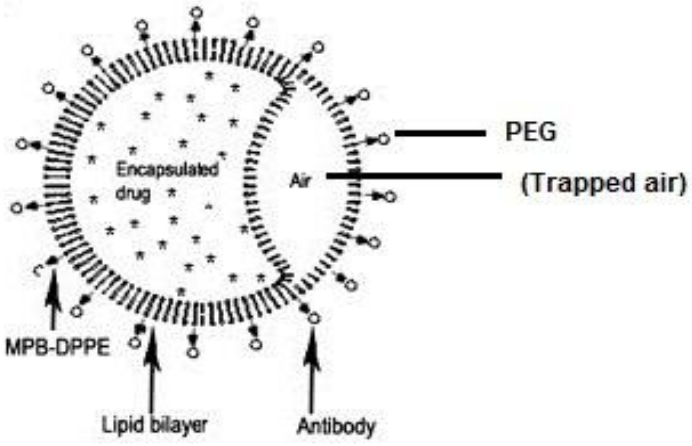


Figure 5. **Liposomal Structure**. Liposomal components and the possible drugs and other molecules that can be encapsulated or conjugated to liposomes as a delivery vehicle .

Drugs and other molecules to be delivered to the targeted sites can be encapsulated inside the liposomes or conjugated to the liposome structure (Alkan-Onyuksel et al., 1996). An efficient method of disassociating and releasing these molecules to the targeted site may be required. Echogenic liposomes (ELIP) have to reflect ultrasound because of air or other gases entrapped in them during the production processes. The entrapped gas in ELIP causes them to serves as an ultrasound contrast (Demos et al., 1999).

Air is usually used as entrapped gas in the liposomal membrane (Kimura et al., 1998). The reflected pulses by the echogenic liposome cause the membrane lipid dispersion. The dispersion of liposomal lipids breaks the membrane linkage and composes a cavitation in the membrane leading to release the encapsulated

components (Huang et al., 2002). The acoustic content of these liposomes can be characterized in order to characterize the echogenic liposome activities. High acoustic content in this echogenic liposome leads to increase the reflected pluses. Thus, the activity will increase in response to the acoustic size increasing (Kimura et al., 1998).

Echogenic liposomes have proven to be very efficient therapeutic delivery agents by optimize action of drug delivery and by application of ultrasound parameter.

Liposome Preparation

In order to prepare liposomes, different types of phospholipids and other compounds are added together in different molar ratios to characterize the liposomal properties. 1,2-dipalmitoyl-sn-glycero-3-phos-phoethanolamine (DPPE) ,1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), L-α-phosphatidylcholine (EPC) ,1,2-dipalmitoyl-sn-glycero-3-phosphor-rac-1-glycerol (DPPG) and cholesterol (CH) are commercially purchased and stored in a chloroform. The polarity of the liposomes can also be determined by choosing the proper type of phospholipids. The liposomal bilayer membrane stability is maintained by cholesterol. In some cases, anti-oxidants such as α-tocopherol or β-hydroxytoluidine are added to the formulation to protect the polyunsaturated lipids.

The afore components mentioned are diluted in chloroform s and mixed Argon is used to evaporate remnant which is removed the chloroform completely under vacuum. After the lipids are dried, then are rehydrated in a warm water bath and sonicated to form the liposomes. The liposome then rehydrated with 0.32M mannitol contained the diluted drug required to encapsulate. The mannitol's partial dispersion of the liposomal membrane provides a method for drugs entrapped in the liposome. The final formulation is then frozen on dry ice and lyophilized for 24 to 48 hours and stored at 4^{o}C. Lyophilized ELIP are reconstituted in deionized water when need (Buchanan et al., 2008; Huang et al., 2001).

The biggest challenge of liposome preparation is maintaining stability. There are many factors that interfere with the chemical stability and cause liposomal degradation or low quality liposome production. Liposomes undergo an oxidation process or hydrolysis due to the double bond of the polyunsaturated lipids and the phospholipid chemical properties. The double bonds of polyunsaturated lipids are subject to oxidation and cleavage of the double bond that leads to liposomal degradation (Kemps and Crommelin, 1988; Logaini and Davies, 1980). It is always recommended to use high purity lipids that minimize the oxidation damage. Also, the liposomes should be protected from light to prevent the cleavage of the double bonds in the unsaturated lipid (Crommelin and Brommel, 1984).

Hydrolysis of liposomal lipid bonds is another challenge that is difficult to manage because of the aqueous environment in the body where the liposomes are injected. Hydrolysis mostly occurs in the four ester bonds present in a phospholipid

when it is exposed to water, but the carboxyl ester bond hydrolyzes faster than the phosphate esters bond (Kemps and Crommelin 1988). During the hydrolysis, the hydrocarbon chains cleave from the lipid backbone, forming fatty acids and lysophospholipids. The lysophospholipid is subjected to another hydrolysis process in which it is the transformed to glycerophospho compound (Grit et al., 1989).

Clinical Use of liposomes present another challenge represented by immune resistance that leading to degradation of the injected liposomes in the circulation. This degradation will reduce liposomal efficacy and reduce the liposomal life time in the circulation. Thus, liposomes are often coated with polyethylene glycol (PEG). The presence of PEG on the liposomal membrane will increase the liposome circulation time and overcome of immunological degradation. PEG reduces liposome uptake by the lymphoreticular endothelial system (LRES) protects the liposomes from the phagocytic uptake, thereby increasing the liposomal life time in vivo (Immordino et al., 2006).

II. MATERIALS AND METHODS

A. Enhance Stem cell delivery conjugated to echogenic liposome by ultrasound

Overview

Many clinical studies have shown that CD 34+ stem cells hold a lot of promises in cardiovascular disease and atheroma progression inhibition (Padfield et al., 2013). In regenerative medicine, stem cells delivery is considered a major challenge. The injected stem cells are subject to dispersion in the circulation or degradation by the immune system. BF-ELIP has the ability to serve as CD34+ stem cell vehicles having anti-CD34 antibodies and anti-ICAM-1 antibodies conjugated to the liposomal membrane. The antibody targets ICAM-1 expressed by vascular endothelial cells under inflammatory condition. BF-ELIP is an echogenic liposome that can optimize targeting efficacy by exposure to ultrasound. In this project, we will design an in vitro system that mimics atheroma conditions to demonstrate the efficiency of the stem cell delivery methods. BF-ELIP can form a link between CD34+ cells and endothelial cells that express ICAM-1 because of binding of these molecules to the liposomal anti bodies. Also, we will test the hypothesis that ultrasound application enhances both the liposome cell targeting and delivery invitro.

Objectives

- Design a transwell culture that can serve as an in vitro system to test ultrasound enhancement of the targeting and delivery of stem cells to atheroma.

- Determine whether US enhances adherence of stem cells to transwell endothelial cell (EC) monolayers and their passage through the monolayers using fluorescently labeled monocytes as source of CD34+ cells.

- Optimize stem cell delivery by using bifunctional liposomes and enhance the delivery by using ultrasound application.

Experimental Design

1: a) Use a transwell system to model monocyte infiltration during atherosclerosis

b) Enhance monocyte infiltration by elevating ICAM-1 expression by TNFα.

2: Demonstrate ultrasound effect on the stem cells adherence and migration.

3: Demonstrate BF-ELIP targeting efficacy.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) (BD bioscience) are seeded in 75cm^2 flasks (Falcon) with Medium 200 (Invitrogen) supplemented with Low Serum Growth Supplement (BD bioscience) for 3 days at 37^{o}C and harvested with Trypsin EDTA (sigma Aldrich). The cells are transferred to 6 transwell plates (Corning). The 6 transwell plates are pre -incubated with endothelial cell medium (ECM) (Invtrogen) for 30 min; 4 x 10^5 HUVEC are added to each insert and incubated for 3 days at 37^{o}C. The final ECM medium volume is 1.5 ml in the insert and 2.4 ml in the well.

The transwell plate cell culture insert used in this project consist of microporous polyester membrane that allows the medium and monocytes to pass through it. The 6-well plate insert diameter is 24 mm with a growth area of 4.67 (fig 6).

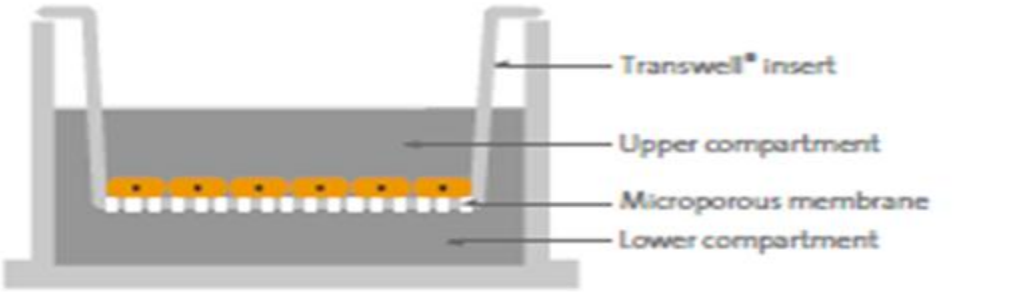


Figure 6. **Transwell Plate Design.** The design of the Corning transwell plate and the structure of the insert.

