CO-CULTURE OF UCMSC DIFFERENTIATED TO SMC'S AND HUVEC ON A GELATIN VINYL ACETATE COPOLYMER BLENDED WITH POLY (ε-CAPROLACTONE) (GeVAC PCL) SCAFFOLD FOR VASCULAR TISSUE ENGINEERING –THE ROLE OF NITRIC OXIDE SYNTHASE ACTIVITY

A DISSERTATION SUBMITTED

BY

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DECLARATION

I, **Anupama Nair**, hereby declare that I had personally carried out the work depicted in the thesis entitled "**Coculture of UCMSC differentiated to SMCs and HUVEC on a GeVAc-PCL scaffold for Vascular Tissue Engineering- The role of Nitric Oxide Synthase activity**" under the direct supervision of **Dr. Prabha D. Nair, Scientist G, Division of Tissue Engineering and Regeneration Technologies,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Coculture of UCMSC differentiated to SMC'S and HUVEC on A GeVAc-PCL scaffold for Vascular Tissue Engineering- The role of Nitric Oxide Synthase activity**" submitted by **Anupama Nair** in partial fulfilment for the degree of Master of Philosophy Technology in Biomedical Research to be awarded by this Institute. The entire work was done by her under my supervision and guidance at **Division of Tissue Engineering and Regeneration Technologies**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, 695012.

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COCULTURE OF UCMSC DIFFERENTIATED TO SMC'S AND HUVEC ON A GeVAc-PCL SCAFFOLD FOR VASCULAR TISSUE ENGINEERING- THE ROLE OF NITRIC OXIDE SYNTHASE ACTIVITY

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LIST OF ABBREVIATIONS

ABAM	Antibiotic/antimycotic
α-SMA	Alpha smooth muscle actin
CD	Cluster of Differentiation
Chol-PCL	Cholesterol-Poly (ε-Caprolactone)
CNN1	Calponin 1
Dex-MA-LA	methacrylated dextran-graft-lysine
DMEM-HG	Dulbecco's Modified Eagle's Minimal media High Glucose
DMEM-LG	Dulbecco's Modified Eagle's Minimal media –Low Glucose
EC	Endothelial cell
ECM	Extra Cellular Matrix
EDNO	Endothelial Derived Nitric Oxide
EDRF	Endothelial-derived Relaxing Factor
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
ePTFE	expanded Polytetrafluroethylene
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
Gel-MA	methacrylamide-modified gelatin

GeVAc	Gelatin Vinyl Acetate Co-polymer
GFAP	Glial Fibrillary Acid Protein
HLA	Human Leukocyte Antigen
hSMC	human Smooth Muscle Cell
HUVEC	Human umbilical vein endothelial cell
IAP	Inhibitor of Apoptosis Protein
IBMX	isobutylmethylxanthine
IFN-β	Interferon beta
IL-1b	interleukin 1-b
iNOS	induced Nitric Oxide Synthase
JAG1	Jagged 1
LDL	Low Density Lipoprotein
MA	Macrophages
MHC	Major Histocompatibility complex
MSC	Mesenchymal Stem Cell
MVEC	Microvascular Endothelial Cells
MYOCD	Myocardin
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSE	Neuron Specific Enolase

PBS	Phosphate Buffered Saline
PCL	Poly(ε-caprolactone)
PDO	Poly (p-dioxanone)
PET	Polyethylene terephthalate
PGA	Poly(glycolic acid)
PIGF	Placenta Growth Factor
PLGA	Poly (lactide-co-glycolide)
PLLA	Poly (L-lactic acid)
S1P	sphingosine 1-phosphate
SMC	Smooth Muscle Actin
SM-MHC	smooth muscle-myosin heavy chain
TE	Tissue Engineering
TEVG	Tissue Engineered Vascular Graft
TF	Tissue Factor
TGF-β1	Tumour Growth Factor beta1
UCMSC	Umblical cord mesenchymal stem cell
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
vSMCs	Vascular Smooth Muscle Actin
vWF	Von Willibrand Factor

WJMSC	Wharton jelly mesenchymal stem cell
XIAP	Xenopus Inhibitor of Apoptosis

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SYNOPSIS

Cardiovascular diseases are major cause of mortality and disease in the Indian subcontinent causing more than 25% death. Heart diseases are found to occur in India 10 to 15 years earlier than in west. Current projections suggest that India have the largest cardiovascular disease burden in the world. One fifth of the deaths in India are from coronary heart disease. Natural synthetic grafts like collagen has been used but they cause low burst pressure due to lack of mechanical integrity. Synthetic scaffolds like expanded polytetrafluroethylene (ePTFE) and Dacron has been used with reasonable patency as large diameter vascular grafts (>6mm diameter). But in the case of small diameter vascular graft constructs their limitations include lack of growth potential, compliance mismatch between prosthesis and native arteries, stenosis, thrombo-embolism ,calcium deposition and infection.

Small diameter vascular graft has been made with natural lecithin incorporated into cholesterol-poly ε-caprolactone) (Chol-PCL) by solution blending in order to modify the performance of the hydrophobic and bio-inert PCL. Polymer nanofibers scaffolds fabricated by electrospinning are among the most promising biomaterials for native extracellular matrix (ECM) analogs.

. A blood vessel consists of tunica externa (including connective tissue, smooth muscle cells etc), tunica media (including smooth muscle cells) and tunica intima (including endothelial cells). Tunica intima serves as an anti-thrombogenic surface and a structural barrier between the circulation and the surrounding tissue. ECs control the entry of leukocytes into tissues where NO helps to block leukocyte adhesion onto the intima. SMCs were found to control the tone of the vessel wall. They are normally in quiescent state and express high levels of contractile proteins like smooth muscle cell α-actin and γ-actin, calponin, myosin, and myosin heavy chain kinases.

Vascular tissue engineering applies the principles and methods of engineering to biological sciences in an attempt to create viable replacements of deficient natural blood vessel, like a properly aligned smooth muscle and endothelial cell layer. For a successful

replacement ‘‘artery’’, it must be infection-resistant, biocompatible (noninflamatory, nontoxic, non-carcinogenic, nonimmunogenic) and biologically stable, leak-proof and thromboresistant, but with adequate porosity for healing and angiogenesis. Various types of stem cells like adipose derived stem cells, mesenchymal stem cells, bone marrow stem cells etc have been used in vascular graft study. Studies showed that TGF-b1 upregulated the Notch ligand Jagged 1 (JAG1) in MSCs, which was necessary for the upregulation of various smooth muscle markers, which suggest that JAG1 ligand expression, and hence, Notch activation, mediates TGF-β1 signaling during MSC differentiation into SMC. Hence **the first hypothesis deals with the differentiation of umbilical cord derived mesenchymal stem cells to smooth muscle cells under the influence of a biochemical signal (TGF β1) on GeVAc PCL82 scaffold.**

Several co-culture systems have been developed to study EC–SMC interactions on a vascular graft or without. These include (1) culture of SMCs and ECs on opposite sides of membranes; (2) culture of ECs on collagen gels containing SMCs; (3) micro carrier/spheroid-bound ECs or SMCs ; (4) conditioned media; and (5) culture of ECs directly on SMCs**. Thus the second hypothesis deals with the direct co culture of HUVEC with UCMSC differentiated to SMCs on a GeVAc PCL82 scaffold.**

Nitric oxide is of much concern on dealing with coculture studies which in turn is related to the VEGF production in angiogenesis. Hence **the third hypothesis deals with the role of nitric oxide in direct co culture model involving HUVEC and UCMSC differentiated to smooth muscle cell on a GeVAc PCL82 scaffold.**

In this thesis, the **first chapter** deals with statistics of cardiovascular disease, challenges in vascular tissue engineering, anatomy of blood vessel, major complications regarding cardiovascular system, cells used in cardiovascular tissue engineering, various coculture models and the role of nitric oxide synthase.

The **second chapter** mainly involves the materials and methods employed in this study. This includes fabrication of GeVAc PCL82 scaffold, isolation of HUVEC and UCMSC;

characterization of UCMSC by FACS (Fluorescent Activated Cell Sorting),immunostaining (for actin) and bilineage study by differentiation to chondrocytes(safranin O staining) and adipocytes(oil red O staining);Biocompatibility of GeVAc PCL82 by *In vitro* cytotoxicity test by direct contact assay, Scanning Electron Microscope (SEM) Analysis, cell proliferation assay of HUVEC and UCMSC on GeVAc PCL82 scaffold. Differentiation of UCMSC to SMC by analyzing via immunostaining for alpha SMA markers, SEM analysis and RT PCR; Characterisation of HUVEC by immunostaining for actin, VCAM, PECAM, von willibrand and Ac LDL uptake; characterization of co culture of HUVEC with UCMSC differentiated to SMCs by immunostaining for vWF and alpha SMA, SEM analysis and RT PCR; nitric oxide based studies using NO assay and RT PCR.

The third chapter deals with the results and discussion. GeVAc PCL82 scaffold was fabricated and analyzed via SEM imaging and diameter of the scaffold was found to be 1.07±0.36 µm and pore size was 3.75± 0.75 µm which can infiltrate within the scaffold; characterization of UCMSC showed the stemness is more prominent in P4,its ability to go to bilineage (chondrocyte and adipocyte) and actin expression via immunostaining; biocompatibility of GeVAc PCL82 was proven by direct contact assay giving a score of 0 and SEM analysis to UCMSC and HUVEC and cell proliferation assay which showed no significant change in DNA content on day 3,5 and 7. UCMSC differentiation to SMC was proven by immunostaining where 5ng/ml of TGF beta 1 gave good alpha SMA staining results, SEM images gave typical hill and valley morphology of differentiated UCMSC to SMCs and RT PCR showed the increased fold expression of SM22 and calponin on day 7 of differentiation proving an increased SMC marker expression on day 7 of differentiation. Coculture was characterized via confocal imaging which showed vWF and alpha SMA by HUVEC and UCMSC differentiated to SMC respectively and SEM imaging on GeVAc PCL82 in direct coculture model. Real Time PCR showed lower expression of VEGF on day 7 of coculture due to short half life of the same. Nitric oxide assay which detected the NO release by single and coculture models. Real time PCR showed increase in iNOS and eNOS when compared to UCMSC control on

differentiation showing higher angiogenic capability whereas in coculture eNOS was highly

The fourth chapter deals with the summary and conclusion where GeVAc PCL82 was synthesized successfully and differentiation of UCMSC to SMCs was illustrated on this scaffold. These differentiated cells were grown successfully in coculture with HUVEC on GeVAc PCL82 scaffold and eNOS and iNOS expressions were found to be higher in coculture model proving the capability to hold direct coculture along with angiogenesis. VEGF was also quantified and angiogenesis capability of this direct coculture model was confirmed.

Future directions can be towards development of a mechano-stimulation model using a bioreactor and the assessment of NOS activity as well as angiogenic markers like VEGF etc. Also we can see the differentiation pattern of UCMSC in the presence of HUVEC without any growth factor under mechanical stimulation via quantifying through RT PCR.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

According to World Health Organisation (WHO), Cardio Vascular Diseases (CVD's) are the number one cause of death globally. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths per year according to WHO statistics. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. It has been reported that the number of people who die from CVDs, mainly from heart disease and stroke, will increase to 23.3 million by 2030.

Many therapeutic and surgical approaches have been put forward in order to combat the disease, such as atherectomy, stenting, vascular bypass graft surgery etc of which vascular grafts are found to be the most widely used technology. Natural synthetic grafts like collagen has been used but they cause low burst pressure due to lack of mechanical integrity. Synthetic scaffolds like expanded polytetrafluroethylene (ePTFE) and Dacron has been used with reasonable patency as large diameter vascular grafts (>6mm diameter). But in the case of small diameter vascular graft constructs their limitations include lack of growth potential, compliance mismatch between prosthesis and native arteries, stenosis, thromboembolism ,calcium deposition and infection. Treatment of risk factors and a plethora of drugs are planned to be replaced by the tissue engineering (TE) which applies the principles of biology and engineering to develop functional substitutes for damaged tissue, such as generating new cardiac tissue, blood vessels, cardiac tissue and heart valves which would become commercially available with the proper FDA approval prior to a clinical application. (Thomas *et al,* 2003*;* Conklin *et al,* 2002*)*

For a proper graft development, one needs to know about the factors responsible for the formation of a blood vessel *in vivo* and *in vitro* (Isner,J.M, 1998). In the recent years, scaffold dependant and scaffold free techniques are being used among which the scaffold system provides a basement or a microenvironment for the cells of the artificial vascular system to grow .The biodegradation rate of the system should be such that it gives enough time for the cells to produce new tissue, provides sufficient mechanical stability to the vascular graft, and its complete degradation after the new vessel is formed. To develop a biomaterial with such properties, electro spinning seems to be the most useful technique due to the possibility to obtain nanofibrillar scaffolds starting from polymeric solution, which is mostly applicable for small diameter vascular graft (Florica *et al,* 2012).

At present there is no FDA approved small vessel graft of 5mm or smaller. Small diameter grafts currently in use are Dacron, expanded polytetraflouroethylene, autologous sephenous vein, umbilical vein graft which generally perform very poorly. The problems include increased risk of thrombosis and infection, limited durability, lack of compliance both of the graft and around the anastomosis, and failure due to restenosis, thus necessitating further interventions (Bhat VD *et al,* 1998). Pre-seeding synthetic graft materials such as expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (PET) with endothelial cells (EC) has been examined in various *in vitro* and *in vivo* models (Kannan *et al*, 2005; Rashid *et al,* 2004). PCL (ε-Polycaprolactone), another synthetic matrix for growing vascular cells has been proven to form more uniform endothelial cells compared to expanded PTFE (ePTFE) (Pektok E *et al,*2008). Small diameter vascular graft has been made with natural lecithin incorporated into cholesterol-poly ε-caprolactone) (Chol-PCL) by solution blending in order to modify the performance of the hydrophobic and bio-inert PCL. Polymer nanofibers scaffolds fabricated by electrospinning are among the most promising biomaterials for native extracellular matrix (ECM) analogs. The electrospinning technique consists of applying a high voltage to a polymer solution, causing the solvent to evaporate and obtaining this way a continuous jet of nanofibers. The advantage of this process is that fibers can be

collected directly into a rotating cylindrical mandrel to get tubular scaffolds with controlled morphology. Many combinations of vascular graft that have been put forward like surface modification on electrospun nanofibers with natural proteins, such as collagen or gelatin, was found to be an effective way to promote ECs spreading and proliferation. Examples can be found for collagen-grafted PCL (Ma et al., 2005a) and gelatin-grafted polyethylene terephthalate (PET) nanofibers (Ma et al., 2005b).

Currently several groups are working towards the development of ‘living grafts’, seeded grafts and hybrid grafts. Endothelial and smooth muscle cell co-culture system has been adopted to develop a vascular graft which mimics exactly the *in vivo* model (Sakamoto *et al,* 2011), even though a hundred percent exact model cannot be reproduced as such. Major problems to the generation of functional tissues in a co-culture model and their widespread clinical use are related to a limited understanding of the regulatory role of specific physicochemical culture parameters on tissue development, and high manufacturing costs of the few commercially available engineered tissue products. Bioreactor systems enable to reproduce and control changes of specific environmental factors, providing means to reveal basic mechanisms of cell function in a 3D environment, and the ability to improvise the quality of engineered tissues. In addition, by automating and standardizing tissue manufacture in regulated closed systems, bioreactors could lower production costs and thus facilitating a broad use of engineered tissues (Martin *et al,* 2004).

Stem cells have come into the scene with much more advantageous effects in developing the vascular graft model. Various types of stem cells like adipose derived stem cells, mesenchymal stem cells, bone marrow stem cells etc have been used in vascular graft study. Development of a procedure using stem cells and progenitor cells and switching these into smooth muscle cells and endothelial cells is an important and exciting advance in the field of tissue engineering. Kurpinski, Lam, Chu et al.(2010) reported that both TGF-beta1 and Notch activation induced the upregulation of smooth muscle gene/protein expression in bone marrow MSCs. Their studies showed that TGF-β1 upregulated the

Notch ligand Jagged 1 (JAG1) in MSCs, which was necessary for the upregulation of various smooth muscle markers, which suggest that JAG1 ligand expression, and hence, Notch activation, mediates TGF-b1 signaling during MSC differentiation into SMC (Kurpinski *et al*,2010). Mesenchymal stem cells are used much in therapeutics just because of their high proliferation rate, self-renewal and immense potential to differentiate have found to contribute much to the tissue architecture maintenance and healing properties. They lack specific tissue related characteristics and dynamics have been found much interesting, especially their ability to differentiate into specialized cell types. Thus keeping all this together we have used umbilical cord derived mesenchymal stem cells as well as human umbilical vein endothelial cells (HUVECs) to in a coculture model on a PCL GVAC scaffold to develop an in vitro model for blood vessel.

In this study we focus onto the differentiation of UCMSC into SMCs on a GeVAc PCL82 scaffold and affect of coculture model in nitric oxide synthase (NOS) activity which can be related to vasorelaxation as well as prevention of thrombosis.

1.2 REVIEW OF LITERATURE

The cardiovascular system transports the blood throughout the body and in turn is a nutrient transporting system and removes gaseous waste from the body. In general this system is comprised of the heart and the circulatory system which include blood vessels, and blood. The lymphatic system is also closely associated with the cardiovascular system. The blood vessels that carry blood away from the heart are called arteries, and their very small branches are arterioles.

