CO-CULTURE OF STEM CELL DERIVED ISLETS & ENDOTHELIAL CELLS ON A THREE DIMENSIONAL BIOMATERIAL TOWARDS A TISSUE ENGINEERED BIO-HYBRID PANCREAS

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NEENA ALOYSIOUS

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DOCTOR OF PHILOSOPHY

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DECLARATION

I, **Neena Aloysious,** hereby certify that I had personally carried out the work depicted in the thesis entitled, "**Co-culture of stem cell derived islets & endothelial cells on a three dimensional biomaterial towards a tissue engineered bio-hybrid pancreas",** except where due acknowledgement has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that **Ms. Neena Aloysious** in the **Division of Tissue Engineering and Regeneration Technologies** of this Institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled ***"Co-culture of stem cell derived islets & endothelial cells on a three dimensional biomaterial towards a tissue engineered bio-hybrid pancreas"*** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from Institutional Animal Ethics Committee for carrying out the study.

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The thesis entitled

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ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
BSA	Bovine serum albumin
cDNA	Complimentary Deoxyribonucleic acid
DDA	Dextran dialdehyde
DEXGEL	Dextran Gelatin
DMEM HG	Dulbecco's Minimal Essential Medium-High glucose
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ehylene diamine tetraacetic acid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
hASC	Human adipose stem cells
hILC	Human islet like cells
IgG	Immunoglobulin
MACS	Magnetic Activated Cell Sorting
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic acid
MSC	Mesenchymal stem cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerthyrin
PEG	Polyethylene glycol
rASC	Rabbit adipose stem cells
RGD	Arginine-Glycine-Aspartic acid
rILC	Rabbit islet like cells
SEM	Scanning electron microscopy
SFM	Serum free medium
VEGF	Vascular endothelial growth factor

SYNOPSIS

Diabetes mellitus the silent epidemic has become one of the most significant non-communicable diseases globally. Diabetes mellitus is a chronic metabolic disease characterized by the elevation of glucose levels in blood either due to inherited and/or acquired deficiency in production of insulin by the pancreatic islets, or by the ineffectiveness of the insulin produced. This condition leads to abnormalities in the metabolism of carbohydrate, protein and/or fat which in turn results in micro-vascular/macro-vascular complications and neuropathies.

Diabetes is mainly treated by administration of oral anti-diabetic drugs or insulin injection. However these approaches cannot mimic the physiological oscillating pattern of insulin release to achieve normoglycemia thereby cannot prevent the long term complication associated with diabetes and hypoglycemic shock. Transplantation of pancreatic islets aims to achieve euglycemia by following the rhythmic oscillating pattern of insulin release as in physiological manner. For transplantation purposes, islets are isolated from donor pancreata by enzymatic digestion which disrupts the islet's extracellular matrix (ECM) and microvasculature generating cellular stress which results in islet cell death, reduced function and survival with poor transplantation outcomes. Islet cells need to be cultured *in vitro* prior to transplantation and long term culture without compromising cell viability and function is challenging. Pancreatic islets *in vitro* culture require a solid matrix to serve as a supporting matrix to promote its survival and substitute for the absence of

native ECM; thus, culturing on a biodegradable scaffold provides a suitable alternative.

Applying the principles of tissue engineering approach which proposes to repair or regenerate damaged organs using a combination of cells, biomaterials and cytokines the longevity as well as function of islet cells could be enhanced. The biomaterial scaffolds used for islet culture should have distinct characteristics. The scaffold should serve as a structural component for maintaining the appropriate three dimensional (3D) architecture of the islet construct. It should have high surface area to volume ratio, high interconnectivity as well as pore geometry for proper orientation of cells, to support cell adhesion and effective oxygen/mass transfer characteristics. The pore size of the scaffold is a critical factor in islet culture, since islets have a diameter of 100-200µm which means that pore size of the scaffold should be greater than islet diameter. The scaffold chosen should also be biodegradable to allow tissue regeneration over time.

Pancreatic islets are one of the most vascularized organs of the body consisting of a framework of capillaries critical for receiving nutrients, mass transfer and intercellular communication between islet cells. Pancreatic endocrine cells strictly require endothelial signals for their differentiation and function. Vascular endothelial cells (EC) induce the development of islets and later islets stimulate EC to form a branching network of capillaries within the islet. Endothelial cells also contribute to the formation of vascular basement membrane of islets which is solely responsible in enhancing beta (β) islet cell proliferation, function and survival.

The need to obtain multiple donor pancreases for each patient and the uncertainty regarding long term side effects from immunosuppression also limit the benefits of islet transplantation to patients with highly uncontrolled diabetes. The use of stem cell differentiated islets could overcome the limited availability of donor islets for transplantation purpose. While embryonic stem cells are known to exhibit unlimited differentiation potential *in vitro* and *in vivo*, their application is limited by ethical, legal, and political concerns, as well as by scientific issues of safety and efficacy. Adult stem cells derived from autologous source offer an alternative approach that circumvents many of these concerns. However, the most abundant and accessible source of adult stem cells is adipose tissue, whose differentiation potential could be exploited to derive pancreatic endocrine lineage.

The first hypothesis of the present study is that adipose stem cells could be a better source to derive pancreatic endocrine lineage cells *in vitro* which could overcome the severe scarcity of donor islets for transplantation purpose. Recognizing the importance of scaffold in tissue engineering islets, the second hypothesis is that a biodegradable and biomimetic 3D scaffold with adequate pore sizes and mechanical strength is a critical requirement to support islet cell survival. Since endothelial cells play an important role in islet neogenesis and function, third hypothesis is that culturing stem cell differentiated islets and EC together as a co-culture model on the scaffold matrix may improve islet cell survival and function.

The thesis has been divided into six chapters. First is the introduction chapter, which sketch out the purpose and significance of the present study. Briefly, introductory chapter comprises of an understanding of the diabetic state, its statistics

and the therapeutic approaches. The study hypotheses, objectives and the relevance of the study in the present global scenario also form part of this chapter.

The second chapter is the literature review that details with causes of diabetes, role of pancreatic islets. Alternative sources of cells and the potential of stem cells to be used in islet transplantation and signaling cues adopted for its differentiation to islets and also the role of EC in neo-islet formation, signals promoting the formation of vascular basement membrane in supporting islet cell function are described in this chapter. The ECM composition of islets, literature review of various scaffolds and extracellular matrix components used for culturing islets, their positive impact on enhancing islet longevity and function as well as their signaling mechanism are explained.

The third chapter comprises the materials and methodology adopted in the present study. First is the enzymatic procedure used for isolating islets from rabbit pancreata. Islets cells were assessed for its purity by dithizone staining. Next is the procedure for isolating stem cells from rabbit and human adipose tissue. The morphology of the isolated cells was analyzed by phase contrast microscopy, characterized by immunofluorescence and flow cytometry analysis. For co-culture system, endothelial cells were isolated from human umbilical vein and characterized by immunofluorescence. Next the methodology adopted for fabrication of the polymeric scaffold using gelatin and oxidized dextran by freeze drying method is discussed in this chapter. Further physicochemical characterization of the scaffold was done by Fourier Transform- Infrared Spectroscopy (FT-IR) to evaluate the functional groups, contact angle assay to study surface characteristics, scanning

electron microscopy to reveal the pore morphology, liquid extrusion porosimetry to evaluate pore geometry, and biodegradation/swelling assays to study the nature of the scaffold. The cytocompatibility of the scaffold was assessed by *in vitro* cytotoxicity assay. The suitability of the scaffold in culturing islets cells was evaluated using rabbit islets. One month cell seeded 3D construct was evaluated for its functionality mainly insulin secretion in response to varying glucose concentrations (5mM and 25mM) compared to islets cultured on 2D tissue culture polystyrene dish (TCPS). Furthermore the viability of cells on scaffold was assessed by live-dead assay and ECM of islet cells was evaluated by immunohistochemistry for collagen IV. Rabbit and human adipose stem cells were differentiated to islet like clusters (ILC) on TCPS dish, TCPS dish coated with scaffold matrix both serving as 2D culture systems and scaffold which serve as 3D culture. To evaluate whether differentiation process resulted in islet specific marker expression on 2-D and 3-D cultures, ILC were subjected to gene and protein expression analysis by polymerase chain reaction (PCR) and immunophenotype respectively. The viability and function of ILC on 2D and 3D cultures were assessed by live-dead assay and insulin quantification respectively. The ILC and EC were co-cultured on 2D and 3D culture systems; further analyzed for its viability and function. Furthermore ECM was analyzed by immunohistochemistry.

The fourth chapter comprising results is divided into six sections. The first section includes the results of characterization of stem cells isolated from rabbit and human adipose for its stemness. Isolated cells from rabbit and human adipose tissue were positive for mesenchymal marker vimentin, stem cell markers like CD105,

CD44 and negative for hematopoeitic marker CD34/CD45. In addition flow cytometry analysis results showed higher population expressing stem cell properties. Islet cells isolated from rabbit pancreas expressed islet specific markers insulin, glucagon and somatostatin. Furthermore this section includes the characterization of endothelial cells from human umbilical vein confirming the presence of endothelial markers like von Willebrand factor and CD31.

The second section deals with the results from characterization of the fabricated freeze dried scaffold. The Scanning electron micrograph showed the morphology of the scaffold with interconnected pores. Liquid extrusion porosimetry data showed that the pore sizes range from 100-400µm. FT-IR data confirmed the efficient crosslinking of oxidized dextran and gelatin by Schiff base linkage. Contact angle data revealed the hydrophilic-hydrophobic nature of the scaffold. The higher swelling ratio of the scaffold is an indication of the efficient nutrient and medium uptake ability of the scaffold. Biodegradation profile revealed that the scaffold had slow degradation rate in the study period of one month. The *in vitro* cytocompatibility assay confirmed that the scaffold was non-cytotoxic.

The third section includes the results from rabbit islets cultured on 3D in comparison to 2D. Insulin gene expression and insulin secretion assay of rabbit islets confirmed that scaffold group islets secreted significantly higher insulin when compared to that of 2D culture. Live-dead assay showed viable islets on day 60 on scaffold whereas islet cells on day 30, 2D was found to be non-viable.

The fourth section includes the results from characterization of 2D and 3D cultured ILC from rabbit. The fifth section includes results from characterization of

2D and 3D cultured ILC from human. The insulin gene expression and secreted insulin in response to high glucose concentrations of ILC was significantly higher on scaffold group in comparison to 2D culture group. The immunofluorescence data confirmed the presence of alpha and delta cells in addition to β cells on differentiated ILC by their simultaneous secretion of glucagon, somatostatin and insulin respectively. Viability data revealed viable islet clusters on scaffold throughout the study period of 30 days in contrast to the 2D cultured ILC.

The sixth section deals with the data obtained from co-culture of EC and ILC on 2D and 3D cultures. Insulin secretion and viability of ILC were significantly higher on the scaffold–ILC-EC groups in contrast to 2D culture and ILC-scaffold culture groups.

In the fifth chapter the implications of the findings from 2D and 3D cultures of ILC and ILC-EC constructs are discussed in detail. The results obtained are interpreted in this section. The importance of scaffold in maintaining the phenotype and structural integrity of ILC as well as its role to serve as a substrate for synthesizing the ECM components to enhance islet survival are discussed.

The sixth chapter summarizes the overall results obtained. The importance of scaffold and endothelial cells in promoting islet function and future aspects of the investigations are proposed.

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus remains a prevalent and challenging health problem in the 21st century. International Diabetes Federation estimated that in 2012, more than 371 million people worldwide are affected with diabetes and is expected to rise to 552 million by 2030. Diabetes mellitus is a group of metabolic disorders either due to defect in insulin secretion or insulin action or both (Zimmet *et al*., 2003). Insulin is a peptide hormone produced by pancreatic islets with a role to regulate carbohydrate and fat metabolism in the body. Insulin secreted by pancreatic islets allows glucose from food to enter the body's cells where it is converted into energy for muscle and tissue function.

A person with diabetes is unable to absorb glucose properly; therefore glucose remains circulating in the blood resulting in hyperglycemia. Chronic hyperglycemia leads to disturbances in carbohydrate, fat and protein metabolism which results in tissue damage over time. The common symptoms associated with diabetes are polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). Untreated diabetes can result in several complications resulting in diabetic ketoacidosis and nonketotic hyperosmolar coma. Long term consequences could end up in life threatening health complications like cardiovascular disease, neuropathy, nephropathy, retinopathy and microvascular

disease. The mean normal blood glucose level in humans is about 5.5 mM (5.5 mmol/L or 100 mg/dL). The normal value for fasting blood glucose level and post prandial value (2 hours after consuming glucose) should be between 70 -100 (mg/dL) and less than 140mg/dl respectively. Fasting blood glucose values between 100-126 mg/dl and post prandial between 140-200 mg/dl are considered to have pre-diabetes. Fasting blood glucose levels greater than 126 mg/dl and post prandial greater than 200 mg/dl are considered to have diabetes.

1.2 Types of diabetes

There are mainly two types of diabetes (Figure 1.1). Type I diabetes also known as insulin dependent diabetes or juvenile onset diabetes, is an autoimmune disease which results in destruction of insulin producing beta (β) islets of pancreas. Due to the reduced number of β cells, insulin production is so less that the person has to solely depend on exogenous insulin to achieve normoglycemia.

Type II diabetes also known as adult onset diabetes is caused either because pancreas does not produce enough insulin or due to insulin resistance. Insulin resistance is an abnormal condition whereby the receptors of the cells that respond to insulin have lost sensitivity and become "resistant" to insulin. In the case of insulin resistance, the pancreas produces insulin, but insulin sensitivity of the cells is impaired, hence glucose cannot enter body cells resulting in hyperglycemia. Central obesity is a major factor contributing to type II diabetes. Abdominal fat secretes hormones mainly adipokines which impair glucose intolerance resulting in insulin resistance. Administration of oral anti-diabetic drugs and medications to improve

insulin sensitivity could be a treatment option to some extent if the β islets are producing insulin. If the β islets are impaired, the patient has to rely on insulin therapy to regulate the blood glucose levels.

Another type of diabetes known as gestational diabetes resembles type II diabetes involving a combination of reduced insulin secretion and insulin responsiveness. It is triggered by pregnancy and may improve or disappear after delivery.

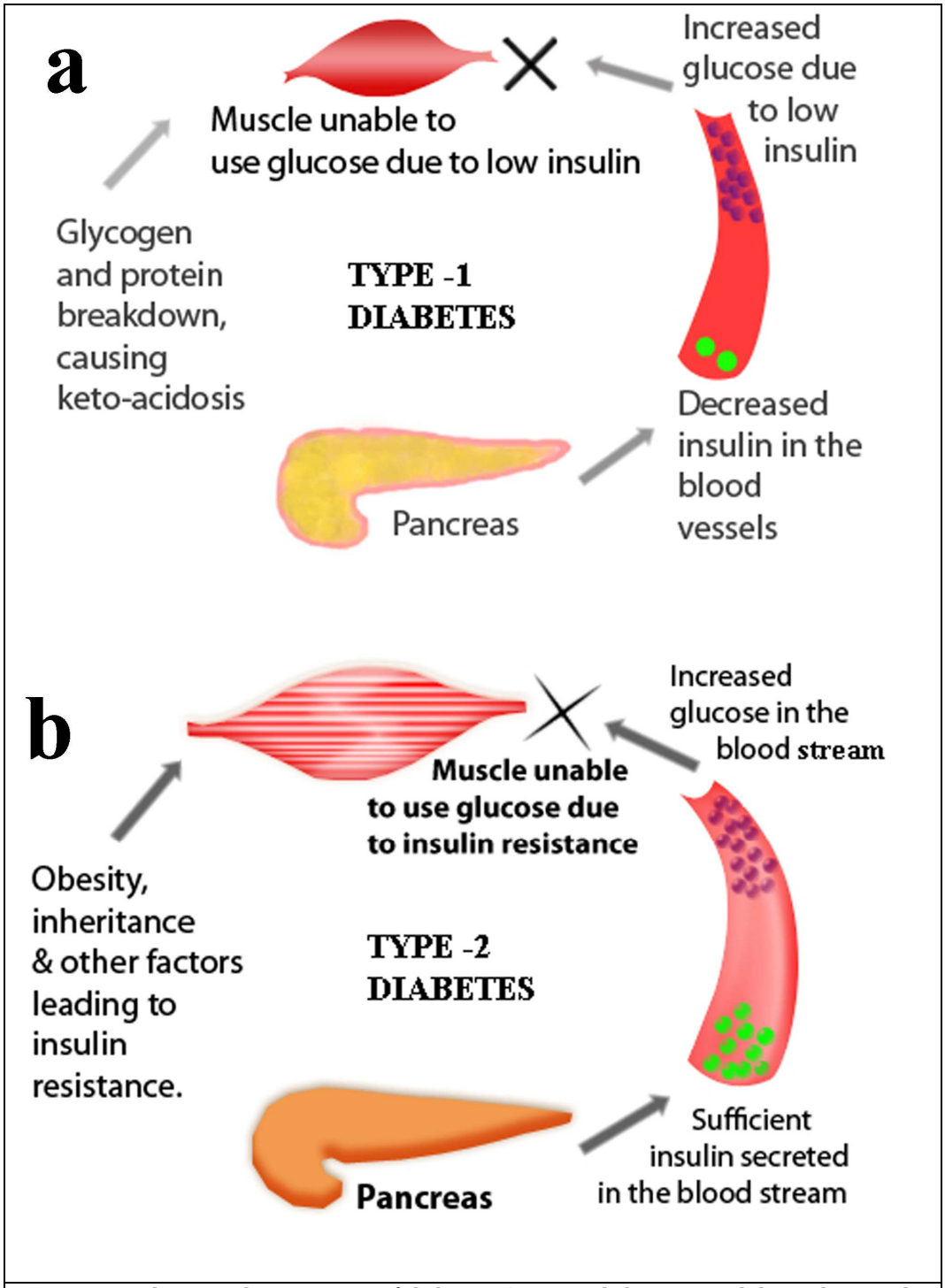

Figure 1.1 Schematic showing types of diabetes. a) Type I diabetes caused due to less insulin production b) Type II diabetes caused due to insulin resistance.

1.3 **Treatment for diabetes**

The major goal in treating diabetes is to minimize any elevation of blood sugar (glucose) avoiding the risk of severe hypoglycemia. Anti-diabetic drugs are used to lower the blood glucose levels in type II diabetic patients. Drugs act in a way that either increases the amount of insulin secretion by pancreas (*eg.* sulfonylurea) or increase the sensitivity of target organs to insulin (*eg.* metformin and thiazolidinediones) or which reduces the rate at which glucose is absorbed from the gastrointestinal tract (*eg*. alpha-glucosidase inhibitor).

Insulin therapy is the treatment of diabetes by administering exogenous insulin, subcutaneously either by injections or by an insulin pump. Insulin could also be given intravenously. Insulin is classified into three types based on the rate at which they are metabolized by the body. They are rapid acting insulin, intermediate acting insulin and long acting insulin. The major drawback associated with administration of drugs and insulin injections are the chances for hypoglycemic shock, besides the end stage complications associated with diabetes cannot be prevented. Although intensified insulin regimen improves glycosylated hemoglobin concentrations and reduces the rate of long-term complications, it does not prevent them.

Pancreas and islet transplantation offers the potential therapeutic option for achieving euglycemia. Pancreas transplantation involves a surgical procedure implanting a healthy pancreas from a deceased donor to a recipient. The side effects of pancreas transplant could be significant; therefore it is usually advised for those

who have serious diabetic complications and is done along with kidney transplant. In order to prevent rejection by the immune system, patients must take a regimen of immunosuppressive drugs. However immunosuppression increases the risk for a number of different kinds of infection and cancer. Over recent years, long-term success of pancreas transplant has improved and risks have decreased, but the severe scarcity of donor organs limits its application.

Islet transplantation involves the transplantation of pancreatic islets from donor pancreas to recipient's liver through hepatic portal vein. Islet transplantation requires minimum 10,000 islet equivalents/kg body weight of the person which demands at least two donor pancreata. Once transplanted, islets sense the blood glucose levels and start producing insulin thereby achieving normoglycemia. Since islets are taken from allogeneic source, immunosuppressive drugs are required to prevent graft rejection. Islet transplantation is advantageous over pancreas transplantation for the reason that islet transplant is a minor surgical procedure, safer and less expensive.

1.4 Tissue engineering approach

The pancreas of deceased donor is subjected to enzymatic digestion to separate islets which disrupts the extracellular matrix (ECM) thus reducing the long term survival of transplanted islets. The use of porous three dimensional (3D) biodegradable scaffolds mainly made of polymeric biomaterials; could substitute the role of native ECM thus providing the structural support for cell attachment and subsequent tissue development. Tissue engineering is an interdisciplinary approach

that applies the principles of engineering, material science and life science towards the development of biological substitutes that restore, maintain, or improve tissue function (Langer and Vacanti, 1993) (Figure 1.2). Cells, scaffolds and growth-stimulating signals are generally referred to as the tissue engineering triad, the key components of engineered tissues.

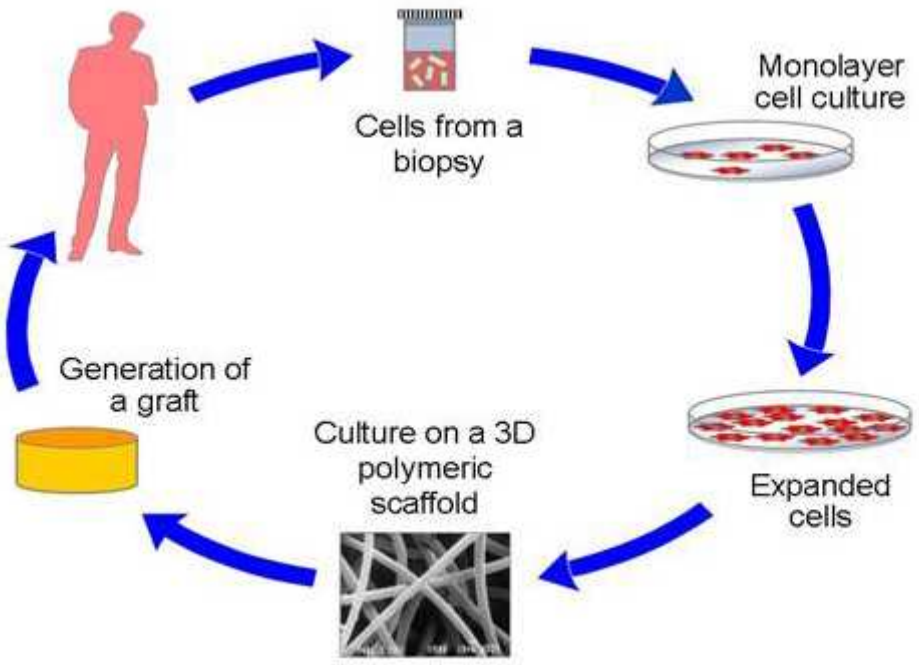

Figure 1.2 Basic concepts of Tissue Engineering. The cells from the patient could be expanded *in vitro* thereafter cultured on a three dimensional scaffold to generate a graft and transplanted back to the patient

The scaffolds could also be modified with extracellular matrix (ECM) ligands, thus promoting infiltration of vascular precursor cells and/or enhancing islet–ECM interactions, an essential factor in maintaining islet function. The scaffold could serve as a vehicle for islet transplantation that promotes engraftment, revascularization, and integration of the implant with the host.

1.5 Islet vasculature

Pancreatic islets are one of the most vascularized organs of the body. Vascular EC not only make up a significant part of the islet volume but also form an integral part of islet function. The islets of Langerhans consist of a framework of capillaries in which endocrine cells are closely associated with islet vasculature, receiving nutrient and hormonal signals across the capillary endothelium. The islet vascular system is critical for intercellular communication between islet cells. The enzymatic digestion procedure, used to isolate islets, partially removes intraislet EC and thereby contributes to transplant failure. Transplanted islets require 10–14 days to revascularize; yet revascularization does not fully restore microvessel density when compared to endogenous islets even at 1 month post-transplant, and by this time frame islets lose their endocrine function.

Endocrine pancreatic β cells require endothelial signals for their differentiation and function. Vascular EC induce the development of islets and later islets stimulate EC to form a branching network of capillaries within the islet. Endothelial cells also contribute to the formation of vascular basement membrane of islets which promotes β islet cell proliferation, function and survival.

1.6 Generation of islets from alternate sources

The inadequate supply of donor pancreas and the high incidence of diabetes make it impossible to achieve insulin independence in diabetic patients. One possibility is to generate islets *in vitro* from stem cells. The potential use of adult stem cells offers the advantage of an autologous model wherein a patient's own cells

can be used thereby circumventing immune rejection. In addition, adult stem cells are less associated with ethical and societal concerns.

1.7 Hypothesis

The first hypothesis is that adult stem cells could be an attractive and alternative source for generating islets under *in vitro* conditions. Secondly, tissue engineering approach utilizing biomaterial scaffold could be employed for culturing islets. The scaffold used could play the role of ECM serving as a supporting framework for cell attachment, function and survival.

The final hypothesis is co-culturing endothelial cells along with stem cell differentiated islets on a biomaterial scaffold could favor islet survival and function. In this case, signals from EC could favor differentiation of stem cells towards islets and contribute to the vascular basement formation which is an important criterion for long term islet survival and function.

Taken together, stem cell derived islets and EC co-cultured on a 3D biomaterial scaffold are expected to overcome the problem of vascularization and may enhance islet viability, survival and insulin secretion.