Mononuclear Extraction and Labeling

Mononuclear (MNC), which cells serve as source CD34+ cells are isolated from whole blood purchased from Gulf Coast Regional Blood Center. Buffy coats were isolated from the blood sample by Ficoll Plus (GE Lifesciences). The MNC are usually found in the buffy coat layer of the centrifuged blood sample (Fig 7), which is located in the intermediate layer of the centrifuged blood sample. After gently extracting the buffy coat layer from the centrifuged test tube, it is diluted with PBS in a ratio of 1:2; 25 ml ficoll (density 1.077 g/ml) in 50 ml test tubes and will fill with diluted buffy coat ill gently overlay the ficoll with an equal amount of the mixture from above. The test tube will Centrifuge for 30 min with 700 x g without brake at 4 degree ^{o}C. The white interphase between the plasma fraction and the ficoll fraction is transferred into a fresh tube. After washing twice with PBS the cell pellet is resuspended with ECM medium. The cells are transferred to flask at a concentration of $5x10^{6}$ cells per ml followed by Incubation of the flask at $37C^{o}$ for 1 h during which the MNC adhere to the flask. The flask is then washed with PBS to remove non-adherent lymphocyte and trypsinized to harvest adherent cells, which are representing pure mononuclear culture.

Oregon Green 488 (Invitrogen) is diluted in a test tube containing PBS to obtain 10 uM concentration. The isolated mononuclear resuspended in 10 uM Oregon Green solution, followed by 30 min incubation at $37C^{o}$. After the incubation period, the labeled monocyte centrifuge for 5 min and resuspended with ECM medium protected from light.

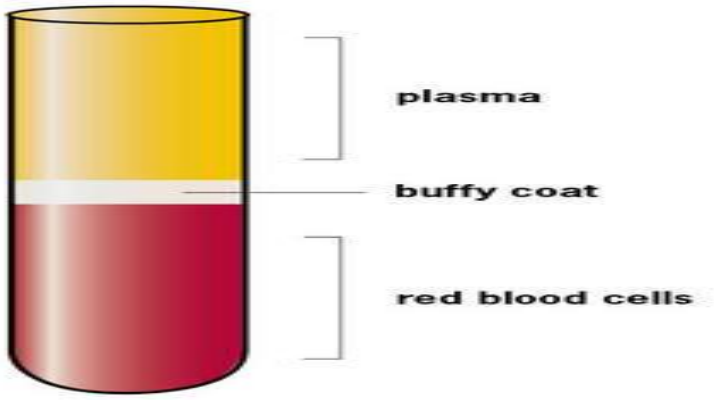


Figure 7. M**ononuclear Cell Layers in Centrifuged Blood Sample.** Centrifuged fresh blood sample in the intermediate layer represent, buffy coat in the blood sample.

Monolayer Treatment

On the third day of endothelial monolayer incubation in the transwell plate insert, the HUVEC monolayer is treated with 20 nM TNFα (kindly provide by Dr. Michael Wassler) overnight for incubation of ICAM-1 inducing. Mononuclear cells are labeled with 10 µM Oregon Green 488 dye diluted in pre-warmed PBS described above.

ELIP is prepared by an evaporation-rehydration-sonication-lyophilization method. As described above, BF-ELIP are prepared by conjugating polycolonal, anti-human/ mouse CD34 antibody and monoclonal ICAM-1 antibody to ELIP using a thioether coupling method (Herbst et al., 2010).

Pretreated HUVEC monolayers on transwell (6 wells/plate) insert membranes are incubated with nonspecific IgG-ELIP or BF-ELIP (1mg/well) for 15 minutes at

37C^{o}, followed by human mononuclear labeled with Oregon Green. After adding the labeled mononuclear, half the inserts are subjected to 6 MHz color Doppler imaging pulses (pulse duration= 3.33 us, pulse repetition frequency= 5 kHz) ultrasound with mechanical index (MI) = 0.4 for 5 minutes in an anechoic chamber fashioned from a gel standoff over a rhoC rubber pad (Smith et al., 2010). (See Fig 8) .

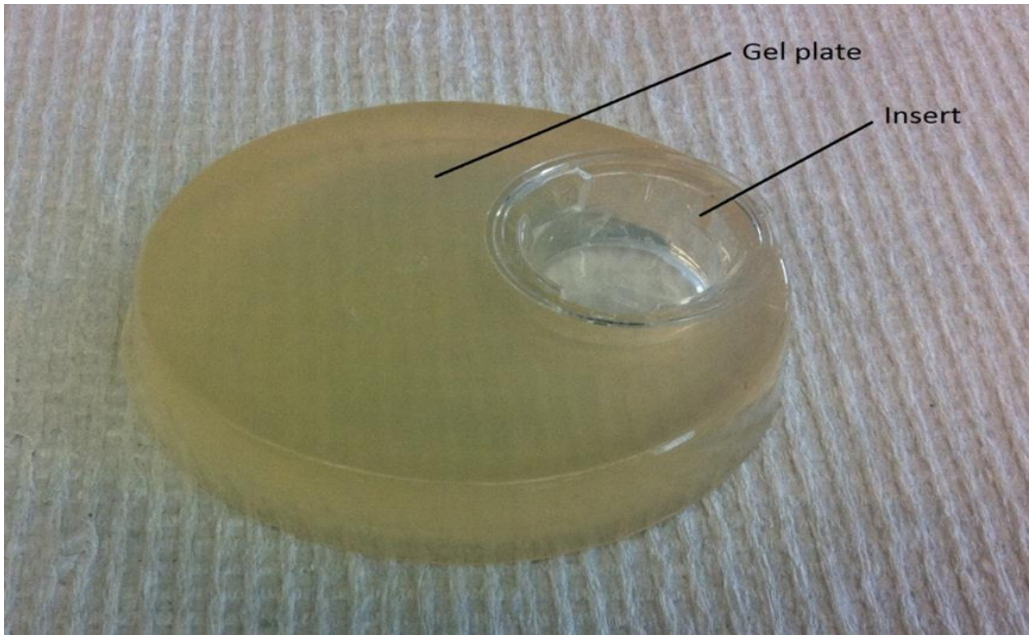


Figure 8. **Insert Exposure to Ultrasound Design.** The insert is placed in a gel standoff over a RhoC rubber pad.

The gel is cut to hold the insert while preventing the medium from leaking out. The insert is filled with ECM medium to optimize the US application.

Cell Harvesting

The transwell plate is incubated for 24 hr. The insert and well of the transwell plate are then treated simultaneously with 0.25% trypsin/0.1% EDTA (Sigm-Aldrich). The trypsinized solutions are transferred to 96 well microplatesd (Corning) and Oregon Green 488 fluorescence is measured Spectramax M5 plate reader at an emission wavelength of 450nm (Fig. 9).

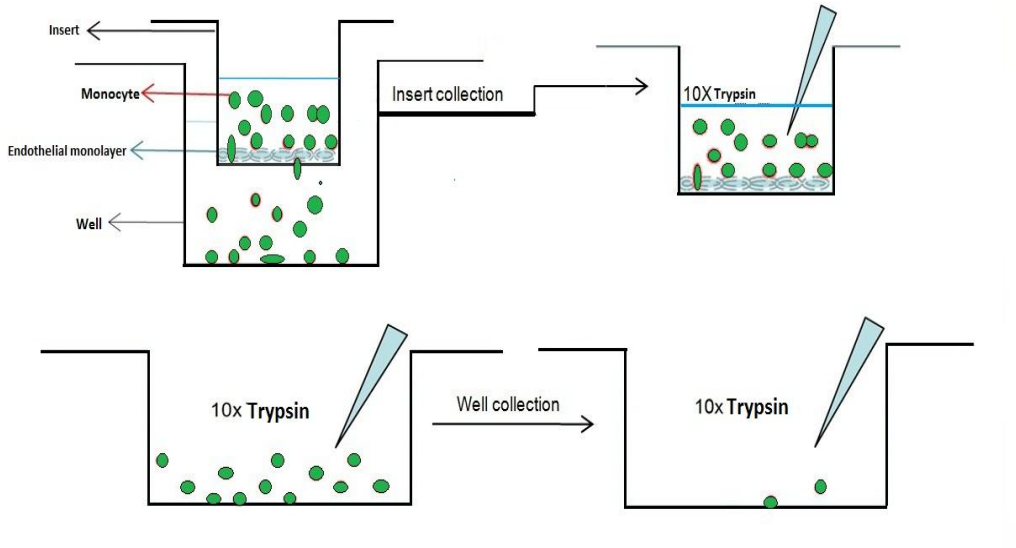


Figure 9. **Diagram of the In Vitro Transwell System Cells Harvesting**. The insert membrane has pores that allow mononuclear to migrate through the endothelial cell layer and pass through into the well.

A standard curve is run with a series of Oregon Green-labeled MNC amount to convert the fluorescence emission data to cell numbers.

B. Bevacizumab Loaded ELIP Release Enhanced by Ultrasound for Atheroma Progression

Overview

Clinical data indicates that VEGF is highly expressed in atherosclerotic conditions. They also show that VEGF mediates atheroma progression by inducing neovascularization in the atherosclerotic arteries while increasing the vascular permeability, which leads to LDL accumulation and plaque formation (Celletti et al., 2001; Sakellarios et al., 2012) . Color Doppler ultrasound has been shown to enhance the release of bevacizumab from bevacizumab-loaded echogenic liposome, providing a basis optimization of VEGF inhibition by bevacizumab-loaded ELIP exposed to ultrasound. VEGF-mediated endothelial cell proliferation can be induced by phorbol -myristate 13-acetate (PMA) and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA) through protein kinase activation pathways that increase VEGF mRNA transcription (Bergslanda et al., 2004; Cai et al., 2006; Xu et al., 2007; Broughman et al., 2006).