1.2.1Anatomy of blood vessel

These blood vessels consist of three layers, the intima, media and adventitia:

1. **Tunica externa** (Latin for outside coat) or tunica adventitia (Latin for coat that comes first): is the outermost layer and forms a connective tissue sheath. In arteries, this layer contains collagen fibers with scattered bands of elastic fibers. In veins, it is generally thicker than the tunica media and contains networks of elastic fibers and bundles of smooth muscle cells. The connective tissue fibers of the tunica externa typically blend into those of adjacent tissues, stabilizing and anchoring the blood vessel.

2. **Tunica media** (Latin for middle coat) is the middle layer: Contains concentric sheets of smooth muscle tissue in a framework of loose connective tissue. The collagen fibers bind the tunica media to the tunica intima and tunica externa. Commonly the thickest layer in the wall of a small artery, the tunica media is separated from the surrounding tunica externa by a thin band of elastic fibers called external elastic membrane. The smooth muscle cells of the tunica media encircle the endothelium lining the lumen of the blood vessel. When these smooth muscles contract, the vessel decreases in diameter; when they relax, the diameter increases. Large arteries also contain layers of longitudinally arranged smooth muscle cells.

3. **Tunica intima** (Latin for innermost coat) is the innermost layer: Includes the endothelial lining and an underlying layer of connective tissue with a variable number of

elastic fibers. In arteries the outer margin of the tunica intima cointains a thick layer of elastic fibers called the internal elastic membrane.

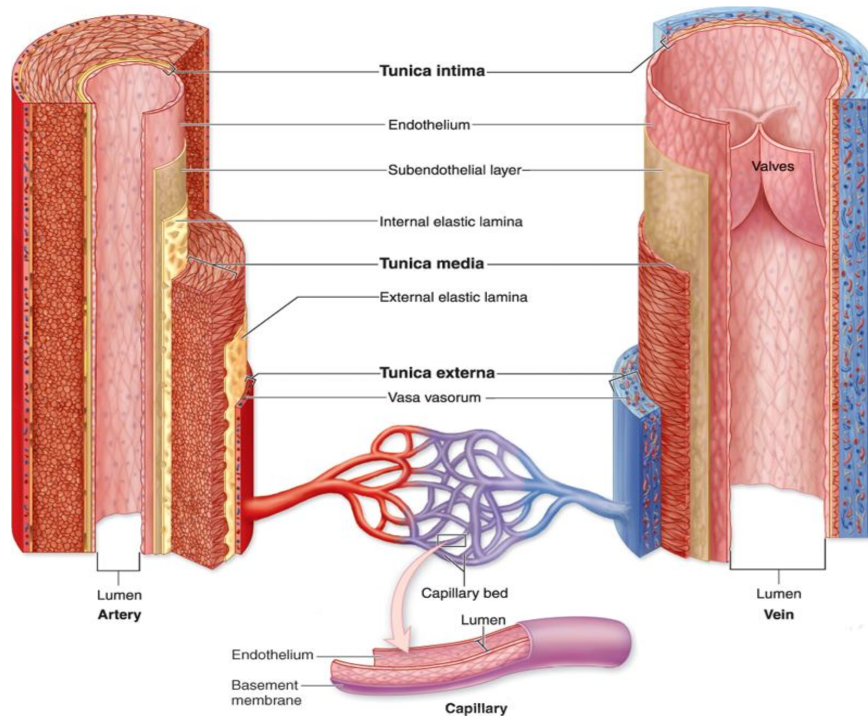


Figure 1.1: Anatomy of blood vessel

Very small branches that collect the blood from the various organs and parts are called venules, and they unite to form veins, which return the blood to the heart. Capillaries are minute thin-walled vessels that connect the arterioles and venules; it is through the capillaries that nutrients and wastes are exchanged between the blood and body tissues.

These blood vessels comprise of cells that include endothelial cells (ECs) and smooth muscle cells (SMCs), forms the major cellular components of arteries and veins. ECs continuously line the interface between blood and tissue and are present in all blood vessels. The intima is the layer closest to blood and the endothelium forms a continuous lining at this region in contact with blood. They serve as an anti-thrombogenic surface and a structural barrier between the circulation and the surrounding tissue. ECs control the entry of leukocytes into tissues where NO helps to block leukocyte adhesion onto the

intima. EC's inhibit platelet deposition and aggregation (clot formation) because of the secretion of molecules like tissue factor (TF) pathway inhibitor, tissue plasminogen activator, prostacyclin and nitric oxide (NO) and also express anticoagulants on their cell membrane, which include heparin sulfate, ADPase, and thrombomodulin. During vessel injury ECs promote thrombosis which activates tissue factor and secretion of type-1 plasminogen activator inhibitor (PAI-1) and von Willebrand factor (vWF) (Thomas *et al,* 2003).

On the other hand, SMCs were found to control the tone of the vessel wall. They are normally in quiescent state and express high levels of contractile proteins like smooth muscle cell α-actin and γ-actin, calponin, myosin, and myosin heavy chain kinases (Conklin *et al , 2002*). Multifunctional SMC's vary in their functions during different stages of vascular development. During early stages of vasculogenesis SMCs are highly migratory and undergo very rapid cell proliferation. It has been reported that SMC progenitor cells participate in complex morphogenic events that lead to the formation of cardiovascular system during which SMCs also exhibit very high rates of synthesis of extracellular matrix components including elastin, collagen, proteoglycans and integrins that comprise a major part of the blood vessel. At this developmental stage, SMCs form much more gap junctions with endothelial cells, and this process is critical for vascular maturation and vessel remodeling (Bhat VD *et al,* 1998). The fully differentiated SMC has been found to express specific receptors, signal transduction molecules, ion channels, calcium regulatory proteins, and contractile proteins which are required for the unique contractile properties of the SMC (Kannan *et al,* 2005).

1.2.2 Complications in blood vessel

Most important reason for the long-term failure of small-diameter (<6mm) vascular grafts is intimal hyperplasia, which is characterized by a cascade of immunoreactions caused by contact between the blood and foreign materials, ultimately leading to atherosclerosis. These immunoreactions, including formation of thrombus; infiltration of macrophages, neutrophils, and monocytes; secretion of cytokines; ingrowth

and proliferation of smooth muscle cells (SMCs) and production of extracellular matrix (ECM); and so on, lead to abnormal tissue growth inside the vascular grafts, thereby blocking blood flow and finally causing the failure of the grafts(Zuwei Ma *et al,*2005). Atherosclerosis is one of the major complications in cardiovascular disease. It is the usual cause of heart attacks, strokes, and peripheral vascular disease, together called as “cardiovascular disease”. One fifth of the deaths in India are from coronary heart disease. By the year 2020, it will account for one third of all deaths. "Atherosclerosis starts when high blood pressure, smoking, or high cholesterol damages the endothelium," says Richard Stein, MD, national spokesperson for the American Heart Association. "At that point, cholesterol plaque formation begins."Cholesterol invasion. Bad cholesterol, or LDL, crosses damaged endothelium. The cholesterol enters the wall of the artery leading to plaque formation. White blood cells stream in to digest the LDL cholesterol. Over years, the accumulating mess of cholesterol and cells becomes a plaque in the wall of the artery. During atherogenesis, activated SMCs migrate into the intima, where they come into close contact with endothelial cells (ECs) lining the vessel wall (Zhou *et al,* 2013). In the atherogenic vessel, SMC apoptosis occurs and is followed by proliferation of the remaining cells, thus inducing destabilization and vulnerability of the lesion plaques. Endothelium-dependent vasodilation of conduit vessels is abnormal in the presence of atherosclerosis because the thickened intima associated with atherosclerosis may act as a barrier to vaso-active substances released from the endothelium as well as because progression of atherosclerosis leads to an injury of the endothelium itself.

Endothelium is found to be the main site of action in most vascular graft failures which it is the main target for antibodies in autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis and Kawasaki disease. Aberrant EC function can therefore lead to atherogenesis, bleeding disorders, autoimmune disorders, graft rejections or even embryonic lethality (Diane Bouis *et al*, 2001).

Native blood vessels consist of several layers with vascular smooth muscle cells (vSMCs) embedded into ECM and an EC layer on the luminal surface. Autologous transplantation of conduits such as saphenous veins and mammary arteries is often used

in arterial bypass. Patients requiring bypass surgery, however, may not have vessels available due to disease or previous surgery. Synthetic, non-degradable vessels have been used in such cases. Small-diameter vascular grafts are more challenging than the large diameter grafts regarding its patency. Success rates in large diameter grafts (>6mm) have been satisfactory with patency rates of 95% after 5 years. Conversely, the patency rate of small-diameter grafts (< 6mm) was reported to be only 30% in the same study.11 Other studies demonstrated patency rates such as 0% to 25% after only weeks or months of implantation in various animal models Complications resulting from noncompliance, thrombogenicity, intimal hyperplasia, aneurysms, and calcium deposition contribute to these low patency rates. While much graft research has focused on coronary artery bypass procedures, the need for small-diameter vascular grafts extends far beyond coronary artery disease (Melchiorri *et al*, 2013).

1.2.3 Role of Tissue engineering

Vascular tissue engineering applies the principles and methods of engineering to biological sciences in an attempt to create viable replacements of deficient natural blood vessel, like a properly aligned smooth muscle and endothelial cell layer. The option of creating living blood vessels from autologous cells offers many potential advantages compared to traditional synthetic is the main idea behind vascular tissue engineering (Hoerstrup *et al*, 2001). For a successful replacement ''artery'', it must be infection-resistant, biocompatible (noninflamatory, nontoxic, non-carcinogenic, nonimmunogenic) and biologically stable, leak-proof and thromboresistant, but with adequate porosity for healing and angiogenesis. These attributes are usually provided within an artery by an intact endothelium, which also acts as a secretory tissue and selective permeability barrier; have appropriate mechanical properties (strength, kink resistance, compliance, good suturability). The vessel must be strong enough to withstand considerable internal pressure before bursting, without kinking when in position. It must also allow sutures to hold under longitudinal and circumferential tension and retain axial and radial compliance and pulsatility; possess appropriate vasoactive physiological properties like the ability to constrict or relax in response to neural or chemical stimuli; and be able to be manufactured cheaply in a relatively short space of time, and in sufficient numbers with differing specifications (diameter, length, etc.) to meet commercial demand and fit the graft (Anitha *et al*, 2003).

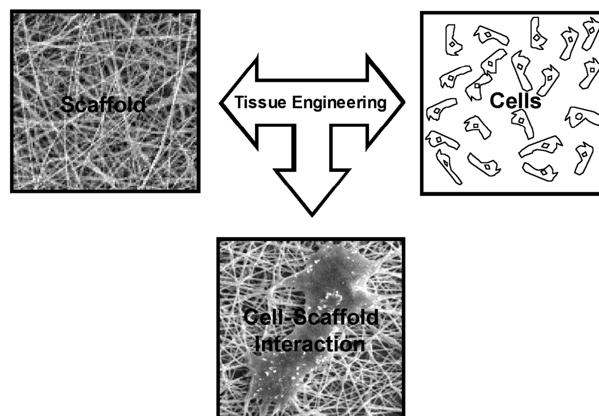


Figure 1.2: Role of Tissue Engineering

The three major categories involved in tissue engineering concept include :

- Scaffold
- Cells
- Growth factors cues

1.2.4 Vascular tissue engineering Scaffold

Scaffolds play an important role in supporting the cells to accommodate and guide their growth into a specific tissue; therefore, designing scaffolds that are favorable to cellular growth is of great importance. Electrospinning is a straightforward, cost-effective, and versatile technique that has been applied recently for the fabrication of nano-featured scaffolds suitable for tissue engineering. By mimicking a natural extracellular matrix more efficient artificial tissue can be constructed.

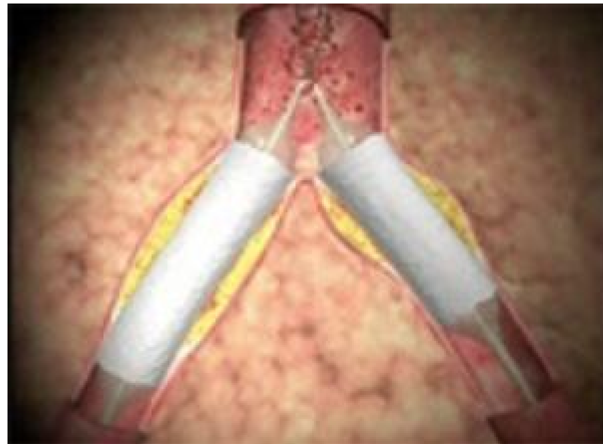


Figure 1.3: Implanted vascular graft

Various 2D and 3D scaffolds have been implied to grow these cells in close proximity and to replicate a blood vessel model. Scaffolds are platform that mimics the functions of native Extra cellular matrix (ECM) (Lavendera *et al,*2005). The fundamental stages in the fabrication of a tissue engineered vascular substitute are: (1) expansion of cells and infiltration into a scaffold, (2) maturation of the cell-scaffold construct to achieve the necessary mechanical and biological properties and (3) surgical implantation into the host (Alperin *et al,* 2005). Bioresorbable materials are most chosen

for the applications in scaffold for various implants. Thus the most commonly used polymers for these applications include poly (L-lactic acid) (PLLA), poly (glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA), poly(p-dioxanone) (PDO),collagen, Polyurathane and poly(ε-caprolactone) (PCL). The degradation rate of these polymers is: 6-8 months for PLA, 3-6 months for PGA, 12 months for PCL, 3-6 months for PDO. Scaffold design can be improved by controlling scaffold parameters such as pore size and pore distribution, as well as incorporating an artificial vascular system, thereby increasing the mass transport of oxygen and nutrients into the interior of the scaffold and supporting cellular growth in that region(Tang *et al,* 2010).

Synthetic polymers applied in vascular tissue engineering, polyglycolic acid (PGA) has been the most frequently used, along with polycaprolactone (PCL) and poly-4-hydroxybutyrate. These degradable synthetic scaffolds serve to provide temporary mechanical stability until new tissue has been synthesized, organized, and cross-linked into a stable structure by seeded cells (Tschoeke *et al,* 2008).

Current biodegradable grafts are damaged by inflammation, setting the scene for thrombosis or long-term aneurysm formation mainly due to byproducts leached out during degradation process. Therefore the search for biocompatible scaffold is still ongoing in the area of small diameter vascular grafts (Aper *et al*, 2004).

1.2.5 Cells in vascular tissue engineering

Tissue engineered vascular grafts should fulfill the ideal characteristics present in the artery. Various endothelial and smooth muscle cell sources have been used. Endothelial sources mostly in use are bovine aorta, EPC (endothelial progenitor cells from blood), microvascular endothelial cells, rat aortic endothelial cells, HUVEC etc. Vascular SMC progenitors have been identified in the developing embryo, indicating substantial variation in derived SMCs and features within the vascular system. Biopsied skin samples may serve as alternative vascular SMC sources for vascular engineering. Skin-derived precursor cells (SKPs) can be manipulated to differentiate into SMCs. human MSCs cultured in the presence of TGF-β1 were also able to differentiate into

SMCs (Chen *et al,* 2011). In recent years, umbilical cord tissue has gained attention as a source of multipotent cells. Due to its widespread availability as these tissues are usually discarded as biological waste, the umbilical cord may be an excellent alternative source of cells for regenerative medicine. Wharton's jelly is the major component filling the inner part of the umbilical cord tissue, and it has been commonly used as a source of obtaining multipotent stem cells from umbilical cord (Jeschke *et al* 2011). These cells obtained from the sub-endothelial layer of the umbilical vein has the capacity to differentiate in vitro into adipocytes and osteoblasts and when isolated from umbilical cord jelly they can also differentiate in vitro and in vivo into a myogenic lineage, confirming the presence of plasticity in this population of foetal-derived tissues (Burra *et al* ,2012).

In cardiac tissue engineering using UCMSC represents a promising approach for the repair of the injured heart. Functional regeneration of heart tissue after cardiomyodegenerative diseases should be demonstrated by the integration of UCMSC-seeded implants and/or their interaction with resident cardiac stem cells. In addition, survival of implanted constructs, tissue-specific differentiation and vascularisation have to be verified. Moreover, electric integration resulting in functional reconstitution of the injured muscle tissue is a key step in the evaluation of safety and efficiency of UCMSC-seeded implants (Hollweck *et al*, 2012).

Among many tissue-originated multipotent stem cells, UCMSC are very usable due to their abundance, low immunogenicity, lack of CD34 and CD45 expression, and simplicity of the methods for harvest and *in vitro* expansion. UCMSC immune properties of hypoimmunogenicity and immune suppression is mainly contributed by UCMSC ability to express human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I, but not HLA MHC class II and co-stimulatory molecules such as CD40, CD80 and CD86. The hypo-immunogenic character of MSC allows their use in xenotransplantation studies without obvious immune response(Jun Sun, 2011).These properties of UCMSC encourage their development as therapeutic tools or agents because they can potentially be used for allogeneic transplantation. In addition, human UCMSC

engineered to express IFN-β induce death of human breast adenocarcinoma cells and bronchioloalveolar carcinoma cells *in vitro* and *in vivo*.