1.8 Objectives

- To isolate stem cells from adipose tissue of rabbit/human and to characterize it for various stem cell markers and differentiation to at least two lineages under *in vitro* conditions.

- To fabricate a 3D scaffold for culturing islets and to characterize its physicochemical properties.
- To differentiate stem cells to islet like clusters (ILC) on two dimensional (2D) culture plate and on 3D scaffold and study its functionality.
- To optimize co-culture system of stem cell differentiated islets and EC and to study the functionality and survival of islets in co-culture system.

  Furthermore to study the mechanism of interaction between EC and stem cell differentiated islet like cells.

1.9 Significance

The present study is based on tissue engineering concept for generating pancreatic ILC from adult stem cells. Here we have fabricated a novel 3D porous and biodegradable scaffold comprised of natural polymers for culturing islet cells. The scaffold fabricated has shown to support islet survival and function by maintaining its phenotype. Our long term goal is to create a biohybrid pancreas by tissue engineering approach which has the ability to secrete islet hormones as the native islets thereby regulating glucose metabolism. The importance of scaffold and EC in islet culture has been demonstrated in the present study. The approaches and findings used in the present study present potential strategies for preparing transplantable islets with enhanced function and survival prospects.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus occurs as a result of imbalance between demand and production of insulin which results in hyperglycemia. The importance of the pancreas in carbohydrate metabolism had been known since experiments in 1890. The entire pancreas from dog was removed and the symptoms of severe diabetes, namely, high blood sugar (hyperglycemia), glycosuria, and finally death involving ketosis and coma were observed in 2 or 3 weeks. This finding was the first experimental proof that diabetes may be of pancreatic origin (Mering and Minkowski, 1890). The elevated fasting and post-prandial blood glucose levels exposes the patient to acute and chronic complications (micro- and macro-vascular) leading to blindness, kidney failure, heart disease, stroke and amputations(American Diabetes Association, 2012) .

The inadequate production and secretion of insulin results in altered blood glucose dynamics and insulin deficiency is the hallmark of diabetes, with insulin resistance being associated with some forms of the disease. Insulin (Latin: '*insula*' for 'island') is a naturally occurring peptide hormone secreted by pancreatic islet cells.

2.2 Pancreatic islets

Islets of Langerhans commonly referred to as "islets" are cell clusters discovered by Paul Langerhans, German anatomist in 1869 which constitute approximately 1% to 2% of total mass of pancreas. There are several cell types within islets which work together to regulate blood glucose levels (Figure 2.1). Pancreatic alpha (α) cells (15–20% of total islet cells) produce glucagon; *β* cells (65–80%) produce insulin and amylin; gamma (γ) cells (3–10%) produce somatostatin; PP or delta (δ) cells (3–5%) produce pancreatic polypeptide and epsilon (ε) cells (<1%) produce ghlerin.

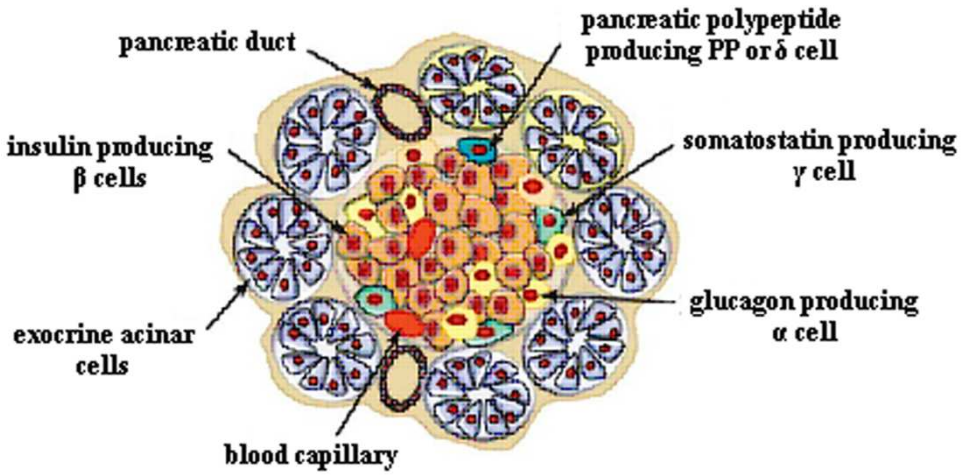

Figure 2.1 Cytoarchitecture of pancreatic islets showing different cell types. Islets of Langerhans comprises of mainly alpha (α), beta (*β*), gamma (γ) and delta (δ) cells each of them having its own endocrine function.

2.3 Insulin hormone

Insulin produced by pancreatic *β* islets allow glucose from food to enter the body's cells where it is converted into energy for muscle and tissue function. Human

insulin is composed of 51 amino acids having molecular weight of 5808 Da. It is a dimer of an A-chain and a B-chain, which are linked together by disulfide bonds. Insulin binds to specific cellular receptors that facilitate entry of glucose into the cell, which uses the glucose for energy. The increased insulin secretion from the pancreas and the subsequent cellular utilization of glucose results in lowering of blood glucose levels. Lower glucose levels then result in decreased insulin secretion.

2.4 Current approaches for treatment of diabetes

The therapeutic goal for treatment of diabetes should aim towards achievement of normoglycemia. Insulin and/or oral hypoglycemic drugs are administered depending on the nature of disease.

2.4.1 Oral hypoglycemic drugs

Oral hypoglycemic drugs are classified based on their function (Krentz and Bailey, 2005) as illustrated in Table 2.1.

2.4.2 Insulin therapy

Insulin therapy is an efficient tool to improve glycemic control in both types of diabetes. Since the discovery of insulin in 1921 by Banting and Best, a variety of insulin formulations have been developed (Bliss, 1993). Researchers first gave an active extract of the pancreas containing insulin to a young diabetic patient in 1922, and the Food and Drug Administration (FDA) first approved insulin in 1939. The first recombinant human insulin was approved by the FDA in 1982 (Miller and Baxter, 1980). The introduction of human insulin by recombinant technology paved

the way to alter insulin molecule for improved pharmacokinetics (Vajo and Duckworth, 2000). Insulin analogues such as Humalogue and Aspart are available that can be absorbed more easily (Garg *et al*., 1999).

Insulin is usually administered subcutaneously, either by injections or by an insulin pump. Insulin could also be given intravenously. Insulin is classified into three types based on the rate at which they are metabolized by the body. They are rapid acting insulin, intermediate acting insulin and long acting insulin.

The discovery of insulin was initially thought to represent a cure for the disease; a concept that remains common among those unfamiliar with the disorder. However, this is not the case. Acute morbidity and mortality as well as a series of chronic complications still occurs (Podar *et al*., 2000). The risk of severe hypoglycemia limits the chance of the patient achieving euglycemia with insulin therapy.

Table 2.1 Mechanism of action of oral hypoglycemic drugs

Drug	Mechanism of action	Side effects
α-Glucosidase Inhibitors (acarbose, miglitol, voglibose)	Reduces glucose absorbance by acting on small intestine to cause decrease in production of enzymes needed to digest carbohydrates	Flatulence Abdominal bloating
Insulin Sensitisers-Thiazolidinediones (Pioglitazone, Rosiglitazone)	Reduce insulin resistance	Fluid retention weight gain
Insulin Sensitisers Biguanides (metformin)	Acts on liver to cause decrease in insulin resistance	Nausea, Diarrhea Anorexia, Lactic acidosis
Insulin Secretagogues Sulfonylurea (glyburide, glimepiride, glipizide)	Stimulate insulin release by pancreatic β cells	Late hyperinsulinemia hypoglycemia and weight gain

2.4.3 Pancreas transplantation

Pancreas is a gland organ in the digestive and endocrine system of vertebrates. The pancreas is located deep in the abdomen, sandwiched between the stomach and the spine. The endocrine portion which comprises islets of Langerhans produces several hormones mainly insulin whose role is to regulate absorption of glucose into cells.

The goal of pancreas transplantation is to safely restore normoglycemia by providing sufficient β cell mass and the improved glucose control could reduce the long-term complications of insulin-dependent diabetes. The first attempts using sheep xenografts without immunosuppression was reported as failure (Williams 1894). The first clinical pancreas transplantation was attempted in 1966 simultaneous with kidney transplantation in a uremic diabetic patient at the

University of Minnesota, (Kelly *et al*., 1967). The initial pancreas graft and patient survival rates were dismal but increased dramatically in the 1980s. Patients are eligible for a pancreas transplant if they have or are at high risk of secondary complications of diabetes, have disabling or life-threatening hypoglycemic unawareness, or are likely to develop these and are judged to be fit enough to survive the operation. Immediate complications that can occur with all types of pancreas transplant include rejection, thrombosis, pancreatitis, and infection. Pancreas transplantation is now performed as a routine treatment for uremic diabetic recipients of kidney transplants, either simultaneously or after the kidney transplant. Such patients are obligated to immunosuppression and, with a successful pancreas transplant, can achieve insulin independence as well as a dialysis free state. The use of lifelong immunosuppressive drugs and the severe shortage of donor organs are the limitations of pancreas transplantation.

2.4.4 Islet transplantation

Islet transplantation has also evolved as a therapeutic approach for improving glycometabolic control in type I diabetic patients (Figure 2.2). Primitive islet transplantation as a treatment for diabetes was first reported in Bristol (U.K.) in 1894, where portions of a sheep pancreas was transplanted subcutaneously into a 15-year-old boy suffering from diabetic ketoacidosis (Williams, 1894) without immunosuppression and the result was graft rejection. Later islet isografts from normal rats were reported to reverse streptozotocin-induced diabetes in rats (Ballinger and Lacy, 1972).

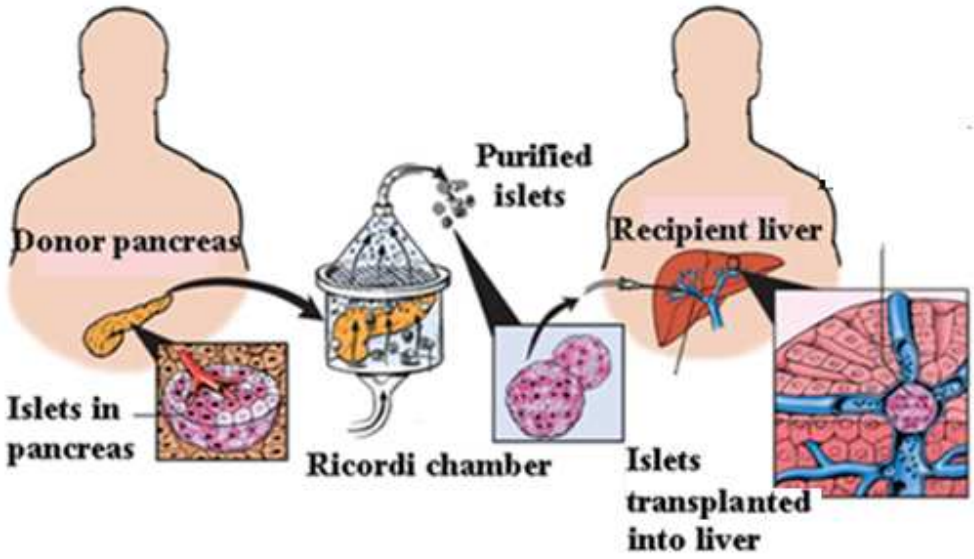

Figure 2.2 Procedure for islet transplantation-The islets from donor pancreas are transplanted into recipient's liver through hepatic portal vein

The first attempts at allogeneic islet transplantation into humans emerged during the late 1970s. In its early days the procedure remained largely unsuccessful, later by 1980s successful transplantation of an isl*et al*lograft was reported in human (Largiadèr *et al*., 1980). Liver was considered to be the best implantation site for islet transplantation and in humans; this could be achieved by percutaneous transhepatic intraportal injection, making it an invasive procedure (Alejandro *et al*., 1987).

With introduction of the automated method for human islet isolation, the panorama of islet transplantation changed dramatically (Ricordi *et al*., 1988). The cell yield after isolation increased significantly using this procedure and along with the use of conventional immunosuppression, allogeneic islet transplantation became successful in patients with type 1 diabetes (Scharp *et al*., 1990, Warnock *et al*.,

1991) with achievement of insulin independence for 1 month following islet transplant. It was reported in islet autografts that 265,000 islets were sufficient to establish insulin independence (Pyzdrowski *et al*., 1992). Later, an insulin-independence rate of 74 percent was achieved two years after autologous islet transplantation in 14 patients who had undergone total pancreatectomy and who had received a portal-vein infusion of more than 300,000 islets (Wahoff *et al*., 1995). In some cases, islet cell function for more than 6 years has been reported (Alejandro *et al*., 1997). However, the need for intense immunosuppression to prevent graft rejection has, limited this approach to patients who are already immunosuppressed either for a previous organ graft or because of simultaneous kidney transplantation. The immunosuppressive regimen itself limited success in some cases, because most protocols use agents that inhibit islet cell function or induce peripheral insulin resistance (Zeng *et al*., 1993).

Encouraging results were obtained from Edmonton group, where large amounts of islet cells from cadaveric pancreases that were not HLA matched were transplanted, with the avoidance of corticosteroids into seven patients with type 1 diabetes who had multiple hypoglycemic episodes or uncontrolled diabetes despite compliance with the prescribed insulin treatment (Shapiro *et al*., 2000). The immunosuppressive regime was steroid free, consisting of dacluzimab [an anti-CD25mAb (monoclonalantibody)], antirejection drug sirolimus and low-dose of calcineurin inhibitor tacrolimus. The islets were prepared in the absence of xenogeneic proteins using human albumin instead of bovine albumin and approximately 10000 IEs (islet equivalents)/kg of body weight was the minimum

islet transplant administered to each patient, often administered as two or sometimes three infusions from sequential donors. Islet culture offers several advantages which include ensuring the quality of islet preparations to decrease immunogenicity of the allograft tissue and also allowing additional time for subject preparation, possible pre-transplant interventions and the opportunity to ship processed islets to remote sites for transplantation (Goss *et al*., 2004).

With recent improvements to the initial Edmonton protocol and the immunosuppressive cocktail, insulin independence has increased to 5 years post-transplantation in 50% of patients (McCall and Shapiro, 2012, O'Connell *et al*., 2013).While significant progress has been made in the islet transplantation field, many obstacles remain that currently prevent its widespread application. Persistent hurdles include the need for multiple donor pancreata to achieve insulin independence, procedure-associated complications, imperfect immunosuppressive regimens; lower rates of insulin independence and loss of function over time (Hirshberg *et al*., 2003). The decreased function and survival of transplanted islets are mainly due to the lack of extracellular matrix and vasculature disrupted during isolation procedures. These hurdles limits the applicability of islet transplantation to only the most severe cases of type 1 diabetes, in which patients having severe problems with hypoglycemia or are unable to achieve acceptable glycometabolic control, despite implementing intensive insulin regimens (Ryan *et al*., 2002).

2.5 Alternative cell sources for islet transplantation

The results from islet transplantation demonstrated that a cure for diabetes is possible through replenishment of the β cell mass. Several alternative forms of insulin-secreting tissue have been suggested to substitute human islets via β cell-replacement therapy (Rieck *et al*., 2012, Titus *et al*., 2000). One possibility is the use xenogenic islets and research focus has rested on porcine islets (Kin *et al*., 2005, Dufrane and Gianello, 2012) which could be an ideal islet source, since pigs are physiologically similar to humans, breed rapidly and produce large litters. In addition, neonatal islets have the potential benefit of proliferation however many obstacles remain, which include its antigenicity owing to the presence of α-galactosyl antigen, zoonotic infections and the reluctance of patients towards xenotransplantation (Deschamps *et al*., 2005, Rios *et al*., 2004). Another approach currently being explored is generation of insulin-producing cells *in vitro*, either by genetic engineering of β cells (Ravassard *et al*., 2011) or by utilizing various potential β cell precursor cells (Guz *et al*., 2001) or stem cells (Hebrok, 2012), with the ability to grow *in vitro* and to differentiate into β cells.

Stem cells with the potential to differentiate into insulin-producing cells include pluripotent stem cells (embryonic stem cells) (Jaramillo *et al*., 2014), induced pluripotent stem cells (Takeuchi *et al*., 2014), as well as multipotent (adult stem cells) from various tissues including the pancreas (Zulewski *et al*., 2001), liver (Yang *et al*., 2002), bone marrow (Xie *et al*., 2009) and adipose tissue (Karaoz *et al*., 2013).

2.5.1 Embryonic stem cells

Embryonic stem cells (ESC) derived from inner cell mass of pre-implanted blastocysts can differentiate into a wide variety of cell types (Martin, 1981). The predominant characteristic of ESC include its ability to self-renew and the potential to differentiate into all embryonic cell types, under *in vivo* and *in vitro* conditions (McKay, 2000).The pluripotent nature and the unlimited expansion potential make ESC an attractive source for cell replacement therapies. Although several researchers have attempted to generate cells with some level of insulin production from mouse (Soria *et al*., 2000, Lumelsky *et al*., 2001) monkey (Lester *et al*., 2004) and human (Assady *et al*., 2001, Segev *et al*., 2004) none of the studies has confirmed the *in vitro* functionality of β cells that can secrete physiologically sufficient amounts of insulin in response to glucose (Basford *et al*., 2012, Van Hoof *et al*., 2011). One of the reasons for this failure is the fact that although pancreatic β cells are the main source of insulin production in mammals, they are not the only cell type that can synthesize and release insulin. Other insulin-producing cells can be found in yolk sac, fetal liver, and certain neuronal cell types (Devaskar *et al*., 1994) which expresses several other genes in common with true β cells, and a β cell type specific profile has therefore been difficult to define. Earlier nestin was used for positive selection of endocrine progenitor cells (Blyszczuk *et al*., 2003), but later it was proven to be marker for neural and pancreatic exocrine progenitors (Delacour *et al*., 2004). The ESC became a potential source for generating β like cells by exploring the signals that regulated embryonic endoderm and pancreas formation (D'Amour *et al*., 2006, Kroon *et al*., 2008). The ESC differentiated into β-cell

precursors, characterized by transcription factors Pdx1 (Pancreatic and duodenal homeobox 1) and Nkx6.1, that are critical to *β*-cell development and maturation, have shown promise *in vivo*. When implanted into immunodeficient mice, these precursor cells were shown to mature into functional *β*-cells and reverse hyperglycemia (Schulz *et al*., 2012). The obstacles that remains challenging the application of ESC derived islet cells include, risk of teratocarcinoma formation, immune rejection and social/legal/ethical issues.

2.5.2 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSC) are pluripotent cells that are generated directly from adult cells by introducing a specific set of pluripotency-associated genes, or "reprogramming factors" (Yamanaka, 2012). Few studies were carried out on deriving insulin producing cells from iPSC. Human induced pluripotent stem cells (hiPSC) were differentiated to insulin producing cells by using a combination of activin A and retinoic acid. More than 10% of the cells became insulin positive and differentiated cells secreted human C-peptide (Kunisada *et al*., 2012). Murine and rhesus monkey derived iPSC differentiated pancreatic precursor cells were found to reverse diabetes in immunodeficient mice after a month long maturation period *in vivo* (Jeon *et al*., 2012, Zhu *et al*., 2011). The safety issues regarding the use of iPSC generated by viral mediated systems should be further investigated prior to clinical application since they may activate related oncogenes (Ye *et al*., 2013).

2.5.3 Adult stem cells

The potential use of adult stem cells offers the advantage of an autologous model whereby a patient's own cells can be used, thereby circumventing immune rejection.

2.5.3.1 ***Pancreatic stem cells***

Regeneration of pancreatic tissue, including *β*-cell mass, has been reported to occur in adult rats subsequent to a 90% pancreatectomy (Bonner-Weir *et al*., 1993). However, the origin of new *β* cells, whether derived from mitotic division of existing *β*-cells or from the differentiation of pancreatic stem cells is controversial. Genetic lineage tracing studies have demonstrated that existing *β*-cells are the primary source of new *β*-cells *in vivo*, although other studies have also found evidence of the involvement of pluripotent stem cells in *β*-cell regeneration. The studies have reported that in mice, pre-existing *β*-cells retained a proliferative capacity which represents the major source of new *β*-cells in adult life (Dor *et al*., 2004).

Stem/progenitor cells which have the ability to differentiate into insulin-producing cells *in vitro* and/or *in vivo* were described in pancreatic islets (Guz *et al*., 2001, Abraham et al., 2002), pancreatic ducts (Bonner-Weir and Sharma, 2002, Katdare, 2004), pancreatic acinar cells (Minami *et al*., 2005) and within adult or fetal pancreas (Ramiya *et al*., 2000). In the case of acinar cells a de-differentiation occurs first followed by re-differentiation into *β*-cells.

2.5.3.2 ***Mesenchymal stem cells***

Mesenchymal stem cells (MSC) are multipotent progenitor cells found in the perivascular spaces of many adult tissues which have the ability of self renewal and differentiation to various lineages (Hematti *et al*., 2013, Uccelli *et al*., 2008). Stem cells with the potential to differentiate into insulin-producing cells have been also reported in liver (Sapir *et al*., 2005), central nervous system (Hori *et al*., 2005), placenta (Kadam and Bhonde, 2010), dental pulp (Govindasamy *et al*., 2011) and bone marrow (Ianus *et al*., 2003).

The MSC from human bone marrow and adipose tissue represent a very similar cell population with comparable phenotypes (Lee *et al*., 2004). The MSC from mouse and rat bone marrow cultured under high glucose condition along with *β* cell stimulating growth factors transdifferentiated to insulin producing cells, capable of expressing pancreatic *β*-cell genes, including insulin, GLUT2 and Pdx-1 *in vitro* were shown to reverse hyperglycemia in an animal model of diabetes (Tang *et al*., 2004). Human bone marrow MSC differentiated insulin producing cells upon genetic manipulation by viral transduction were shown to express low levels of islet transcription factors (Moriscot *et al*., 2005, Milanesi *et al*., 2012).

The studies with streptozotocin-induced irradiated mice have demonstrated that immunosuppressive properties of MSC contribute to *β* cell regeneration. Moreover, MSC had significantly suppressed *β*-cell specific T-cell proliferation in pancreas, thereby shielding neo *β*- cells from destruction by T-lymphocytes thus, overcoming the inherent autoimmune pathology associated with type 1 diabetes (Franquesa *et al*., 2012).

Human adipose stem cells were shown to differentiate to pancreatic endocrine characteristics when treated with pancreatic extract. The study also demonstrated that human adipose stem cells express ESC markers including OCT-3/4, Nanog, and REX-1 that also have the potential to differentiate into pancreas cell lineages (Lee *et al*., 2008). Differentiation of stem cells towards functional islet like cells with the potential to secrete insulin in response to various glucose concentrations is challenging. Though human umbilical cord blood stem cells were differentiated to insulin producing cells; they were not able to respond to glucose challenge (Gao *et al*., 2008). The functionality of islet like cell clusters from wharton jelly stem cells through stepwise culturing in neuron-conditioned medium were demonstrated *in vitro* and *in vivo*. These islet-like cell clusters were shown to have human C-peptide and secrete human insulin in response to physiological glucose levels (Chao *et al*., 2008, Kadam and Bhonde, 2010). The potential of human adipose stem cells were exploited to differentiate towards physiologically competent functional islet like cell aggregates via stage specific differentiation protocol (Chandra *et al*., 2011). The ease in availability, autologous source and abundance of adipose tissue makes it an attractive candidate for generating alternative islets for cell replacement therapy in type 1 diabetes.

2.6 Role of endothelial cells in pancreatic islet function

Pancreatic islets are one of the most vascularized organs, having a blood perfusion of about 10% of that of whole pancreas, despite representing only 1% of the gland; this reflects high exchange demand with the endocrine cells, and high metabolic supply. Experiments on early pancreatic development demonstrated that

blood vessel endothelium in the dorsal aorta provides inductive signals for the differentiation of the primitive endoderm into islet cells (Yoshitomi and Zaret, 2004). A successive study on pancreatic organogenesis has shown that aortal endothelial cells induce the expression of pancreatic transcription factor Ptf1a, in the dorsal pancreatic endoderm which is essential for the development of endocrine and duct cell lineages.

A two step-model for islet development has been proposed: the first step involves signals from the endothelium to the pancreatic epithelium; the second involves signals in the opposite direction, with islets expressing VEGF-A (Vascular endothelial growth factor) at later stages of their development to attract capillaries (Figure 2.3) (Konstantinova and Lammert, 2004). VEGF signaling profoundly affects both vascularization and innervation of the pancreatic islets. In addition the endocrine cell-derived VEGF directs the patterning of intra-islet capillaries during embryogenesis, forming a scaffold for the postnatal ingrowth of essential autonomic nerve fibers (Reinert *et al*., 2014).