Thus, we hypothesize that bevacizumab can be used as anti-atherosclerotic agent by inhibiting VEGF expression and ultrasound that can be used to enhance the release of bevacizumab from bevacizumab-loaded ELIP to optimize the inhibition. In this project we will demonstrate the efficiency of liposomes for both cellular delivery and drug release to inhibit the atheroma progression. In this project we will demonstrate the efficiency of ELIP for both stem cell delivery and drug release to inhibit atheroma progression.

Objectives

- Induce VEGF expression by using an independent activator in HUVEC cell cultures, detectable by western blot of the cellular extracts.
- Design VEGF ELISA to determine VEGF expression in HUVEC cell culture and demonstrate bevacizumab inhibition of VEGF expression in vitro.
- Design a proliferation assay to demonstrate the relationship between the endothelial cell proliferations mediated by VEGF expression for the purpose of demonstrating bevacizumab-loaded liposome (BEV-ELIP) inhibition efficacy of HUVEC proliferation mediated by VEGF.
- Demonstrate BEV-ELIP efficacy to inhibit VEGF expression and determine ultrasound effect on the bevacizumab release enhancement from the loaded liposome in both the VEGF ELISA and proliferation assays.

This project was conducted in Dr. Klegerman's lab as an in vitro model for the inhibition efficacy of bevacizumab/BEV-ELIP of atheroma progression mediated by VEGF.

Experimental design

- Induce PKA, and PKC, mediated VEGF expression by PMA/DOPPA as protein kinase activators.

- Design cell proliferation assay for control and treated groups to characterize the relationship between cell proliferation mediated by VEGF and inhibition by Bevacizumab/BEV-ELIP.

- Use Western blot to detect VEGF expression and PKC/PKA activation (translocation) in control and treated groups.

- Design VEGF ELISA for control and treated groups to determine VEGF expression level and inhibition efficacy of Bevcizumab/BEV-ELIP.

- Determine ultrasound enhancement of BEV-ELIP release by both VEGF ELISA and proliferation assay on HUVEC cells.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) (BD bioscience) are seeded in 75cm^2 flasks (falcon) with Medium 200 (Invitrogen) supplemented with Low Serum Growth Supplement (BD bioscience) for 3 days at 37^oC in an tuner place of 5% CO_2. HUVEC are trypsinized with 0.25% trypsin/0.1% EDTA (Sigma-Aldrich). The harvested HUVEC are transferred and seeded into 24-well plates (Falcon) with Medium 200 (8x 10^4 cells per well).

The seeded cells are incubated overnight after which the plate is divided into three separate groups. The first group is treated with 10nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). The second group is treated with 100 nM 12-deoxyphorbol 13-O-phenylacetate (DOPPA) (Sigma- Aldrich), while the third group is left untreated as a control group. The plate is incubated for 12 hours and harvested after 12 hours of treatment and approximately 30 hr. of total incubation.

BEV-ELIP Preparation

Bevacizumab loaded ELIP can be prepared by adding the following components (molar ratio) to round bottom flask (provided by Dr. T Peng): Dipalmitoylphosphatidylcholine (46%) ; Dioleoylphosphatidylcholine (23%); Dipalmitoylphosphatidylglycerol (8%); Dipalmitoylphosphatidylethanolamine (8%); Cholesterol (15%).

After mixing the components, the flask is rotated in a warm water bath and evaporated under a stream of Argon. The flask is then placed in a vacuum desiccator connected to a pump overnight for complete durations after which the flask contents are rehydrated with 0.2M D-mannitol (Fisher Scientific) containing 5 mg/ml of Bevacizumab (Genetech). After through mixing BEV-ELIP, the flask then will kept overnight in low temperature air vacuum (lyophillizaition) and rehydrated with water next day transferred to small vial as its ready to use.

To expose The BEV-ELIP to ultrasound ,BEV-ELIP contained in rubber condom evacuated from the trapped air and submerged in water bath and exposed to ultrasound for 5 min (30 sec off/on) for bevacizumab release followed by UV exposure for 30 to 60 min for sterilization. The ultrasound set for a parameter of 6 MHz color Doppler imaging pulses (pulse duration= 3.33 us, pulse repetition frequency= 5 kHz) ultrasound with mechanical index (MI) = 0.4 for 5 minutes in an anechoic chamber fashioned from a gel standoff over a rhoC rubber pad (Smith et al., 2010).

Protein Preparation and Western Blotting

Three groups of HUVEC seeded in 24 well plates (untreated or incubated with 10 nM PMA or 100 nM DOPPA) are placed on ice and homogenized on homogenization buffer for resuspending and collecting .The homogenization buffer consists of 50mM Tris HCl, pH 7.5 with 0.5 uM EDTA, 0.5 mM EGTA, 10% glycerol, 10 ug of both inhibitor A and inhibitor B that has 0.2 nM fluoride associated with 50mM of Triton 100X detergent (all provided by Dr. E. Martin).

For detection of VEGF expression total proteins isolated from HUVEC. Briefly, micro plates wells are washed with phosphate buffer solution, pH 7.4 (Hyclon co.), followed by 100 ul of homogenization buffer, freezing the plate for 30 min at -80 ^{O}C. After freezing the sample collected from each group placed in small vials. Samples are then lysed by rapid freeze-thaw cycles using dry ice of three repetitious followed by centrifugation at low temperature (0 ^{o}C) for 10 min at 14000 RPM. The pellet is discarded and the protein contained in the supernatant.

To investigate the mechanism of VEGF expression mediated by PKC and PKA pathways, western blots for PKA and PKC are run in order to determine the translocation of the protein kinases for the cytosol to the endoplasmic reticulum membrane in response to VEGF activation factors.

PKC and PKA can be extracted from the cytosol and the membrane fraction using protein fraction methods. The three groups of HUVEC washed with PBS are placed on ice, followed by add on of 100ul homogenization buffer. Samples were lysed by freeze-thaw cycles (3X) in dry ice and water at room temperature. The vials

are centrifuged at 100,000X g for 1hr.The supernatant represents the cytosolic protein which is then removed to new vials while the pellet is resuspended with 1 M NaCl in cold homogenization buffer followed by centrifugation for 30 min at 100,000X g. The supernatant is discarded and the pellet dissolved in homogenization buffer containing 2% Triton X100. The dissolved pellet represents the membrane protein while the supernatant extracted at the beginning represent the cytosolic protein.

Bradford Protein Assay

Following cell harvest with lysis homogenization buffer, the protein concentration of each sample is determined by the Bradford technique. Bovine serum albumin (BSA) (provided by Ms. E. Golunski) is used to construct protein standards ranging from 200 to1000 ug/μL. The homogenized samples are diluted as needed and triplicate determinations were made by pipetting 5μL of sample into 100 ul of distilled water in a microplate. The absorbance of the plate is measured at 595 nm in a Spectromax M5 plate reader. The protein concentration of the unknown sample was determined using a standard curve of BSA concentrations verses the absorbance.

Western Blot

One volume of homogenized protein is added to three volumes of loading buffer (provided by Ms. E. Golunski) and denatured at 90^{o}C for 5 min. The denatured samples are added to 12% acrylamide gel. Protein bands in SDS-PAGE gels are transferred to nitrocellulose membranes, which are then incubated with a 100X dilution of primary antibody (bevacizumab kindly provided by Dr. Klegerman), washed and followed by 4000X secondary IgG antibody anti-human (IRDye 800CW, Sigma Aldrich). Human VEGF standard is run on the same gel .The primary and secondary antibody are diluted in Li-Cor Odessy blocking buffer (Bioscience) provided by (Dr. T Peng). The nitrocellulose membrane is assayed for infrared florescence with a Li-Cor Odyssey instrument.

For PKCα, the nitrocellulose membrane is incubated with Primary antibody: 1 µg/ml anti-PKCα (Cell Signal) followed by 4,000X anti-rabbit secondary antibody (IRDye 800CW, Sigma Aldrich). For PKAα, the membrane is incubated with primary antibody of 1 ug/ml anti PKAα (Santacluz) followed by 4000X of anti-rabbit secondary antibody (IRDye 800CW, Sigma Aldrich). The membrane is incubated overnight with the primary antibody at 4^{o}C, followed by 1 hour of secondary antibody. Protein bands are detected was with the Odyssey LI-COR IR fluorescence imaging system.

VEGF ELISA

VEGF expression can be detected in vitro by VEGF ELISA of cultured endothelial cells in microplate wells. The ELISA is performed by seeding 2 x 10^4 HUVEC in 96-well microplate wells incubated overnight with Medium 200. After 12 hours of incubation, 10 nM PMA or 100 nM DOPPA is added to the treated groups.

After 30 hours of total incubation, cells are fixed with formalin (Sigma Aldrich) and incubated with 5 µg/ml of bevacizumab (primary antibody) for 2 hours at 37^{o}C. Wells are washed with PBS for 3 times, followed by 10,000X secondary anti-human IgG-HRP (Sigma Aldrich) for 1 hour at room temperature. After wells a 3X PBS washes for three time color development with is pretreated ABTS substrate is for 30 minutes at room temperature. Plate absorbance is measured at 405 nm with a SpectraMax M5 reader.

Proliferation Assay

The endothelial proliferation can be characterized by VEGF expression and bevacizumab inhibition. The proliferation assay is used to investigate the relationship between VEGF expression and cell proliferation. Fresh HUVEC are added to 96-well plates 2 X 10^4/well and incubated for 18 hours in Medium 200. The wells treated with 10 nM phorbol 12-myristate 13-acetate (PMA), 100 nM 12-deoxyphorbol 13-O-phenylacetate (DOPPA) or medium alone (no PMA, no DOPPA) 4 hours later and incubated for 24 hours.