Recent research has demonstrated that Wharton jelly MSC express mesodermal markers like vimentin and smooth muscle actin. Endodermal markers like Gata-4, Gata-5, Gata-6, HNF4-α; and neuro-ectodermal markers like nestin, neuron specific enolase (NSE) and glial fibrillary acid protein (GFAP) are also expressed. Thus these cells can differentiate towards different mature cell types derived from all three germ layers (Melania Lo Iacono *et al*, 2011). As for other MSC populations, according to the Position Statement of the International Society for Cellular Therapy, WJ-MSC is defined as multipotent stem cells since they can differentiate into at least three different cellular lineages: osteoblasts, adipocytes and chondrocytes (Dominici M, Le Blanc K, Mueller I, *et al,* 2006).

Differentiation of Mesenchymal Stem Cells (MSCs) towards vascular phenotypes can be distinguished by the expression of experimentally identified specific markers or by functional assays. For smooth muscle cell (SMC) differentiation, markers specific to this cell type include cytoskeletal proteins α-smooth muscle actin (α -SMA), smooth muscle myosin, SM22 and calponin (C. H. P. Arts *et al*, 2002). On the other hand, endothelial cell (EC)-specific markers include platelet-endothelial cell adhesion molecule 1 (CD31), vascular endothelial (VE)-cadherin and EC-synthesized glycoprotein von Willibrand Factor (vWF). Functionally, ECs are also characterized by their ability to form tube-like structures in matrigel and uptake acetylated low-density lipoproteins. Studies have shown that culturing ESCs on collagenIV induced ESC differentiation into vascular precursor cells that could differentiate into endothelial cells and SMCs (Nishikawa SI, Nishikawa S, Hirashima M *et al*,1998; Yamashita J, Itoh H, Hirashima M *et al* ,2000). In this study UCMSC differentiation was done based on the SMC marker mRNA expression levels and sustained SMC marker expression by MSC's cultured on the EC-matrix by Lozito *et al.* and Ball et al. who demonstrated the direct co-culture of bone marrow MSCs with

endothelial cells resulted in increased smooth muscle alpha-actin mRNA and protein (Stephen *et al,* 2004; Lozito *et al,* 2009).

The cells which form the most part of tunica media and some part of tunica externa are **smooth muscle cells (SMCs)**. Their phenotype ranges from the growth arrested contractile phenotype present in the normal vessel wall to the proliferating and synthetic phenotype seen in culture or during atherosclerosis (Lavender *et al,* 2005). SMCs control the tone of the vessel wall. They are normally in quiescent state and express high levels of contractile proteins like smooth muscle cell α-actin and γ-actin, calponin, myosin, and myosin heavy chain kinases (Owens GK, 1995). Vascular SMCs are multifunctional and their functions vary during different stages of vascular development. During early stages of vasculogenesis SMCs are highly migratory and undergo very rapid cell proliferation. SMC progenitor cells participate in complex morphogenic events that lead to the formation of cardiovascular system. During vascular system development, SMCs also exhibit very high rates of synthesis of extracellular matrix components, which include elastin, collagen, proteoglycans and integrins that comprise a major part of the blood vessel. At this developmental stage, SMCs form much more gap junctions with endothelial cells, and this process is critical for vascular maturation and vessel remodeling (Owens *et al* ,2004).However, the fully differentiated SMC expresses specific receptors, signal transduction molecules, ion channels, calcium regulatory proteins, and contractile proteins required for the unique contractile properties of the SMC (Owens *et al*, 1988) .

Endothelial Cells (ECs) which comprise the tunica intima mostly determines the success of a vascular graft. Endothelial cells form a confluent monolayer of thin flattened, rhomboid-shaped cells lining the intimal surface of all blood vessels, and thus are located at the vital interface between the circulating blood and the body's tissues. By controlling events related to hemostasis, thrombosis, and inflammation, endothelial cells constitute an important regulatory organ (Wu *et al,* 1992). They play a major role in angiogenesis research which forms the innermost lining of the blood vessel and the most

important one. The functions of these cells vary depending upon the size and location of the blood vessel. Endothelial cells do not form a passive barrier but they transport small molecules macromolecules and hormones like insulin, and degrade lipoprotein particles. They play a role in regulating blood pressure, blood coagulation and fibrinolysis, adhesion and transmigration of inflammatory cells to the target and most importantly angiogenesis (Diane Bouis *et al* , 2001). Most EC functions are studies via HUVEC (human umbilical vein endothelial cell) which can be taken upto 5-10 passage and can be maintained in culture upto 5 months. Results obtained from different human source are not comparable. Furthermore ECs tend to lose their primary characteristic and responsiveness beyond passage (Dominici M, Le Blanc K, Mueller I, *et al,* 2006).

For the basic understanding of the endothelial cells, its typical characteristics can be divided into its phenotype and function. Phenotype includes presence of Weibel-palade-bodies (WP bodies are rod shaped organells specific for EC's) which store large amounts of von Willebrand factor ,also called as FactorVIII-related antigen that can be quickly released upon activation of cells. vWF also occurs as secretion which is a large adhesive glycoprotein or megakaryocyte. In blood vWF helps in the stabilized regulation of Factor VIII in circulation. ICAM, VCAM and E-selectin are expressed upon activation(Diane Bouis *et al,* 2006).

Along with HUVEC there are many other sources of endothelial cells found. Subcutaneous fat derived microvascular endothelial cells (MVEC) have been found to improve graft patency in animals. In humans the results were disappointing, due to thrombogenicity and progressive intimal hyperplasia (C. H. P. Arts *et al,* 2002). Patient-derived ECs, called EPCs, can be isolated noninvasively from small samples of peripheral blood, expanded to higher cell densities, and sodded onto the lumen of small diameter grafts to enhance graft patency in a thrombotic animal model (JD Stroncek *et al,* 2012).

Interestingly, it is widely recognised that a rapid confluent endothelial layer protects a synthetic graft from thrombosis and early occlusion. However, extensive endothelialisation has never been shown in clinical practice, despite the literature being

replete with animal models demonstrating endothelialising grafts. This has contributed to the impetus behind endothelial seeding of grafts before implantation, because physiological shear stress is a hostile environment for EC adherence and function. Endothelial cells have intimate interaction with SMC, expressing transforming growth factor genes, which are also responsible for SMC organisation and collagen secretion. The same growth factor signals EC apoptosis. Asano and colleagues have demonstrated pore extrusion of EC via morphological changes and realignment through a collagen scaffold as a preliminary angiogenic bud (Asano *et al,* 2005). This was controlled by the shear forces exerted on EC by the blood flow (Sandip Sarkar *et al*, 2007).

1.2.6 MODELS OF SMC-EC CO-CULTURE

ECs-SMCs co culture system has been more recently developed and it's the preferable model of culture because ECs and SMCs are in close apposition in smaller arteries and arterioles and thus can be used in tissue-engineered blood vessels which help to form a vascular graft similar to that *in vivo*. Here in this system, contacts have been observed between ECs and SMCs either directly or indirectly. The attachment of the endothelial cells (ECs) on cultured blood vessels is often sub-optimal, and adherent ECs may be pro-coagulant (Strauss *et al,* 2000). Several co-culture systems have been developed to study EC–SMC interactions. These include (1) culture of SMCs and ECs on opposite sides of membranes [38-42]; (2) culture of ECs on collagen gels containing SMCs [43]; (3) micro carrier/spheroid-bound ECs or SMCs [44, 45]; (4) conditioned media [46]; and (5) culture of ECs directly on SMCs (Cui *et al, 200*3). A Vertical-step flow (VSF) chamber was also being used, in which ECs were co cultured with SMCs in collagen gels (Strauss *et al,* 2000). Another method of encapsulation has been attempted where smooth muscle cells are encapsulated in a biomimetic hydrogel based on methacrylated dextran-graft-lysine (Dex-MA-LA) and methacrylamide-modified gelatin (Gel-MA) and is exposed to a monolayer of endothelial cells. The formation of smooth muscle phenotype determining contractile genes like α-actin, calponin, smooth muscle-myosin heavy chain (SM-MHC), and smoothelin and elastin by EC's and the denser cell

network suggest it to be an upcoming promising model for the vascular graft (Mulder *et al*, 1994).

A bi-layer type I collagen membrane has been fabricated under vacuum suction and lyophilization methods, where the smooth muscle cells were inoculated into the lower side of the porous membrane and the endothelial cells were seeded onto upper smooth side of the membrane. This tissue engineered vascular substitute was proven to have enough tensile strength and biocompatibility as well as advanced vascular regeneration (Hsi-Chin Wu *et al,* 2007). A co-culture model using EC seeding directly on quiescent SMCs has been established, which reproduces the close physical proximity of ECs and SMCs observed *in vivo* and maintains SMCs in a quiescent state. Results demonstrated that such co-culture can alter EC function. This co-culture model is of great importance in understanding EC/ SMC interaction in the TEVGs (Lavender *et al,* 2005). SMC and EC co-cultures were successfully established and maintained in long-term culture (7 wk) on an enclosed perfused bundle of semipermeable polypropylene capillaries (Eileen.M.Redmond *et al,* 1995).

In another system, bovine aortic ECs have the normal abluminal orientation toward bovine aortic SMCs and are separated only by a 13-mm-thick semipermeable membrane. They maintain their contact using cytoplasmic projections and through various soluble factors like TGF beta 1 which helps to maintain them in coculture with their typical phenotype (MARK F. FILLINGER *et al,* 1997). Numerous coculture systems of EC and SMC have been developed to study paracrine interactions in the vessel wall. These include planar coculture models of cells cultured together in the same dish, bilayer coculture, two-compartment filter systems, and agarose cocultures (Fillinger *et al,* 1997; Bonin *et al,* 1994). These studies have shown that EC and SMC regulate each other's quiescent phenotype. Korff *et al* studied blood vessel maturation processes in a novel 3-dimensional spheroidal coculture system of EC and smooth muscle cells (SMC) found that coculture spheroids differentiate spontaneously in a calcium dependent manner to organize into a core of SMC and a surface layer of EC, thus mimicking the

physiological assembly of blood vessels with surface lining EC and underlying mural cells (KORFF *et al,* 2001).

These techniques have their own advantages and disadvantages. Direct contact between the two cells reduces the distance between them which provides a platform for the short-lived intermediates like NO. Using the perfused transcapillary co-culture model, it has been also reported that ECs protect against flow-induced SMC migration and flow-induced EC plasminogen activator inhibitor type 1. A parallel plate EC/SMC co-culture system has been adopted where the laminar shear stress significantly inhibits SMC-induced adhesion molecule gene expression. Furthermore, SMCs has shown to induce an up regulation of pro-inflammatory gene expression in ECs that are located in close proximity to SMCs. In direct co culture system, it has been reported that quiescent state SMCs are mostly adopted because the proliferating ones interfere with the contraction of SMC and inturn fails to produce a normal blood vessel function (Lavendera *et al,* 2005). Evensen et al. developed a very useful microplate co-culture system to quantify angiogenesis. Human umbilical vein ECs and pulmonary artery vascular smooth muscle cells were mixed together and then cultured in 96-well plates. The principal output was network formation which was measured by expressing either green fluorescent protein or a derivative in the ECs. The tube length was measured over time using image analysis software and found to be stable after 3 days. As a proof of principle, the assay was sensitive to tyrosine kinase inhibitors of the VEGF receptor 2. Further, this method can be used to generate dose response curves. Thus it has been noted that EC-SMC contact is essential to maintain the blood vessel function and for a successful graft.

Powell *et al* discovered that SMCs cocultured with ECs as a bilayer had lower amounts of active TGF-b1 protein compared with SMCs cultured alone and SMCs cocultured with ECs as a monolayer. SMCs cocultured with ECs as a bilayer migrated at a greater rate than SMCs cultured either alone or cocultured as a monolayer. The EC effect on SMC migration was inhibited by the addition of 5 ng/ml of TGF-β1. Yuji Narita *et al.* showed that the gene expression in MSCs in response to TGF-β induced Notch ligand Jagged 1 (JAG1) and SMC markers, including smooth muscle a-actin (ACTA2),

calponin 1 (CNN1), and myocardin (MYOCD), which were dependent on the activation of SMAD3 and Rho kinase.

1.2.7 Nitric Oxide Synthase (NOS)

Nitric oxide, a gaseous ligand is of great concern when the mechanisms in vascular coculture are discussed. Nitric oxide (NO) is a short-lived messenger molecule(highly unstable with a half life of just a few seconds) that is formed from r-arginine by the enzyme, NO synthase (Goran K. Hansson *et al,* 1994).NO is a potent vasodilatator contributing to the maintenance of low basal tone and attenuating the action of vasoconstrictors (Myatt *et al,* 1992). NO is involved in the regulation of diverse biological functions and is enzymatically generated by three different isoforms of NO synthases (NOS) (Nakatsubo *et al*, 1998), nNOS (neuronal NOS, NOS I), iNOS (inducible NOS, NOS II),and eNOS (endothelial NOS, NOS III). Endothelium-dependent relaxations to acetylcholine are mainly of pharmacological interest since few blood vessels are innervated by cholinergic nerves. However,a number of more physiological stimuli [physical forces, circulating hormones (catecholamines, vasopressin, aldosterone), plasma constituents (thrombin, sphingosine 1-phosphate (S1P)), platelet products (serotonin, adenosine diphosphate), and autacoids (histamine, bradykinin, prostaglandin E4)] share with acetylcholine the ability to evoke the release of EDNO (Endothelial Derived Nitric Oxide). NO plays a key role in the protection exerted by the endothelium against coronary disease which is produced by the endothelial constitutive isoform of NO synthase (endothelial nitric oxide synthase (eNOS), NOS III) (2A].NO is synthesized in the placenta and eNOS has been localized in syncytiotrophoblast, stem villous blood vessels and the endothelium of arteries and veins of the umbilical cord and chorionic plate (G Scho¨nfelder *et al,* 2004).

iNOS has been found to be higher in atherosclerotic disease, where VSMCs comes to a synthetic phase and proliferate and migrate to neointima where they increase the extracellular matrix in the blood vessel leading to wall thickening. In such cases, endothelial depended responses are attenuated and the total NO is contributed by VSMC

iNOS.3 .In balloon-injured rat carotid arteries, iNOS expression is induced by interleukin 1-b (IL-1b) (Joly GA *et al,* 1992).

By the initial identification of endothelial-derived relaxing factor (EDRF) in 1980 by Furchgott and Zawadzki, and the subsequent discovery that EDRF was NO by eNOS, NO has been attributed to a number of biochemical processes, including the induction of vasorelaxation of vascular smooth muscle,the contraction of gastrointestinal organs, neurotransmission, and activation of soluble guanylate cyclase. At high concentrations, NO is bactericidal and cytotoxic. NO regulates numerous physiological pathways and many disease processes are related to the impairment of these pathways, for example, as seen in diabetes and atherosclerosis. Recently, the Nobel Prize was awarded to three investigators for their discovery and research related to NO, so the science of NO is timely. Therefore, the perfusionist needs to have an adequate understanding of the regulation of NO and NOS (D Bradford Sanders *et al,* 2000).

Nitric oxide is produced at basal levels in endothelial cells by endothelial nitric oxide synthase (eNOS) 20–22 at 50 nM per hour. NO is a potent inhibitor of platelet and leukocyte adhesion, and inhibitor of SMC proliferation6 and is largely responsible for vessel homeostasis. eNOS (or NOS3, 135-kDa protein, encoded by *NOS3* gene) is most abundantly expressed in the endothelium. Evidences are established showing its expression in other cell types composing cardiovascular tissues, e.g., in cardiac myocytes. Although nNOS was traditionally considered as a neuronal-specific isoform, recent work demonstrated its expression in vascular smooth muscle cells as well as cardiac myocytes (J.-L. BALLIGAND *et al,* 2009). NO released from contracting skeletal muscle indicates that the repetitive skeletal muscle contractions lead to activation of a Ca21/calmodulin-dependent NOS; therefore, both nNOS and eNOS may be activated . However, NO produced in muscle fibers during contractile activity may be predominantly nNOS derived (Goran K. Hansson *et al,* 1994). eNOS transfection of vessel wall after balloon injury has been reported to cause a 70% reduction of intimal hyperplasia 14 days after injury (von *et al,* 1995).

In short vascular effects of nitric oxide include:

- Direct vasodilatation (flow dependent and receptor mediated)
- Indirect vasodilatation by inhibiting vasoconstrictor influences (eg:inhibits angiotensin II and sympathetic vasoconstriction)
- Anti-thrombotic effect- inhibits platelet adhesion to vascular endothelium
- Anti-inflammatory effect- inhibits leukocyte adhesion to vascular endothelium; scavenges superoxide anions
- Anti –proliferative effect-inhibits smooth muscle hyperplasia

Because of the above actions of NO, when its production is impaired or its bioavailability is reduced, the following can result:

- Vasoconstriction (eg: coronary vasospasm. elevated systemic vascular resistance, hypertension)
- Thrombosis due to platelet aggregation and adhesion to vascular endothelium
- Inflammation due to up-regulation of leukocyte and endothelial adhesion molecules
- Vascular hypertrophy

1.3 HYPOTHESIS

It is hypothesized that UCMSC may differentiate to SMCs on a GVAc-PCL82 (Gelatin-Vinyl Acetate copolymer and poly-ε-caprolactone blend) electrospun scaffold under the influence of biochemical signals and further support endothelial cells when grown in direct co-culture model. It is also a part of hypothesis that the expression levels of nitric oxide synthase (eNOS and iNOS) have a critical role in endothelial and smooth muscle cell direct co-culture model.