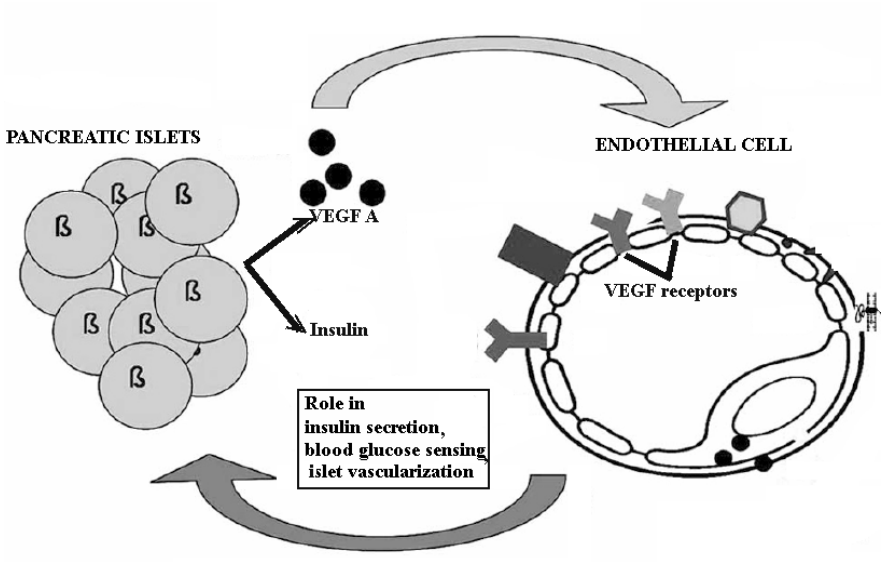

Figure 2.3 Schematic representation of cross talk between islets and endothelial cell. Islets secrete VEGF A which binds to its receptors on endothelial cells thereby enhancing islet vascularization, basement membrane formation and helps in blood glucose sensing. Endothelial cells also play an important role in enhancing islet cell function.

Intra-islet endothelial cells are fenestrated, and the density of the capillary network in the islets is ~10 times higher than that of the exocrine tissue. Islet endothelium is crucially involved in fine-tuning blood glucose sensing and regulation. Besides providing oxygen and nutrients to the endocrine cells, islet endothelium is in fact involved in the trans-endothelial rapid passage of secreted insulin into the circulation.

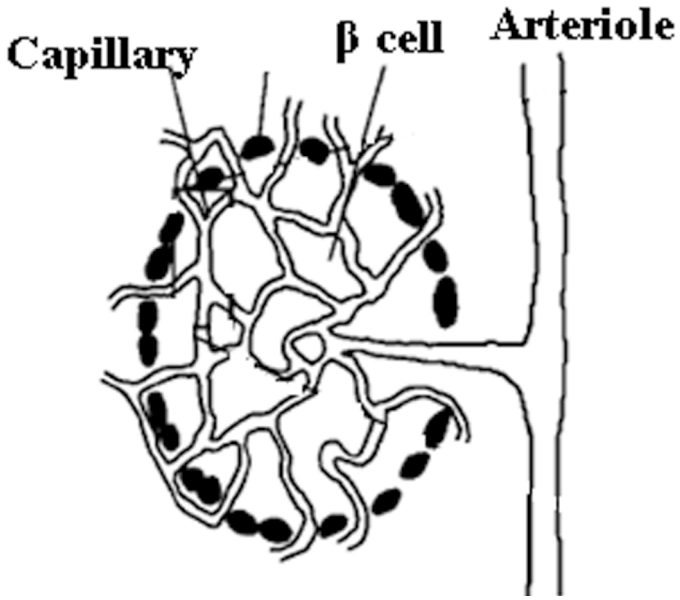

Figure 2.4 Morphology of pancreatic islet vasculature showing capillaries interspersed between beta islet cells.

Pancreatic islets have a dense capillary network (Figure 2.4) which is important for islet cell differentiation, maturation, function and survival (Lammert *et al*., 2001).Deletion studies indicate that VEGF-A is responsible for this dense islet vascularization, being more expressed in the endocrine than the exocrine pancreas (Henderson and Moss, 1985). Pancreatic β cells express high levels of VEGF-A regulating vasculogenesis, the process of formation of new blood vessels from endothelial cells. The VEGF-A bind to endothelial cells via VEGF receptor2 (VEGFR2) thereby forming vascular network within islets. The β cells sequester VEGF-A to regulate microvessel permeability and facilitate rapid uptake of released insulin into the blood (Kuroda *et al*., 1995). Another study indicated that mice with β cell reduced VEGF-A expression show impaired glucose-stimulated insulin secretion, related to vascular alterations of the islets (Brissova *et al*., 2006). Islet

endothelium is also involved in the activities of endothelial nitric oxide (NO) synthases, forming the vasoactive mediator NO, which are specifically regulated by the glucose level (Kolb and Kolb-Bachofen, 1992, Suschek *et al*., 1994). In addition, islet EC express genes encoding for a number of other factors involved in angiogenesis, including potent pro-angiogenic factors, such as VEGF, and angiostatic factors, such as endostatin and pigment epithelial-derived factor (Lammert *et al*., 2001).

The vascular endothelial cells are also responsible for synthesizing the intraislet basement membrane comprising of laminin, collagen IV and fibronectin which are essential for proper functioning of pancreatic islets (Lammert *et al*., 2003). The signals from vascular basement membrane mainly laminins are known to enhance insulin gene expression in pancreatic β cells. The basement membrane proteins has shown to enhance insulin gene expression, β cell proliferation and insulin secretion via interaction with integrin β1 subunit with various α chains (Nikolova *et al*., 2006).

One of the main factors limiting the success of islet transplantation is slow rate of revascularization. When avascular islets are transplanted they depend on the host vasculature and even after 1 month complete revascularization could not be achieved which subjects islets to chronic hypoxia and ischemia (Kuroda *et al*., 1995). Survival and function of transplanted islets depends upon the extent and period of revascularization, which usually occurs within 12–14 days (Jansson and Carlsson, 2002), yet the new vascular supply to grafted islets is significantly less than native pancreatic islets and has lower oxygen tension than normal

islets(Mattsson *et al*., 2002, Carlsson *et al*., 2001). Enhanced revascularization of islets immediately after transplantation may overcome the challenge of poor islet survival and function.

On an *in vivo* study involving transplantation of freshly isolated mouse islets under kidney capsule, donor endothelial cells were shown to participate in early process of vessel formation contributing to revascularization of fresh islets, however neither the vascular density nor the endocrine function were improved (Nyqvist *et al*., 2011). Various approaches have been suggested to enhance the process of revascularization of transplanted islets. Certain growth factors secreted by islet cells as well as fibroblast growth factor and endothelial cell growth factor, are suggested to increase neo-vascularization by attracting endothelial cells at the transplantation site. Another approach involves the use of angiogenic factors such as VEGF-A, angiopoeitin-1 or inhibiting angiostatic factors (Lai *et al*., 2005). To increase the VEGF-A at the site of transplantation, VEGF producing transfected EC were co-transplanted along with the islets (Zhang *et al*., 2004). The β cells producing VEGF-A or islets with transient VEGF expression also promoted revascularization and prolonged islet survival after transplantation (Narang *et al*., 2004).

Another study demonstrated that controlled production of VEGF by transfected EC immediately after transplantation is more beneficial than continuous VEGF producing EC as the uncontrolled VEGF secretion can lead to tumor formation (Mathe *et al*., 2006). Though, VEGF can enhance the process of revascularization, it is also essential to maintain the pool of intra-islet EC before transplantation (Olsson and Carlsson, 2005).

Establishment of new vasculature in grafted islets was shown to arise from intra-islet EC, recipient EC or bone-marrow derived cells. In an *in vitro* system, purified islet EC was shown to stimulate β cell proliferation, through secretion of hepatocyte growth factor (HGF). The VEGF-A and insulin are the islet-derived factors that induce HGF secretion. The *in vivo* experiments, using pancreas of pregnant rats in which a high physiological proliferation of β cell occurs, showed prominent expression of HGF, coinciding with the peak of β cell proliferation (Johansson *et al*., 2006).

The *in vitro* studies on .co-transplantation of endothelial progenitor cells (EPC) derived from cord blood cells and islets have shown to enhance islet engraftment in islet transplantation. Furthermore EPC co-transplanted islets accelerated the rate of revascularization at the early stage of transplantation (Kang *et al*., 2012). Another approach involves coculture of MSC and EC with islets before transplantation, which enhances vessel formation and revascularization *in vitro*. In this approach islets were coated with bone marrow derived MSC and dermal microvascular endothelial cells which resulted in migration of endothelial cells within and around the islets thus facilitating more vessel formation and better vascularization of islets (Johansson *et al*., 2008).

The *in vivo* studies have demonstrated that co-transplantation of MSC with transplanted pancreatic islets is more effective with respect to pancreatic islets alone in ensuring glycemic control in diabetic rats. The MSC were both able to prolong the survival of pancreatic islets, as well as directly differentiated into “insulin-

releasing" phenotype thereby potentiating pancreatic islets functionality and feasibility (Scuteri *et al*., 2014).

In another approach, islet survival and graft engraftment was improved by co-culturing mouse islets along with human dermal fibroblasts and human umbilical vein EC on a poly (lactide-co-glycolide) (PLGA)-poly-L-lactic acid (PLLA) 3D scaffold. Moreover gene expression profiles of endothelial cell growth factors, ECM and islet specific markers were also significantly higher on 3D culture (Kaufman-Francis *et al*., 2012).

2.7 Tissue engineering approach to enhance islet microenvironment

Pancreas organogenesis is an interactive process in which tissues from different germ layers secrete and respond to growth signals. During *in vitro* differentiation, cells are grown in 2D cultures as monolayers. Therefore, 3D interactions between cell types as they occur during islet formation *in vivo* are lacking (Nyitray *et al*., 2014). In addition, the destruction of the islet microenvironment and the loss of supporting matrix that occur during isolation, purification and in the pre-transplant culture period, subject the islets to a cellular stress. The lack of cell-cell interaction and optimal microenvironment results in impaired β islet function and survival (Bissell *et al*., 1982, Alismail and Jin, 2014). To overcome the present limitations, tissue engineering approach has evolved which utilizes the principles of life science, engineering and material science, using living cells manipulated through their extracellular environment to develop biological substitutes for implantation into the body to either repair, replace or to restore

tissue/organ function (Langer and Vacanti, 1993). Tissue engineering approach has the potential to overcome many of the shortcomings of the Edmonton protocol leading to increased longevity of islet transplantations (Amer *et al*., 2014). Cells, scaffolds and signaling cues are referred to as tissue engineering triad, the key components of engineered tissues (Figure 2.5). The concept of scaffolding in tissue engineering is to mimic the functions of native ECM, at least partially (Salvatori *et al*., 2014).

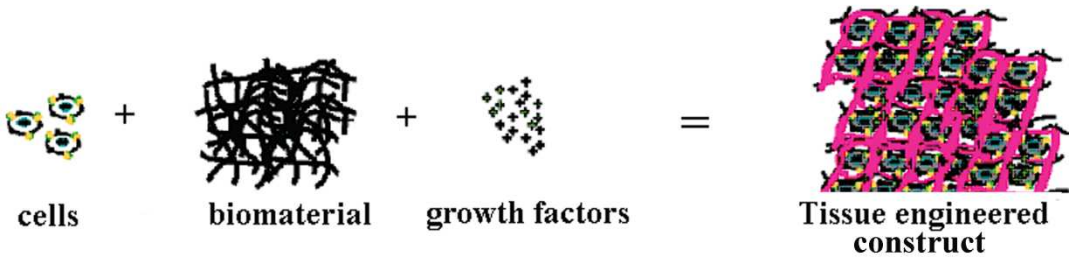

Figure 2.5 Key components of Tissue Engineering. Cells, biomaterial scaffold and growth factors are known as the tissue engineering triad.

2.7.1 Extracellular matrix and its role in islets

The importance of matrix on pancreatic islet cell differentiation, attachment, and proliferation was first realized in the early 1980s (Thivolet *et al*., 1985). Extracellular matrix the most important component of the islet microenvironment is a solid matrix made up of a complex mixture of carbohydrates and proteins whose role is to act as a cellular scaffold providing structural support and physical environment for cells residing in the tissue to attach, grow and migrate. The ECM

may provide bioactive signals which are important for pancreatic islet development and function (Jiang and Harrison, 2002, Hersel *et al*., 2003).

Adult human islets are encircled by an incomplete capsule namely peri islet comprising of a single layer of fibroblasts and collagen fibers. The peri islet capsule is in close proximity to an additional layer of matrix proteins known as peri insular basement membrane (BM) (Meyer *et al*., 1998a) (van Deijnen *et al*., 1992). The BM is mainly composed of laminin (Banerjee *et al*., 2012) and collagen (Col) IV (Jiang *et al*., 1999, Meyer *et al*., 1998b) however, fibronectin (Leite *et al*., 2007), Col I, Col III, Col V, Col VI (Van Deijnen *et al*., 1994) nidogen and proteoglycans has also been reported (Goh *et al*., 2013). The ECM composition varies between species. Collagen fibers have great tensile strength that not only provide structural support for cells internally and externally, but are also required for cell growth, adhesion, migration, differentiation, morphogenesis, and injury repair. Although Col IV have shown to promote the survival of intact islets when compared to collagen I (Pinkse *et al*., 2006), another study has shown it to decrease insulin production and secretion in purified *β*-cells (Kaido *et al*., 2006).

Table 2.2 The extracellular matrix components of islet cells and its specific integrin receptors (Cheng *et al*., 2011)

Extracellular matrix of islets	**Integrin**
Collagen	α1*β*1, α2*β*1, α10*β*1, α11*β*1
Laminin	α1*β*1, α2*β*1, α3*β*1, α6*β*1, α7*β*1, α9*β*1, αv*β*3 αv*β*5 αv*β*8, α6*β*4
Fibronectin	α3*β*1, α4*β*1, α5*β*1, α4*β*7, α8*β*1, αv*β*1, αv*β*3, αv*β*5, αv*β*6, αIIb*β*3
Nidogen/Entactin	α3*β*1, αv*β*3
Vitronectin	αv*β*1, αv*β*3, αv*β*5, αIIb*β*3
Perlecan	α2*β*1

Several studies have reported that the ECM proteins mediate β cell function via integrin receptors (Table 2.2) which results in increased insulin production (Bosco *et al*., 2000) and decreased apoptosis (Hammar *et al*., 2004). Integrins are a diverse class of $\alpha\beta$ heterodimeric receptor proteins through which cells interact with matrix proteins like collagen, fibronectin and laminin mediating cell adhesion (Juliano and Haskill, 1993). The integrins predominant in islets are α3, α5, αv and β1 (Wang *et al*., 1999). There are studies demonstrating the presence of αvβ5, αvβ3 and β1 integrins in developing islets (Cirulli *et al*., 2000). Most of the integrin receptors of islets have affinity to bind RGD (Arginine-Glycine-Aspartic acid) motif. The absence of integrin mediated signals has shown to trigger apoptosis through activation of stress activated protein kinase JNK (c-Jun-NH_2-terminal kinase) (Meredith Jr and Schwartz, 1997).

The ECM has also proven to play an essential role in maintaining the differentiated state of cells (Lin and Bissell, 1993). Integrin β1-ECM interactions are important for promoting islet survival, maintaining islet structural integrity and enhancing glucose stimulated insulin secretion (Wang *et al*., 2005).

2.7.2 Scaffolds and islets

Exact duplication of the ECM *in vivo* would be the ideal microenvironment to culture islets, but the complexity of the ECM in islets makes this challenging. Both biological and synthetic scaffolds have been used for islet tissue engineering including collagen, fibrin glue, MatrigelTM, alginate polylactic-co-glycolic acid (PLGA), polyglycolic acid (PGA), and polycaprolactone. Collagen matrix cultured

with dissociated pancreatic islet cells underwent reassociation to mimic their precise topographical distribution in rat islets *in vivo* (Montesano *et al*., 1983). Porous scaffolds can facilitate efficient human islet transplantation and provide a platform for modulating the islet microenvironment (Gibly *et al*., 2013).

Another group conducted a co-culture study of rat islets and collagen hydrogel mixture comprising of collagen type I mixed with collagen type III, type IV, and laminin. In the co-culture group viability of islets were enhanced but there was no significant increase in the expression of mRNA level of insulin, glucagon, and somatostatin (Nagata *et al*., 2002).

Mouse islets cultured in collagen type I matrix populated with fibroblast cells was shown to improve islet viability and function post transplantation in mice. The composite scaffold reduced the critical islet mass required for diabetes reversal by half and enhanced islet cell proliferation (Jalili *et al*., 2011). Rat islets cultured in polyglycolic acid (PGA) fibrous scaffold for 5 days transplanted into the leg muscles of streptozotocin-treated diabetic Wistar rats normalized the blood glucose concentration by secreting insulin. The islets in scaffold were found to be superior in function and morphology (Hou *et al*., 2009).

Human islets were cultured on different ECM components namely collagen I and IV, fibronectin, and laminin to study its adhesion, survival, and functionality. The results demonstrated the importance of integrin mediated effects and behaviour. Collagen I/IV and fibronectin were found to induce islet cell adhesion, whereas fibronectin played role in maintaining islet structural integrity and insulin content distribution however, in long term culture islet phenotype was gradually lost. The

insulin gene expression and glucose metabolism was highest in islets cultured on collagen I and IV in contrast; insulin release was highest on fibronectin. Taken together, the findings suggest that various ECM proteins have its own individual role in eliciting diverse islet responses and integrin-mediated behaviour (Daoud *et al*., 2010).

Another group demonstrated the formation of nano-thin, poly (ethylene glycol) (PEG)-rich functional coatings on individual islets via layer-by-layer assembly technique. The islet surface was modified with biotin-PEG-N-hydroxysuccinimide (NHS) and further covered by streptavidin and biotin-PEG-peptide conjugates. Glucagon-likepeptide-1, an insulinotropic ligand was conjugated to biotin-PEG-NHS. Surface modified islets functionalized with PEG conjugate were capable of secreting more insulin in response to high glucose levels compared to control islets (Kizilel *et al*., 2010).

A peptide amphipile nanostructured gel-like structure was engineered comprising of cell adhesion binding domain, Arginine-Glycine-Aspartic acid (RGD) and Matrix metalloproteinase- 2 (MMP-2) cleavable sequences to impart degradation. Rat pancreatic islets encapsulated in these hydrogels, had higher insulin secretion and viability when compared to suspension culture. After 14 days, nanofiber encapsulated islets remained insulin positive and released insulin in response to glucose stimulation, while those cultured in suspension were weakly insulin positive (Lim *et al*., 2011). A novel saccharide-peptide hydrogel was also developed wherein encapsulated rat islets remained viable up to four weeks, while

the unencapsulated islets were mostly non-viable and lost their 3D morphology after two weeks (Liao *et al*., 2013).

A biomimetic hydrogel was designed using immobilized cell-cell communication cues to enhance the survival and function of encapsulated pancreatic β-cells to treat type 1 diabetes. The pancreatic β-cell lines when encapsulated in poly (ethylene glycol) (PEG) hydrogels, their survival and glucose responsiveness to insulin were highly dependent on the cell-packing density. The thiolated EphA5-Fc receptor and ephrinA5-Fc ligand were conjugated into PEG hydrogels via a thiol-acrylate photopolymerization to render the hydrogel bioactive.Along with cell-adhesive peptides, the immobilized fusion proteins (EphA5-Fc and ephrinA5-Fc) synergistically increased the survival of pancreatic β cell lines (Lin and Anseth, 2011).

Mouse islets co- encapsulated with ECM proteins (collagen IV and laminin) and mesenchymal stromal cells were cultured in silk hydrogel for 7 days. The islets encapsulated with collagen IV, or laminin had increased insulin secretion at day 2 and day 7 respectively. In addition, 3.2-fold increased insulin secretion was observed in islets co-encapsulated with stromal cells and ECM proteins. An up regulated expression of functional genes namely insulin I, insulin II, glucagon, somatostatin, and PDX-1, and down regulation of the de-differentiation genes like cytokeratin 19 and vimentin were observed in contrast to the non-encapsulated islets (Davis *et al*., 2012).

A biostable, macroporous scaffold fabricated from poly(dimethylsiloxane) (PDMS) was investigated for its application in islet tissue engineering. The

metabolic function and glucose-dependent insulin secretion of islets from multiple sources including rodents, nonhuman primates, and humans within these scaffolds were studied *in vitro*. Islets loaded within PDMS scaffolds exhibited viability and function comparable to standard culture conditions when incubated under normal oxygen tensions, but displayed improved viability compared to standard 2D culture controls under low oxygen tensions. The *in vivo* efficacy of scaffolds to support islet grafts was evaluated after transplantation in the omental pouch of chemically induced diabetic syngeneic rats, which promptly achieved normoglycemia (Pedraza *et al*., 2013). Later a combination of fibrin-based proangiogenic hydrogel having platelet derived growth factor and major integrin binding sites loaded within a macroporous poly (dimethylsiloxane) (PDMS) scaffold was used for islet transplantation. The results showed that there was a significant decrease in the time required to achieve normoglycemia for syngeneic mouse islet transplants. Mature intraislet vessels were observed and the hydrogel, positively influenced the efficiency of engraftment (Brady *et al*., 2013).

The effect of substrate architecture on insulinoma organization and function was studied by culturing insulinomas on 2D gelatin substrates and 3D fibrous gelatin scaffolds with three distinct fiber diameters and fiber densities. Small, closely spaced gelatin fibers promoted the formation of large, rounded insulinoma clusters, whereas monolayer organization and large fibers prevented cell clustering and reduced glucose-stimulated insulin production (Blackstone *et al*., 2014).

The current review highlights the importance of scaffold matrix and endothelial cells in development, maturation and function of pancreatic endocrine

lineage specific β cells. Based on the literature review, the present work aims toward formation of glucose responsive functional β islet cells from adipose stem cells. In the present study we attempted co-culture of stem cell differentiated islet cells along with EC on a scaffold matrix to mimic the native microenvironment of pancreatic islets for prolonging the survival and enhancing its function.

CHAPTER 3

MATERIALS & METHODS

3.1 Isolation and characterization of cells

The islet cells were isolated from rabbit pancreas and characterized. Stem cells isolated from rabbit and human adipose tissues were also characterized. Endothelial cells were isolated from human umbilical vein and characterized.

3.1.1 Isolation and characterization of rabbit islets

3.1.1.1 *Isolation of rabbit islets*

Pancreata were obtained from cadaveric rabbits (New Zealand white) after approval from Institutional Animal Ethics Committee (IAEC). The pancreatic tissue was digested using 1 mg/ml collagenase V (Sigma, USA) at 37^0C for 10-15 minutes in an orbital shaker (Shewade *et al*., 1999). Islet cells were purified using Ficoll (Sigma, USA) (Scharp *et al*., 1987)) method and the purity was assessed using dithizone (Sigma, USA) stain (Latif *et al*., 1988).

3.1.1.2 *Diphenylthiocarbazone staining for rabbit islets*

Dithizone (DTZ) (Sigma, USA) stock was prepared at a concentration of 39mM in Dimethylsulfoxide (Sigma, USA) and stored briefly at -15^0C. Dithizone working solution was prepared fresh by mixing 100µl DTZ stock, 10ml Krebs Ringer Bicarbonate HEPES (KRBH) buffer (Appendix A-1). Rabbit islets were washed with Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, USA) and

incubated with filtered DTZ working solution for 20 minutes at 37^0C. The stained islets were observed with phase contrast microscope (Leica, Germany).

3.1.1.3 ***Immunocytochemistry of rabbit islets***

The isolated islets were used for immunocytochemical studies. Islets were washed with PBS and fixed with 4% (w/v) paraformaldehyde (Lab Rasayan, India) for 20 minutes at room temperature. After several washing with PBS, cells were permeabilized with chilled methanol (Spectrochem, India) for 20 minutes. Permeabilization was done for visualization of intracellular antigens. Two percent (w/v) bovine serum albumin (BSA) (Sigma, USA) was used as the blocking agent. Islet cells were incubated for 1 hour with the following cell specific primary antibodies: anti rabbit –insulin (Abcam, USA), glucagon and somatostatin (Dakocytomation, Denmark). After several washing with PBS, cells were incubated with Fluorescein isothiocyanate/Phycoerythrin (FITC/PE) labeled secondary antibodies (Santa Cruz Biotechnology, USA)**.** The cells were counterstained with Hoechst stain (Sigma, USA).

3.1.2 Isolation and culture of rabbit adipose stem cells

Three to seven months old New Zealand white rabbits were chosen for the experimental study. The animal was anaesthetized and a minor incision was made on the supra scapular region. Subcutaneous fat tissue (approximately 2g) was surgically removed under aseptic condition and collected in PBS (Gibco, USA) with 10X antibiotic and antimycotic. Fat tissue was washed three times with PBS and suspended in an equal volume of PBS containing 0.1% collagenase type II (Sigma,

USA) and 1% (v/v) antibiotic-antimycotic (Gibco, USA). The tissue was placed in an orbital shaker at 37°C with continuous agitation for 30-45 minutes and centrifuged for 10 minutes at 2500 rpm at room temperature. The pellet containing cells were resuspended in maintenance medium consisting of Dulbecco's Modified Eagle Medium (DMEM HG, Gibco,USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, USA) , 1X antibiotic-antimycotic .The primary cells were plated in cell culture flask (25 cm^2 flask, BD (Becton Dickinson) Biosciences, India) in a humidified atmosphere at 37^0C with 5% CO_2. Medium changes were done at 2 days interval. The cells were subcultured with 0.25% (w/v) trypsin/0.038% (w/v) ethylene diamine tetraacetic acid (EDTA) (Gibco, USA) and seeded at a density of $2x10^4$cells/cm^2 and cultured in the same conditions for cell expansion. Morphology of cultured cells was observed by phase-contrast microscope (Nikon, USA).