The wells either have the medium alone, bevacizumab or BEV-ELIP added to control groups or to groups pretreated with PMA/DOPPA. After the final incubation, cells are enumerated by trypsinization and counting with a hemacytometer. Colorimetric assay can be used to obtain accurate results for many groups. This assay can be done by adding 10 ul (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT assay, Sigma Aldrich) to 100 ul of wellplate medium incubated for four hours. After this incubation, 75 ul of medium is gently removed and 25 ul of DMSO is added to each well, followed by 1 hr. of incubation 37^{o}C. Absorbance of the wells is measured by a SpectraMax M5 reader at 540nm.

III. RESULTS AND DISCUSSION

A. Enhance Stem Cell Delivery Conjugated to EchogenicLiposome by Ultrasound

The in vitro transwell system estimated through the labeled monocyte adherence and migration endothelial monolayer. Based on the growth area of the insert provided by the transwell manual, 400,000 of HUVEC added to the insert can pass through the membrane without obstacles (See Fig10).

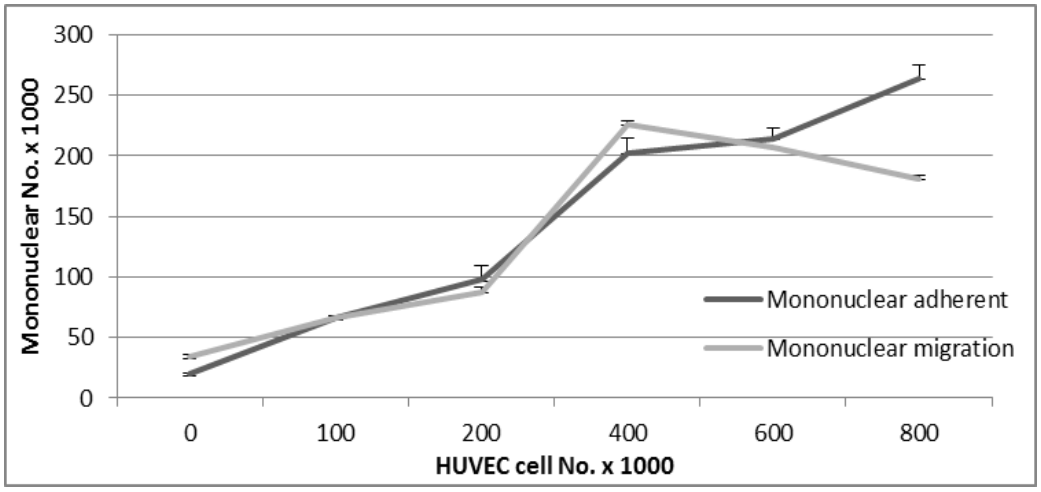


Figure 10. **HUVEC Number and Mononuclear Adherence and Migration Rate.** The labeled mononuclear migration and adherence varied with different HUVEC numbers, 400,000 cultured HUVEC in the insert gives the highest number of migrated mononuclear (well), a good adherence rate (insert) and the lowest rate of mononuclear loss generally. The added mononuclear are700,000,each point represents a (mean± SD, n= 6.)

After we tested the HUVEC number that provides a confluent monolayer giving the highest total control mononuclear number adherent to and passing through the monolayer, we then induced the expression of ICAM-1 as an inflammatory signal. TNFα upregulates ICAM-1 expression by endothelial cells, providing method

to characterize the atherosclerotic condition invitro (Bernot et al., 2005). Endothelial permeability increases in response to increasing TNFα concentration (Watsky et al., 1996). TNFα causes an reduction in the density of the endothelial monolayer and increases its permeability in vitro (Friedl et al., 2002). In these studies, a series of TNFα concentrations was added to the endothelial monolayer to optimize ICAM-1 expression, while limiting cell death (Fig. 11).

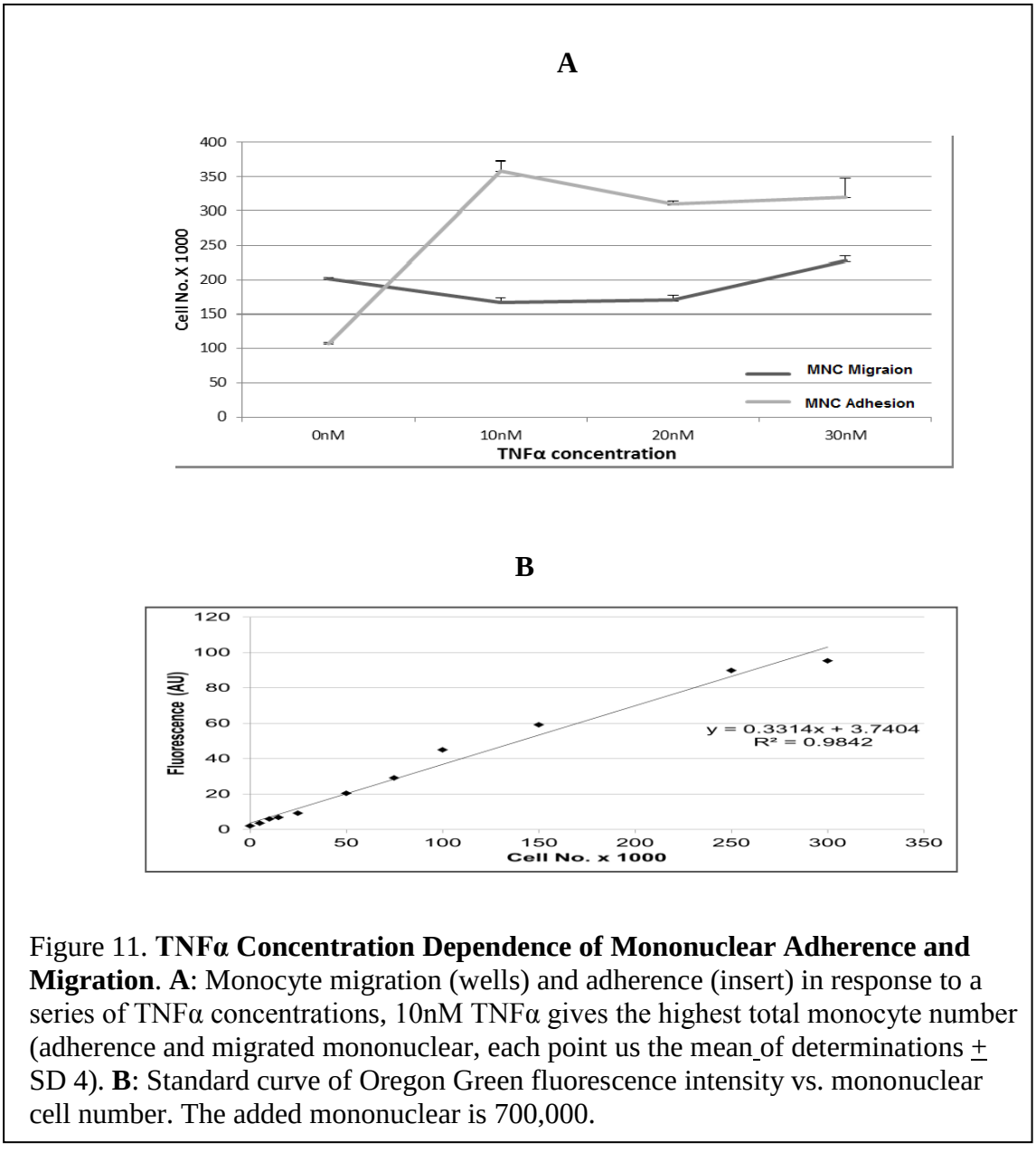
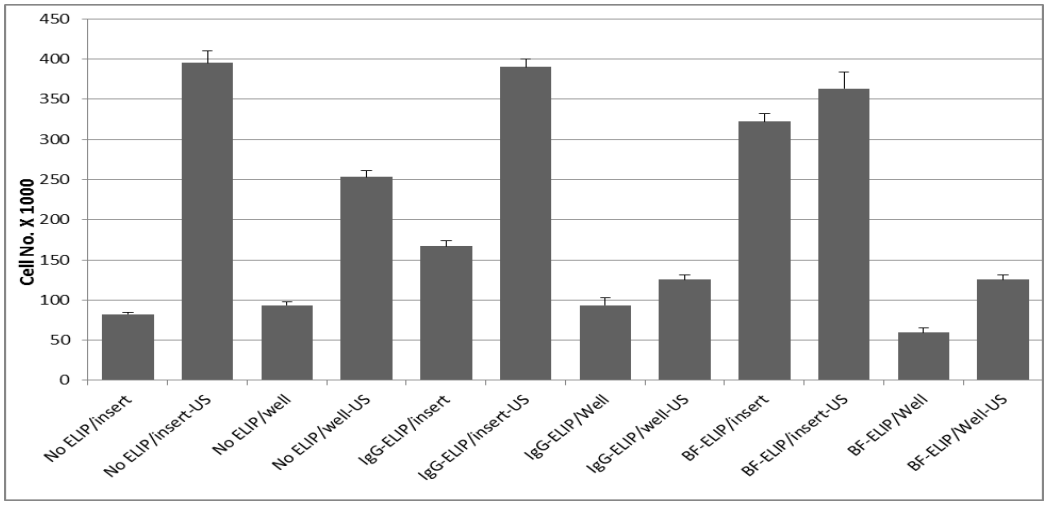


Figure 11. **TNFα Concentration Dependence of Mononuclear Adherence and Migration**. **A**: Monocyte migration (wells) and adherence (insert) in response to a series of TNFα concentrations, 10nM TNFα gives the highest total monocyte number (adherence and migrated mononuclear, each point us the mean of determinations ± SD 4). **B**: Standard curve of Oregon Green fluorescence intensity vs. mononuclear cell number. The added mononuclear is 700,000.