1.4 OBJECTIVES

1. Construction of natural hybrid scaffold using a blend of Gelatin Vinyl Acetate co-polymer (GeVAc) and Polycaprolactone (PCL)–GeVAc PCL82.
2. Isolation and characterization of Human Umbilical Vein Endothelial Cells.
3. Isolation and characterization of Umbilical cord mesenchymal stem cells.
4. Biocompatibility of HUVEC and UCMSC on GeVAc PCL82.
5. Differentiation of UCMSC to SMCs
6. Characterization of co-culture model using differentiated UCMSC to SMCs and HUVEC on GeVAc PCL 82.
7. Co-culture studies based on nitric oxide.

1.5 SIGNIFICANCE OF THE STUDY

This study has aimed to see the possibility of differentiation of UCMSC to SMCs on a GeVAc PLC 82 scaffold and its ability to hold the direct co-culture model comprising HUVEC and UCMSC differentiated to SMCs without altering the cell morphology. The result of the study has potential applications in the development of tissue engineered vascular graft. The role of nitric oxide production via endothelial as well as induced nitric oxide synthase in response to UCMSC differentiation to SMC is also investigated in this direct coculture model.

CHAPTER 2

MATERIALS AND METHODS

2.1 FABRICATION OF GeVAc PCL82 SCAFFOLD

7.5% Gelatin Vinyl Acetate (GeVAc) copolymer and 30% poly-ε-caprolactone (PCL) were dissolved in 1, 1, 1, 3, 3, 3 -Hexafluoro isopropanol (HFIP) mixed in the ratio 8:2 (GeVAc:PCL 82) respectively. This was shaken in a magnetic stirrer for two days. The electrospinning apparatus used was custom made nanofibre electrospinning station (Holmarc,optomechatronics,Cochin,India) with high voltage (14KV) power supply (Gamma high voltage Research Inc.,Ormord Beach,FL). 8ml of the mixture of GeVAc PCL82 was loaded in 10ml syringe and was electrospun with a flow rate of 2ml/hr. Tip to collector distance was maintained at 14cm with a rotating mandrel speed of 300rpm. The resultant nanofibrous scaffold was crosslinked in a chamber with 50% aqueous Glutaraldehyde vapors for 3hrs. The resultant scaffold (GeVAc-PCL 82) was dried and stored at room temperature.

SEM analysis of the scaffold was done with 1x1cm GeVAc PCL 82 scaffold. Sample was then placed on a stub and underwent gold sputtering. After processing the sample was placed on the stage of scanning electron microscope and images were obtained at various magnifications like 1500X and 3000X. Pore dimensions were measured using Image J software.

2.2 ISOLATION OF HUVEC

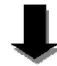
Umbilical cord was cleaned thoroughly to remove all the blood stain, two ends of the cord was chopped off to avoid contamination and a cannula with a three way stop cork was inserted into the large vein. Ice cold PBS was flushed through the large umbilical vein for further cleaning.

HUVEC were isolated from umbilical cord innermost layer as follows:

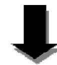
2% collagenase type I (163.00units/mg, Gibco USA.) made in serum free M199 was injected into the cord after tying both the sides tightly without any leak. The cord with collagenase type I was incubated at 37°C and 5% CO_2 for 12 minutes followed by a thorough massage to let the detached cells to eject out and was collected in a 50ml centrifuge tube. To this, equal amount of serum containing media was added quickly to stop the enzyme action. This was then centrifuged at 1850 rpm for 10minutes at 4°C. Supernatant was removed carefully and 1ml of complete M199 (M199+10% FBS+1% Antibiotic) was added to the pellet. This was then seeded into a T25 flask with 5ml media. Media change was given the very next day of the isolation followed by changes every 2 days.

The schematic diagram represents the isolation of HUVEC

Umblical cord was washed thoroughly with PBS through the one end connected to cannula and a three way stop cork and injected ice cold PBS to clean the clot and blood stain inside



Collagenase I (.2%) was injected into the umbilical cord tied at both ends



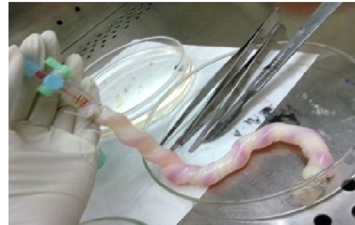
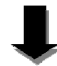
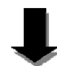


Figure 2.1: Human umblical cord with collagenase type 1

Incubated for 12 minutes at 37°C and the cord was massaged for 3 minutes, added equal amount of serum containing media and centrifuged at 1850rpm for 10min



Seeded into a T25 flask with M199 with 20% serum and 1% antibiotic-antimycotic and incubated



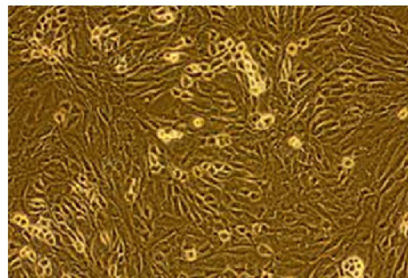


Figure 2.2: Isolation of HUVEC

2.3 ISOLATION OF Umbilical cord mesenchymal stem cell (UCMSC)

Umbilical cords were cut into two or three pieces to wash and remove blood stains thoroughly. Then they were chopped into very fine pieces with the help of sterile scalpel and forceps and were put into a screw capped conical flask. Approximately 25-50 ml of collagenase type II from *Clostridium histolyticum* (422units/mg, Sigma co.) solution (50ml DMEM without serum+0.1gm collagenase II) was added into the conical flask with chopped pieces of umbilical cord followed by 1hr 30 min digestion in a shaking incubator at 37°C. This was filtered through a funnel with a gauze cloth folded thrice and more to minimize the pore size and acquire a proper filtration. It was filtered into 50ml centrifuge tubes. This was then centrifuged at 2000rpm for 10minutes at 4°C and the supernatant was carefully removed. The pellet was suspended in 1 ml media and seeded into T25 flasks with 5ml of serum containing media (DMEM+ 20%FBS+1% ABAM (Gibco, USA) , according to the cell number obtained. Medium was changed in alternative days to allow cell proliferation followed by medium change in every two day interval after the initial passage.

2.4 CHARACTERISATION OF UCMSC (Umbilical cord mesenchymal stem cells)

2.4.1 FACS (Fluorescent Activated Cell Sorting):

FACS analysis was done to check for the purity of umbilical cord mesenchymal stem cells. Four set of samples were made. Sample with 1x10E6 umbilical cord mesenchymal stem cells were taken as a control. FACS was done to check the percentage of mesenchymal stem cell specific marker expression at passage 1 and passage 4. Positive markers (CD44 and CD105) and negative markers (CD 34/45) were used. The procedure done was as follows:

Umbilical cord MSCs were trypsinised and pelleted down from 5 T25 flasks at 500g for 10 minutes, and the pellet was suspended in PBS. Pellet from each flask was centrifuged again in 2.5ml centrifuge tubes of which two were taken for control, two for

positive marker (CD105-PE, CD44-FITC (Santa cruz, Biotechnology)) and one for negative marker (CD34-PE/45-FITC (Santa cruz, Biotechnology)). Antibodies were added at the dilution of 1:50 for 1 hour. After incubation, the samples were centrifuged at 400g for 10min. Supernatant was discarded and to the pellet obtained 1ml of PBS was added. These were then centrifuged at 400g for 10min twice to remove the unbound antibodies. The pellet was suspended in 1ml PBS finally and transferred to a Polypropylene tube (PP tubes) and analyzed via FACS.

2.4.2 Immunostaining

UCMSC were seeded onto GeVAc PCL 82 electrospun coverslips in a 6 well plate. Immunostaining was done to show the cell morphology on 3D cultures on the scaffold (UCMSC on GeVAc PCL 82). Phalloidin was prepared by adding 10µl of phalloidin working solution to 500µl of PBS.UCMSC were stained for actin filaments with phalloidin as follows:

Media was removed and UCMSC in between passage 1 and 4 were permeablized with 50% ice cold methanol for 30 minutes. Washed with PBS and added phalloidin. Incubated for 40minutes at room temperature and again a PBS wash was given. Cells were finally fixed with 4% paraformaldehyde (SD fine chemicals, India) for 15minutes and viewed under inverted fluorescent microscope (DMIL generic, Leica, Switzerland).

2.4.3 Bi-lineage study

To assess the stemness of UCMSC, bi-lineage study was done by inducing UCMSC to adipocyte and chondrocyte leneages.

Adipocyte differentiation:

To a four well plate containing subconfluent UCMSC ,adipogenic differentiation media was added with the following composition: DMEM-Low Glucose with 1% antibiotic-antimycotic solution (50ml) ;0.9Mm IBMX (3-isobutyl-1-methylxanthine) made into working concentration of 0.5µM in 1:50 dilution (27.7µl); 10Mm Dexamethazone (5µl);9mM Indomethacin H8 made into working concentration of 200µM (0.5µl). Medium change was given in every 2 days and was stained on 20th day.

On 20th day differentiated cells were fixed with 4%paraformaldehyde overnight at 4°C. Adipocyte staining was done as follows: Cells were washed with PBS and fixed with formalin free fixative for 10 minutes. This was washed off with PBS and 700-1000µl of Oil Red O (ORO) working solution was added and incubated for 10minutes at room temperature. Red debris was removed to obtain a clear picture via phase contrast microscope at various magnifications.

Chondrocyte differentiation:

To a four well plate containing subconfluent UCMSC ,adipogenic differentiation media was added with the following composition: DMEM-Low Glucose with 1% antibiotic-antimycotic solution (48.35ml) ;sodium pyruvate (500µl); 200Mm L-glutamine (350µl) ; non essential amino acid (500 µl) ; 40 µg/ml proline (50 µl) ;10mg/ml stock ascorbic acid made into 50 µl/ml working solution (250 µl) ;0.1 Mm Dexamethazone (0.5 µl). Medium change was given in every 2 days and was stained on 21st day.

On 21st day differentiated cells were fixed with 4%paraformaldehyde overnight at 4°C. Safranin O Staining was done to stain the cells differentiated to chondrocytes as follows: 5minutes incubation was followed by 10 minutes haemetoxylin incubation. Washing was given distilled water followed by acid alcohol for 10 seconds. This was then immediately washed with tapped water and stained with 0.02% aqueous Fast Green for 3minutes. Rinsing was given quickly with 1%acetic acid for 10-15 seconds.

Following this, 0.1% Safranin O was added for 3-10 minutes and washed with distilled water and dried at room temperature. Images were obtained by phase contrast microscope at various magnifications.

2.5 BIOCOMPATIBILITY OF GeVAc PCL82

2.5.1 *In vitro* cytotoxicity test by direct contact assay

The *in vitro* cytotoxicity test was done to test the cytotoxicity of the materials in contact with HUVEC and UCMSC. The sterilized samples, negative control namely High density polyethylene (HDPE) and positive control -0.1% phenol in triplicate were placed on a confluent monolayer of umbilical cord mesenchymal stem cells (UCMSC) and human umbilical vein endothelial cells (HUVEC). After incubation cells with the test samples at 37± 2˚C for 24±1 hour, cell culture was examined microscopically for cellular response and test samples. Morphology of cells was assessed in comparison with negative and positive control materials. Since this study involves UCMSC and HUVEC, the same cells were used for the assay. The cellular response of the test samples were scored as 0, 1, 2 and 3 according to non-toxic, mildly toxic, moderately toxic and severely toxic.

2.5.2 Scanning Electron Microscope (SEM) Analysis

UCMSC and HUVEC were seeded on 1x1cm2 GeVAc PCL 82 scaffold in a Polypropylene tubes (PP tubes) with 2ml 20%DMEM-HG medium and incubated at 37°C Carbon dioxide incubator. Medium change was given at alternative days for 7 days. Sample was then fixed in formalin free fixative for overnight or for even 2-3 days. Series of alcohol treatment was given as follows: sample was treated with 30%,50%,70%(sample can be stored at this concentration of ethanol for overnight and more at 4°C),80%,90% and 100% ethanol giving two changes in each ethanol concentrations 15 minutes each. After these steps the sample was dipped in iso-amyl alcohol and wrapped in a filter paper. Critical point drying (Hitachi HCP2, Hitachi Japan) was done followed by gold sputtering in vacuum (Hitachi E101). After processing the

samples were placed on stub using two sided tape and examined by secondary imaging under 15KV scanning electron microscope (Hitachi S2400). Images were obtained at various magnifications like 1500X and 3000X

2.5.3 Cell Proliferation Assay on GeVAc PCL82

Proliferation assay of HUVEC and UCMSC on GeVAc PCL82

Isolated HUVEC at passage 1 and UCMSC at the passage 3 were seeded at a density of 1X104 cells per 1X1cm **GeVAc PCL82** scaffold and incubated in M199 with 20%FBS at 37°C and 5%CO2 in Polypropylene tube (PP tubes)s in an incubator. Total 12 samples were kept of which 3 samples each were retrieved on 1st, 3rd, 5th and 7th days respectively. These were then lyophilized overnight. Each sample was digested with 1ml proteinase k solution (Sigma Chemical co.) (7.5mg proteinase k+ 1.5mg SDS +15ml tris HCl) at 57°C for 12-24 hours and was thoroughly vortexed. DNA standards (Sigma Chemical co.) were made with 10X fluorescent assay buffer at the concentrations of 30µg/ml (30µl DNA standard stock+970µl of DEPC water), 15 µg/ml, 7.5 µg/ml, 3.75 µg/ml and 1.88µg/ml. Each standard was taken 50µl each in quadruplicates in a 96 well plate. The digested sample was then vortexed well to remove the cells from the scaffold. These samples were then transferred to a microflour 96 well plate (Dynex Technologies,Chantilly, VA) in triplicates. A blank was set with just Hoechst 33258 (Sigma Chemical co.) dye alone along with the standards and the sample. 200µl of Hoechst 33258 solution was added to every well with DNA standard and samples in dark and the fluorescence were read at an excitation wavelength of 360nm and an emission wavelength of 460 nm using a plate reader (Hidex Chameleon Plate Reader, Finland) at 25˚C.

A graph was plotted with microgram of DNA per ml on Y axis and incubation time on X axis and the standard deviation was calculated.

2.6 DIFFERENTIATION OF UCMSC TO SMCs

Based on the studies of Vaza˜o *et al*. who demonstrated human embryonic stem cells (hESCs) differentiation to hSMC (human Smooth Muscle Cells) at 10ng/ml of TGF beta 1 and also based on the studies of Kurpinski, Lam, Chu et al. who tried MSC differentiation to SMC with 5ng/ml TGF beta1, differentiation with these concentrations of TGF beta1 was tried on UCMSC to differentiate into SMCs. The media used were DMEM-HG with 1%FBS and 5ng/ml TGF beta 1(medium 1) and DMEM-HG with 1%FBS and 10ng/ml of TGF beta 1(medium 2). UCMSC were seeded onto 4 well plates and media change was given on every alternate days. Differentiation was assessed at 4th and 7th days and the best concentration required was confirmed based on immunostaining results.

2.6.1 Immunostaining

Immunostaining was done to show the cell morphology of the differentiated cells with 10ng/ml and 5ng/ml of TGF beta1 on TCP. On day 7, UCMSC differentiated tentatively to SMCs were fixed with 4% paraformaldehyde overnight at 4°C, washed with PBS and permeablized with 50%ice cold methanol for 20 minutes. Again washed with PBS and blocked with 2% bovine serum albumin for 30minutes. Human specific primary antibody, α smooth muscle actin (α SMA (santa cruz,biotechnology) with 2%BSA in the ratio 1:50 was added followed by 1.30hr incubation. After incubation the cells were given a PBS wash followed by addition of anti-mouse FITC tagged secondary antibody. Incubated with secondary antibody for 1 hr. PBS wash was given which was followed by incubation with Hoechst for 10 minutes. Images were obtained under inverted fluorescent microscope (DMIL generic, Leica, Switzerland).

2.6.2 SEM analysis

SEM analysis was done to study the differentiated cell morphology on GeVAc PCL82 scaffold.

2.6.3 RT PCR analysis

RT PCR was done to quantify the gene expression levels of differentiation of UCMSC to SMCs on GeVAc PCL82 scaffold. UCMSC were seeded on GeVAc PCL82 scaffold (1x1cm) and incubated in differentiation media (with 5ng/ml TGFβ1) for 7 days in sterile Polypropylene tube (PP tubes) giving a media change on alternate days. The samples were then retrieved in 1 ml trizol and then stored in -80°C.