3.1.2.1 ***Population doubling time***

The time of population doubling (Td) for rabbit adipose stem cells was calculated using the formula (Equation 1)

$$T_d = \frac{T \times \log 2}{\log N_t - \log N_o} \qquad \textbf{Equation 1}$$

T is the total number of hours in culture, No is the initial number of cells seeded and N_t is the number of cells at time T. Doubling time was calculated in cultures until passage 10. Cells from passage 2-4 were used throughout the study.

3.1.2.2 ***Cytoskeletal organization***

The cytoskeletal organization of adipose tissue derived cells was studied using phalloidin stain. The trypsinized cells were seeded on surface of tissue culture polystyrene (TCPS) (BD Biosciences, India) dish and at subconfluent stage fixed with formalin free fixative (Sigma, USA) for 15 minutes, permeabilized with 50% (v/v) chilled methanol for 10 minutes and stained with FITC conjugated phalloidin (Sigma, USA) for 30 minutes in the dark. Hoechst (Sigma, USA) counterstained nuclei and FITC tagged actin filaments were observed with fluorescent microscope (Leica, Germany).

3.1.2.3 ***Immunocytochemistry and Flow cytometry***

Immunostaining was performed on monolayer of adipose derived cells grown on coverslips. Cells were washed with PBS and fixed for 20 minutes at 37^0C in formalin free fixative (Sigma, USA). For visualization of intracellular antigens, cells were permeabilized with chilled methanol for 20 minutes at 37^0C. Bovine serum albumin (2% w/v) (Sigma, USA) in PBS was used as the blocking agent. The slides were then incubated for 60 minutes at room temperature with different mouse anti rabbit antibodies: vimentin (Santa Cruz Biotechnology, USA), CD105-PE (phycoerythrin) (Santa Cruz Biotechnology, USA), CD44-FITC (BD Biosciences, India), CD45-PE/CD34-FITC (BD Biosciences, India). For vimentin, FITC conjugated secondary anti-mouse IgG antibody (Santa Cruz Biotechnology, USA) was used. Hoechst (10μg/ml) (Sigma, USA) was used to counterstain nucleus. The coverslips were then mounted with fluorescent mounting medium (Sigma, USA) and viewed with a phase contrast fluorescent microscope (Leica, Germany) via 20x

objective lens and an appropriate filter. For immunophenotyping by fluorescence activated cell sorting (FACS), cells were detached with trypsin-EDTA, washed in PBS and stained with the following antibodies: vimentin, CD105, CD44, CD34 and CD45. Flow cytometry was performed using a FACS Calibur (Becton and Dickinson, USA) and for each measurement a total number of 10,000 cells were recorded.

3.1.2.4 *Induction of Adipogenic or Osteogenic Differentiation*

Cultured cells (2×10^4 / cm^2) at passage 2-4 were incubated in adipogenic differentiation medium (DMEM HG (Gibco, USA) supplemented with 10% (v/v) FBS (Gibco, USA), 10^{-7} M dexamethazone (Sigma, USA), 0.5mM isobutylxanthine (Sigma, USA), 50µM indomethacin (Sigma, USA) and osteogenic medium (DMEM HG supplemented with 10% (v/v) FBS, 10^{-7} M dexamethazone(Sigma, USA), 20mM β glycerolphosphate (Sigma, USA), 50 µg/ml ascorbate 2 phosphate (Sigma, USA)) for 21 days. Cells cultured in DMEM HG supplemented with 10% FBS was used as control and medium change done at an interval of 2 days. Osteogenic differentiation and adipogenic differentiation was assessed by Alizarin red (Sigma, USA) /Von Kossa (Sigma, USA) and Oil red O (Sigma, USA) stains respectively. Control cells without differentiation stimuli were also stained in the same manner.

3.1.3 Isolation and expansion of human adipose stem cells

Fat tissue samples were collected from patients during lipectomy/liposuction after approval from Institutional Ethics Committee. Adipose tissue samples were washed with PBS and digested using 0.075% collagenase type I (w/v) (Gibco, USA) for 30-

45 minutes (Zuk *et al*., 2002). Fetal bovine serum (Gibco, USA) was added to digestion mixture and the cells were centrifuged at 2000 rpm for 10 minutes at 4^0C. The pellet containing adipose stem cells were resuspended in expansion medium comprising of DMEM-HG (Gibco, USA) supplemented with 10% (v/v) FBS. The cells were plated onto T25 culture flask at a density of 10^4 cells/cm^2. Human adipose stem cells from passage 4 were used throughout the study.

3.1.3.1 ***Cell Sorting***

The adipose derived cells were sorted using magnetic activated cell sorting. The CD105 magnetic bead was used for positive selection. Mini MACS (Magnetic activated cell sorting) MS column (Miltenyi Biotech, Germany) was used along with separation buffer comprising of PBS without calcium and magnesium along with 2mM EDTA (Invitrogen, USA) and 0.5% (w/v) BSA (Sigma, USA) according to manufacturer's protocol. The sorted cells were subjected for flow cytometry analysis to assess its purity.

3.1.3.2 ***Immunocytochemistry***

The cells from passage 2-3 were used for immunocytochemical studies. The isolated adipose cells were characterized for various extracellular and intracellular markers to confirm they are of mesenchymal origin and possess stem cell properties. The cells after reaching subconfluence stage were washed with PBS and fixed with 4% (w/v) paraformaldehyde for 20 minutes at room temperature. After several washings with PBS, cells were permeabilized with chilled methanol for 20 minutes. Permeabilization was done for visualization of intracellular antigens only. Two percent (w/v) BSA was used as the blocking agent. Adipose derived cells were

incubated for 1 hour with the following cell specific primary antibodies: vimentin, CD105, CD44, CD90 and CD45/CD34. After several washings with PBS, cells were incubated with FITC/PE labeled secondary antibodies**.** The cells were counterstained with Hoechst stain (Sigma).

3.1.3.3 ***Differentiation to adipogenic and osteogenic lineage***

Cells from passage 2-4 were used for the differentiation studies. The cells were seeded at a density of 5 x 10^3 / cm^2 in DMEM HG supplemented with 10% (v/v) FBS, until the cultures reached subconfluence. Then the cultures were treated with osteogenic medium (10^{-7} M dexamethazone, 20mM β glycerolphosphate, 50 µg/ml ascorbate 2 phosphate) (Sigma, USA) for 24 days and adipogenic medium (10^{-7} M dexamethazone, 0.5mM isobutylxanthine, 50µM indomethacin) (Sigma, USA) for 21 days. Medium change was done at an interval of 2 days. Osteogenic differentiation and adipogenic differentiation was assessed by Alizarin Red (Sigma, USA) and Oil Red O (Sigma, USA) staining respectively. Control cells without differentiation stimuli were also stained in the same manner.

3.1.4 Isolation of endothelial cells from human umbilical vein

Umbilical cords were obtained after caesarian term deliveries under aseptic conditions after approval from Institutional Ethics Committee and collected in PBS containing antibiotics and antimycotic. The umbilical vein was canulated and washed with PBS. The distal end of the cord was clamped and the vein was filled with 0.2% (w/v) collagenase type I enzyme in M199 (Medium 199, Gibco, USA) supplemented with antibiotics. The proximal end was also clamped and the cords

were kept for incubation at 37^0C for 15 minutes. The vein was washed with M199, followed by gentle massaging of the cord. The suspension of EC were collected and centrifuged at 1800rpm, 4^0C for 10 minutes (Jaffe *et al*., 1973). The pellet was resuspended in M199 supplemented with 20% (v/v) FBS, 1% (v/v) L glutamine (Gibco, USA) and 1X antibiotic/antimycotic and the cells were seeded in 0.1% (w/v) gelatin (Sigma, USA) coated 35mm tissue culture treated petridish. Cells were maintained at 37^0C in a humidified atmosphere containing 5% CO_2. Non adherent cells were removed from culture after 3 days and to the adherent cells fresh medium was fed at 2 days interval. After reaching confluency cells were trypsinized with 0.25% trypsin EDTA and passaged into new flask for further expansion and onto coverslips for immunostaining. Cells from passage 2 and 3 were used throughout the study.

3.1.4.1 ***Immunocytochemistry***

The cells cultured on coverslips were washed with PBS and fixed with 4% (w/v) paraformaldehyde (Lab Rasayan, India) for 20 minutes at room temperature. After several washing with PBS, cells were permeabilized with chilled methanol for 20 minutes. Permeabilization was done for visualization of intracellular antigens only. Two percent (w/v) BSA was used as the blocking agent. Cells were incubated with for 1 hour with the following cell specific primary antibodies: polyclonal rabbit anti human von willebrand factor (vWF) and monoclonal mouse anti human CD31. The secondary antibodies used were chicken anti rabbit IgG FITC and chicken anti mouse IgG FITC respectively. The cells were counterstained with hoechst stain.

3.2 Synthesis and fabrication of scaffold

3.2.1 Oxidation of Dextran

Dextran oxidation using periodate is a classic method used to incorporate functional aldehyde groups and prepare dextran dialdehyde (Draye *et al*., 1998). Dextran dialdehyde can serve as a crosslinker for polymers (like gelatin) bearing free amino groups.

Briefly 10g of dextran (Mw 35,000-45,000, Sigma, USA) was dissolved in 100ml of distilled water after which 6.6 g of sodium meta- periodate (Sigma, USA) was added. The mixture was stirred in the dark for 6 hours. Dextran dialdehyde (DDA) with 50% oxidation could be obtained by this procedure. The percentage of oxidized dextran was calculated by titration method (Appendix A-2).The reaction mixture was dialyzed (Sigma, USA, dialysis tubing, Mw cut off 8303) against distilled water at room temperature for 3 days and thereafter lyophilized for 24 hours and stored at 4^0C till use.

3.2.2 Fabrication of Dextran-Gelatin (DEXGEL) scaffold

Gelatin (5g, type A, porcine skin, bloom value- 175, Sigma, USA) dissolved in 50 ml of 0.1M sodium borate buffer (pH 9.4) was stirred at 2500 rpm for 20 minutes at room temperature, thereafter 50 ml of dextran dialdehyde solution (5 g dextran dialdehyde dissolved in 0.1M sodium borate buffer) was added and the stirring continued for another 20 minutes. The mixture was poured onto cylindrical vials (Tarsons, polystyrene 10ml) and kept at 37^0C overnight after which it was freeze dried at -80^0C for 48 hours. The crosslinking between oxidized dextran and gelatin

occurs by the formation of Schiff base links between free amino groups of lysine and hydroxylysine residues present in gelatin and aldehyde groups of oxidized dextran. The freeze dried scaffolds named as DEXGEL (Dextran Gelatin) were cut into desired shapes followed by final rinsing in distilled water for 1 hour at 37^0C after which it was freeze dried (-80^0C for 24 hours) and sterilized by ethylene oxide treatment .

3.3 Physicochemical characterization of DEXGEL scaffold

3.3.1 Morphology and Porosity Analysis

The microstructure of the scaffold was characterized using scanning electron microscopy (SEM) .The freeze dried scaffolds (5mm diameter, 3mm thickness) were sputter coated with gold and examined with SEM (JEOL JSM 6390LV, Japan) operated at 10 kV under high vacuum mode. The porosity of scaffold was determined using liquid extrusion porosimetry (PMI, model No.LEP-1100A, New York). Porous sample of specific mass and density was taken and placed on a 5 µm Millipore membrane. The pore characteristic of scaffold was evaluated in the wet state using water as the solvent which fills the pores of the sample and the membrane. Pressure of a non reacting gas, Nitrogen, is slowly increased over the sample so as to displace the liquid from the pores of the sample. From the displaced liquid the porosity measurements were taken. Pressure required to displace liquid from a pore is related to the size of the pore as per the formula:

$$p = 4\gamma\cos(\theta / D) \qquad \textbf{Equation 2}$$

where p is the pressure difference across the pore, γ is the surface tension of the liquid, θ is the contact angle of the liquid with the sample and D is the pore diameter.

3.3.2 Fourier Transform Infra-Red (FT-IR) Spectroscopy

For structural Analysis, FT-IR spectra (Attenuated total reflectance -ATR mode) of gelatin, dextran, dextran dialdehyde (DDA) and DEXGEL scaffolds were taken using Thermo Nicolet 5700 FT-IR with Diamond ATR Accessory. The spectra were taken in the frequency range of 4000 - 400cm^{-1}. The FT-IR spectra of DEXGEL in 7 days, 30 days and 60 days culture were taken to study the degradation pattern.

3.3.3 Water Contact Angle Measurement

Surface wettability of DEXGEL film was characterized with water contact angle measurement by sessile drop method. The images of the droplet on the film were visualized through the image analyzer (Optical Contact Angle System, Data physics, OCA15 plus, Germany).

3.3.4 Swelling studies

For determining the swelling characteristics, samples (known weight scaffolds) were immersed in PBS solution at 37^0C. The swollen samples were taken out from PBS solution, wiped the surface with tissue paper followed by immediate weighing at definite intervals till equilibrium swelling reached.

The swollen weight was recorded and swelling ratio was calculated using the following formula:

$$\text{Swelling ratio} = [(W_f - W_i) / W_i] \qquad \textbf{Equation 3}$$

where ,W_i is the initial weight of dry scaffold and W_f, the weight of swollen scaffold.

3.3.5 Enzymatic degradation assay

The *in vitro* degradation of the scaffold was studied by incubating preweighed sterilized scaffolds (5mm diameter, 3mm width) in PBS supplemented with 60μg/ml collagenase (Gibco, USA) at 37^0C and monitoring the weight loss of the scaffolds. The samples were drawn at regular intervals, washed with distilled water and freeze dried. The final weight of the samples was recorded and the percentage degradation rate was calculated using the following formula:

$$\text{Percentage degradation rate} = [(Wi - Wn) / Wi] \times 100 \qquad \textbf{Equation 4}$$

where Wi is the initial dry weight of the scaffold, Wn is the final dry weight of the degraded sample after n days in PBS.

3.3.6 Cytotoxicity tests

3.3.6.1 ***Direct method***

The *in vitro* tests for cytotoxicity were done to assess the response of cells in culture to direct contact with biomaterial (International Organization for Standardization -ISO 10993-5 guidelines-"Tests for *in vitro* cytotoxicity"). Sterilized

DEXGEL scaffolds (5mm diameter, 3mm width) were positioned on rabbit adipose stem cell monolayer and incubated at 37^0C in CO_2 incubator. After 48 hours, culture plates were visualized under phase contrast microscope (Leica, Germany).

3.3.6.2 ***Extraction Method***

The *in vitro* cytotoxicity of DEXGEL scaffold was evaluated by an indirect extract method. Twenty four hour extracts of DEXGEL scaffolds (extraction ratio- 60cm^2/20ml medium) were collected. Seeding density was 10^4 rabbit adipose stem cells per well in 96- well plate. Culture medium was removed after 24 hours and cells were incubated with test extract for 24 hours. The control cells were fed with normal maintenance medium. After treatment as indicated, 20µl of 5mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, USA) was added and plates were incubated at 37^0C for 4 h. Wells were drained and formazan crystals were solubilized in 150µl Dimethyl sufoxide. The optical densitometry was measured at a wavelength of 550 nm (reference 630 nm). Percentage survival was determined as per the formula [(Absorbance of test extract treated cells / Absorbance of Control cells) ×100] and compared with control, untreated cells regarded as 100%. Data shown are the mean ± standard deviation of three similar experiments each performed in triplicate.

3.4 Studies with rabbit islets on DEXGEL scaffold

3.4.1 Islet cell seeding and culture

Rabbit islets were divided into two groups (n=4 wells or scaffold, per group). The test group, with islets cultured on scaffold and control group, with islets

cultured on TCPS (tissue culture polystyrene, Becton Dickinson, India) dish. Islets were seeded at a density of 100-200 clusters per scaffold or well of 24 well plate. Both group of cells were supplemented with DMEM-HG with 10% (v/v) FBS, 1x antibiotic- antimycotic and cultured in a 37^0C constant temperature incubator with 5% CO_2. Medium changes were done at two days interval.

3.4.2 Islet cell morphology

The morphology of islet cells on TCPS dish and scaffold were analyzed using phase contrast microscope (Leica, Germany) and scanning electron microscopy (Hitachi S 2400) respectively. For SEM analysis, samples were fixed with 3% (v/v) glutaraldehyde for 2 h, after which dehydration was carried out using graded ethanol changes, later critical point dried (Hitachi, Japan), and gold sputtered in vacuum (Hitachi, Japan).

3.4.3 Islet viability

The viability of islets on scaffold and TCPS dish were analyzed using live/dead calcein AM/ethidium homodimer-1 stains (Invitrogen, USA). Islet cells on scaffold and TCPS were visualized using Confocal Laser Scanning Microscope (Carl Zeiss 510 Meta, Germany) and fluorescent microscope (Leica, Germany) respectively.

3.4.4 Gene expression analysis

Total RNA was extracted from day 10 islets cultured on scaffold and TCPS dish using TRIzol reagent (Gibco, USA) according to manufacturer's instruction.

The resulting mRNA was reverse transcribed to cDNA with Revert-Aid reverse transcriptase (Fermentas, PCR kit) for 60 minutes at 42^0C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers (Integrated DNA Technologies, USA) whose sequences are tabulated in Table 3.1. Sybr Green Master mix (Invitrogen, USA) was used for the reaction and 40 cycles were performed in BioRad System programmed with specified conditions for denaturation and annealing. The quantitative Real time PCR (qRT-PCR) results were normalized to β actin (reference gene).

Table 3.1 List of primer sequences for rabbit islets

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
β actin	GCGGGACATCAAGGAGAA	GGAAGGAGGGCTGGAAGAG
Insulin	ATCGTGGAGCAGTGTTGC	CGTGGAGGGGTTTATTGG
Integrin β1	CAGTGAATGGGAACAACG	TTTCCCTCATACTTCGGATT
Integrin αv	CATTTCCGAGTCTGGGCCA	GGCATCAGAGGTGGCTGGA

3.4.5 Immunohistochemistry

Immunohistochemistry was done on scaffold–islet constructs to analyze islet specific protein expression. The scaffold-islet constructs were fixed in formalin free fixative for 24 hours before histological analysis and consequently samples were dehydrated through a series of graded ethanol, embedded in paraffin and sectioned as 6μm sections using microtome (Leica, Germany). For histological evaluation, sections were deparaffinized, rehydrated through series of graded ethanol. The sections were stained with mouse anti-rabbit primary antibodies: insulin, glucagon and somatostatin; after which streptavidin peroxidase labeled secondary antibody

(Ultratech DAB kit, USA) was added. Counter staining was done with Mayer's hematoxylin (Sigma, USA). Immunocytochemistry was performed on control islets.

3.4.6 Islet cell function

Quantification of the protein level expression of insulin secreted was carried out for islets on control and test groups. Samples were pre-incubated for 3 hours in glucose free KRBH buffer (Appendix A-1), followed by incubation with KRBH containing 5.5mM and 25mM glucose for an additional 1 hour respectively. Insulin assay was performed by enzyme immunoassay (Rabbit Insulin kit, myBiosource, USA), according to the manufacturer's instruction. After glucose challenging, islet cells were lysed using cell lysis buffer (20mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen, USA), 10mM Tris (Invitrogen, USA), 200mM Sodium chloride (NaCl) (SD Fine Chemicals, India), 0.2% Triton X-100 (Gibco, USA), and 100μg/ml Proteinase K (Invitrogen, USA)) for 1.5 hours at 37^0C. The mixture was centrifuged at 14,000 rpm for 5 minutes at room temperature and the supernatant was collected. Equal volume of isopropanol and 4mM NaCl were added to the supernatant which was then incubated at -20^0C overnight. After centrifugation DNA pellet was dissolved in milliQ water. The RNA interference was inhibited by addition of RNase A, followed by incubation at 37^0C for 1 hour. The DNA was quantified using Quant-iT Picogreen double stranded DNA reagent (Invitrogen, USA). The amount of insulin secreted quantified by enzyme-linked immunosorbent assay (ELISA) was expressed relative to DNA concentration.

3.4.7 Analysis of ECM

To assess the formation of ECM on scaffold islet constructs, immunohistochemistry was performed using mouse anti rabbit collagen IV (Abcam, USA) antibody.

3.5 Studies of rabbit adipose stem cell to islet like cells

3.5.1 *In vitro* differentiation of adipose stem cells to islet like cells in culture plate and on scaffold

Stem cells were seeded at a density of $5x10^5$ to 10^6 cells on a TCPS dish (35mm) (BD Biosciences, India), ultra low attachment culture dish (60mm) (Corning, USA) and DEXGEL coated dish. Stem cells ($5x10^5$ cells) were seeded on DEXGEL scaffold (5mm diameter and 3mm thickness). A three stage protocol used by various research groups (Chandra *et al*., 2011) (Sun *et al*., 2007) was adopted for the differentiation study with a deviation of avoiding Insulin Transferrin Selenium (ITS) from the protocol as ITS is expected to interfere with results of insulin production. On day 1, cells were fed with 'serum free medium (SFM) A' comprising of DMEM HG supplemented with 50µM/L β mercaptoethanol (Sigma, USA), 4nM activin A (Sigma, USA) and 1X antibiotic-antimycotic at 37^0C in a CO_2 incubator. On day 4, SFM A was replaced with 'SFM B' which consisted of 1% (v/v) non essential amino acid (Gibco, USA), 2mM/L L-Glutamine, 1X B27 (Gibco, USA), 20ng/ml basic fibroblast growth factor (bFGF) (Sigma, USA) and 20ng/ml epidermal growth factor (EGF) (Sigma, USA). The SFM B was continued for 7 days with medium change at an interval of 2 days. On day 11, SFM C was added which consisted of 10ng/ml betacellulin (Sigma, USA), 10ng/ml activin A, and 1X B27

and 10mM/L nicotinamide (Sigma, USA) for 10 more days. Media changes were at 2 days interval.

3.5.2 Diphenylthiocarbazone staining to detect *β* cells in islet like cells

Dithizone (Sigma, USA) stock was prepared at a concentration of 39mM in Dimethyl sulfoxide (Sigma, USA) and stored briefly at -15^{0}C. The DTZ working solution was prepared fresh by mixing 100µl DTZ stock, 10ml KRBH buffer. The differentiated rabbit islet like cells (rILC) were washed with PBS and incubated with filtered DTZ working solution for 20 minutes at 37^{0}C. The stained cell clusters were observed with phase contrast microscope.

3.5.3 Assessment of Cell Viability

The viability of rILC on 2D cultures and DEXGEL scaffold were analyzed with live/dead calcein AM/ethidium homodimer-1 stains (Invitrogen, USA). Calcein AM (excitation 495 nm, emission 515 nm) are retained within live cells and ethidium homodimer (excitation 495 nm, emission 635 nm) are excluded by the intact plasma membrane of live cells. On day 30, rILC on scaffold and on 2D cultures were stained by calcein and ethidium bromide. The samples were incubated with 50µl of live- dead staining solution (2µl calcein (4mM stock) and 4 µl of ethidium homodimer (2mM stock) in PBS) for 15 minutes at 37^{0}C. After several PBS washes, cells on 2D culture were visualized using fluorescent microscope (Leica, Germany) and on scaffold were visualized using Confocal Laser Scanning Microscope (Carl Zeiss 510 Meta, Germany).

3.5.4 Quantitative Real - Time (qRT) Polymerase Chain Reaction

Total RNA was extracted from triplicates of rILC cultured on DEXGEL coated dish and DEXGEL scaffold using TRIzol reagent (Gibco, USA) according to manufacturer's instruction. The resulting mRNA was reverse transcribed to cDNA with Revert-Aid reverse transcriptase (Fermentas, USA) for 60 minutes at 42^0C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers (Integrated DNA Technologies, USA) whose sequences are listed in Table 3.1. Sybr Green master mix (Invitrogen, USA) was used for the reaction. Forty cycles were performed in BioRad System programmed with specified conditions for denaturation and annealing. The qRT-PCR results were normalized to β actin (reference gene) carried out in parallel to correct differences in RNA input. Rabbit islets on 2D and 3D cultures were used as control.

3.5.5 Immunocytochemistry of rabbit islet like cells

Immunostaining of day 20 rILC and rabbit islets on 2D cultures was performed to assess the protein level expression of insulin. The cells were fixed with formalin free fixative for 20 minutes at room temperature, permeabilized with chilled methanol for 20 minutes and blocked with 2% (w/v) BSA. Cells were incubated for 1 hour with the following cell specific primary antibodies for insulin (Santa Cruz biotechnology, USA), glucagon and somatostatin (Dakocytomation, Denmark). After PBS wash cells were incubated with phycoerythrin/FITC conjugated anti-mouse IgG secondary antibodies and images analyzed using fluorescent microscope (Leica, Germany). Nucleus was counterstained with DAPI (Gibco, USA).

3.5.6 Histology and immunohistochemistry

The scaffold constructs with differentiated rILC (day 20) was washed in PBS and fixed in formalin free fixative for 24 hours before histological analysis. Samples were dehydrated through a series of graded ethanol, embedded in paraffin and sectioned as 6μm sections using microtome (Leica, Germany). For histological evaluation, sections were deparaffinized, rehydrated through series of graded ethanol. To identify pancreatic endocrine hormones by immunohistochemistry, sections were stained with mouse anti-rabbit primary antibodies: insulin, glucagon and somatostatin; after which streptavidin peroxidase labeled secondary antibody (Ultratech DAB kit, USA) was added. To further confirm the evidence of ECM formation rILC-scaffold constructs were stained with mouse anti rabbit collagen IV antibody followed by labeling with secondary antibody. Counter staining was done with Mayer's hematoxylin (Sigma, USA).