The optimal total mononuclear number detected in both well and the insert by seeding that can be obtained at 400,000 of HUVEC and adding 10nM TNFα to the transwell plate. We used this optimized system to demonstrate bifunctional ELIP efficacy compared to a control preparation (IgG-ELIP) in addition to untreated HUVEC. We also assessed the effect of ultrasound on the study groups (Fig.12).

A



B

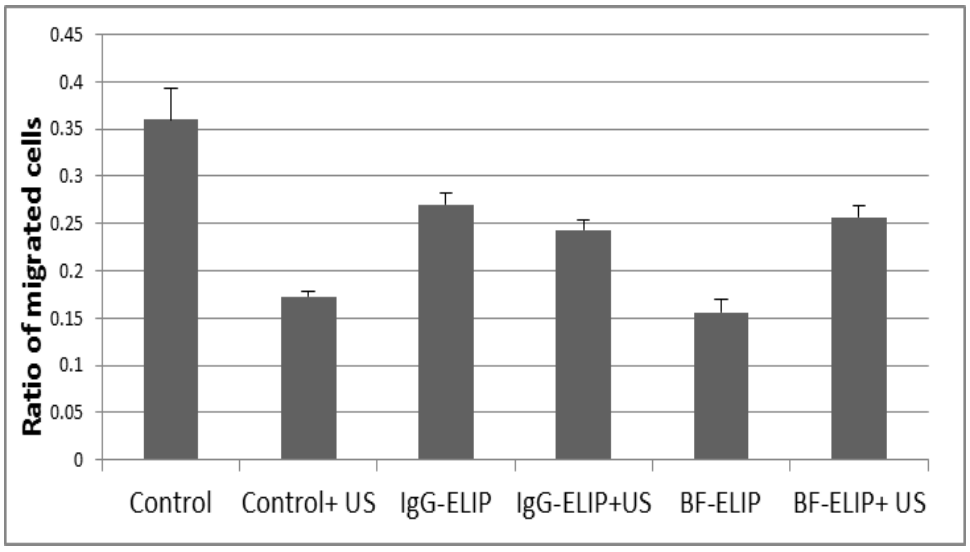


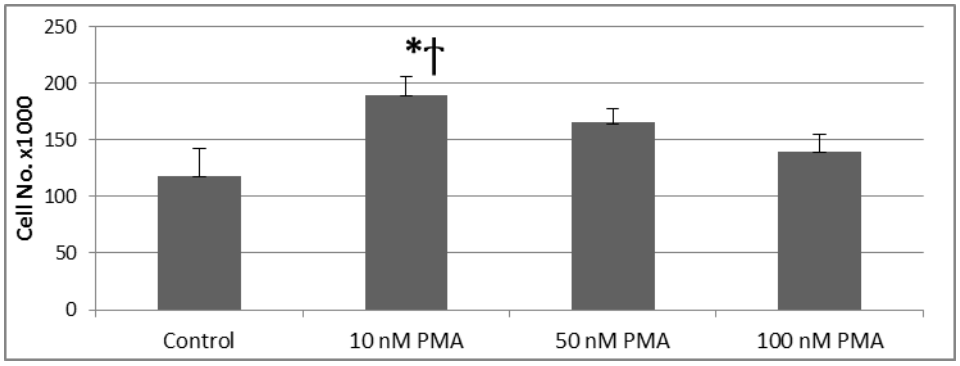
Figure 12. **BF-ELIP Efficacy with and without Ultrasound. A** Ultrasound greatly enhanced the mononuclear adherence to the endothelial monolayer and the migration level through the monolayer in all exposed groups. Mononuclear 700,000, mean± SD; n=6. **B** Proportion of mononuclear passing through the HUVEC monolayer; Mean ± SD, n=6.

BF-ELIP enhanced adherence of mononuclear to the ICAM-1expressing HUVEC monolayer relative to untreated controls and IgG-ELIP, but did not increase the number of monocytes traversing the monolayer. The monolayer was treated with 10nM of TNFα overnight to express ICAM-1. US greatly increased the number of monocytes adhering to and passing through the monolayer in all groups. The greatest increase of monocytes passing through the monolayer was without ELIP, but only BF-ELIP caused an increase in the proportion of monocytes passing through the monolayer

B. Bevacizumab Loaded ELIP Release Enhanced by Ultrasound for Atheroma Progression

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to provide evidence that induced VEGF could mediate HUVEC proliferation. The expressed VEGF can also be detected by VEGF ELISA using bevacizumab as anti-VEGF antibody. It has previously been shown that VEGF expression is mediated by PKC and PKA. Thus, phorbol -myristate 13-acetate (PMA) and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA) activators of PKA and PKC will induce VEGF expression, which is by the protein kinase pathways that stimulate VEGF mRNA transcription. These two activators could be toxic at high concentrations, so it is necessary to determine the optimal concentrations of dPPA and PMA that induce the highest HUVEC cell proliferation rate without appreciable cell death. (Fig. 13).

A



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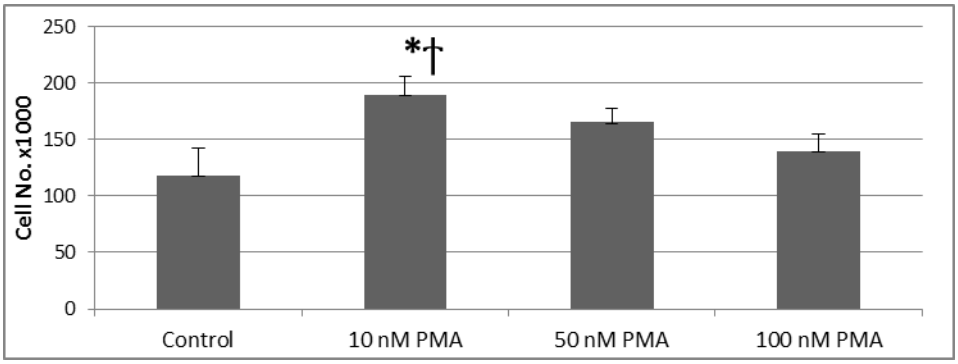


C

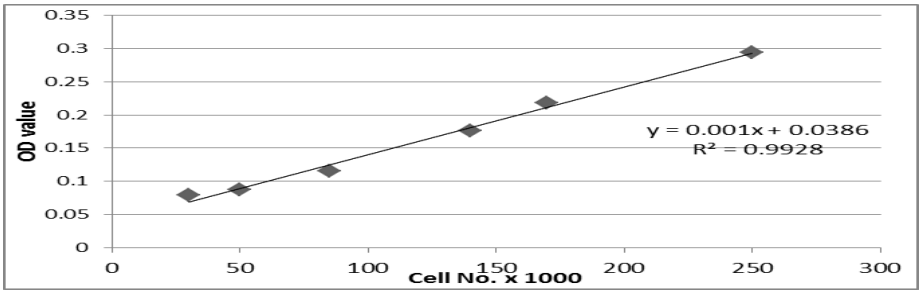
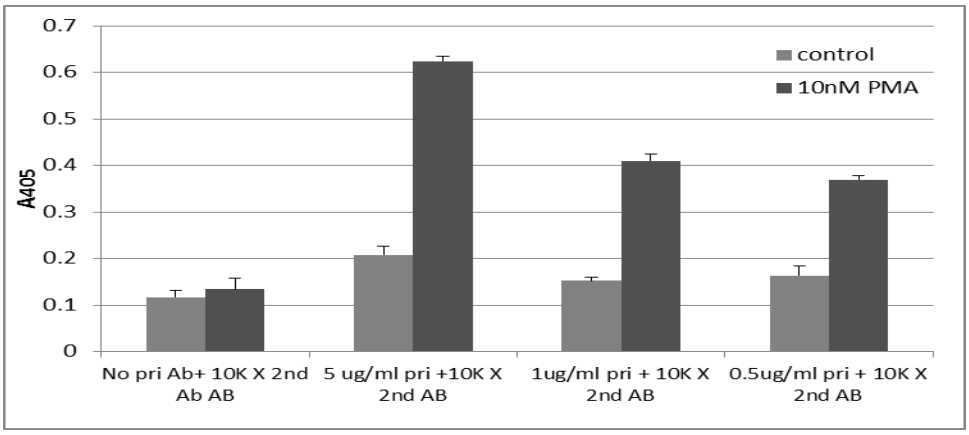


Figure 13. **Proliferation Assay for PMA and dPPA**. The MTT assay was used to detect cell proliferation. **A**: The highest proliferation rate can be obtained when HUVEC were treated with 10nM of PMA compared to series of PMA concentrations, (curve = Mean ± SD;n=4;*P<0.01 vs Control , †P<0.05 vs. 50nM PMA).**B**: The highest proliferation rate can be obtained when HUVEC treated with 100nM of dPPA compared to series of dPPA concentrations started as non-treated cells (control) and 500 nM as highest concentration. (Curve = mean ± SD; n = 4.* p < 0.01 vs. Control; † p < 0.01 vs. 50nM dPPA). **C**: MTT assay standard curve.

The fig. 13 also shows that cell proliferation is induced by protein kinase activators via protein kinase pathways. To prove the hypothesis that VEGF mediates protein kinase-induced cell proliferation, the VEGF ELISA was used to detect VEGF expression in response to protein kinase activators. The optimal concentrations of PK activators determined above that gave the highest proliferation rate were added to HUVEC microplate, followed by anti-VEGF and secondary anti body treatment (Fig14).