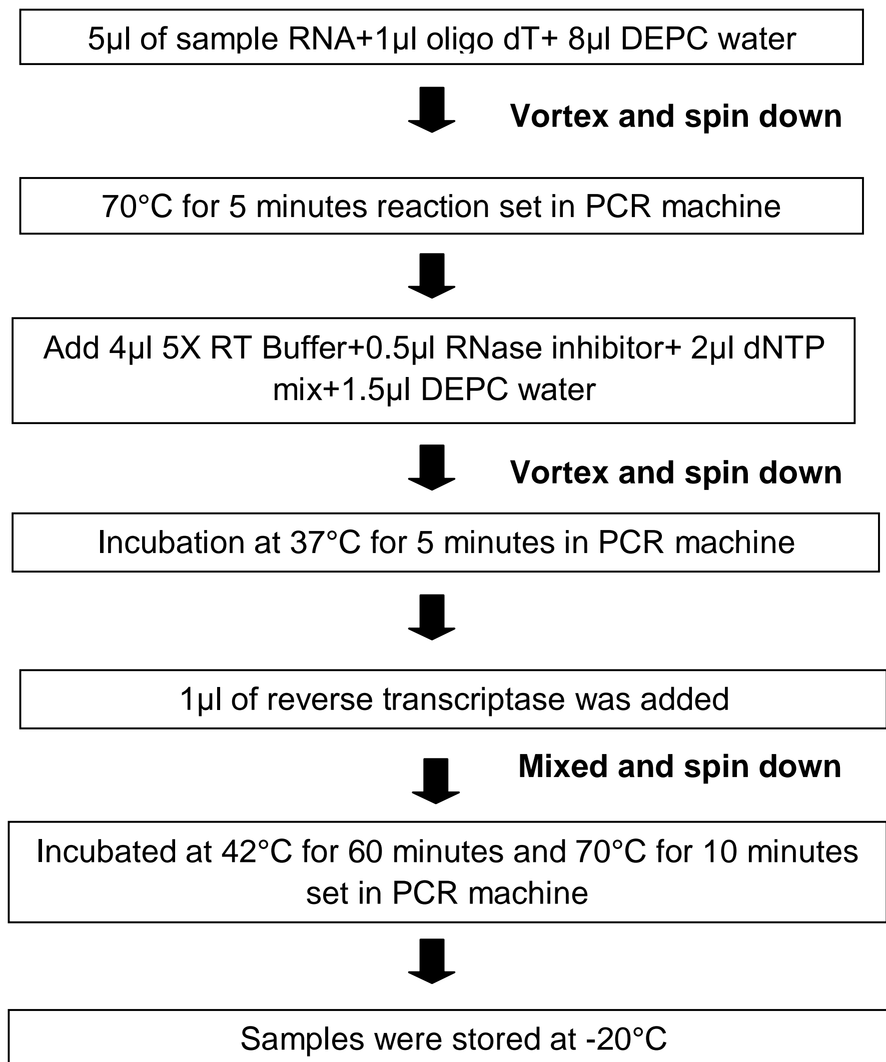
RNA isolation was done as follows:

Sample was thawed for 5-15minutes.0.2ml of chloroform of high purity was added and vortexed for 15min. Samples were then kept at room temperature for 5-15 min and were centrifuged at 12000g for 15min at 4°C. Three layers were formed of which the colorless aqueous phase was transferred to a fresh tube. Added 0.5ml of isopropanol and stored at

4°C for 10 minutes. Centrifuged the samples at 12000g for 10 minutes at 4°C, supernatant was removed and 1 ml of ethanol (75%) was added. The samples were centrifuged at 7500g for 5min at 4°C. The supernatant was removed and the samples were air dried for 3 minutes, dissolved in 20µl of DEPC water and stored at -80°C. The sample was quantified by quant it kit.

cDNA synthesis

5µl of RNA was aliquoted into four tubes for each sample.



Real time PCR was done for the expression of markers like humann SM22 and human calponin in day 1 and day 7 of differentiation to hSMC. UCMSC at passage 4 was used as the control cell. Real time mix used was 2.5µL of forward and reverse primers (100nM); 5µl of DEPC water, 1µl of sample cDNA and 12.5µl of sybr green.

TABLE 2.1: List of SMC specific Primers for RT PCR

Human Primers	**Sequence**	**Annealing temperature**
GAPDH	Sense:5'ACATCATCCCTGCCCCTAGT3' Antisense:5'CTAAGTGTCCCCCAGCAGTG3'	57.75˚C
SM22	Sense: 5'CTCTGCTTTGGGGATGGGAA3' Antisense:5'TCAACAGATTCCTTGGCCCC5'	57.4˚C
Calponin	Sense: 5'-TGGGGTCATGGGACAAAGC3' Antisense:5' GTGCATGCTGGGGTTTGTAG3'	57.05˚C
eNOS	Sense :5'TGGGGTCATGGGACAAAGC3' Antisense:5'GTGCATGCTGGGGTTTGTAG3'	57.3˚C
iNOS	Sense: 5'GGCCTCGCTCTGGAAAGA3' Antisense:5'TCCATGCAGACAACCTT3'	54.15˚C

Melting curve and Ct values were obtained and the fold increase graph was plotted compared to the expression of SM22 and calponin markers by UCMSC cells alone, thus giving the fold extent of differentiation.

2.7 CHARACTERISATION OF HUVEC

2.7.1 Immunostaining

TABLE 2.2: List of primary and secondary antibodies specific for HUVEC immunostaining

<table>
<tr><th>Cell culture condition</th><th>Primary antibody</th><th>Secondary antibody</th></tr>
<tr><td>On 2D</td><td>PECAM</td><td>Chicken Anti- Mouse-FITC</td></tr>
<tr><td rowspan="3">On 3D</td><td>Von Willibrand factor</td><td>Chicken anti-rabbit-FITC</td></tr>
<tr><td>PECAM</td><td>Chicken Anti- Mouse-FITC</td></tr>
<tr><td colspan="2">VCAM tagged with FITC</td></tr>
</table>

2D- Cells on TCT (Tissue Culture Treated) plate.

3D-Cells on GeVAc PCL82 scaffold

Phalloidin staining was done to immunostain actin filaments of HUVEC on GeVAc PCL82 scaffold spun on coverslips. Acetylated LDL staining was done by adding 1X AcLDL in M199 and incubating for 4hours at 37˚C in dark and then viewed under inverted fluorescent microscope. Then the cells were washed with PBS and fixed with 4% PFA for 30min in dark. VCAM staining was also done with HUVEC on GeVAc PCL82 scaffold spun on coverslips. Cells were fixed overnight with 4%PFA and washed with PBS. This was then permeabilised with ice cold methanol (50%) and blocked with 2%BSA and 1:50 (anti VCAM-FITC: 2% BSA) was made and added 300µl to scaffold spun coverslips with HUVEC. This was then incubated for 1hour, washed with PBS and viewed by inverted fluorescent microscope.

2.8 CHARACTERISATION OF COCULTURE OF HUVEC WITH DIFFERENTIATED UCMSC TO SMCs

2.8.1 Immunostaining

Umbilical cord mesenchymal stem cells (P4) were seeded onto PCL-GVAC scaffold spun onto coverslips (2x2cm) and grown for 2days. 5ng/ml of TGFβ in 1% HG DMEM was given for 7 days and they were seeded with HUVECs (P1). Further endothelial specific media was given and the cells were grown for 5 days and fixed with 4%PFA. Immunostaining was done as follows: differentiated Smooth muscle cells were stained first with α SMA (primary antibody) and chicken anti-mouse-PE (secondary antibody), exciting PE with HeNe543 (543nm) laser according to the previously explained procedure. This was followed by HUVEC immunostaining with von Willibrand factor (Santa Cruz, Biotechnology) (primary antibody) and chicken anti-rabbit-FITC with excitation at 490 nm(secondary antibody)(santa cruz ,biotechnology). PBS was then added to the stained samples and viewed via confocal laser scanning microscope-LSM510-META (Carl Zeiss, Germany).

2.8.2 Real Time PCR

Real time PCR was done using sybr green to assess the levels of VEGF expression by the cells in direct co-culture model on GeVAc PCL 82 scaffold. HUVEC on 2D tissue culture treated plate was taken as the control. This was done to have a quantitative assessment of VEGF expression in response to direct co-culture of HUVEC with hSMC.

2.9 NITRIC OXIDE BASED COCULTURE STUDIES

2.9.1 Nitric oxide assay

Nitric oxide assay is a procedure for the measurement of cell associated free nitric oxide and Nitric Oxide Synthase (NOS) activity in living cells under physiological conditions. Sigma's Fluorometric cell associated nitric oxide synthase detection system measures the intracellular production of nitric oxide by a non radiometric method. This system utilizes a cell permeable diacetate derivative of 4, 5- diaminofluorescein (DAF-2DA). This penetrates the cells and rapidly ,where it hydrolysed by intracellular esterase activity to DAF-2 that, in turn, reacts with Nitric Oxide produced by NOS to form a fluorescent triazolofluorescein.

Nitric oxide assay was done with hSMC (UCMSC differentiated to hSMCs), UCMSC differentiated to hSMCs and HUVEC on day 7 on GeVAc PCL82 scaffold in a 96 well non tissue treated plate (BD Biosciences). The reaction mixture was added to the cell seeded on the scaffold (GeVAc PCL82), 200 µl /well.

TABLE 2.3: List of Nitric oxide assay reaction mix

Reaction	**Arginine substrate solution (A4344)**	**DAF-2DA solution (D225)**	**Reaction buffer (R2525)**
Control (minus dye)	10µl	-	190µl
Blank no cells	10µl	0.1µl	190µl
Induced cells	10µl	0.1µl	190µl

Two hours incubation was given in dark at room temperature with this mixture on the cell samples. After incubation the reaction mixture was transferred to a 96 well black colored microtitre plate. Reading was taken using plate reader (Hidex Chameleon Plate

Reader, Finland) at an excitation wavelength of 490nm and an emission wavelength of 520nm.

2.9.2 Real Time PCR

Real Time PCR was done to quantify the intracellular markers expressed in response to coculture on GeVAc PCL82 in comparison with HUVEC.

TABLE 2.4: List of HUVEC specific primers for RT PCR

Human primers	SEQUENCE	Melting temperature
GAPDH	Sense:5'ACATCATCCCTGCCCCTAGT3' Antisense : 5'CTAAGTGTCCCCCAGCAGTG3'	57.75˚C
VEGF	Sense:5'CTGTTGTAGTCCCAGGGTGC3' Antisense:5'ACCAGGCTCCATGCTGATTT3'	57.95˚C
eNOS	Sense :5'TGGGGTCATGGGACAAAGC3' Antisense:5'GTGCATGCTGGGGTTTGTAG3'	57.3˚C
iNOS	Sense: 5'GGCCTCGCTCTGGAAAGA3' Antisense:5'TCCATGCAGACAACCTT3'	54.15˚C

CHAPTER 3

RESULTS AND DISCUSSION

3.1 FABRICATION OF GeVAc PCL82 SCAFFOLD

Most of the ECM components that support the cells on a matrix are in the nanometer range (50-500nm). It is also known that cells attach and proliferate by means of ECM. Mimicking such a 3D matrix is the challenge in tissue engineering technology. Thus electrospinning has emerged as a promising direction for producing artificial tissues. This process utilizes an electrostatic field to control the formation and deposition of polymer nanofibers [8–10]. The procedure, which is technically feasible for the fabrication of filaments ranging in the nanometer to micrometer scale with a certain degree of alignment (S. Srouji *et al.*2008).

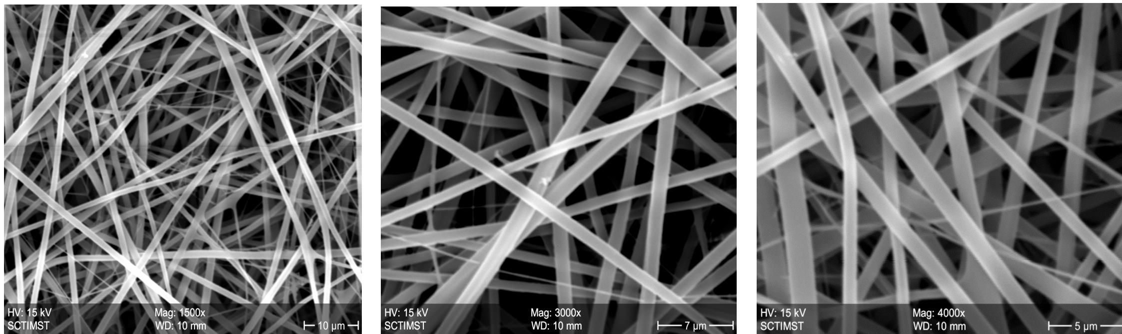


Figure 3.1: SEM images of the electrospun GeVAc PCL82 were obtained at 15000X, 3000X and 4000X magnification

Porous scaffolds of GeVAc and PCL blend was fabricated using electrospinning technique in the ratio 8:2. SEM images were observed and the diameter and pore size of the electrospun GeVAc PCL82 scaffold was obtained by analyzing SEM images in ImageJ software. Diameter of the scaffold was found to be 1.07±0.36 µm and pore size was 3.75± 0.75 µm which can allow for infiltration of cells within the scaffold.

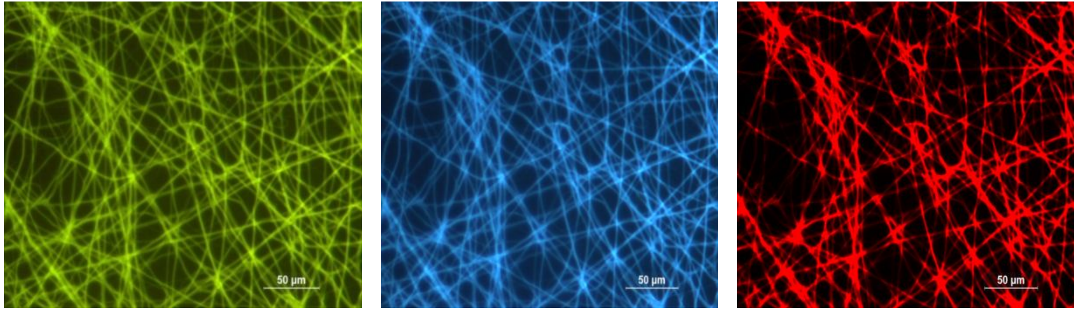


Figure 3.2: Auto fluorescence property of GeVAc PCL82 scaffold was shown under inverted fluorescent microscope.

GeVAc PCL82 was found to fluoresce under all the filters of inverted fluorescent microscope. This property was contributed by both the gelatin copolymer as well as the PCL.

3.2 ISOLATION OF HUVEC

HUVEC were isolated from human umbilical cord with 0.2% collagenase type1 and the cells were grown in M199 with 20%FBS. They are the source of macro endothelial cell and found to be really successful cell source in vascular tissue engineering which has been already proven by *AU et al* (2008). It has been also reported that engineered blood vessels derived from human umbilical cord vein endothelial cells and hMSCs remained stable and functional for more than 130 days *in vivo*.

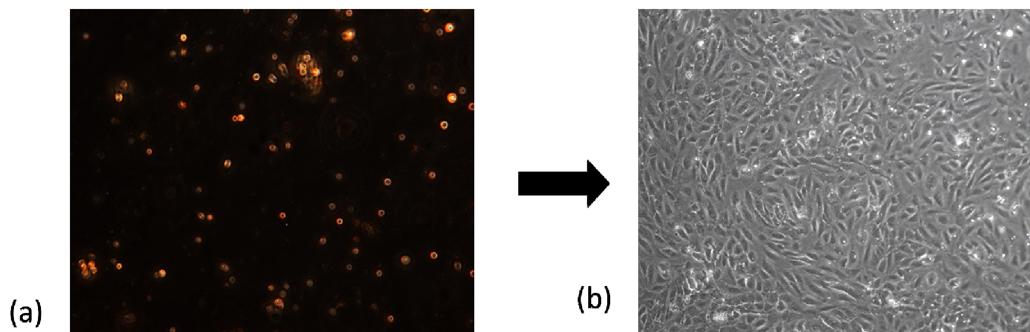


Figure 3.3: Phase contrast microscopy :(a) the HUVEC isolated from umbilical cord (b) shows the proliferated HUVEC in 24 hours (P0) on 2D culture flask.

Isolated HUVEC (Human Umbilical Vein Endothelial Cells) were found to attach and proliferate in 24 hours and form a typical cobble stone morphology forming a monolayer of cells on the tissue culture treated plates. Cells were grown till 80-90% confluence before taking it forward to the next passage. HUVECs preferably in between passage1 and passage 3 were taken for the study as they were not found to be stable on higher passages. At higher passages cells lose its cobble stone morphology and tend to spread giving spindle shaped morphology.

3.3 ISOLATION OF UCMSC

UCMSC are found to be a good source of stem cells available in plenty as compared to that of mesenchymal stem cells isolated from the other sources. Passage 3-5 was selected for the following studies. These cells are selected by plastic adherence and display a fibroblast-like morphology. As compared to BMMSC, UCMSC are thought to have a higher proliferative potential and differentiation capacity into cells of multiple mesenchymal lineages, such as osteocytes, chondrocytes, and adipocytes, as well as endothelial cells (Wu et al., 2007).

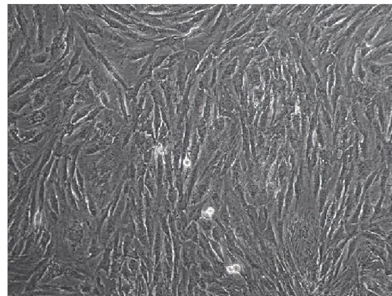


Figure 3.4: Phase contrast microscopy: the proliferated UCMSC at P0 stage

Isolated UCMSC were found to attach and proliferate on a T25 tissue culture treated flask with 10% FBS containing F12 medium and 1%antibiotic-antimycotic (ABAM) in 48hours. These cells were given media change in every 3 days and taken to 4th passage and more.