3.5.7 Insulin secretion assay and DNA estimation

The amount of insulin secreted by differentiated rILC on 2D culture plates and on 3D scaffold (day 20) in response to glucose challenge was quantified. Samples were pre-incubated for 3 hours in glucose- free KRBH (Appendix A-1) buffer, followed by incubation with KRBH containing 5mM and 25mM glucose for an additional 1 hour respectively. Rabbit islets used as controls were also challenged with similar concentrations of glucose. The supernatants were collected and frozen at -80^0C till the assay was performed. Insulin quantification was performed by ELISA (Rabbit insulin kit, myBiosource, USA), according to the manufacturer's instruction. After glucose challenge, the cells on 2D and scaffold were lysed using

cell lysis buffer. The concentration of DNA was estimated using Quant-iT Picogreen dsDNA reagent (Invitrogen, USA) according to manufacturer's instruction. The amount of insulin secreted was expressed relative to DNA concentration. The given results are representative data confirmed from 4 different experiments.

3.6 Studies on human adipose stem cell differentiated islet like cells

3.6.1 Differentiation of human adipose stem cells to islet like clusters

Differentiation was carried out using a three stage protocol. Adipose stem cells were seeded at a density of 10^5 cells/cm^2 and 10^7 cells/cm^{3}on DEXGEL coated dish and DEXGEL scaffold respectively. On day 1, cells were fed with 'serum free medium (SFM) A' comprising of DMEM HG supplemented with 50μM/L β-mercaptoethanol and 4nM activin A at 37^0C in a CO_2 incubator. On day 4, SFM A was replaced with 'SFM B' which consisted of 1% (v/v) non essential amino acid, 2mM/L L-glutamine, 1X B27, 20ng/ml basic fibroblast growth factor (bFGF) and 20ng/ml epidermal growth factor (EGF). The SFM B was continued for 7 days with medium change at an interval of 2 days. On day 11, SFM C was added which consisted of 10ng/ml betacellulin, 10ng/ml activin A, and 1X B27 and 10mM/L nicotinamide for 10 more days. Media changes were at 2 days interval.

3.6.2 Gene expression analysis

Total RNA was extracted from day 20, 2D and 3D cultures of human islet like cells (hILC) using TRIzol (Invitrogen) reagent and the concentration of RNA was estimated using Qubit RNA assay kit. The cDNA was prepared from RNA by reverse transcription using oligo dT primers and reverse transcriptase (Fermentas,

USA). The sequences of human specific primers for Pdx1, Ngn3 (Neurogenin 3), insulin, glucagon, somatostatin, pancreatic polypeptide and somatostatin (Integrated DNA Technologies, USA) are tabulated in Table 3.2. Quantitative gene expression was analyzed by Real Time PCR. The samples were run in duplicates and PCR results were normalized to β-actin (reference gene).

Table 3.2. List of primer sequences for human islet like cells

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
β actin	CAGAGCCTCGCCTTTGCC	TCAGGGTGAGGATGCCTCT
Ngn3	CGACTTGCTGCTCAGGAAAT	GAGGTTGTGCATTCGATTGC
Pdx1	GTTGAATGGGGCGGCAATTG	TGTAGGAGGGCAGGGATGTG
Insulin	GGCTTCTTCTACACACCCAA	AGGGCTTTATTCCATCTCTCT
Glucagon	AAGTGAGTGGGAGAGGGAA	GCAGGTGAAGAGAGAGCAAG
Somatostatin	TGAGCAGGATGAAATGAGG	GAAGAGAGATGGGGTGTGG
Pancreatic polypeptide	ACCTGCGTGGCTCTGTTA	GCGTGTCCTCTTTGTGTCTT
Ghlerin	TCTGGGCTTCAGTCTTCTCC	CCTTCTGCTTGACCTCCATC

3.6.3 Immunofluorescence analysis

The differentiated hILC on 2D culture were characterized for its protein expression by immunocytochemical analysis. Cells were fixed with formalin- free fixative for 20 minutes at room temperature, permeabilized with chilled methanol for 20 minutes and blocked with 2% (w/v) BSA. The hILC were incubated for 1 hour with the following cell specific primary antibodies for insulin (Abcam, USA), glucagon and somatostatin (Dakocytomation, Denmark). After PBS wash, cells were incubated with PE/FITC conjugated anti-mouse or anti-rabbit IgG secondary

antibodies and images analyzed using Fluorescence microscope (Leica, Germany). Nucleus was counterstained with DAPI (Sigma, USA).

3.6.4 Immunohistochemistry

The differentiated hILC on scaffold were fixed in formalin-free fixative. The samples were dehydrated using graded ethanol series, paraffin embedded and 6µm sections were taken using microtome (Leica, Germany). For immunohistochemical analysis, sections were deparaffinized, rehydrated and stained with islet and/or endothelial specific markers: insulin, glucagon and somatostatin after which streptavidin peroxidase labeled secondary antibody (Ultratech DAB kit, USA) was used.

3.6.5 Viability assay

The hILC on 2D and 3D culture were assessed for its viability by live-dead calcein AM/ethidium homodimer-1 stains (Invitrogen, USA). The samples were incubated with 50 µl of live- dead staining solution (2µl calcein (4mM stock) and 4 µl of ethidium homodimer (2mM stock) in PBS) for 15 minutes at 37^0C. After several PBS washes, cells on 2D culture were visualized using fluorescent microscope (Leica, Germany) and on scaffold were visualized using Confocal Laser Scanning Microscope (Carl Zeiss 510 Meta, Germany).

3.6.6 Glucose challenge assay

To assess the functionality of hILC, glucose stimulated insulin secretion assay was used. Day 20 hILC were challenged with 5mM and 25mM glucose

concentrations and the secreted insulin was collected and subjected for ELISA assay (Insulin ELISA kit, Invitrogen, USA).

3.7 Studies on co-culture of human islet like cells and endothelial cells

3.7.1 Coculture study

Endothelial cells were seeded along with day 10 hILC on 2D and 3D at a density of 10^5 cells/cm^2 and 10^7 cells/cm^3 respectively. The co-cultured cells were fed with hILC medium for 4 weeks.

3.7.2 Gene expression analysis

Total RNA was extracted from day 20, 2D and 3D cultures of ILC-EC constructs using TRIzol (Invitrogen) reagent and the concentration of RNA was estimated using Qubit RNA assay kit. The cDNA was prepared from RNA by reverse transcription using oligo dT primers and reverse transcriptase (Fermentas). Specific primers for insulin and VEGF-A were purchased from IDT (Integrated DNA Technologies, USA) whose sequences are tabulated in Table 3.3. Quantitative gene expression was analyzed by Real Time PCR. The samples were run in duplicates and PCR results were normalized to β-actin expression and values were expressed relative to 2D cultured hILC.

Table 3.3. List of primers used for co-cultured human islet like cells and endothelial cells

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Insulin	GGCTTCTTCTACACACCCAAG	AGGGCTTTATTCCATCTCTCT
VEGF- A	GCTTGCCATTCCCCACTTGA	GCGGTGTCTGTCTGTCTGTC

3.7.3 Immunophenotype analysis

Protein expression on hILC-EC constructs were analyzed by immunocytochemical analysis. The hILC-EC co-culture constructs were fixed with formalin- free fixative for 20 minutes at room temperature, permeabilized with chilled methanol for 20 minutes and blocked with 2% (w/v) BSA. The hILC-EC constructs were incubated with insulin/vWF antibody for 1 hour. After PBS wash cells were incubated with PE/FITC conjugated anti-mouse or anti-rabbit IgG secondary antibodies and images analyzed using a Fluorescence microscope (Leica, Germany). Nucleus was counterstained with DAPI.

3.7.4 Immunohistochemistry

The hILC-EC 3D constructs after fixation were dehydrated using graded ethanol series, paraffin embedded and 6µm sections were taken using microtome (Leica, Germany). The sections were deparaffinized, rehydrated and stained for collagen IV and integrin β1 after which streptavidin peroxidase labeled secondary antibody (Ultratech DAB kit, USA) was used.

3.7.5 Viability assay

The hILC-EC 3D constructs were stained with live-dead calcein AM/ethidium homodimer-1 stains (Invitrogen, USA) to assess its viability. The samples were incubated with 50 µl of live- dead staining solution (2µl calcein (4mM stock) and 4 µl of ethidium homodimer (2mM stock) in PBS) for 15 minutes at 37^0C. After several PBS washes, cells were visualized using a Confocal Laser Scanning Microscope.

3.7.6 Glucose challenge assay

Glucose stimulated insulin secretion assay was used to assess the functionality of co-culture 3D constructs. Day 20 hILC-EC constructs were challenged with different glucose concentrations (5mM and 25mM) and the secreted insulin was collected and subjected for ELISA assay (Insulin ELISA kit, Invitrogen, USA).

3.7.7 Statistical Analysis

The quantitative results are represented as mean ± standard deviation and were assessed statistically using one way analysis of variance (ANOVA) followed by post-hoc Tukey test. A value of $p < 0.05$ was considered to be statistically significant.

CHAPTER 4

RESULTS

4.1 Isolation and characterization studies

4.1.1 Isolation and characterization of rabbit islets

The islet cells were isolated from rabbit pancreas by collagenase V enzymatic digestion. Islet cells observed under phase contrast microscope exhibited cluster morphology the size of the clusters ranging from 50 to 200µm. Dithizone stained positive islets appeared crimson red as it is a zinc chelating agent which binds to zinc granules in β islets. To confirm the presence of specific markers in isolated islets, immunostaining was done. Rabbit islets were found positive for insulin, glucagon and somatostatin (Figure 4.1).

4.1.2 Isolation and characterization of rabbit adipose tissue stem cells

4.1.2.1 *Isolation of rabbit adipose stem cells*

The adipose tissue obtained from supra scapular region of rabbit (Figure 4.2) was enzymatically digested using collagenase II. The isolated cells were directly plated onto tissue culture treated dish and non adherent cells were separated during medium change. At the primary culture, adherent cells exhibited fibroblast like morphology evident on day 2 and reached confluency by day 8. Actin staining revealed the cytoskelatal organization of adipose tissue derived stem cells on tissue culture treated dish (Figure 4.3).

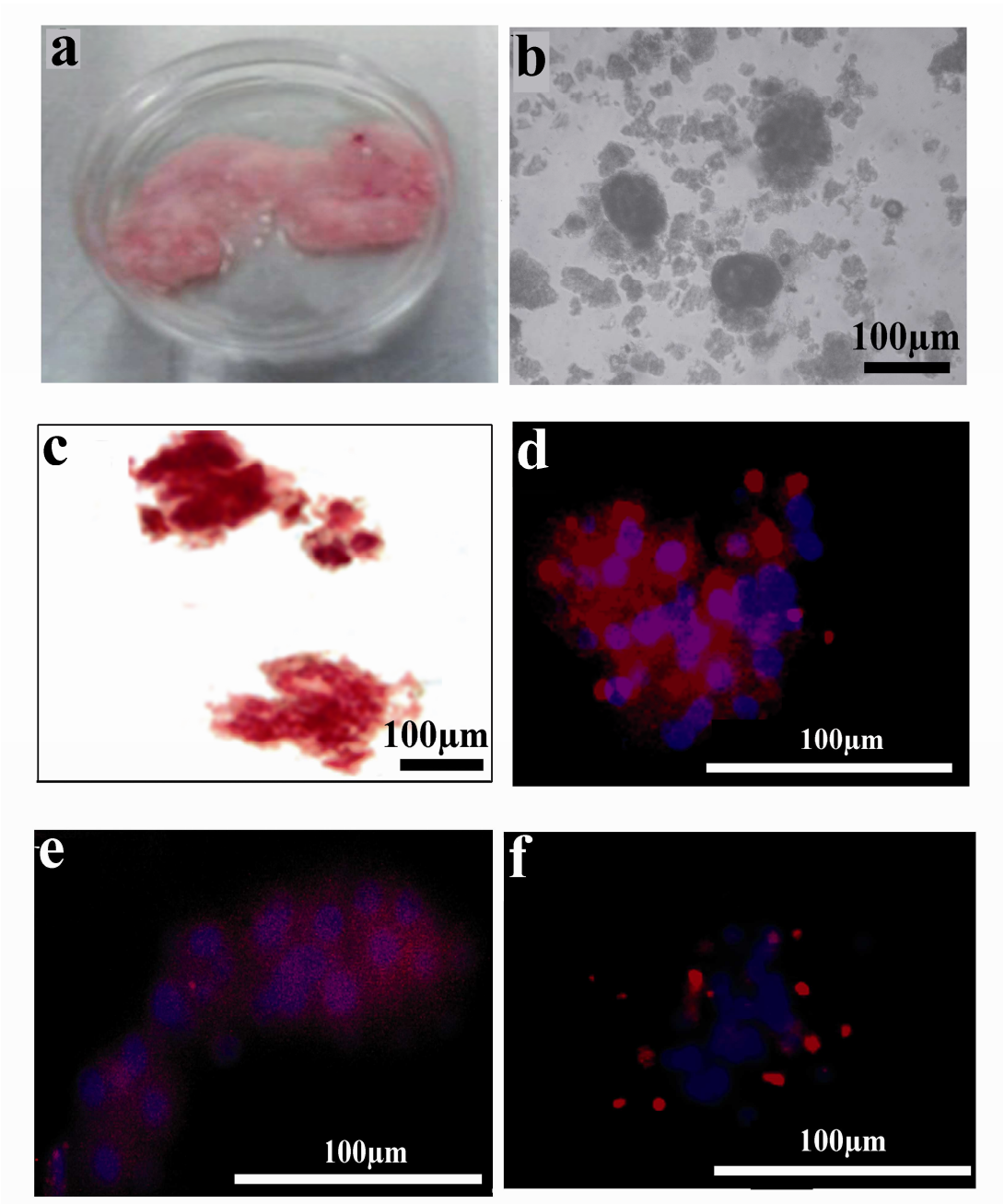

Figure 4.1 Isolation and characterization of rabbit islets. a) Pancreas harvested from New Zealand white rabbit b) Phase contrast micrograph of islet cells isolated from rabbit pancreas by collagenase V digestion c) Dithizone stained rabbit islets appeared crimson red due to the presence of zinc granules. Immunocytochemistry of rabbit islets .Positive for d) insulin e) glucagon and f) somatostatin. Nucleus stained blue.

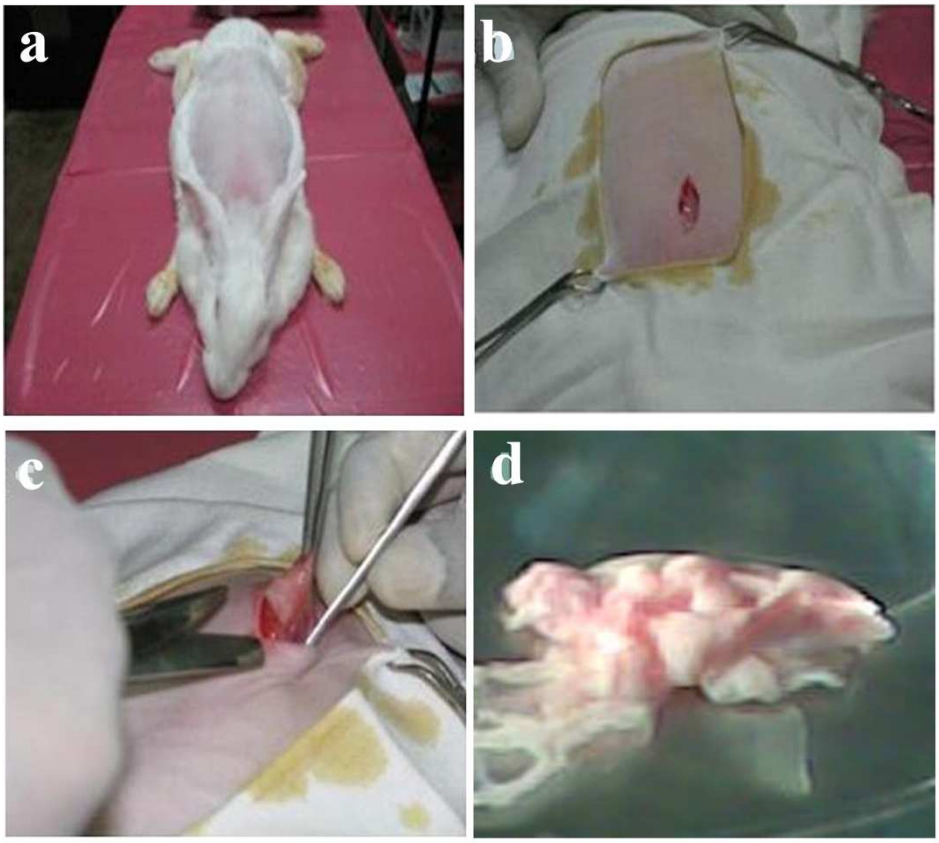

Figure 4.2 Rabbit adipose tissue harvest procedure a) New Zealand white rabbit b) incision made on supra scapular region c) adipose tissue procured using forceps d) adipose tissue collected in sterile bottle containing phosphate buffered saline supplemented with antibiotic and antimycotic.

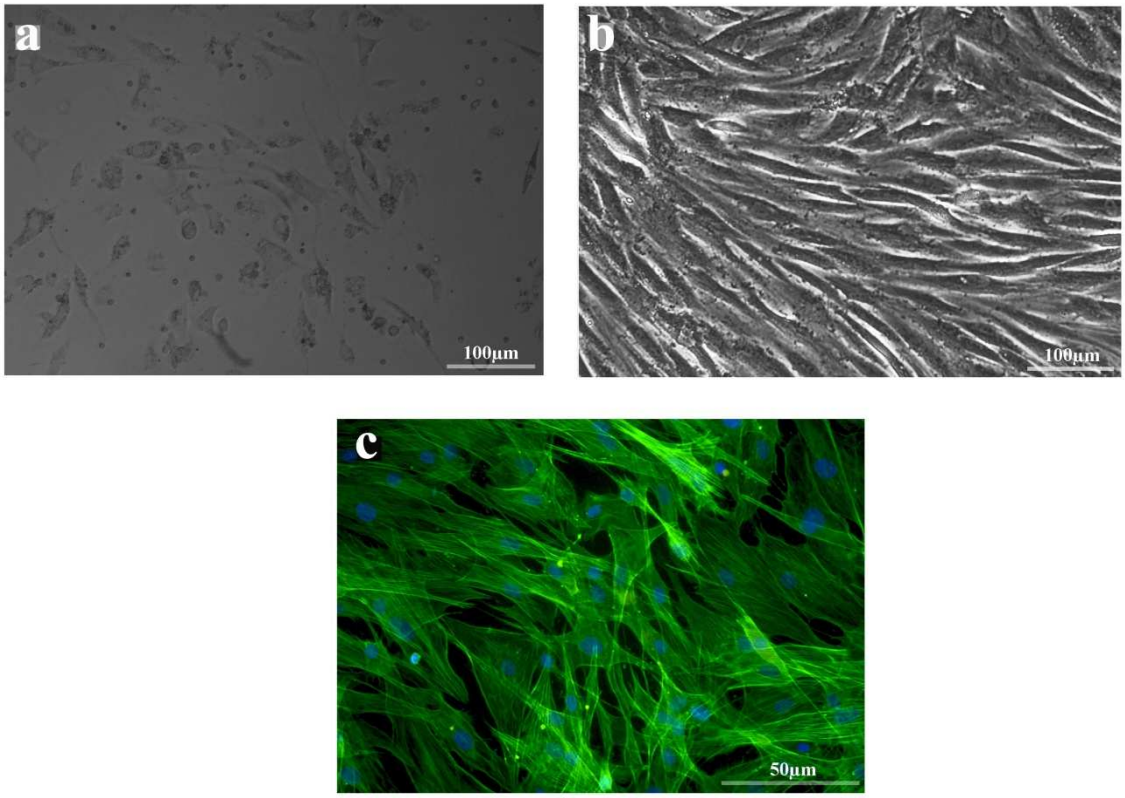

Figure 4.3 Phenotypic characteristics of isolated stem cells from rabbit adipose tissue. Phase contrast image of rabbit adipose stem cells on a) Day 2. b) Day 8. c) Cytoskeletal organization evident by actin stain showing fibroblast phenotype.

4.1.2.2 ***Growth characteristics***

Population doubling times were calculated following the first passage according to the calculation described in 3.1.2.1 (Equation 1) and the results are shown in Figure 4.4 Population doubling time of rabbit adipose stem cells calculated from passage 1-passage 10. Initially after primary isolation and seeding, cells exhibited a lag phase and the doubling time was nearly 51 hours. Cells between passage 3 and 8 exhibited reduced doubling time of approximately 40-44 hours.

From passage 8, the doubling time significantly increased (*p value <0.05) in comparison to passage 3-7 along with the change in morphology of cells in culture.

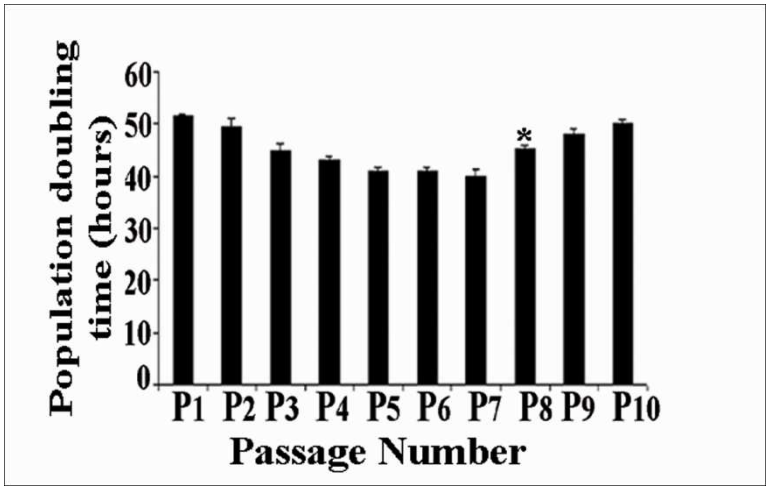

Figure 4.4 Population doubling time of rabbit adipose stem cells calculated from passage 1-passage 10. After an initial lag phase, cells from passages 3 exhibited reduced doubling time. From passage 8, the doubling time was significantly increased in culture (*p value <0.05) with respect to passage 3-7.

4.1.2.3 *Immunocytochemistry*

Immunocytochemical staining revealed that cells derived from adipose tissue were positive for vimentin which confirmed that the cells obtained were mesenchymal in origin and negative result for CD34/CD45 confirmed the absence of hematopoeitic cells. Positive results for CD105 and CD44 indicated that the isolated cells are stem cells (Figure 4.5).

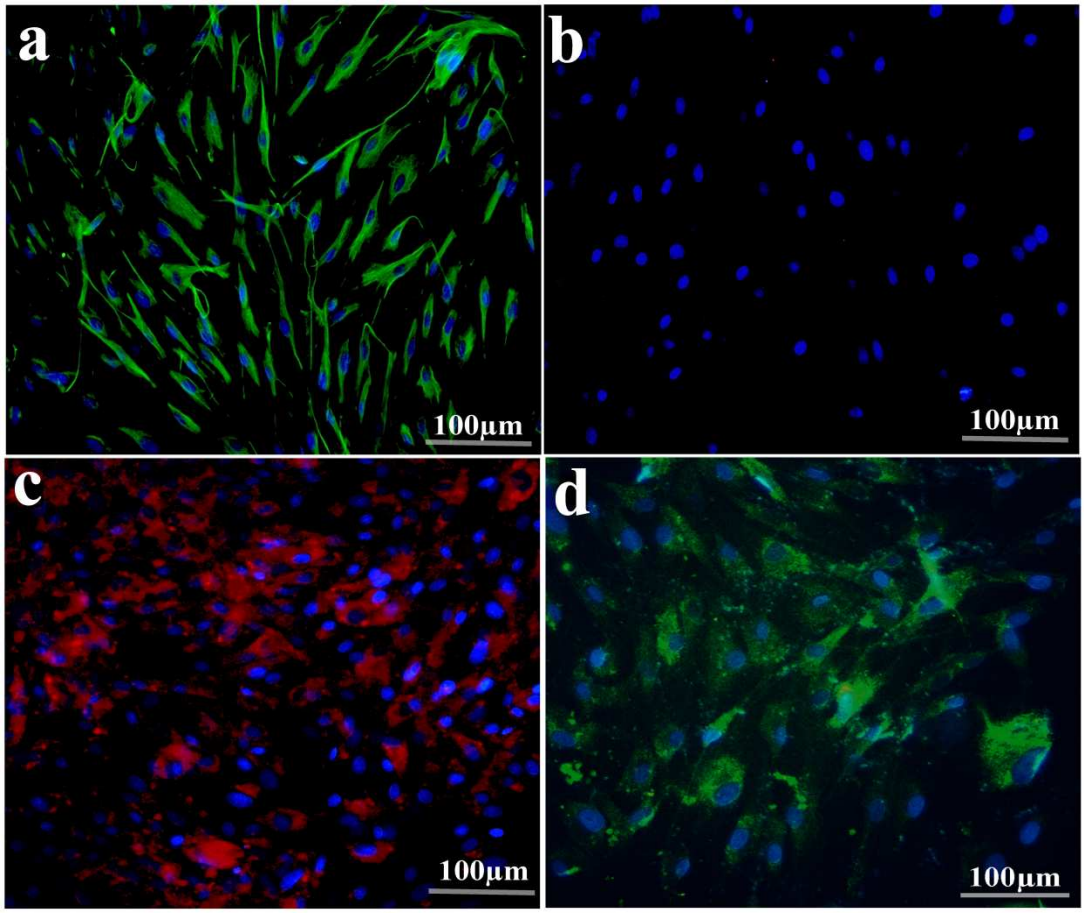

Figure 4.5 Immunophenotype characterization of rabbit adipose derived cells. The cells were a) Positive for mesenchymal marker vimentin and b) Negative for hematopoeitic marker CD45/CD34. The cells were also positive for stem cell markers c) CD105 and d) CD44.