A



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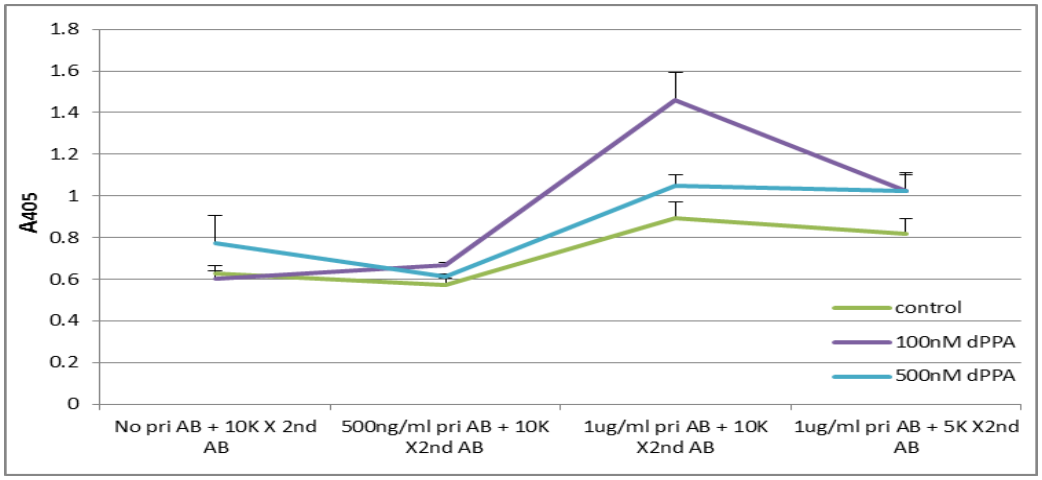

Figure 14. **VEGF ELISA for PMA and dPPA. A**. VEGF expression level of HUVEC treated with PMA and the optical density in response to the primaty antibody concentration increasing compared to control. **B**. VEGF expression levels in response various dPPA concentions compared to control (Curve= mean± SD, n=4).

The results obtained from the VEGF ELISA support the hypothesis that VEGF mediates cells prolifertion caused by activation of protein kinase pathways. The ELISA respouse was optimized relative to primary and secondary antibody

concentrations. The binding of bevacimab increased in response to prior exposure of increasing bevacizumab concentrations, reflected byincreasing OD values. High concentration of dPPA led to inibited cell proliferation and declining VEGF expression because of the toxic effects of dPPA at high concentrations.

Cells pre-treatment with bevacizumab or bevacizumab-loaded ELIP inhibited both cell proliferation and VEGF expression. The induced VEGF expression by protein kinase activators was blocked by bevacizumab or BEV-ELIP .The inhibition of both VEGF expression and cell proliferation varied based on the pretreatment concentration of bevacizumab ,BEV-ELIP (Fig.15).

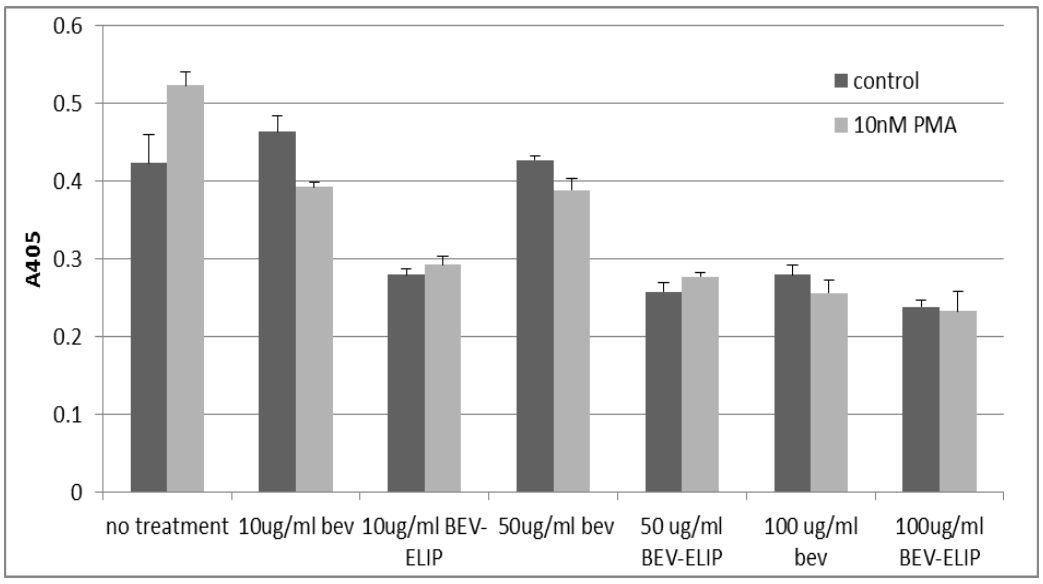

Figure 15. **VEGF ELISA for Bevacizumab/BEV-ELIP Inhibition**.VEGF inhibition increased by increasing the concentration of bevacizumab,BEV-ELIP. The ELISA indcated that BEV-ELIP inhibition efficacy is greater than bevacizumab alone. (Cuve= mean + SD; n=4)

Liposoma exposure to ultrasound increases the release of bevacizumab from the loaded ELIP, which increases the VEGF ihibition and cell proliferation in vitro demonstrated by VEGF ELISA and MTTassay (Fig. 16).

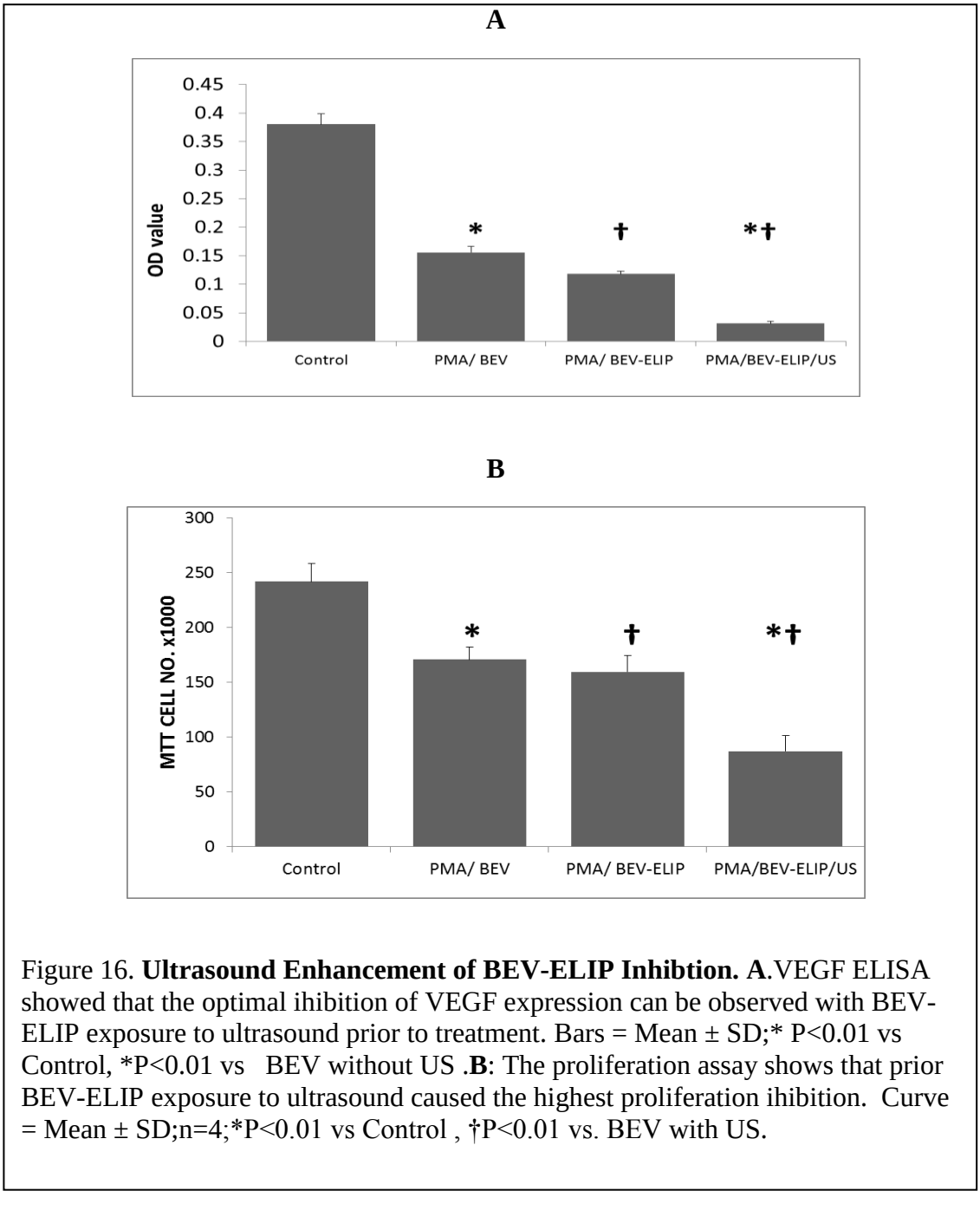

Figure 16. **Ultrasound Enhancement of BEV-ELIP Inhibtion. A**.VEGF ELISA showed that the optimal ihibition of VEGF expression can be observed with BEV-ELIP exposure to ultrasound prior to treatment. Bars = Mean ± SD;* P<0.01 vs Control, *P<0.01 vs BEV without US .**B**: The proliferation assay shows that prior BEV-ELIP exposure to ultrasound caused the highest proliferation ihibition. Curve = Mean ± SD;n=4;*P<0.01 vs Control , †P<0.01 vs. BEV with US.