3.4 CHARACTERISATION OF UCMSC

3.4.1 FACS (Fluorescent Activated Cell Sorting)

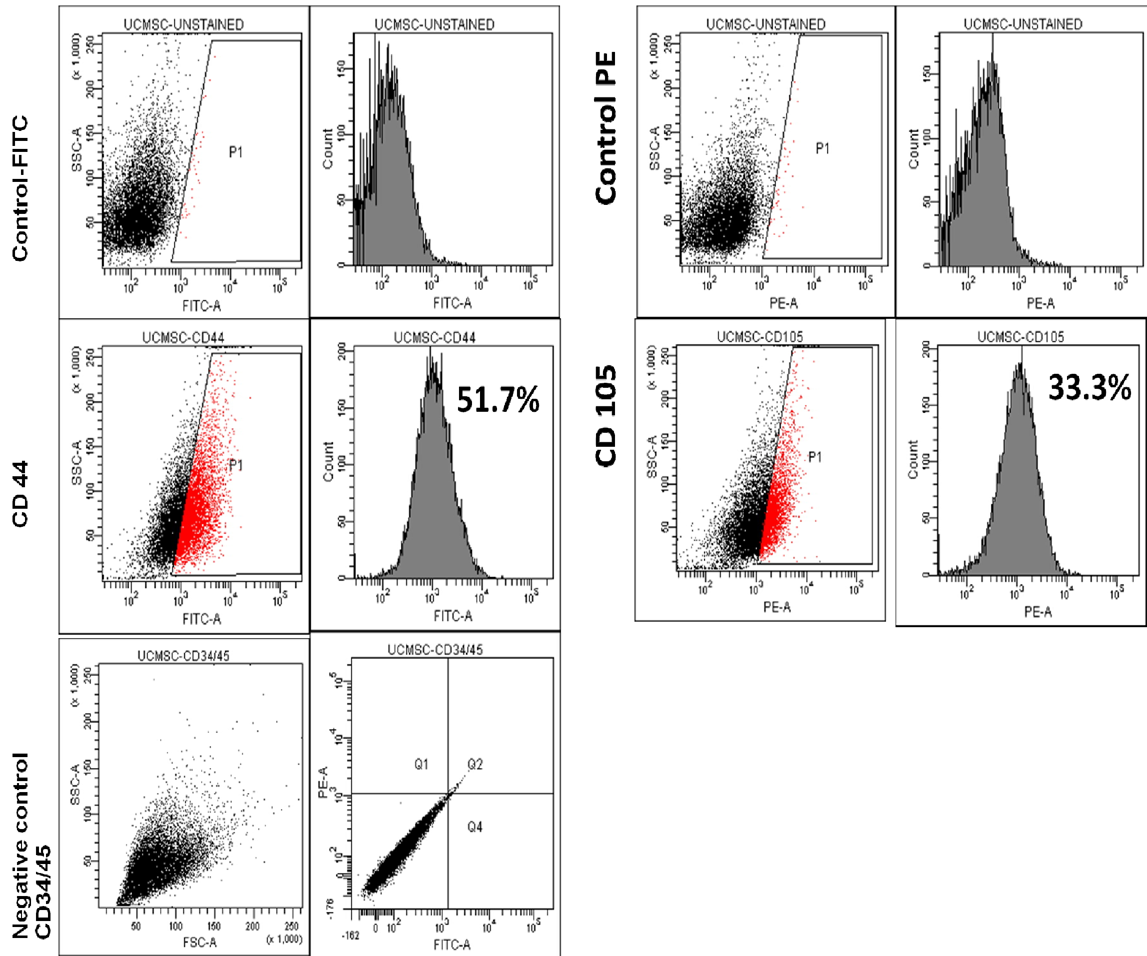


Figure 3.5: Flow cytometric analysis of UCMSC at P1

FACS of UCMSC at passage 1 showed 51.7% positivity for CD44 and 33.3% positivity for CD 105. Negative markers like CD34 and CD 45 were not expressed by the cells showing the percentage of stemness at P1 stage and need to take the cells to higher passages.

P4 UCMS cells were taken for FACS and following results were obtained:

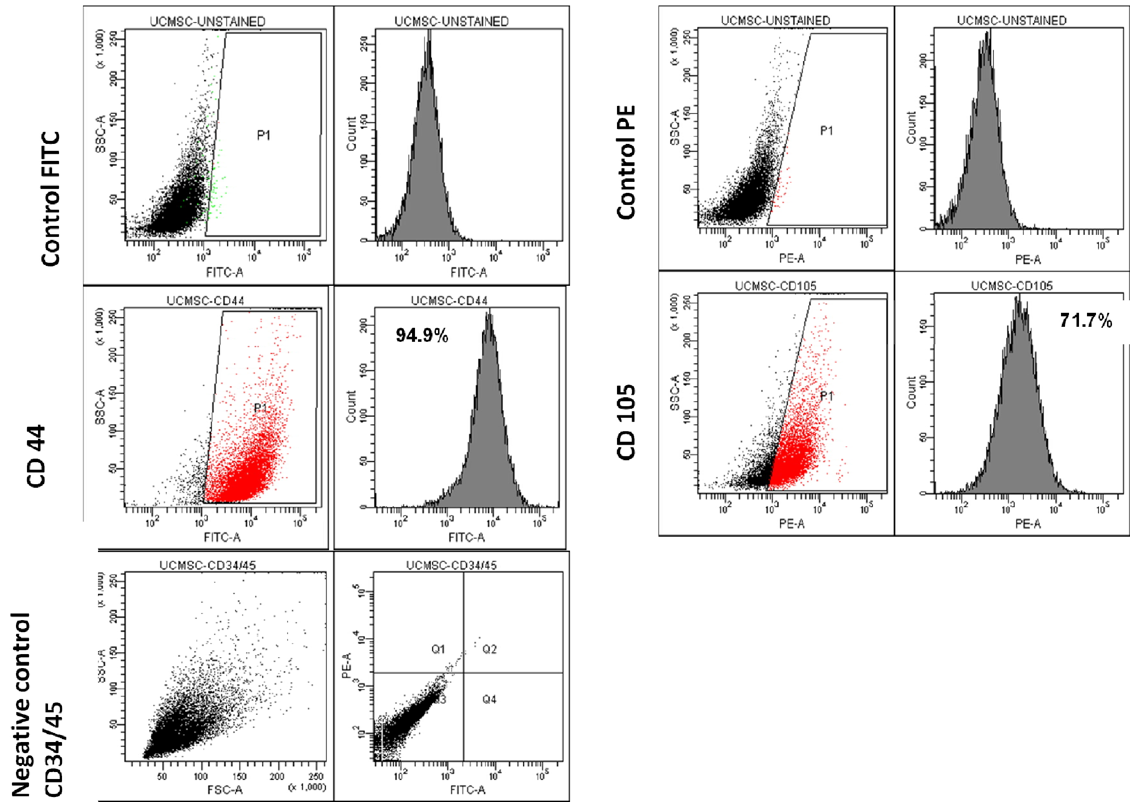


Figure 3.6: Flow cytometric analysis of UCMSC at P4

Passage 4 cells were found to show 94.9% positivity for CD44 whereas 71.7% positivity for CD 105 and hence found to show more stemness which was proven by higher expression of CD44 and CD 105 as compared with passage1 cells. Cells expressing αSMA and several MSC markers at initial passage of isolated MSC from umbilical cord were shown by Romanov *et al, (*2003). Hence passage 4 cells were selected for the study.

3.4.2 Immunostaining

Immunostaining showed the UCMS cell morphology on the GeVAc PCL82 scaffold electro spun on 2x2cm cover slips. UCMS cells were stained with phalloidin tagged with FITC to stain and elucidate actin filaments showing UCMSC s typical fibrous phenotype. Actin filaments of UCMSC were found to spread over and populate the GeVAc PCL82 scaffold and form a surface mimicking the native tissue, thus proving the compatibility of the GeVAc PCL82 scaffold to the UCMS cells.

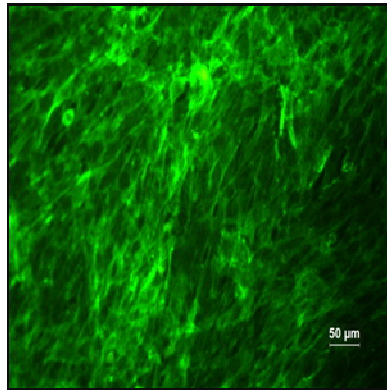


Figure 3.7: Immunostaining: UCMSC on GeVAc PCL82 scaffold stained for actin using phalloidin

3.4.3 Bilineage study

UCMSC have the potential to differentiate into different cell lineages in a controlled condition. UCMSC were found to show more stemness than bone marrow stem cells as reported by Wu et al. (2007). In this study we have tried to differentiate UCMSC into adipocytes and chondrocytes on 21 days incubation by providing an adequate induction medium to prove the multilineage differentiation capability or stemness of UCMSCs.

Adipocyte differentiation

Pierdomenico *et al.* (2011) has already illustrated the changes in the differentiation levels of UCMSC from diabetic as well as normal mothers where both samples were found to show differentiation with a slight increase in adipocyte differentiation in diabetic mothers compared to the normal. P4 UCMSC were found to accumulate lipid droplets on 21days of incubation with adipogenic media as shown by Oil Red O staining. In this study, adipocyte specific medium was given to P4 UCMSC for 21 days followed by Oil Red O staining which gave lipid droplets stained in red.

Chondrocyte differentiation:

UCMSC at passage4 were given chondrocyte specific media to assess the ability of UCMSC to differentiate to chondrogenic lineage on day 21. Safranin O staining showed UCMSC differentiated to chondrocytes are formed in 3 weeks with chondrocyte like cells in a mucopolysaccharide-rich stroma. Wang *et al.* 2009 demonstrated the ability of UCMSC to promote a better chondrocyte specific matrix on a 3D platform and thus enhancing the mechanical integrity of the constructs.

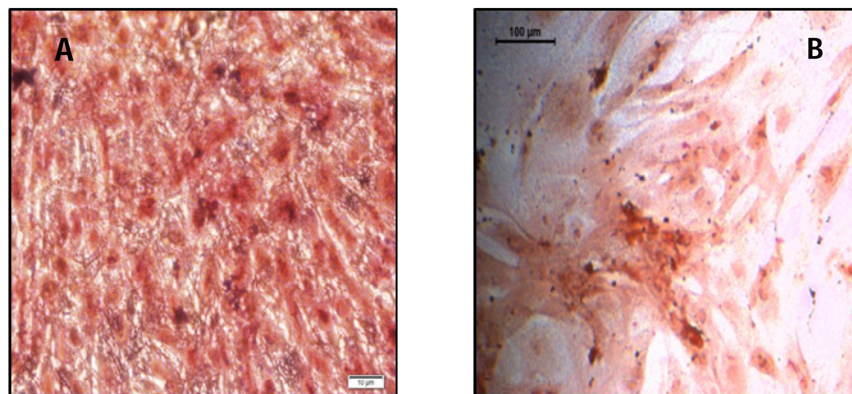


Figure 3.8: (A) safranin O staining for UCMSC differentiated to chondrocytes and (B) Oil Red O staining for UCMSC differentiated to adipocytes.

The ability of UCMSC s to go to bi-lineage thus proves the stemness of UCMSC and shows their capability to go to multileneage.

3.5 BIOCOMPATIBILITY OF GeVAc PCL82

3.5.1 In vitro cytotoxicity test by direct contact

This method showed the effect of diffusible agents or leachants from GeVAc PCL82 scaffold on UCMSC and HUVEC. Figure 8 showed that the direct contact of GeVAc PCL82 scaffold on HUVECs and UCMSC retained their characteristic morphology with a general scoring of 0 when viewed under an inverted phase contrast microscope at 10X magnification. No signs of cellular lysis, intracellular granulation or cell morphological changes were observed. The cells were well spread and properly attached, similar to the cells in contact with non toxic polyethylene control whereas the cells in the direct contact with the positive phenol control underwent lysis and assumed a round shape due to toxicity.

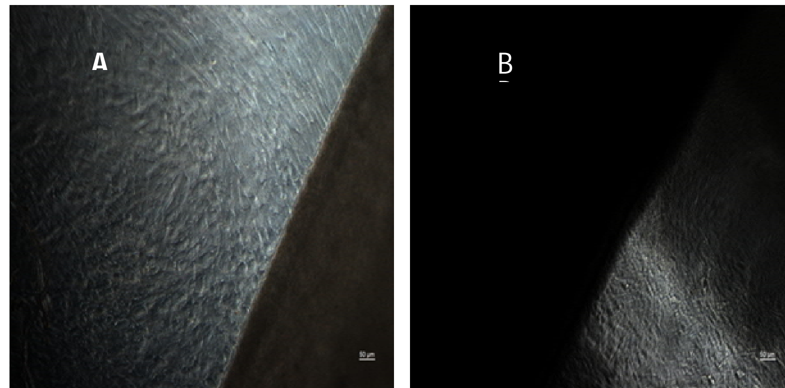


Figure 3.9: Direct contact assay of GeVAc PCL82 with (A) UCMSC and (B) HUVEC

3.5.2 SEM analysis of UCMSC and HUVECs on GeVAc PCL82 scaffold

` Scanning Electron Microscopic analysis was done to show the cell grown on the GeVAc PCL82 scaffold. This method helps to analyze the cell morphology on the scaffold which cannot be viewed by an ordinary phase contrast microscope. SEM helps to analyze the surface morphology using an electron beam. Samples cannot be recovered once taken for SEM imaging but gives the minutest details like scaffold fibre diameter, pore size, cell size etc.

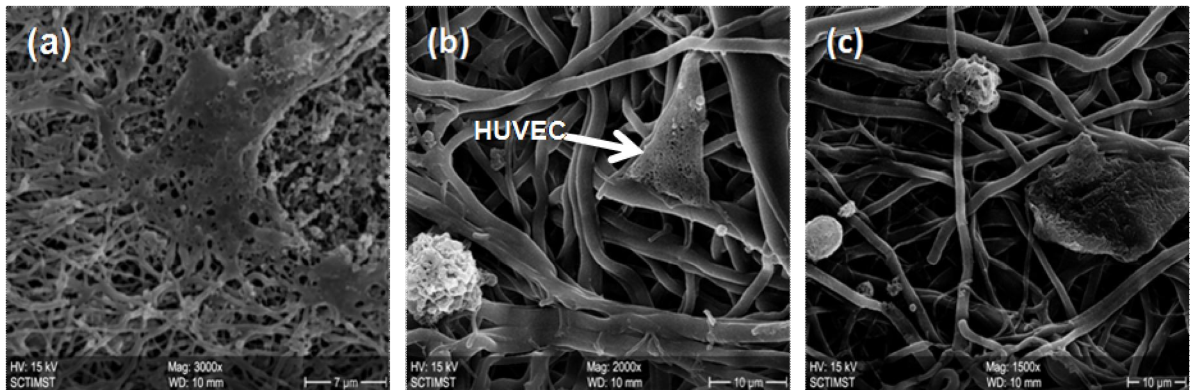


Figure 3.10: SEM IMAGING: (a) 3000X A magnification of UCMSC on GeVAc PCL82 (b) and (c) HUVEC at 2000X and 1500X magnification on GeVAc PCL82

In this study the biocompatibility of GeVAc PCL82 scaffold to HUVEC and UCMSC is assessed by SEM. SEM analysis of HUVEC on GeVAc PCL82 scaffold (b) and (c) showed its typical cobblestone morphology and some cells clustered on the scaffold. Some HUVEC were found to integrate into the scaffold pores and maintaining the cell morphology. UCMSC formed a flat fibrous morphology spreading well on the GeVAc PCL82 scaffold thus found compatible to the scaffold.

Hence considering the results, GeVAc PCL82 scaffold found to be biocompatible to UCMSC and HUVEC and provided a good matrix support for the cells to grow which was proven by the SEM images where cells has attached and proliferated without loosing its characteristic morphology.

3.5.3 Cell Proliferation Assay

Proliferation assay detects the DNA content and inturn helps to assess the cell viability. In this method Hoechst dye binds to the DNA of the cell which is assessed via colorimetric method using a plate reader. Proteinase K treatment was done to lyse cells and let the DNA out. In this study, proliferation assay was done to assess the rate of proliferation of HUVEC and UCMSC on GeVAc PCL82 scaffold at various time intervals.