4.1.2.4 ***Flow cytometry analysis***

For flow cytometric analysis, cells without addition of antibody were used as control. Flow cytometry histogram of antibody labeled adipose derived cells (Figure 4.6) revealed that cells expressed high levels of vimentin, CD44 and moderate levels of CD105 but did not express hematopoeitic lineage markers like CD45 and CD34.

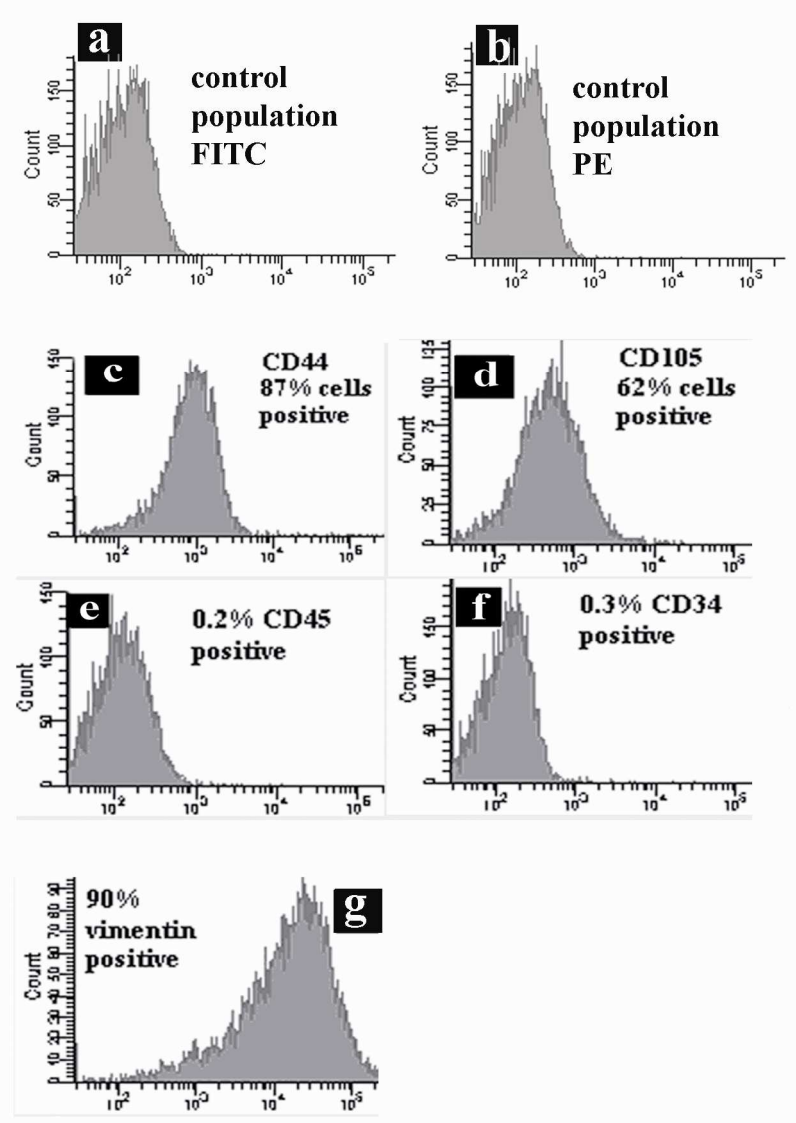

Figure 4.6 Flow cytometry histogram of rabbit adipose derived cells. a) & b) Control population comprising of unstained cells c) 87% positive for CD44 d) 62% cells were positive for CD105. The population was negative for hematopoietic markers e) CD45 and f) CD34. g) 90% of the cells were positive for mesenchymal marker vimentin

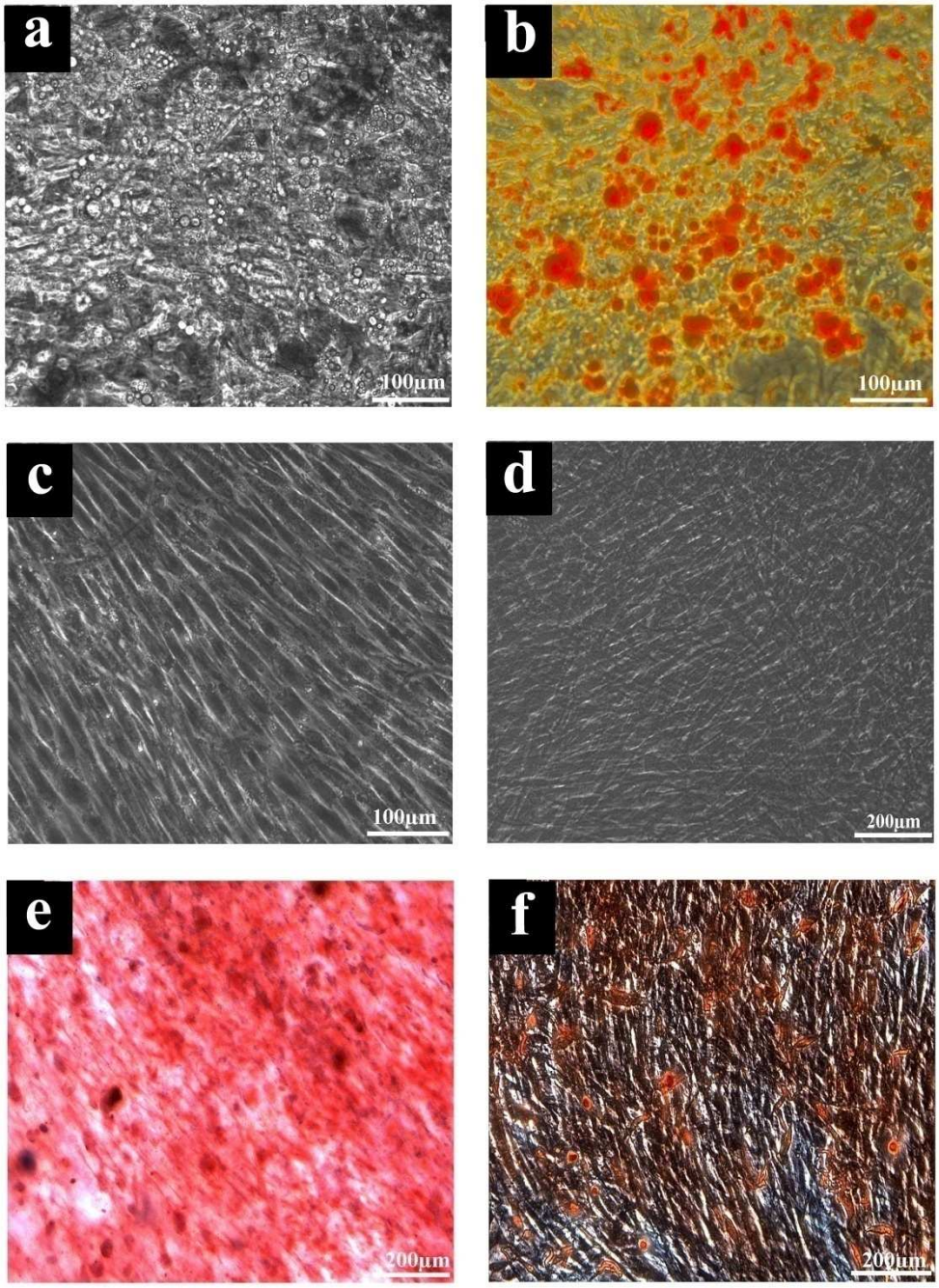

Figure 4.7 Bilineage differentiation of rabbit adipose stem cells: Adipogenic lineage a) Phase contrast microscope of differentiated adipocytes b) Lipid droplets stained red by Oil red O c) Control cells stained negative. Osteogenic lineage: d) phase contrast microscope of differentiated osteocytes. Calcium deposits stained e) Red by Alizarin Red and f) Black by Von Kossa.

4.1.2.5 *Adipogenic and osteogenic differentiation of rabbit adipose stem cells*

To confirm the stemness of isolated cells they were differentiated to adipocytes and osteocytes (Figure 4.7). Differentiation of stem cells to adipocytes was evident by the accumulation of neutral lipid droplets in the differentiated cells that was stained red by Oil Red O. Control cells did not show any significant coloration upon addition of stain. Osteogenic differentiation of stem cells *in vitro* was assessed by Alizarin Red and Von Kossa stains which formed complex with calcium deposits. The mineralized calcium deposits were observed in red colour by Alizarin Red stain and black colour by Von Kossa.

4.1.3 Isolation and characterization of human adipose stem cells

4.1.3.1 *Isolation of human adipose stem cells*

Stem cells isolated from human adipose tissue by collagenase I enzymatic digestion exhibited plastic adherent property and reached confluence by day 7 (Figure 4.8).

4.1.3.2 *Immunofluorescence of human adipose stem cells*

The cytoskeletal organization was revealed by phalloidin stain. Immunophenotype characterization of hASC showed that cells were positive for vimentin, and negative for hematopoeitic marker CD34/CD45. The hASC were positive for stem cell markers CD105, CD90 and CD44 (Figure 4.9).

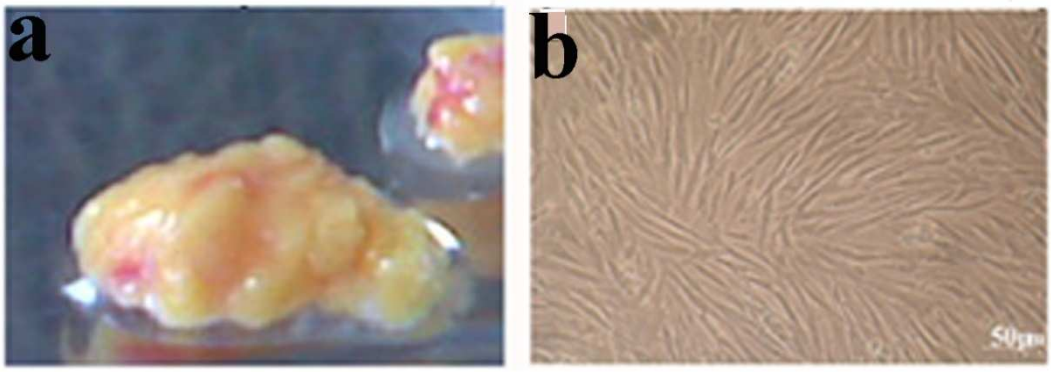

Figure 4.8 Isolation of human adipose stem cells a) Human adipose tissue obtained after lipectomy collected in a sterile bottle containing phosphate buffered saline supplemented with antibiotic and antimycotic. b) Confluent culture of day 7 cells derived from human adipose tissue by collagenase I enzymatic digestion.

4.1.3.3 *Flow cytometry analysis*

The isolated adipose derived cells revealed heterogeneous population by flow cytometry analysis which was sorted by magnetic activated cell sorting to obtain homogenous population of stem cells. The homogeneity of sorted cell population was assessed by flow cytometry analysis which revealed that 99% of the population was positive for CD105 and 88% positive for CD44 (Figure 4.10).

4.1.3.4 *Human adipose stem cell differentiation to adipogenic and osteogenic lineage*

To further confirm stem cell characteristic human adipose stem cells (hASC) were differentiated to adipocytes and osteocytes on induction with adipogenic and osteogenic medium respectively. The differentiated adipocytes were stained positive with Oil Red O evident by presence of red colored lipoid droplets (Figure 4.11a). Differentiated osteocytes were positively stained with Alizarin Red indicating the presence of calcium deposits in red colour (Figure 4.11b).

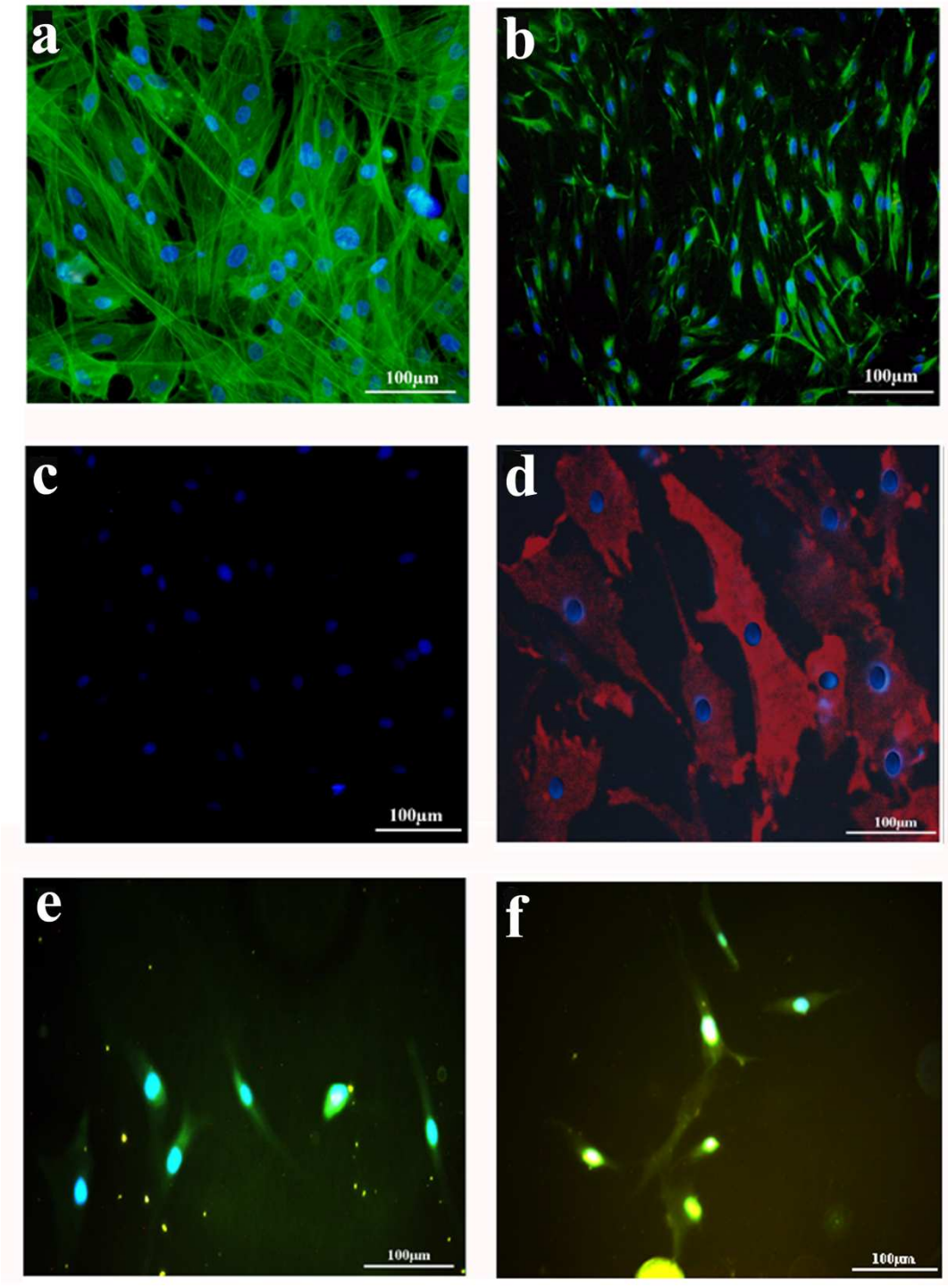

Figure 4.9 Immunophenotype characterization of human adipose derived cells Positive for a) Actin b) Vimentin. Negative for c) CD45/CD34. Positive for stem cell markers d) CD105 e) CD90 and f) CD44.

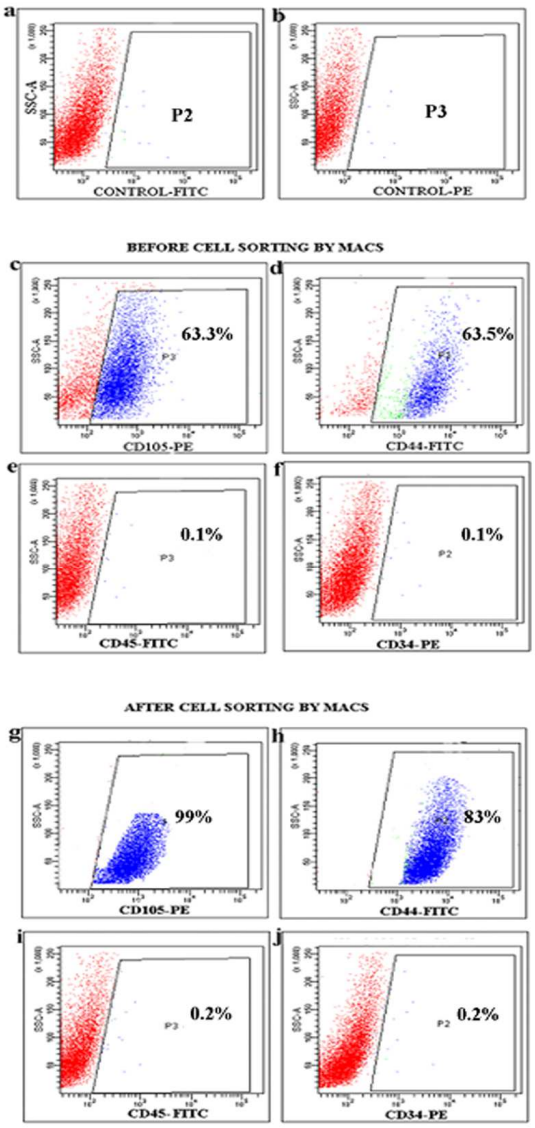

Figure 4.10 Flow cytometry analysis of human adipose derived cells before and after cell sorting based on positive selection by Magnetic activated cells sorting a) control population having unstained cells-FITC b) control population for PE. Percentage of cell population positive for stem cell markers before cell sorting c) 63.3% positive forCD105 d) 63.5% positive for CD44 e)Negative for CD45 f) Negative for CD34. After cell sorting g) 99% of the population positive for CD105 h) 88% positive for CD44 i) Negative for CD45 and j) Negative for CD34.

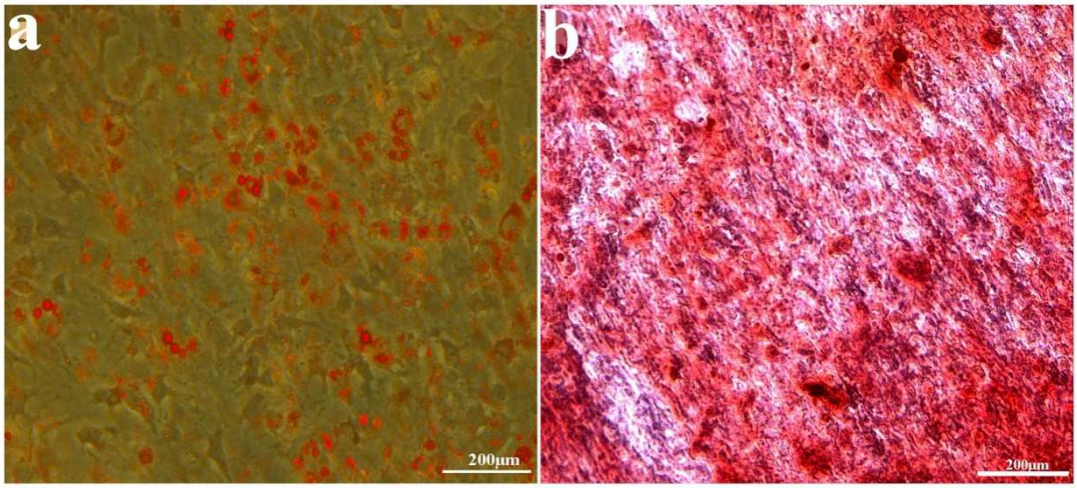

Figure 4.11 Bilineage differentiation of human adipose stem cells a) Oil Red stained lipid vacuoles in differentiated adipocytes and b) Calcium deposits in Alizarin Red stained osteocytes.

4.1.4 Isolation and characterization of human umbilical vein endothelial cells

4.1.4.1 *Isolation of endothelial cells*

Isolated EC adhered onto gelatin coated dishes and showed their characteristic cobblestone morphology (Figure 4.12). The cells after reaching confluency by 4th day were trypsinized and seeded onto gelatin coated coverslips for immunocytochemical studies.

4.1.4.2 *Immunocytochemistry*

To confirm endothelial cell origin immunostaining was performed for endothelial specific markers like vWF and CD31 or PECAM-1 (Figure 4.13). The EC were found to be positive for vWF which is an intracellular antigen and CD31 which is a cell surface marker for EC.

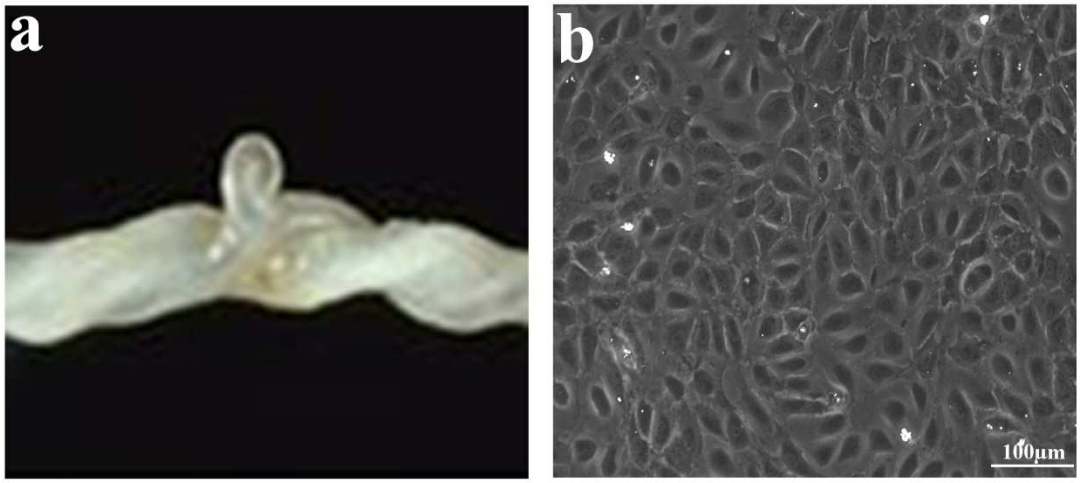

Figure 4.12 Endothelial cell isolation from a) Human umbilical cord by collagenase I enzyme digestion. b) Phase contrast image of endothelial cells showing cobblestone morphology after reaching confluency.

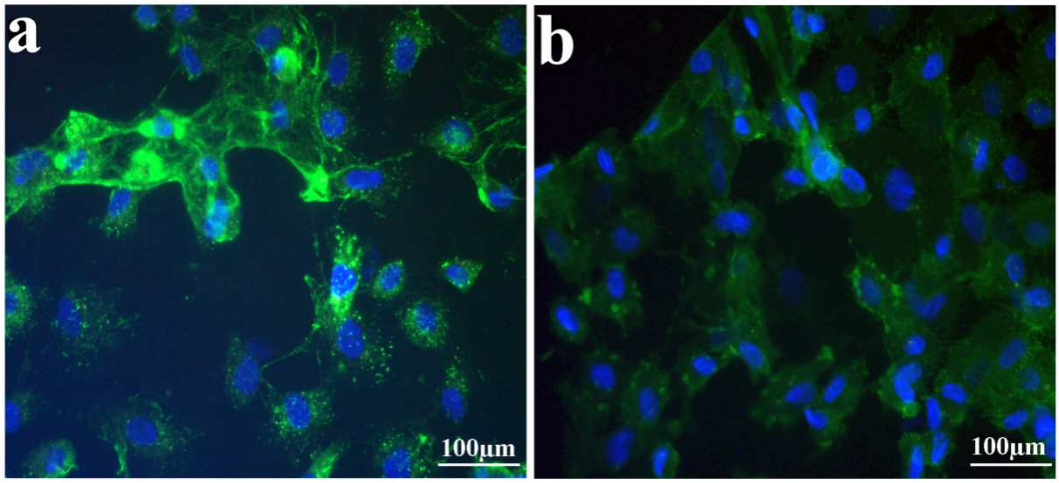

Figure 4.13 Immunophenotype of human umbilical vein derived endothelial cells. The endothelial cells were positive for a) VWF and b) PECAM-1.

4.2 Fabrication and characterization of scaffold

4.2.1 Dextran-Gelatin scaffold

Dextran contains hydroxyl groups that can readily be modified with periodate to form aldehyde groups. A 10% (w/v) solution of DDA having degree of oxidation of 50% was prepared. The actual percentage of oxidized dextran after periodate treatment calculated by titration method (Appendix A-2) was found to be in the range of 40-45%. The DDA was cross linked with gelatin in the presence of 0.1M sodium borate buffer by continuous stirring for 20 minutes without aid of any external crosslinking agents (Figure 4.14). After stirring, DEXGEL was allowed to crosslink at 37 ^{0}C overnight.