VEGF expression induced by PMA can also be detected in cell homogenates by western blot technique. Transposed SDS electrophoretogram was incubated with bevacizumab as primary anti-body for VEGF detection (Fig. 17).

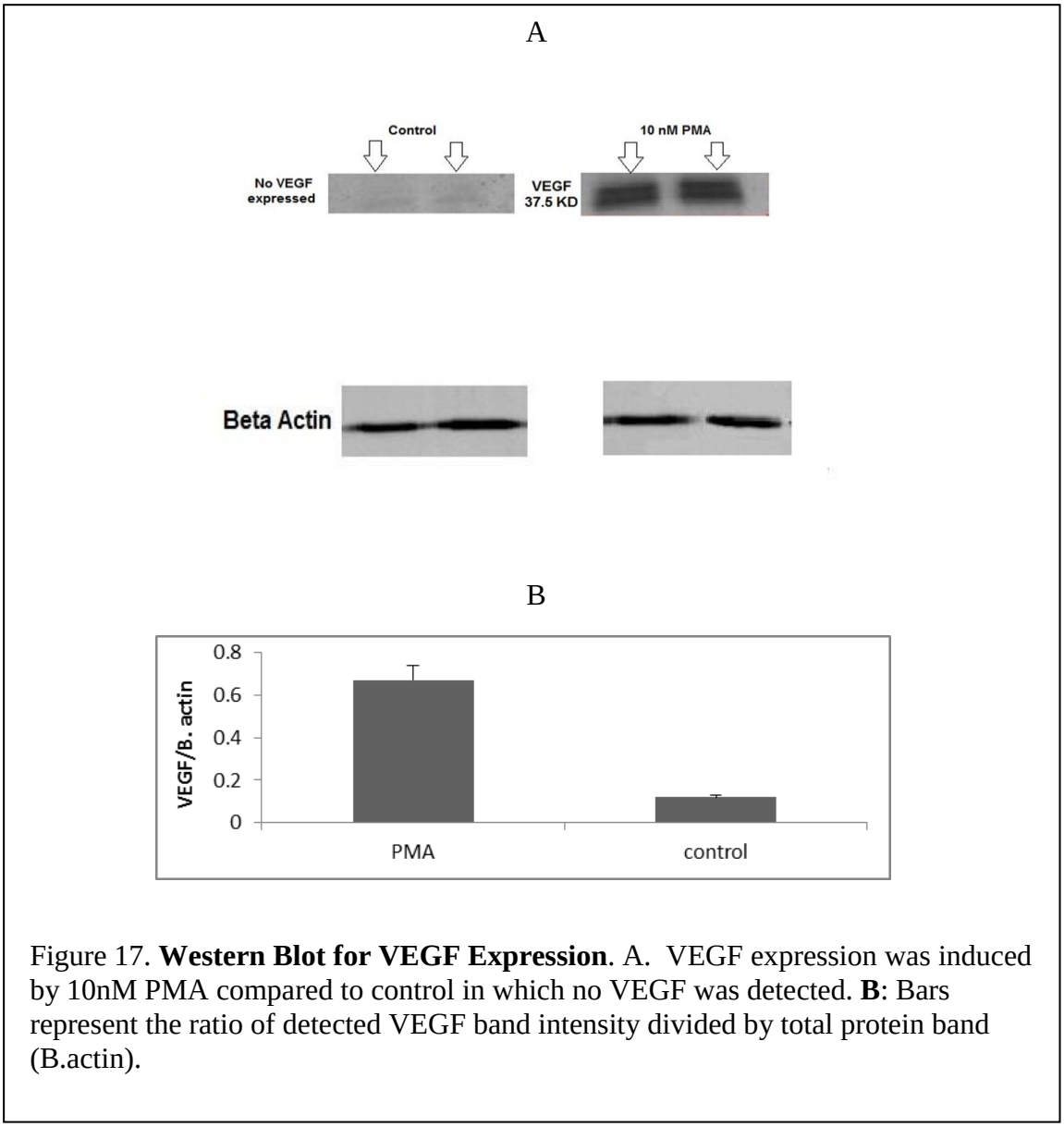

Figure 17. **Western Blot for VEGF Expression**. A. VEGF expression was induced by 10nM PMA compared to control in which no VEGF was detected. **B**: Bars represent the ratio of detected VEGF band intensity divided by total protein band (B.actin).

To demonstrate that PMA and dPPA activate VEGF expression via protein kinase pathway, western blot were ran on protein fraction homogenates for both cytosolic and membrane lysis protein (Fig. 18).

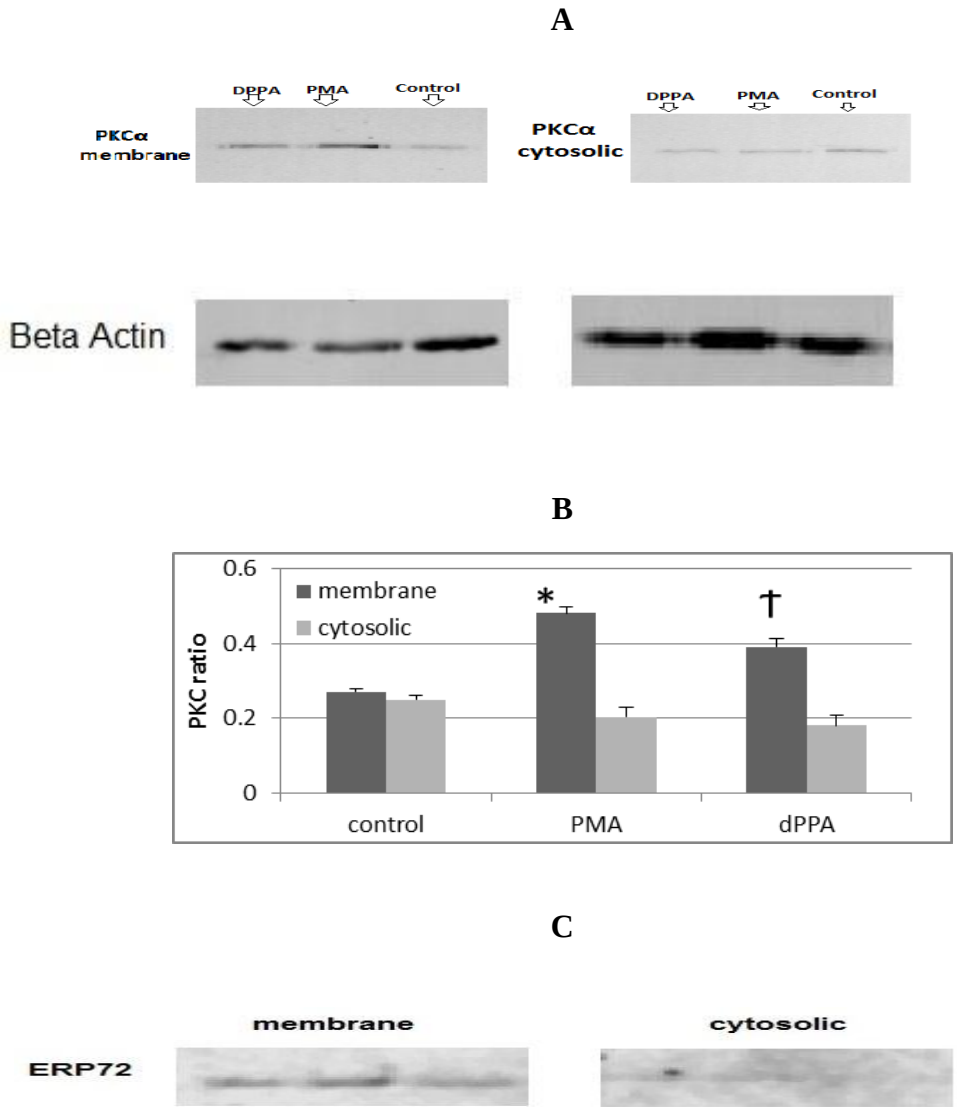

Figure 18. **Western Blot for PKCα Translocation**. **A.** PMA and dPPA induce VEGF expression by Protein Kinase C alpha translocation from the cytosolic to the endoplasmic. PKCα located in the cytosol the none active form of Protein Kinase C. **B**: Bars represent the ratio of detected PKC band intensity divided by total protein band (B.actin).,* P<0.01 vs corresponding control, ǂ P< 0.01 vs. corresponding control, C: ERP72 protein detection for the same SDS page.

ERP72 is an endoplasmic reticulum protein. The detection of this protein in western blots provides evidence that the PKCα homogenates of cells pretreated with PMA and dPPA was located in the endoplasmic reticulum indicating protein kinase C activation.

To prove the hypothesis that VEGF expression can be induced by any activation of protein kinase C or A,

HUVEC pre-treated with 30uM forskolin (cellsignals Co.) for 1 hour after 12 hours of primary incubation were subjected to cell homogenization and protein fractionation for western blot. See fig (19).