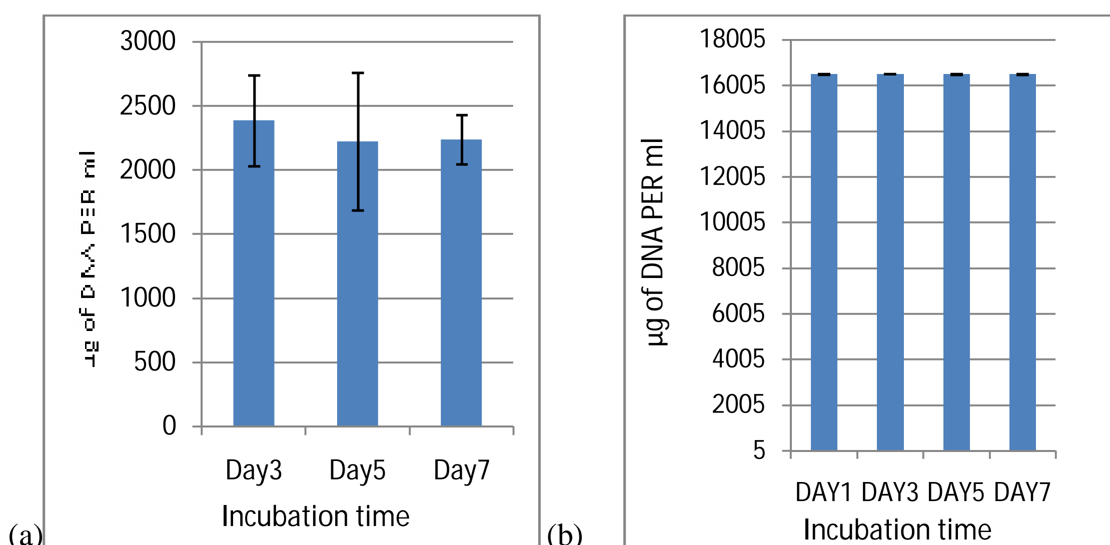


Figure 3.11: Proliferation assay: (a) HUVEC and (b) UCMSC on GeVAc PCL82 Scaffold

$1x10^5$ UCMSC's were seeded and proliferation assay was done for day 3,5 and 7 days which showed that cell number remained almost constant on GeVAc PCL82(1X1cm) scaffold on all these days without any significant decrease. HUVEC were also quantified for DNA content on GeVAc PCL82 (1X1cm) scaffold on day 3, day 5 and day 7. No much significant difference was found in these days.

These results suggest that HUVEC and UCMSC were able to adhere via cell adhesion cites and remain viable efficiently on the GeVAc PCL82 scaffold without any adverse effects. Thus the GeVAc PCL scaffold was found to support cell growth efficiently which was supported by SEM results.

3.6 DIFFERENTIATION OF UCMSC TO SMCs

3.6.1 Immunostaining

Immunostaining mainly detects the marker expression, either intracellular or extracellular on the cells. TGF beta 1 has shown its potential to differentiate stem cells to SMCs in many stem cell types like bone marrow stem cells,

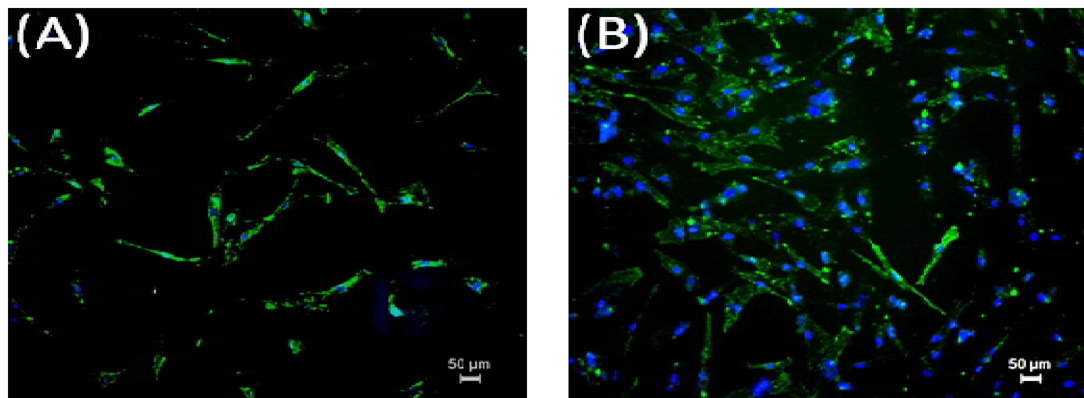


Figure 3.12: Immunostaining: (a) UCMSC differentiation to SMC when given 10ng/ml TGF beta1 and (b) UCMSC differentiation to SMC when given 5ng/ml TGF beta1

According to the literature, differentiation of MSC to SMCs was done using 5ng/ml (Kurpinski, Lam, Chu et al.) and 10ng/ml (Vaza˜o *et al.*) of TGF beta1. Both the studies have shown remarkable expression of alpha SMA marker. In this study both concentration of 5ng/ml of TGF beta1 as well as in 10ng/ml where made in 3%DMEM HG and differentiation was examined using immunohistochemical method with alpha smooth muscle actin primary antibody and FITC tagged anti-mouse secondary antibody. On day 7, remarkable expression of alpha SMA was assessed in both 10ng/ml and 5ng/ml concentration of TGF beta1. Hence 5ng/ml was selected for the study due to its ability to show a differentiation at lower concentrations.

3.6.2 Scanning Electron Microscopic Analysis

Scanning Electron Microscope (SEM) helps to visualize the surface morphology of the specimen using electron beam. The scanning electron microscope (SEM) is becoming one of the most unique and also versatile instruments available for the nondestructive inspection, evaluation, examination or analysis of the microstructural surface condition, configurational and point-to-point characteristics of solid objects.

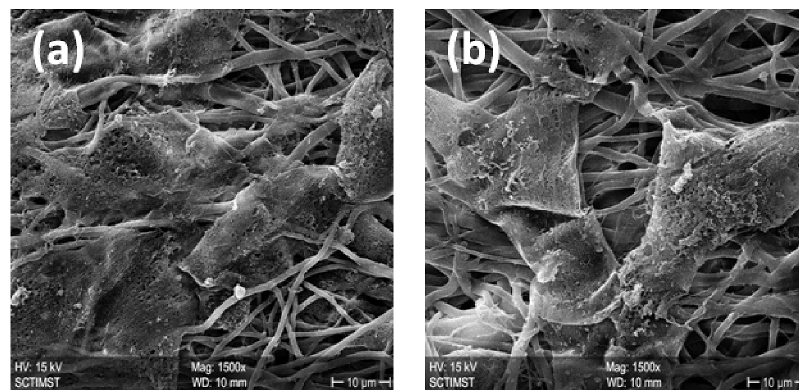


Figure 3.13: SEM analysis: (a) and (b) represents UCMSC differentiated to SMCs on GeVAc PCL82 scaffold.

In this study, the SEM analysis of UCMSC differentiated to hSMCs using 5ng/ml of TGFβ1 showed the umbilical cord mesenchymal stem cells (P4) differentiated to SMCs formed a hill and valley morphology, typical to SMC's and attached well to the GeVAc PCL 82 scaffold. These cells were found to populate and integrate well with the GeVAC PCL 82 scaffold and also the fibrous morphology of UCMSC was found to be replaced by smooth SMC morphology. Thus it was found to form a biomimetic surface which can support the growth of differentiated cells proving its role in regenerative medicine.

3.6.3 Real time PCR

Real time PCR is a quantitative PCR which allows sensitive, specific and reproducible quantitation. SYBR Green-based detection method was adopted here because along with its sensitivity it is the least expensive method too compared to TaqMan method.

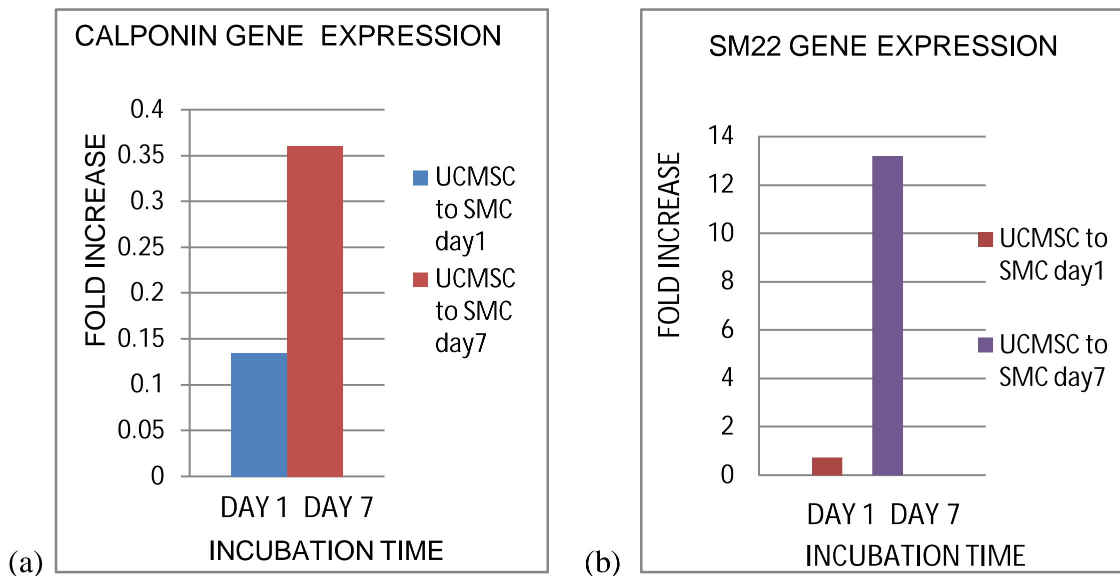


Figure 3.14: (a) Shows the calponin expression on day1 and day7 of differentiation to SMC (b) shows the expression of SM22

Real time PCR analysis showed the quantitative results of UCMSC s differentiated to SMCs on GeVAc PCL 82 scaffolds on day 1 and day 7. UCMSC (without scaffold) were taken as the control cells to assess the marker expression on differentiation. On day 1, calponin marker expression was increased to 0.14 fold and SM22 marker expression was increased to 1 fold as compared to that of the UCMSC cell control. On the other hand when analysed the differentiation on day 7, the calponin expression on the UCMSC differentiated to SMCs was found to increase by 0.36 fold

whereas SM22 marker expression on day7 of differentiation was found to increase by 13 fold. Calponin and SM22 are markers specifically expressed by SMCs and the differentiation of UCMSC to SMCs on day 7 has shown the higher SMC specific marker expression which suggests that day 7 is optimal for the differentiation studies and hence was chosen for the rest of the study.

3.7 CHARACTERISATION OF HUVEC (Human Umbilical Vein Endothelial Cells)

3.7.1 Immunostaining

Immunostaining mainly detects the marker expression, either intracellular or extracellular on the cells. Immunostaining can be considered as a method that visualizes distribution and localization of specific antigen or cellular components in separated tissues, or tissue sections. Compared to other bio-techniques that are based on the antigen-antibody reaction such as immunoprecipitation, or western-blot, immunostaining provides in situ information which promises a more convincing experimental result.

Platelet endothelial cell adhesion molecule (PECAM-1) also known as cluster of differentiation 31 (CD31) is a protein that in human is encoded by the *PECAM1* gene found on chromosome 17. PECAM-1 plays a key role in removing aged neutrophils from the body. In this study, HUVEC were found to express PCAM on 2D and 3D figure 3.15 (a) and (b). This suggests that HUVEC were found to be functional on 2D and 3D matrix.

Phalloidin staining (figure 3.15 (c)) shows the expression of actin on GeVAc PCL 82 scaffold and thus showing the endothelial morphology on the electro spun GeVAc PCL 82 scaffold. Acetylated LDL uptake attaining was done to see the Ac LDL uptake. DiI-Ac-LDL can be used to metabolically label endothelial cells thus facilitating their identification by fluorescence microscopy (VOYTA *et al,* 1984). In this study we showed the Ac LDL uptake by endothelial cells (HUVEC) in figure 3.15 (e) and by HUVEC on GeVAc PCL 82 scaffold in figure 3.15 (f) proving its functionality.

HUVEC are shown to express endoglin (CD105) on GeVAc PCL82 scaffold (figure 3.15 (g)). Endoglin (CD105) is a transmembrane glycoprotein expressed on activated vascular endothelial cells. This factor serves as an accessory protein for the transforming growth factor-b receptor system. Several studies indicate that CD105 is involved in the development of blood vessels and that this molecule represents a powerful marker of neovascularization (Wang *et al,* 2013).

Another important molecule potentially involved in endothelial cell-matrix interactions is von Willebrand factor (vWF), a multimeric glycoprotein produced by these cells that is incorporated into the subendothelial matrix (Cheresh *et at* ,1984). Von Willibrand expression was shown by HUVEC on GeVAc PCL82 scaffold in figure 3.15d.

VCAM or CD106 (Vascular cell adhesion protein) protein mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction. On 2D, thus the HUVEC stained for VCAM shows the presence of these markers in figure 3.15h denoting the healthy and functional cells.

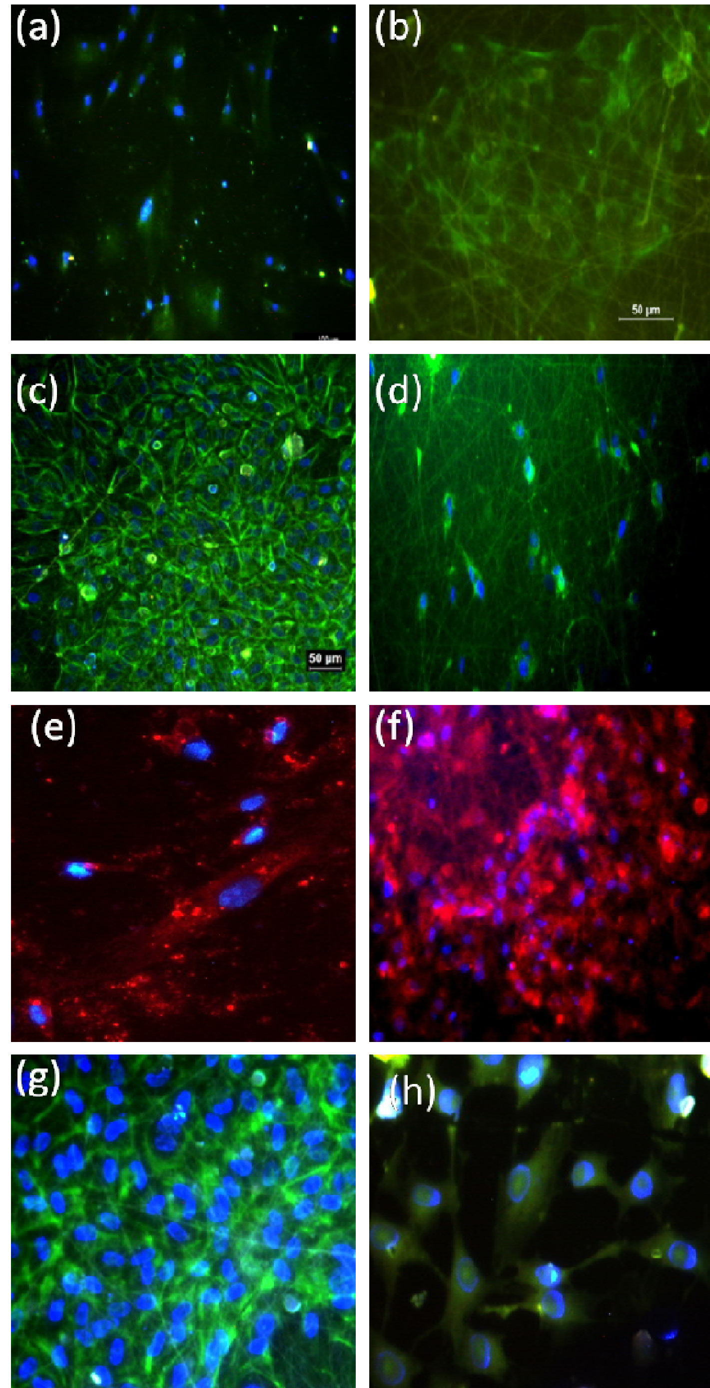


Figure 3.15: Immunostaining: (a) PECAM; (e) Ac LDL uptake (h) VCAM expressed in HUVEC on 2D and (b) PECAM (c) phalloidin for actin (d) von Willibrand (f) Ac LDL uptake (g) endoglin HUVEC on GeVAC PCL 82(on 3D).

3.8 CHARACTERISATION OF COCULTURE OF HUVEC WITH DIFFERENTIATED UCMSC TO hSMCs

3.8.1 Immunostaining

Confocal microscopy was done to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images as the ordinary fluorescent microscope couldn't give a clear image of cells on a 3D matrix as well as in coculture of cells tagged by two different colour like FITC (green) and PE (red). In this study we have illustrated the direct coculture model of HUVEC and hSMCs (UCMSC differentiated to SMC) on a GeVAc PCL 82 scaffold by confocal imaging (a). von Willibrand of HUVECs are well expressed in green (tagged with FITC) on hSMCs in red (tagged with PE) showing that the cells are functional in contact with each other as well as with the GeVAc PCL 82 scaffold.

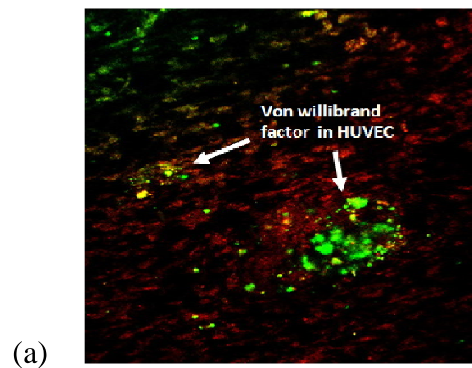
(a) 

Figure 3.16: (a) confocal images of direct coculture of hSMC(differentiated from UCMSC)and HUVEC where red stain denotes alpha SMA whereas green denotes vWF of HUVEC

Here the hSMCs form spread morphology and ECs remain like clusters above the differentiated cells showing the possibility of the co culture model. HUVEC were found to grow in close proximity to hSMC showing the direct coculture model.