4.2.2 Morphology and Pore distribution

Scanning electron micrograph showed the pore morphology of DEXGEL and its interconnectivity in the dry state. Porosity measurement data obtained by liquid extrusion porosimetry demonstrated that more than 70% of the pores are in the range of 100-200μm in size. The macroporous nature of DEXGEL is necessary for providing a better environment for maintaining spherical morphology as well as for the survival and functioning of islets which are in the size of 50-200 μm (Figure 4.15). The stirring speed optimal for generating pores in the range of 100-300 μm was 2000-2500 rpm. Temperature at the time of crosslinking also plays an important role in determining pore size.

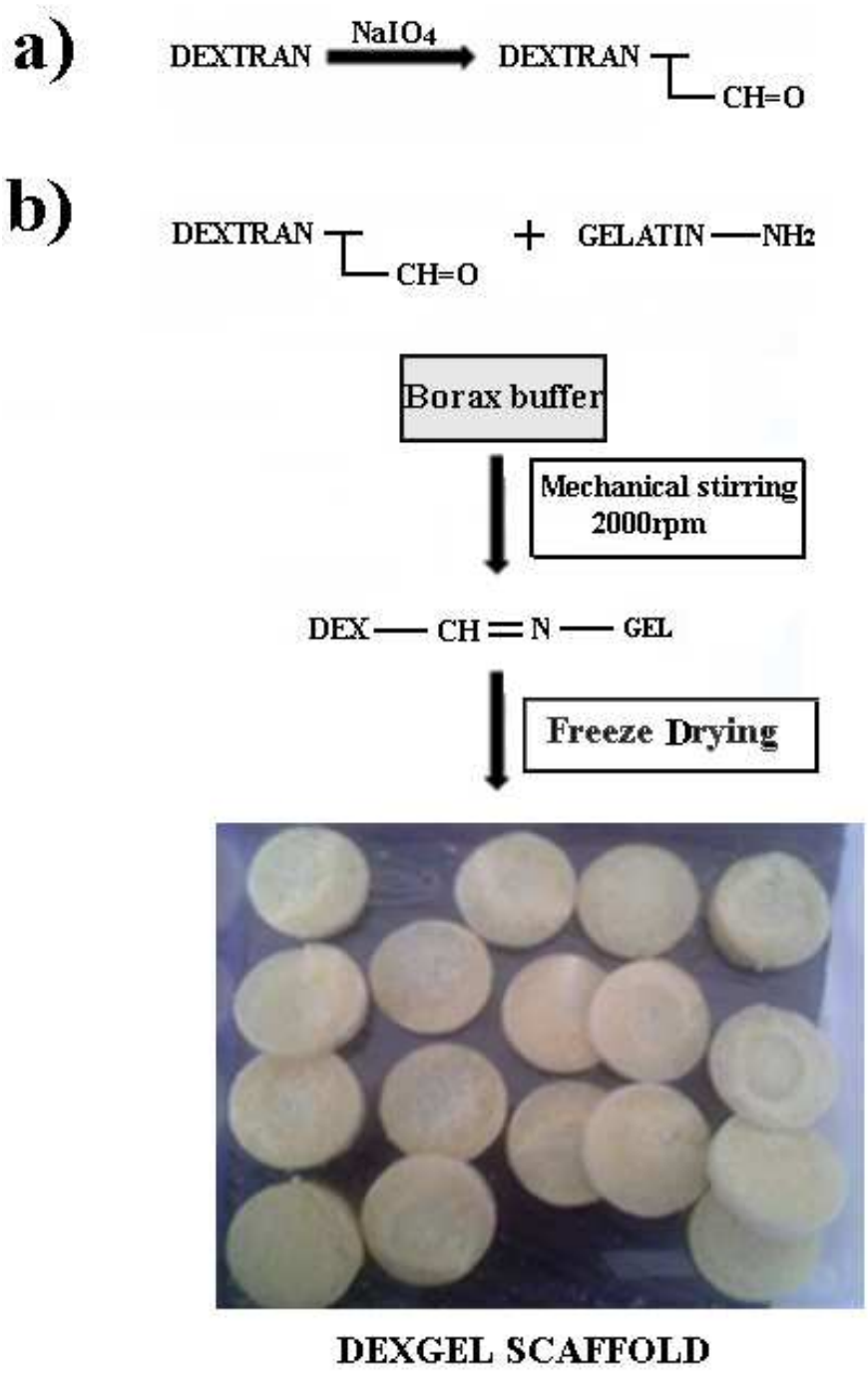

Figure 4.14 Summary of DEXGEL scaffold fabrication method. Dextran is partially oxidized to Dextran dialdehyde using metaperiodate oxidation. Oxidized dextran is reacted with gelatin in the presence of borax buffer (pH 9.4) to form Schiff base linkage which is then subjected to freeze drying to obtain DEXGEL scaffold**.**

Table 4.1 Optimization of pore size on DEXGEL scaffold by varying temperature

Temperature during crosslinking	**Pore size calculated by Liquid extrusion porosimetry**
30-40 ^{0}C	100-300µm
Less than 8 ^{0}C	Smaller pores (less than 100 µm)

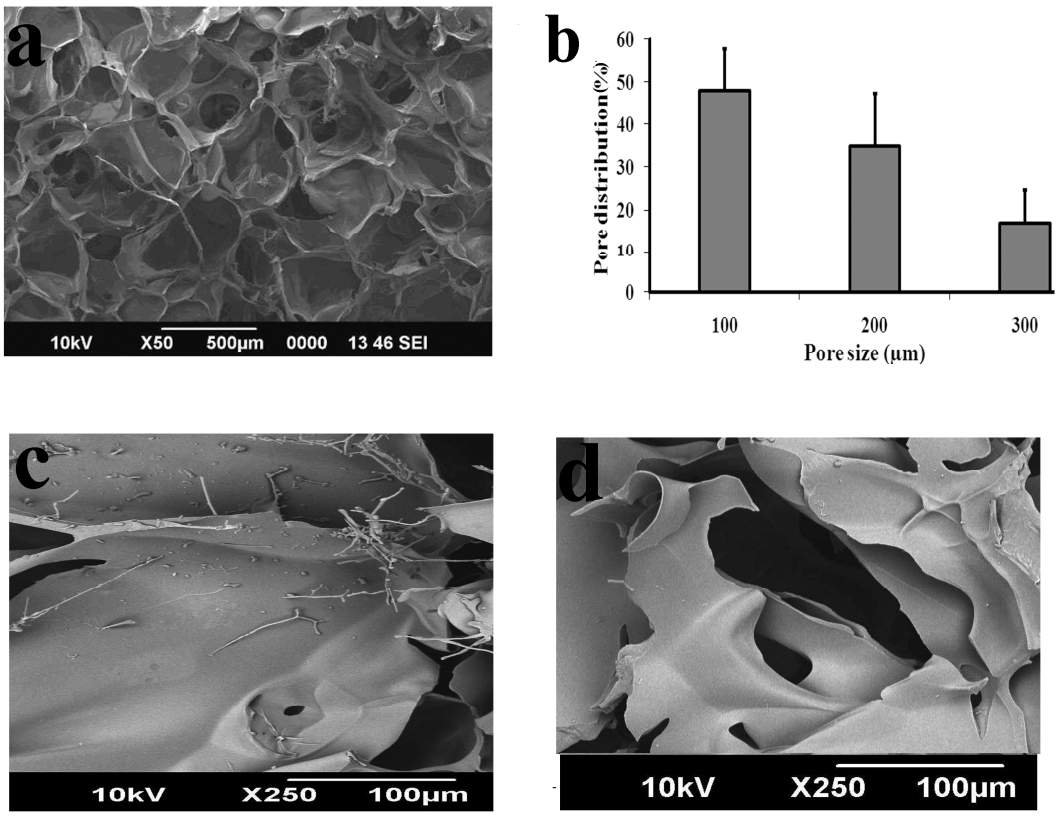

Figure 4.15 Pore analysis of DEXGEL scaffold a) Scanning electron micrograph showing the pore morphology of DEXGEL scaffold .b) Liquid extrusion porosimetry data showing the pore distribution histogram on DEXGEL. Scanning electron micrograph showing pore morphology of DEXGEL prepared by stirring speed c) greater than 2500 rpm d) less than 2500 rpm.

4.2.3 FT-IR Spectroscopic Analysis

The FT-IR spectra of DEXGEL scaffold (Figure 4.16) demonstrated characteristic peaks of both gelatin (amide peaks at 1527 cm^{-1} due to N-H bend and C-H stretch as well as at 3266 cm^{-1} due to N-H stretching vibration) and dextran dialdehyde (1733 cm^{-1}). The aldehyde peak of oxidized dextran was observed at 1733cm^{-1} due to C=O carbonyl group and at 2900cm^{-1} due to C-H stretching .The aldehyde peaks were not significant in freshly prepared DEXGEL. The peak at 1630 cm^{-1} was the characteristic peak for Schiff's base related to the crosslinking between aldehyde group of dextran and amino group of gelatin. The FT-IR spectra of DEXGEL in 1 week culture and 1 month culture did not exhibit any significant absorption peaks at 1743cm^{-1} and 1541cm^{-1}. The FT-IR spectra of DEXGEL after 60 days in culture exhibits absorption peaks at 1743cm^{-1} and 1541cm^{-1} indicating the presence of C=O and NH bending which corresponds to free carbonyl and amino groups respectively resulted due to the degradation at Schiff base linkage.

4.2.4 Contact Angle Measurement of DEXGEL film

Contact angle is a measure of the hydrophobicity of a material. The higher the air-water contact angle, the more is its hydrophobicity. Here the air-water contact angle value for DEXGEL scaffold (Figure 4.17a) was 64.5^0C which displayed a balance between hydrophilic-hydrophobic nature. Gelatin is highly hydrophilic in nature but due to cross linkage with DDA there was a balance between the hydrophilic – hydrophobic nature for DEXGEL which contributes to better cell-matrix interaction in tissue engineering applications.

4.2.5 Swelling Studies

Swelling ratio of DEXGEL was 7.5 to 8 as illustrated in Figure 4.17b. As scaffold swells pore size increase in diameter thus facilitating cells not only to attach but also to migrate inside and grow in a three dimensional fashion, during *in vitro* cultures. In addition, the cells gain maximum internal surface area of the scaffold.

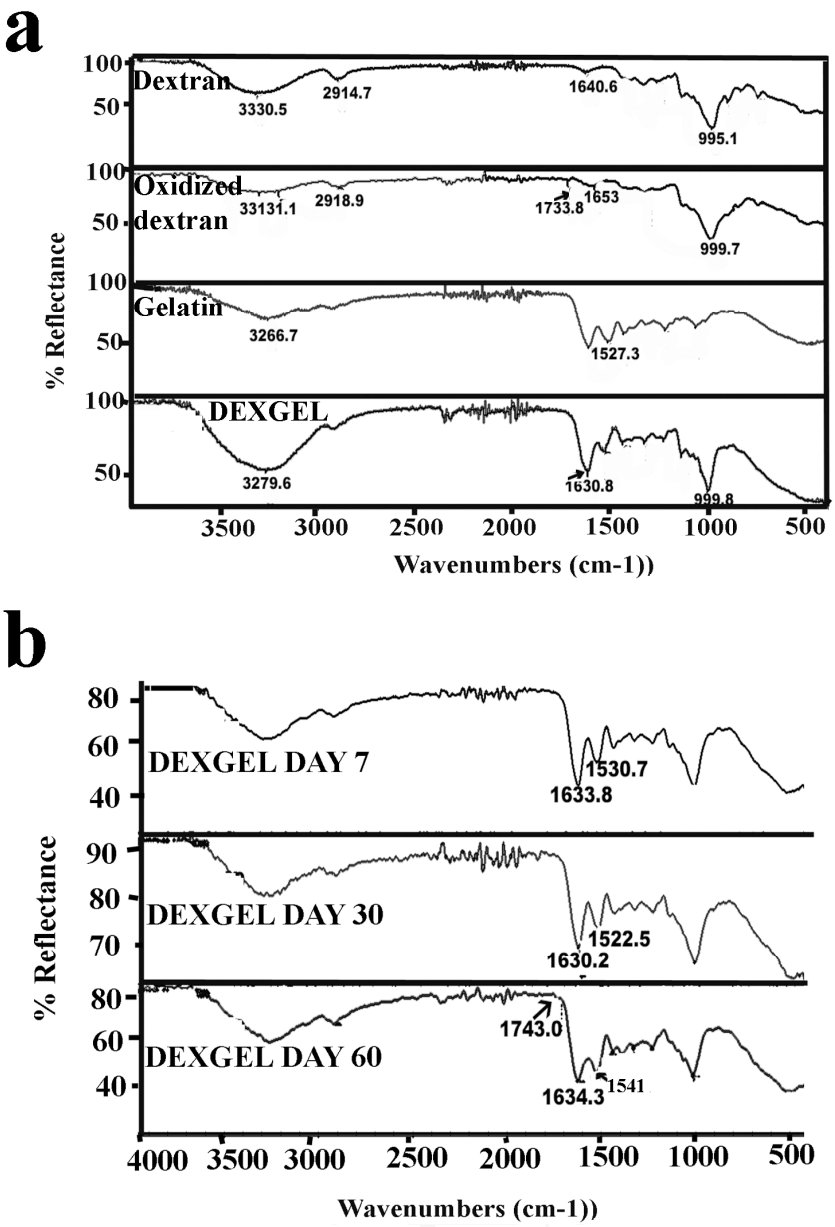

Figure 4.16 a) FT-IR spectrum of dextran, oxidized dextran, gelatin and DEXGEL scaffold. FT-IR data confirmed the presence of aldehyde peak in oxidized dextran, and absorption peak at 1630cm^{-1} due to Schiff base formation in DEXGEL scaffold indicated by arrows. b) FT-IR spectrum of DEXGEL after 60 day culture confirmed the occurrence of degradation products represented by free carbonyl and amino peaks at 1743cm^{-1} and 1541cm^{-1} respectively which was absent in freshly prepared DEXGEL. Day 7 and Day 30 DEXGEL in culture were used for comparison.

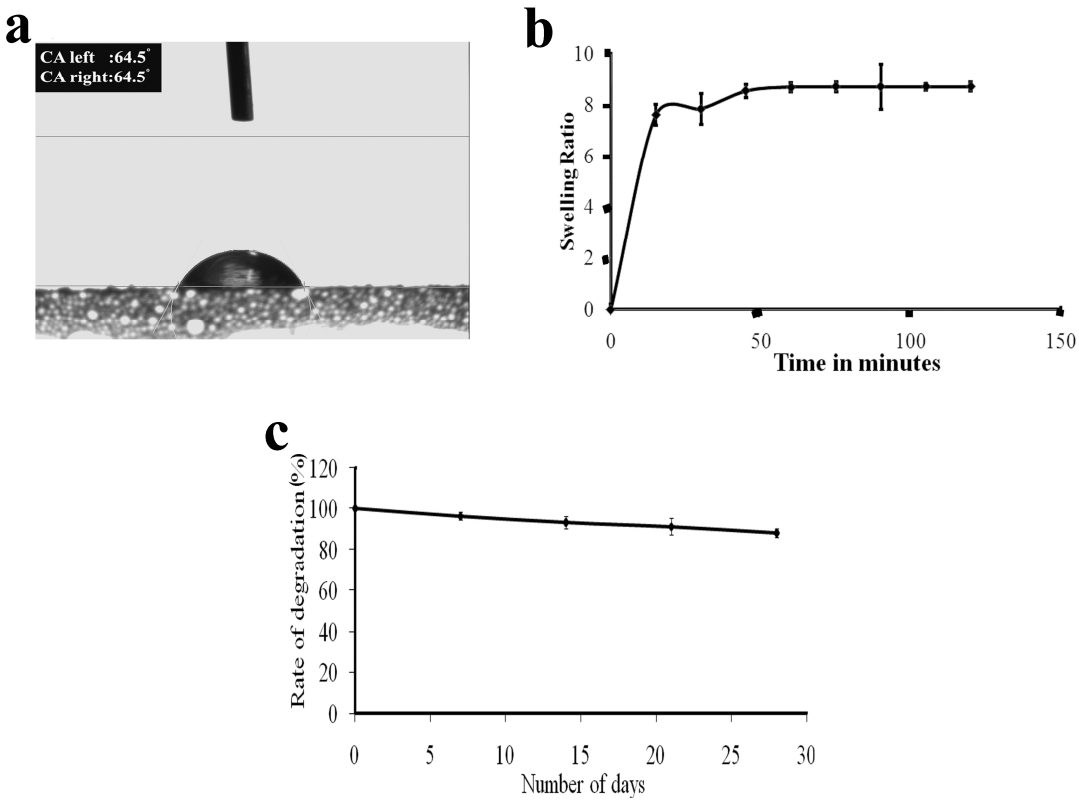

Figure 4.17 Physicochemical characterization of DEXGEL scaffold. a) Air-water contact angle of DEXGEL film revealing the hydrophilic- hydrophobic balance in DEXGEL film. b) Swelling behavior of DEXGEL scaffold in PBS. High swelling ratio enhances cellular infiltration and nutrient uptake. c) *In vitro* degradation rate of DEXGEL scaffold under enzymatic treatment. Slow degradation profile of DEXGEL is due to efficient crosslinking.

4.2.6 Enzymatic degradation study

The assessment of weight loss of the scaffold is a reliable method for investigation of degradation behavior of scaffolds. DEXGEL scaffold had a slower degradation rate (Figure 4.17c) which is comparable with the rate of formation of ILC. The slow degradation rate also confirms the efficient crosslinking between dextran dialdehyde and gelatin groups.

4.2.7 *In vitro* cytotoxicity assay

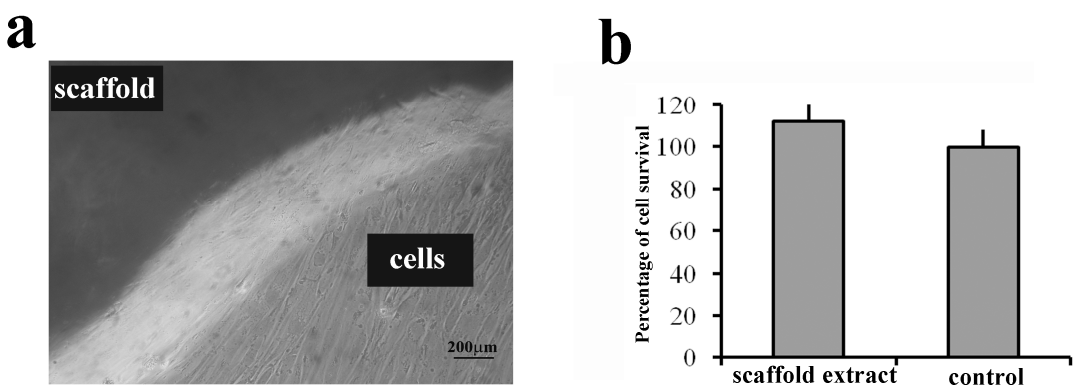

Figure 4.18 Cytotoxicity assay a) Direct contact assay. No evident morphological change in cells cultured in the vicinity of DEXGEL scaffold. c) MTT assay showing cell viability with 100% scaffold extract compared to control group by indirect contact method. The scaffold extract treated group and control group did not show any statistically significant difference in cell viability.

Stem cells cultured with DEXGEL scaffold for 48 hours did not show any change in the morphology. Cell viability on DEXGEL extract was determined by MTT assay. The MTT (thiazolyl blue) is converted from yellow-colored salt to purple-colored formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases, the activity of which is linear to the cell number. The results of MTT assay confirmed that scaffold extract was non-cytotoxic to the cells which were similar to that of the untreated control (Figure 4.18).

4.3 Studies of rabbit islets on DEXGEL scaffold (3D) in comparison to islets cultured on tissue culture dish (2D)

4.3.1 Morphology of islets on tissue culture dish and DEXGEL scaffold

Islets cultured on tissue culture polystyrene (TCPS) dish (2D) and DEXGEL scaffold (3D) exhibited different morphology (Figure 4.19). By day 10, islets on 2D exhibited disrupted cluster morphology, with decreased cell number when observed under phase contrast microscope and after dithizone staining. Meanwhile, islets on scaffold grew in a three dimensional pattern by adhering to it and formed cell clusters with characteristic cluster phenotype and in general formed the 3D islet tissue.

4.3.2 Viability

The viability of the islets cells was confirmed by calcein-ethidium bromide staining (Figure 4.20). The islets cultured on scaffold were viable over the period of 30 days in contrast to islets cultured on TCPS. Islets cultured on TCPS became non viable by day 10 and the number decreased on day 30.

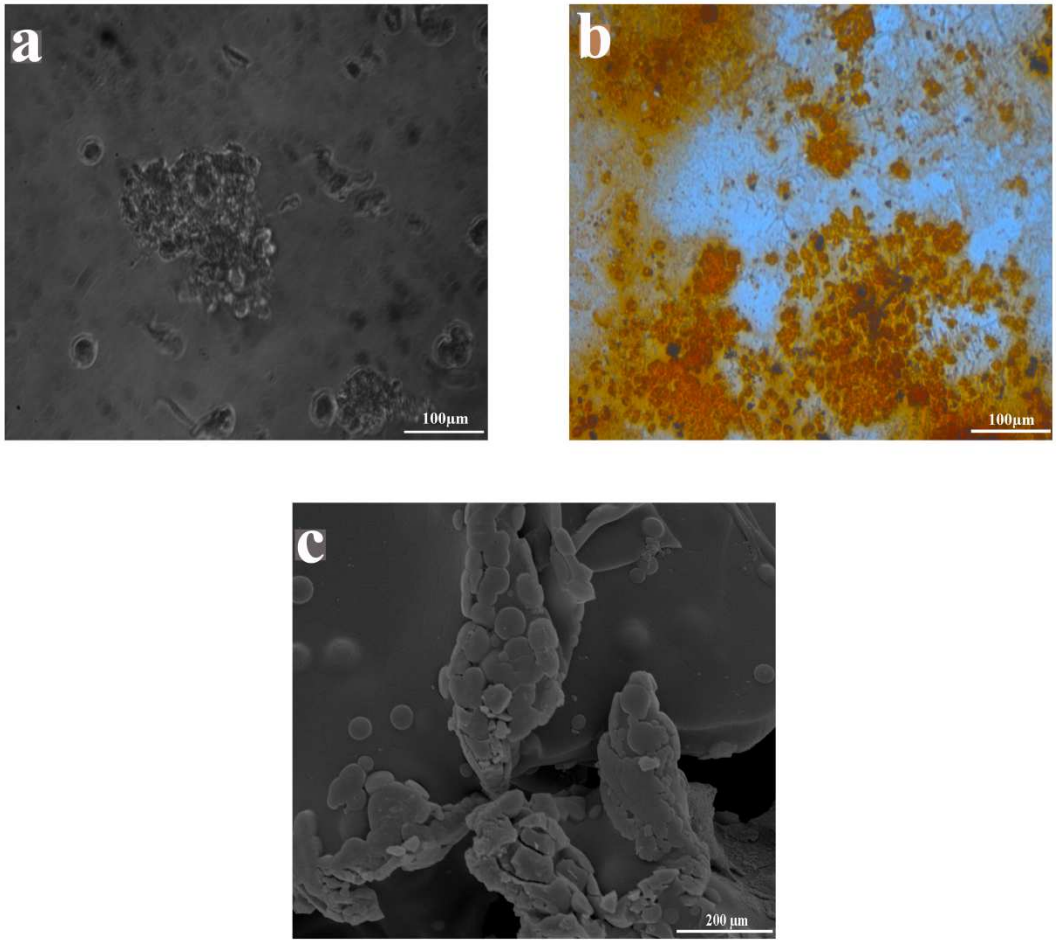

Figure 4.19 Phenotypic characteristics of rabbit islets cultured on tissue culture treated dish and scaffold for 10 days. a) Phase contrast image showing the disrupted cluster morphology of islet cells cultured on tissue culture treated dish. b) Dithizone stained rabbit islets cultured on tissue culture treated dish did not show significant crimson red appearance. c) Scanning electron micrograph showing the intact cluster morphology of islets maintained when cultured on DEXGEL scaffold.