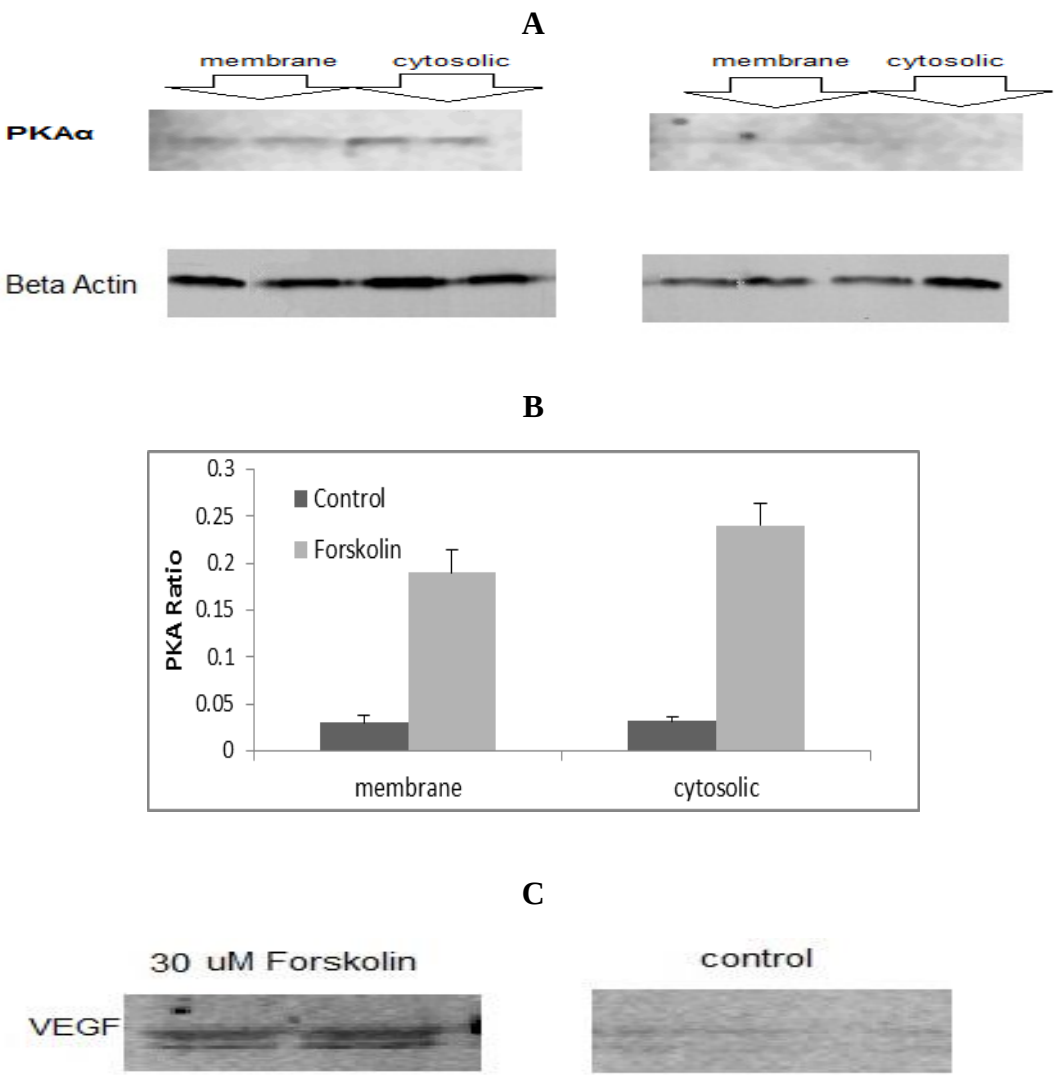

Figure 19. **Western Blot for PKAα and VEGF Expression**. **A.** PKA expression in pre-treated cells with 30uM forskolin compared to non-treated group. **B**: The ratio of detected PKA percentage to the total protein (beta actin). **C**: VEGF expression by forskolin via PKA activation.

Forskolin is a direct activator of PKAα mediated by cyclic AMP. Fig. 19 confirms that Forskolin induces PKA expression. In response to this activation forskolin induce VEGF expression via PKA activation pathway supporting the

hypothesis that VEGF expression could be mediated by any activation of PKA alone.

IV. Discussion

Stem cells have been used previously for vascular regeneration. Atherosclerosis has been defined as arterial cell dysfunction (Luscher, 1997; Kinlay et al., 2001) and can be managed using endothelial progenitor cells. Although using stem cells for vascular regeneration is considered a useful therapeutic approach, it has many limitations (Xu et al., 2003). Stem cells delivery was been a big challenge of vascular regeneration therapy. Using efficient delivery methods such as liposomes provides a feasible method to manage the cellular delivery to the damaged area. We have demonstrated the efficacy of bifunctional liposomes for cellular delivery of stem cells to reduce the atheroma progression (Herbt et al., 2010). Previous studies have shown that bifucntional liposomes reduce stem cells loss and increase stem cells adherence to the endothelial monolayer in vitro.

Treating HUVEC with TNFα simulated the inflammatory conditions associated with atheroma progression that leads to endothelial dysfunction. The TNFα induced I-CAM-1 that is an inflammatory molecule associated with atherosclerosis progression and thus provides plausible target for bifunctional liposome targeting. BF-ELIP has anti ICAM-1 antibody conjugated to the liposomal membrane. The BF-ELIP mediates stem cell adherence to the inflamed endothelial cells. Ultrasound plays a crucial role in stem cell delivery mediated by BF-ELIP. The

results of this project indicated that groups exposed to ultrasound had a higher rate of stem cell migration and adherence.

To understand the role of ultrasound in BF-ELIP targeting enhancement ,previous experiments indicated that ultrasound increased the endothelial permeability by causing temporary disruption of the cellular membrane that makes the delivered component to stream through .The membrane disruption results from the ultrasonic contrast agent that effect on the acoustic components of the cellular membrane causing the intracellular molecules porosity that lead to increase the endothelial permeability.Also intrasound pulses causing proposity in the intracellular matrix Therefore, the migration rate of stem cells is increased due to high permeability endothelial layer exposed to ultrasonic pulses. (Juffermans et al., 2003).

Ultrasound also enhances stem cells adherence. The results of this study indicate that all stem cells samples exposed to ultrasound pulses had a very high rate of adherence to the endothelial monolayer supporting the hypothesis that ultrasound enhances cellular delivery. The contrast ultrasound pulses induce the mononuclear cells pass through and adherent to the endothelial monolayer and it can be optimized by liposomal delivery to the endothelial layer.

Bifunctional ELIP promotes enhanced stem cell adherence with and without ultrasound e because of the conjugated of ICAM-1 antibody which binds to endothelial ICAM-1 that is expressed under inflammatory conditions.

Future work following this project will focus on demonstration of efficacy in vivo since the hypothesis of this project has been supported in-vitro. Further

investigation is also needed regrading mechanism of stem cell adherence enhanced by ultrasound. We also need to investigate the mechanisms of ultrasound mediated the increased cellular permeability and stem cells passage.

Angiogenesis in atherosclerotic arteries is mediated by VEGF. Cell proliferation can be induced by many intracellular pathways. VEGF-induced cellular proliferation can be inhibited by bevacizumab. Although bevacizumab is approved for tumour growth inhibition through blocking of VEGF activity, but it has devastating side effects. PMA and dPPA have been used to induce VEGF expression in vitro through stimulation of Protein Kinase A and C.

Treating cells with high concentrations of these components over time will cause to cells apoptosis and to activation of protease that cause cell proliferation regression (Park et al., 2001).

Therefore, these components were titrated to determine the optimal concentration for mediating cell proliferation with minimal toxicity. The dual cellular treatment with protein kinase activators and bevaciamab as VEGF blocker will severely inhibits cell proliferation because bevaciamab blocked VEGF activity while the protein kinase activator as general activator induce another pathways protease activity.

Protein kinase A and C mediates VEGF expression when they translocate from the cytoplasm to the endoplasmic reticulum. The translocated PKA and PKC alpha type will lead to induction VEGF expression by increasing VEGF mRNA synthesis (Mamputu et al., 2002).

Forskolin has been used to investigate the relationship between VEGF expression and PKA activation alone. Forskolin is considered a Protein Kinase A activator through the cAMP signalling pathway. Short period of treatment with foskolin led to VEGF expression mediated by protein kinase A. Activation of either PKC or PKA is sufficient to induce VEGF expression whether the protein kinase is translocated or not. Thus, forskolin shows that activation of PKA alone is sufficient to induce VEGF expression with no needs for both PKA and PKC.

It is expected that liposomes will provide a method to reduce bevacizumab side effects, while affording a controlled release formulation for delivery of the drug

This project demonstarted that ultrasound enhanced the liposomal release of bevacizumab optimizing VEGF inhibition. The liposomal release enhancement by ultrasound is caused by the cavitation effects of ultrasound pulses. That disrupt the lipid bilayers realeasing the encapsulated bevacizumab.Ultrasonic pulses are also responsible for increasing the endothelial permeability by disrupting the extracellular matrix and induce the porosity of cellular membrane and the intracellular molecules in endothelial cells that lea in the end to increase the drug uptake by the cell (Juffermans et al., 2009; Cho et al., 2002).

Thus, the results obtained with cell based assay provided enough evidences to demonstrate BEV-ELIP efficacy with and without ultrasound in vitro.Furthermore, the results obtained in this project showed that even a low concentration of BEV-ELIP inhibits VEGF expression instead of using a high concentration of bevacizumab to reach the same inhibition rate.

Thus, a low dose of bevacizumab released from ELIP by ultrasound can achieve the same effect as a high concentration of bevaciamab alone with fewer side effects.

As future steps of this work, as vivo investigation is required to demonstrate the efficacy of BEV-ELIP enhanced by ultrasound to reduce atheroma progression in an animal model as part of translational development of a therapeutic approach for bevacizumab-mediated pathway to reduce atheroma progression in the clinic.

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