3.8.2 Real time PCR

Real time PCR is a quantitative PCR which allows sensitive, specific and reproducible quantitation. SYBR Green-based detection method was adopted here because along with its sensitivity it is the least expensive method too compared to TaqMan method. Vascular endothelial growth factor (VEGF) is an endothelial cell–specific mitogen and a key regulator of angiogenesis in a variety of physiologic and pathologic processes (Zygalaki *et al,* 2004). Half life of the VEGF was found to be 90seconds by Elsie S. Place, Nicholas D. Evans & Molly M. Stevens In this we have quantified the VEGF marker expression by HUVEC in direct coculture with hSMC (UCMSC differentiated to SMC) on day 1 and day 7 of coculture.

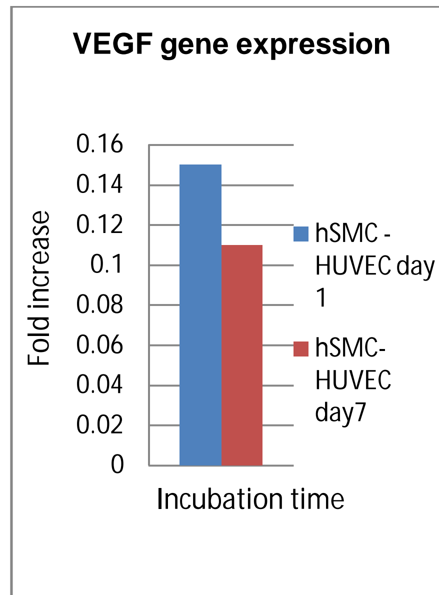


Figure 3.17: VEGF expression by HUVEC in co-culture with hSMC on GeVAc PCL82 scaffold compared to HUVEC in 2D.

VEGF expression via Real Time PCR in direct coculture model on the GeVAc PCL82 scaffold was analyzed. VEGF was found to over express on day 1 compared to day 7 in direct coculture model in 3D. This depicts that in coculture VEGF levels are fold increased compared to HUVEC in 2D culture. The slight decrease in day 7 was not a

significant decrease thus the direct co-culture model on this scaffold was found to support angiogenesis.

3.9 NITRIC OXIDE BASED COCULTURE STUDIES

3.9.1 Nitric Oxide Assay:

NO is a simple diatomic gas which is formed as a byproduct of the conversion of L-arginine to L-citrulline by NO synthases (NOS). In the circulation, the production of NO by vascular cells promotes blood flow by inhibiting smooth muscle contraction and platelet adhesion and aggregation. These effects of NO are mediated by the activation of soluble guanylyl cyclase and the consequent rise in guanosine-cyclic monophosphate (cGMP) in vascular smooth muscle cells (SMCs) and platelets. While endothelial cells continually release small amounts of NO through a constitutive isoform of NOS, SMCs express an inducible isotype, termed inducible NOS (iNOS), which is capable of synthesizing large quantities of NO (Elsie S. Place *et al*,2009).

This study was done to assess the nitric oxide production by co-culture of HUVEC with UCMSC differentiated to hSMC, hSMC (UCMSC differentiated to SMCs and HUVEC on the GeVAc PCL82 scaffold, due to its role in angiogenesis.

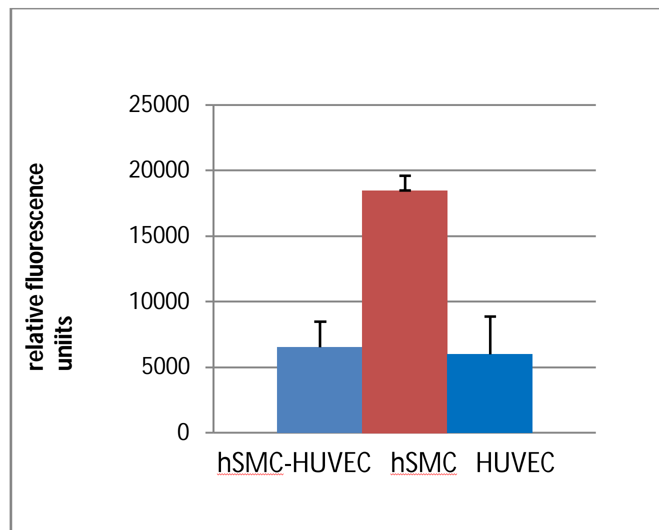


Figure 3.18: Nitric oxide assay of hSMCs co-cultured with HUVEC, hSMC and HUVEC on GeVAc PCL82

Total nitric oxide released in response to direct co-culture (hSMC- HUVEC), hSMCs and HUVEC was analysed according to the fluorescence intensity produced by fluorescent triazolofluorescene. The fluorometric nitric oxide synthase detection system (SIGMA) utilizes a cell permeable diacetate derivative of diaminofluorescein (DAF-2DA) which penetrates the cell and hydrolysed by intracellular esterase activity to DAF2. That in turn reacts with nitric oxide produced by Nitric Oxide Synthase to form a fluorescent triazolofluorescene.

Nitric oxide was found to be highly produced by hSMCs on GeVAc PCL82 scaffold (UCMSC differentiated to SMCs) but lower production was found for HUVEC and hSMC on GeVAc PCL82. This study was done to detect the nitric oxide release inorder to confirm the nitric oxide production by the cells used in this study,

3.9.2 Real Time PCR

In this study, using real time PCR quantification was done to check the iNOS and eNOS marker expression by UCMSC differentiated to hSMCs on GeVAc PCL 82 scaffold. Real Time PCR using SYBR Green was used to quantify the expression of genes in this study.

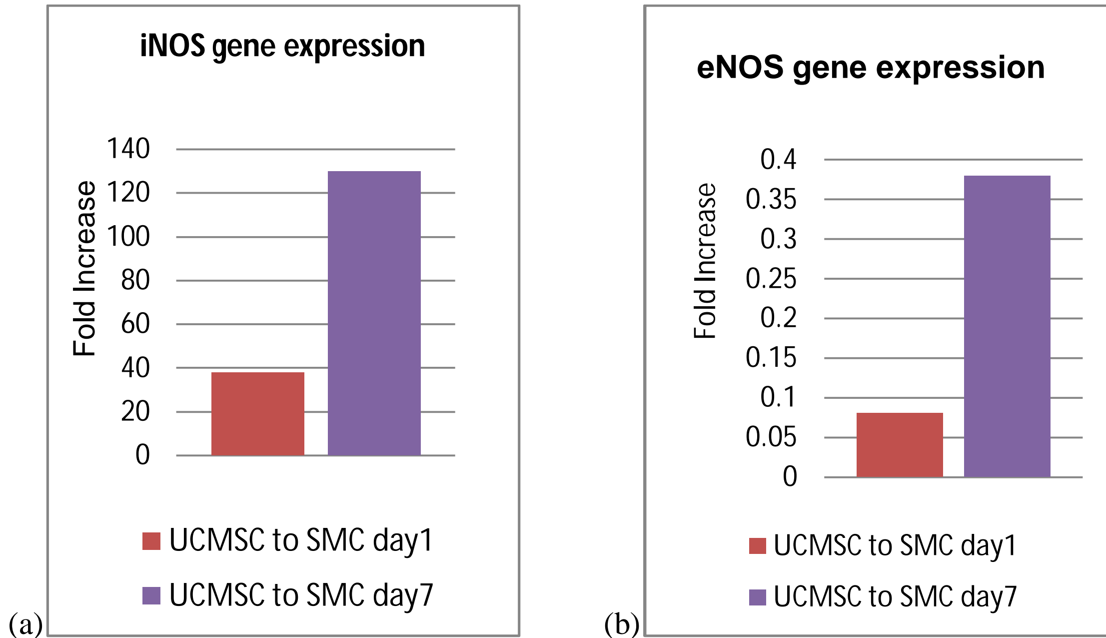


Figure 3.19: Real Time PCR: (a) expression of iNOS by UCMSC differentiated to hSMC on day1 and day 7 ;(b) expression of eNOS by UCMSC differentiated to hSMC on day1 and day 7. UCMSC on 2D were taken as control.

iNOS was originally identified in cytokine-activated macrophages. However, studies have shown that SMCs as well as several other cell types respond to proinflammatory cytokines by transcribing the same iNOS gene as the macrophage. Since iNOS is expressed during neointimal formation but not in the normal arterial wall and since intimal SMCs exhibit a different phenotype and express a set of genes that is not expressed by SMCs of the media, the capacity to express iNOS may be related to the acquisition of the neointimal phenotype where the smooth muscle cells show high proliferation (Yan and Hansson, 1998).

In this study iNOS and eNOS expression was found to increase on day 7 of UCMSC differentiated to hSMC on GeVAc PCL 82 scaffold than on day 2.130 fold increase was observed for iNOS in co-culture model whereas 0.38 fold increase was observed for eNOS when compared to day 1 differentiation of UCMSC to hSMC on GeVAc PCL 82 scaffold. According to the literature, this acquisition of iNOS production by hSMCs can be related to neointimal formation thus showing the highly proliferative SMCs. As we have not given any angiogenic cues, this proliferation indicates the smooth muscle phenotypic characteristic on differentiation with TGFβ1.

Another Real Time PCR experiment was conducted (fig:20) to see the levels of iNOS and eNOS expression in direct co culture model. Synthesis of NO by endothelial NOS (eNOS) is critical for the maintenance of vascular homeostasis, in part due to its inhibitory actions on vasomotor tone. it has now been studied that NO has a number of other important effects on vascular function, such as inhibition of adhesion molecule expression and platelet aggregation, prevention of smooth muscle cell (SMC) proliferation, and modulation of vascular growth (S. Babaei, D.J. Stewart, 2002).

In this study, eNOS was found to show fold increase on day 7 of coculture (HUVEC with hSMCs) on GeVAc PCL 82 scaffold. 2 fold increases was found when compared to the expression in HUVEC without the scaffold. No significant increase in eNOS was found on day 1 of coculture. This suggests the increased tendency of angiogenesis, which was supported by fold increase in VEGF, and inhibitory effect on smooth muscle cell proliferation.

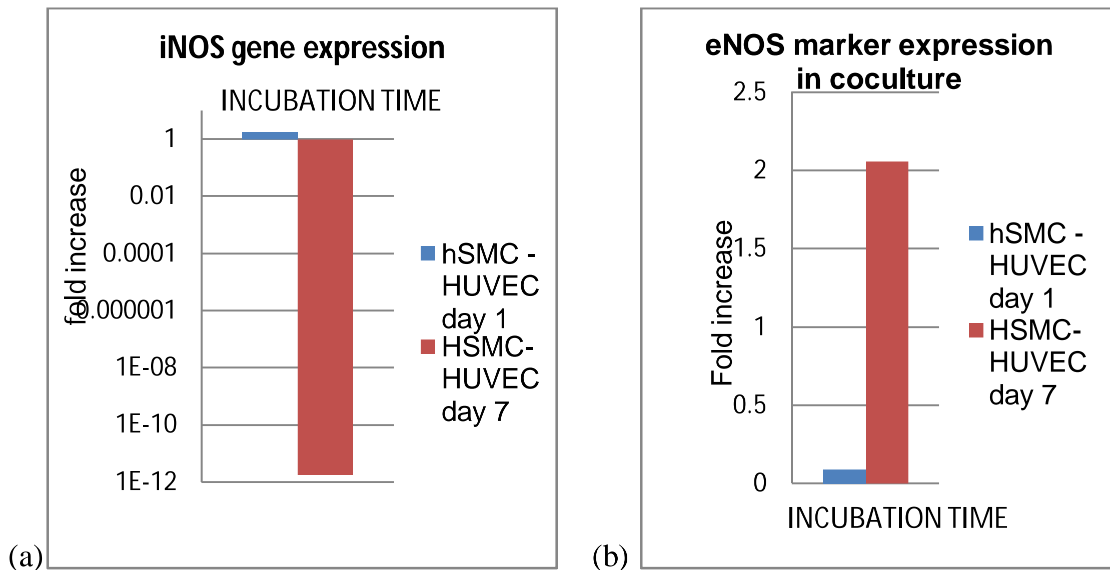


Figure 3.20: Real Time PCR: (a) iNOS expression by hSMC cocultured with HUVEC ON DAY 1 and day 7 when HUVEC was taken as the control.

According to this study, iNOS levels where downregulated to about 10^{12} folds in day 7 of coculture when compared to HUVEC on 2D. In direct coculture, fold decrease in iNOS as compared with the HUVEC in 2D suggested that no inflammatory response was triggered in direct co-culture model of HUVEC and differentiated UCMSCs to SMCs on GeVAc PCL82 scaffold.

CHAPTER 4

SUMMARY AND CONCLUSION

In this study, a biomimetic scaffold using Gelatin colpolymerised with Vinyl Acetate (GeVAc) and blended with Polycaprolactine (PCL) in the ratio 8:2 using electro spinning technology is a novel approach to vascular tissue engineering. A pore size of 3.75± 0.75 μm and 1.07±0.36 μm diameter was obtained on analysing SEM images via Image J software. Cells used for the coculture study like UCMSC and HUVEC were isolated. UCMSC were found to show more stemness at passage 4 expressing 94.3% of CD44 and 71.7% of CD105 stem cell specific markers. Stemness was further proved by bi-lineage differentiation of UCMSC to chondrocytes as well as adipocytes. Biocompatibility of UCMSC and HUVEC were proven by direct contact assay with GeVAc PCL 82, SEM analysis and cell proliferation assay where the cells under study were found to show little significant difference in the proliferation rates on day 3,5 and 7, thus proving the biocompatibility of GeVAc PCL82 scaffold to the cells .HUVEC were able to express PECAM, VCAM, vWF, phalloidin, and Ac LDL uptake on GeVAc PCL 82 scaffold which showed the functional marker expression responsible for angiogenesis, anti thrombosis etc. UCMSC were able to grow and differentiate to SMCs on GeVAc PCL82 scaffold when given a biochemical signal provided with TGF β1 on 7th day. This was further proven by SEM analysis showing typical SMCs hill and valley morphology of the differentiated cells on GeVAc PCL82 scaffold as well as an increased SMC specific marker (SM22 and calponin) expression which was found to increase on day 7 of differentiation. Thus GeVAc PCL82 proves to be a suitable matrix for differentiation studies in graft development.

Direct coculture possibilities was shown on GeVAc PCL82 scaffold using HUVEC and UCMSC differentiated to SMC. This direct coculture model was illustrated by confocal microscopy showing the expression of vWF of HUVEC and alpha SMA expression of hSMCs thus proving the functional cells on the scaffold. SEM analysis showed the cells growing in direct coculture over the GeVAc PCL82 scaffold. It was

further proven by quantifying VEGF expression via RT PCR which showed the angiogenic property of HUVEC in this direct coculture model.

Nitric oxide levels were found to be critical in a coculture model. Nitric oxide assay gave the nitric oxide production higher in UCMSC differentiated to SMC on the GeVAc PCL82 scaffold. On the other hand, HUVEC and HUVEC directly cocultured with UCMSC differentiated to SMC on scaffold showed lesser nitric oxide production. This method helped to detect the nitric oxide release by the cell culture systems used in this study. Further nitric oxide synthases (eNOS and iNOS) gene were quantified using RT PCR where on differentiation of UCMSC to SMCs on GeVAc PCL82 higher levels of eNOS (0.4 fold increase) and iNOS (130 fold increase) were expressed, when compared with UCMSC in 2D. These results showed highly proliferative functional smooth muscle cells giving higher iNOS on differentiation. In direct coculture model on GeVAc PCL82 scaffold eNOS activity was upregulated two fold whereas iNOS activity was downregulated twelve fold, when compared to HUVEC on 2D. This shows a no inflammation, higher angiogenic and vasorelaxation capability of HUVEC in coculture model.

In this study we have demonstrated a novel GeVAc PCL 82 scaffold which could hold the cells (HUVEC and UCMSC) maintaining its functionality. Differentiation of UCMSC to SMC was also supported by GeVAc PCL82 upon providing biochemical cues (TGF-β1). The direct coculture of UCMSC differentiated to SMCs on GeVAc PCL82 scaffold was proven by SM22α and calponin marker expression. Direct coculture of UCMSC differentiated to SMCs and HUVEC on this scaffold proved it as a promising potential scaffold for vascular tissue engineering. VEGF expression along with upregulated eNOS expression in the direct coculture model showed the functionality of HUVEC along with its angiogenic, antithrombogenic and vasodialation property. On differentiation of UCMSC to SMCs, an upregulation in iNOS expression showed the increased proliferation capability of SMCs. Thus this nitric oxide based study has proven

that GeVAc PCL82 is a promising scaffold structure that is found to support coculture of vascular cells, promoting angiogenesis as well as differentiation of UCMSCs to SMCs.

4.1 FUTURE PROSPECTS

The future prospects of this study include:

- UCMSCs differentiated to SMCs can be seeded with HUVEC on tubular GeVAc PCL82 construct in a mechano-stimulation model using a bioreactor.

- Pulsative media flow mimicking the pressure conditions in an *in vivo* blood vessel can be applied to the tissue engineered construct and NOS activity as well as the presence of angiogenic markers like VEGF etc. can be assessed.

- The differentiation pattern of UCMSC in the presence of HUVEC without any growth factor under mechanical stimulation in a bioreactor can be assessed by quantifying SMC marker expression.

- Importance of further investigations is paramount as numerous mechanisms within the various pathways of Nitric oxide remains unsolved and more study on the pathways involved in nitric oxide synthesis in coculture TEVG model is warrented at each stage of vascular graft development.

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