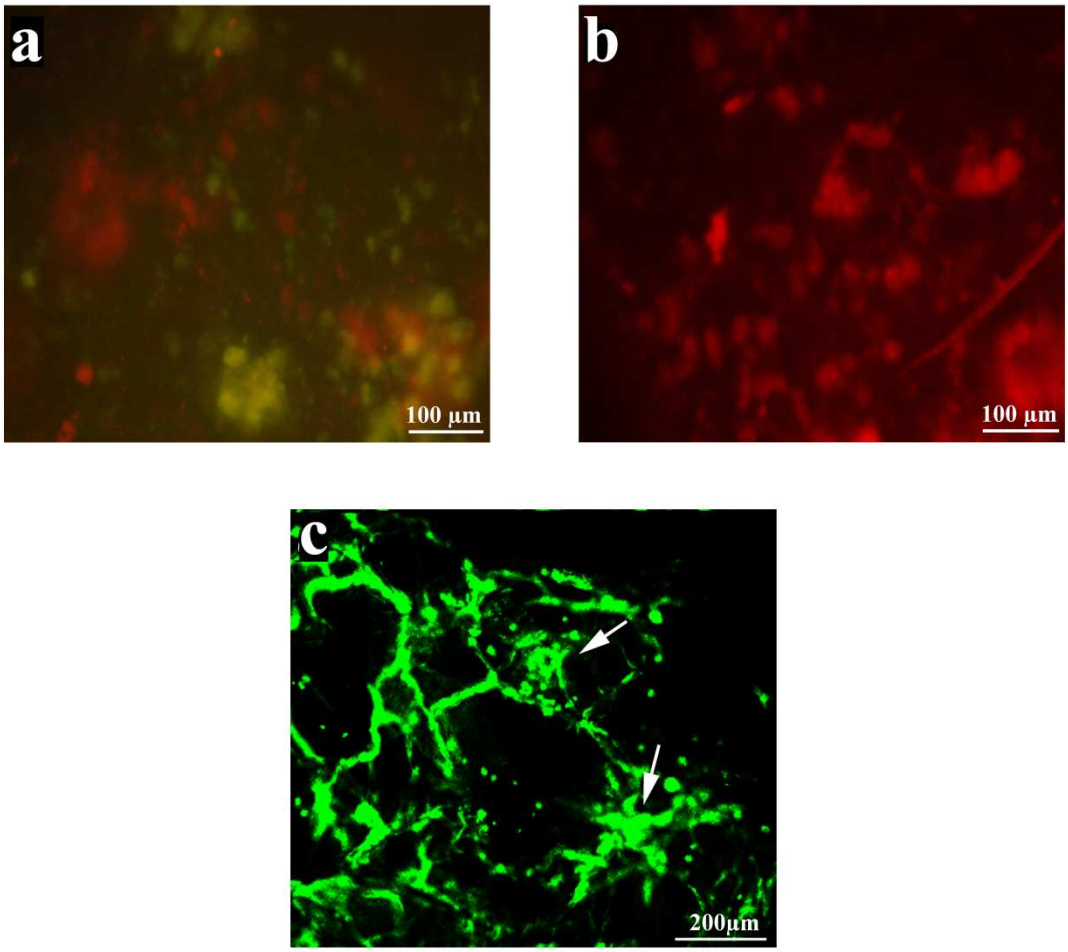

Figure 4.20 Viability of rabbit islets cultured on tissue culture treated dish and DEXGEL scaffold by calcein-ethidium bromide staining. a) Live and dead islets were observed on islets cultured on tissue culture treated dish by day 10. b) Dead islet cells were observed on tissue culture treated islets on day 30. c) DEXGEL cultured islets were found to be live on day 30. Intact islet clusters represented by arrows.

4.3.3 Gene expression

The results from Real time PCR analysis (Figure 4.21a) for insulin gene for islets cultured on scaffold displayed almost 4 fold significant increased expression when compared to that of control islets. The expression of integrin β1 and αv on

scaffold were greater in comparison to 2D cultures which are responsible for the enhanced viability of islets. In comparison, expression of integrin β1 was found to be significantly higher to that of integrin αv expression on rabbit islets cultured on 3D (Figure 4.21). Integrins β1 and αv are important receptors for binding to extracellular matrix molecules like collagen and fibronectin thereby mediating signals required for preventing the apoptosis of islet cells.

4.3.4 Protein expression

To confirm the presence of islet specific hormones in scaffold immunohistochemistry was done. Islets on scaffold expressed specific hormones mainly insulin, glucagon and somatostatin (Figure 4.22).

4.3.5 Islet cell function

Rabbit islets on 3D at 5 mM glucose concentration showed 1.2 fold increased insulin secretion than on 2D. However there was no statistically significant difference observed. At 25mM glucose concentration insulin secretion from rabbit islets on 3D was 2 times significantly greater than islets on 2D (Figure 4.23) indicating the importance of scaffold in enhancing its function.

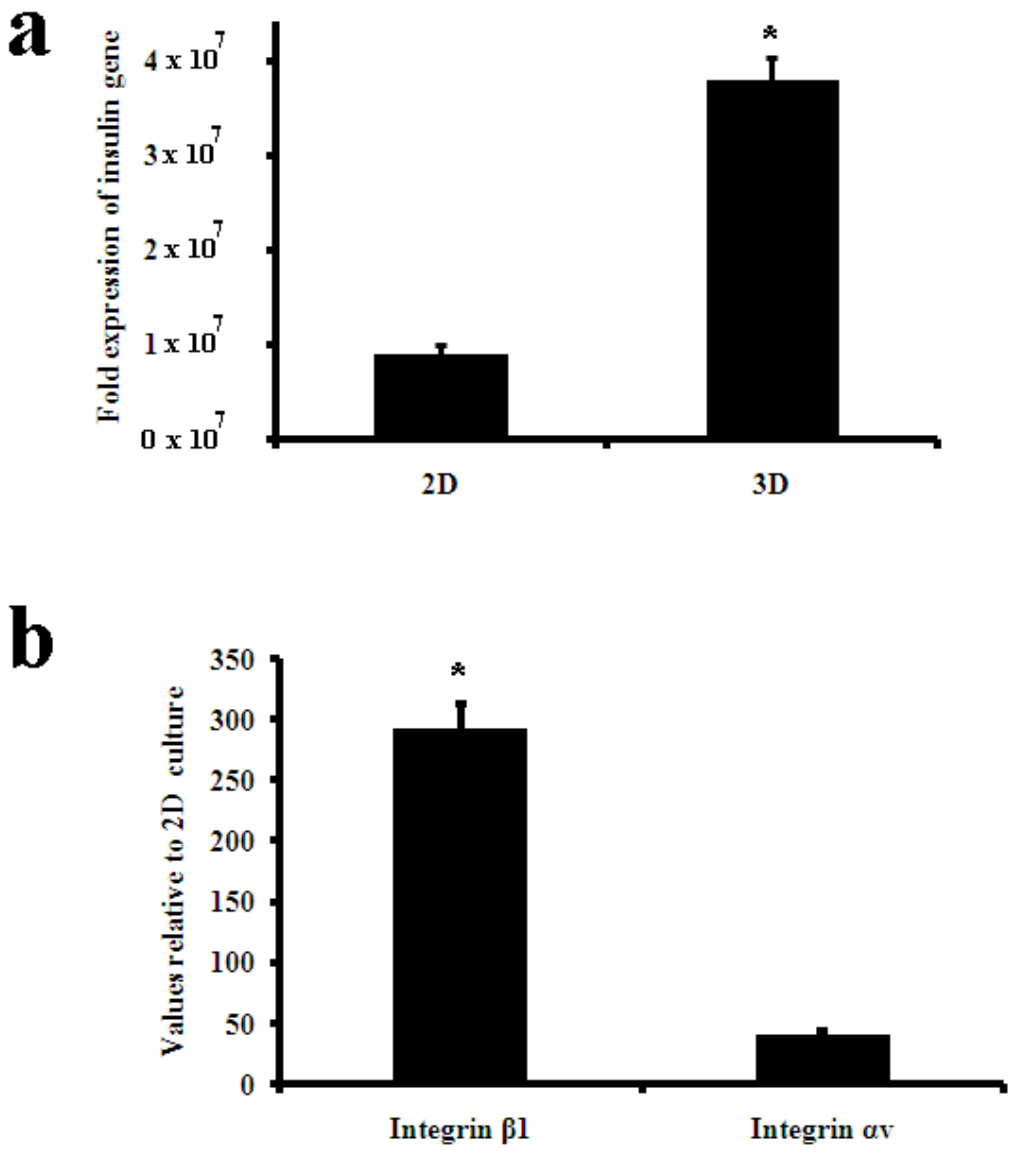

Figure 4.21 Expression of rabbit pancreatic beta islet specific genes studied using Quantitative Real-Time Polymerase Chain Reaction. a) Insulin gene expression was found to be significantly higher DEXGEL scaffold (3D) cultured islets in comparison to islets cultured on tissue culture dish (2D). b) Integrin expression was studied for beta islet specific markers integrin β1 and integrin αv on 3D cultures. Integrin β1 expression was found be significantly higher than integrin αv on 3D cultures. The values are expressed relative to islets on 2D cultures (* p value < 0.05).

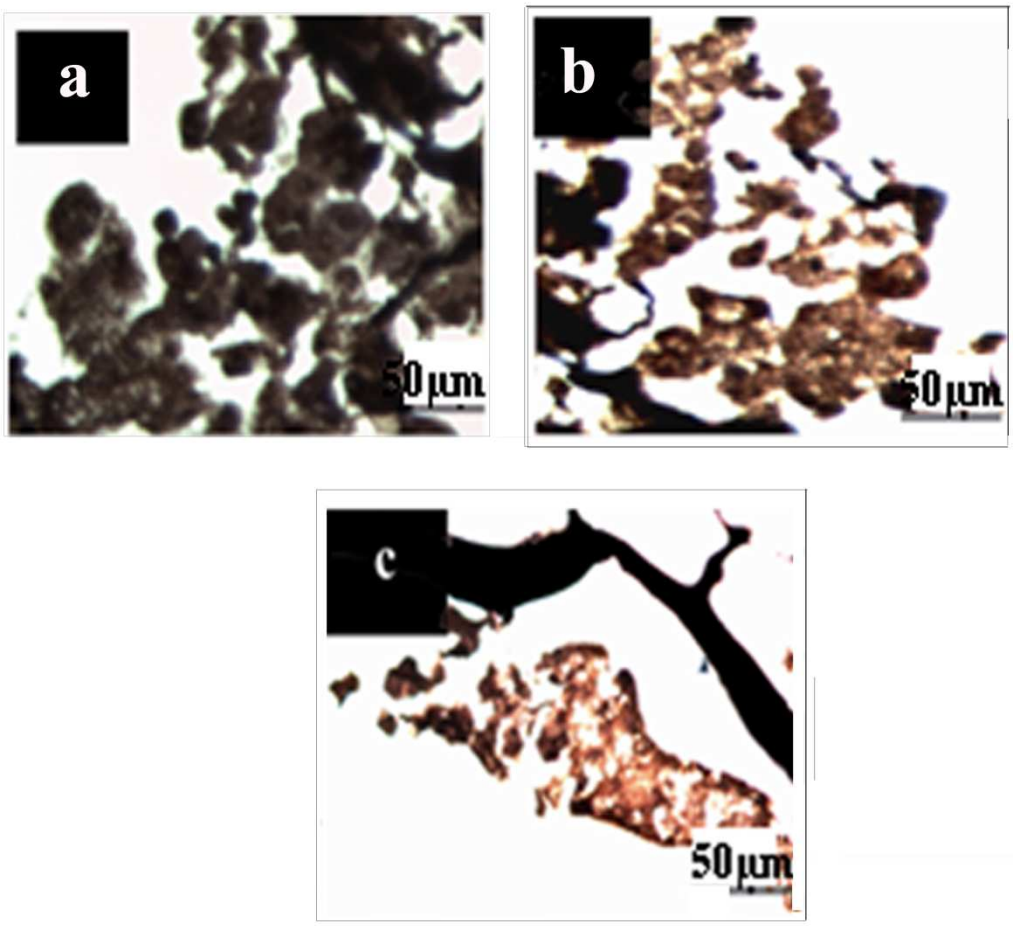

Figure 4.22 Immunohistochemical staining of rabbit islets on DEXGEL scaffold. Islets in scaffold were positive for a) Insulin b) Glucagon c) Somatostatin. Nucleus counterstained with Mayers' hematoxylin.

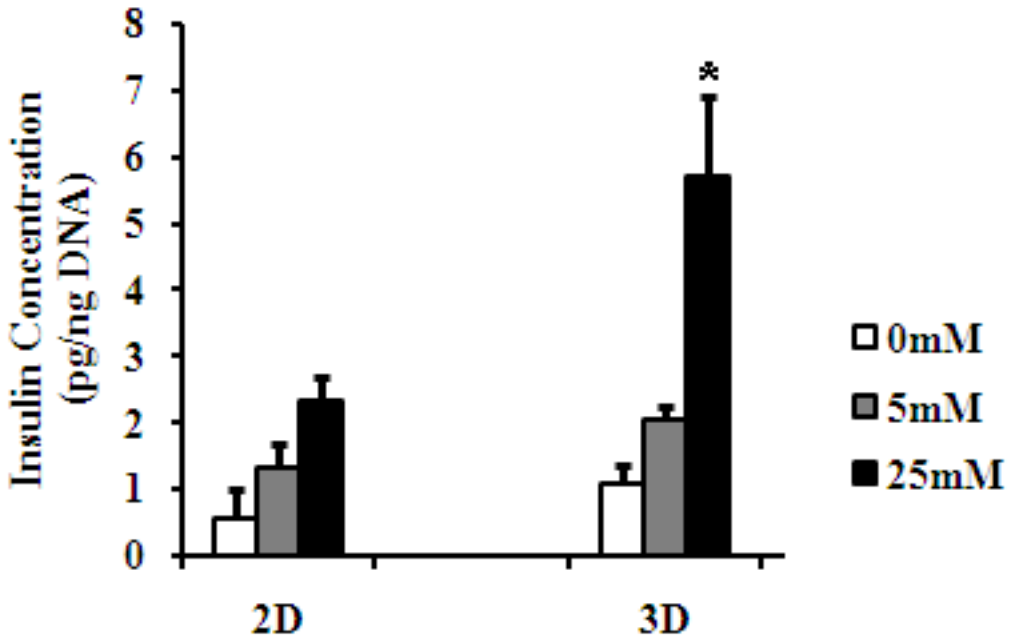

Figure 4.23 Islet cell functionality assessment by glucose challenge assay. Islets were incubated with different glucose concentrations (0mM, 5mM and 25mM) and the insulin secreted was quantified by Enzyme Linked Immuno Sorbent Assay (ELISA). Islets cultured on DEXGEL scaffold (3D) secreted significantly (*p value < 0.05) higher amount of insulin in response to high glucose concentration (25mM) in comparison to islets cultured on tissue .culture dish (2D).

4.3.6 Evidence of ECM formation

The scaffold-islet cultures were positive for collagen on day 30 which increased by day 60 (Figure 4.24). The results highlight the importance of scaffold in serving as a supporting framework for islets to synthesize its own ECM.

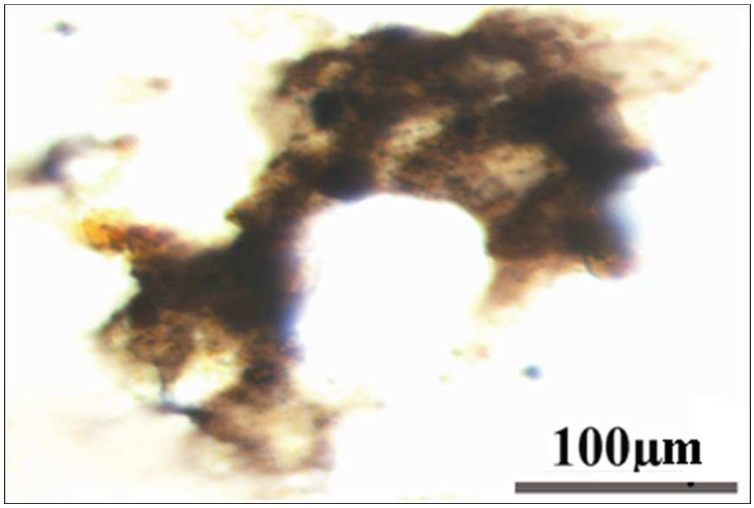

Figure 4.24 Immunohistochemical analysis of islets cultured on DEXGEL scaffold for collagen. Day 60 islet-scaffold constructs were positive for collagen IV expression which is a major extracellular component of native islets.

4.4 Differentiation of rabbit adipose stem cells to islet like cells on 2D and 3D culture

4.4.1 *In vitro* differentiation of rabbit adipose stem cells to islet like cells

In the tissue culture treated dish (Figure 4.25), cells exhibited a clustering morphology by day 10 and by day 15 cell clusters were distinguishable. By day 18, morphology of islet clusters were observed, but the cells were adhered onto the tissue culture dish. The yield of rILC on tissue culture treated dish was approximately 350-450 per 10^6 cells.

Rabbit adipose stem cells differentiated on ultra low attachment dish formed colonies via aggregation of cells by day 3 due to the presence of beta mercaptoethanol which have the property of stimulating colony formation. Beta mercaptoethanol also plays an important role in protection of cells from stress induced by serum free conditions (Figure 4.26). By day 10, the size of rILC increased in size and displayed the spherical morphology and by day 20, rILC resembled islet like morphology. The yield of rILC on ultra low attachment dish was approximately 800-1000 per 10^6 cells. Rabbit islet like cells on DEXGEL coated dish (Figure 4.27) exhibited similar pattern as that of ultralow attachment dish.

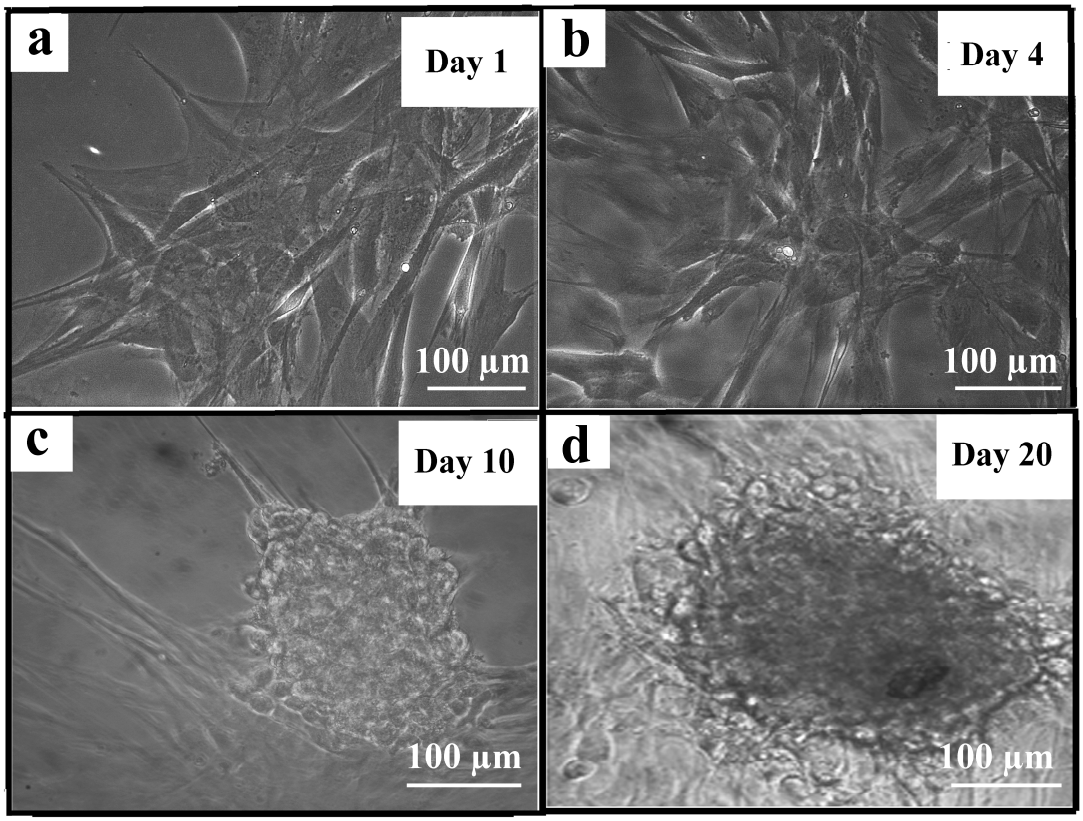

Figure 4.25 Change in morphology of stem cells during differentiation towards islet like cell on tissue culture treated dish a) Day 1 b) Day 4 c) Day 10 d) Day 20. The cells underwent clustering pattern and islet like cluster morphology was observed on day 20.

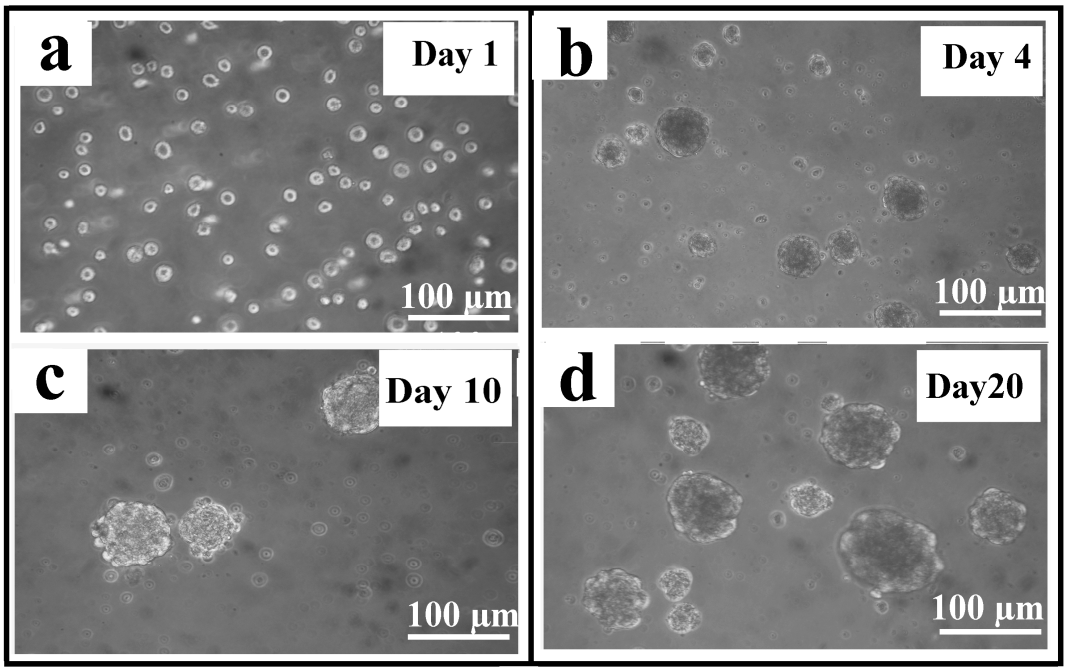

Figure 4.26 Change in morphology of stem cells during differentiation towards islet like cell on ultra low attachment dish a) Day 1 b) Day 4 c) Day 10 d) Day 20. The cells underwent clustering pattern by day 3 and the size of the cell clusters increased by day 20.

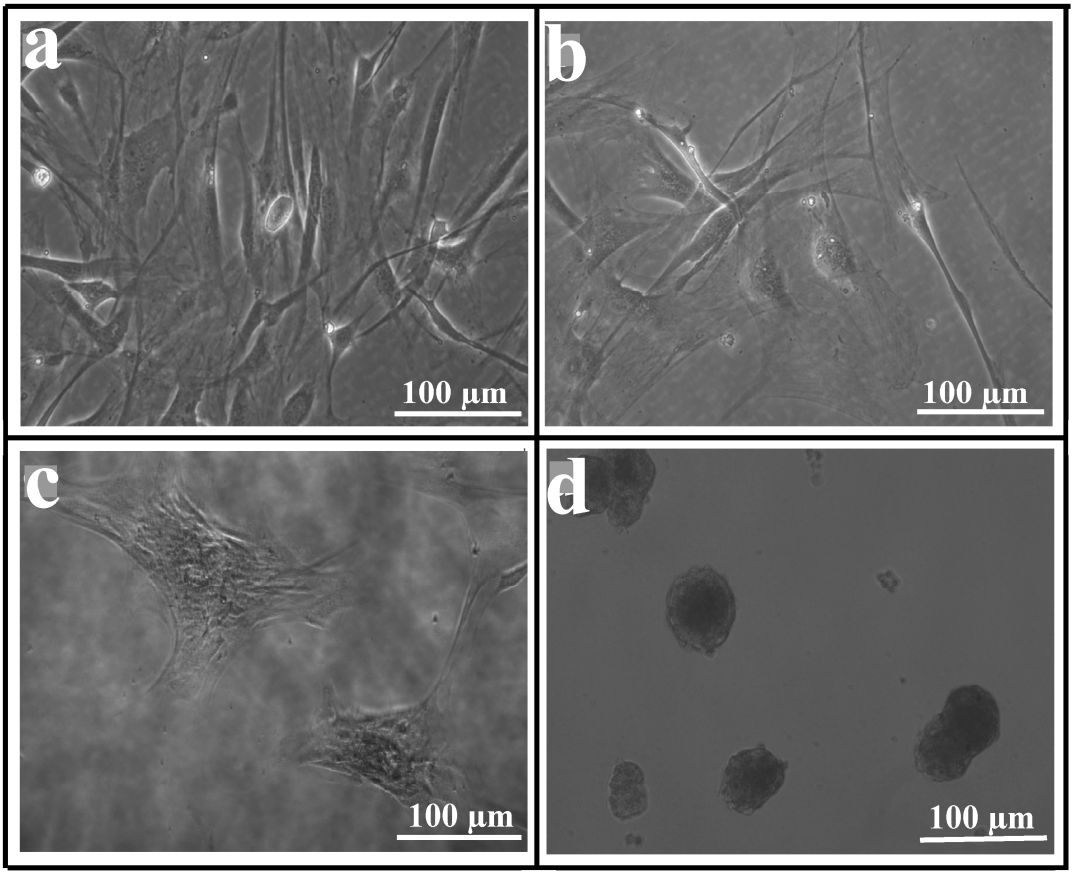

Figure 4.27 Morphology of rabbit adipose stem cells on differentiation towards islet like cells on DEXGEL coated dish. a) Day 1 b) Day 4 c) Day 10 d) Day 20. Typical islet like clusters was observed on day 20.

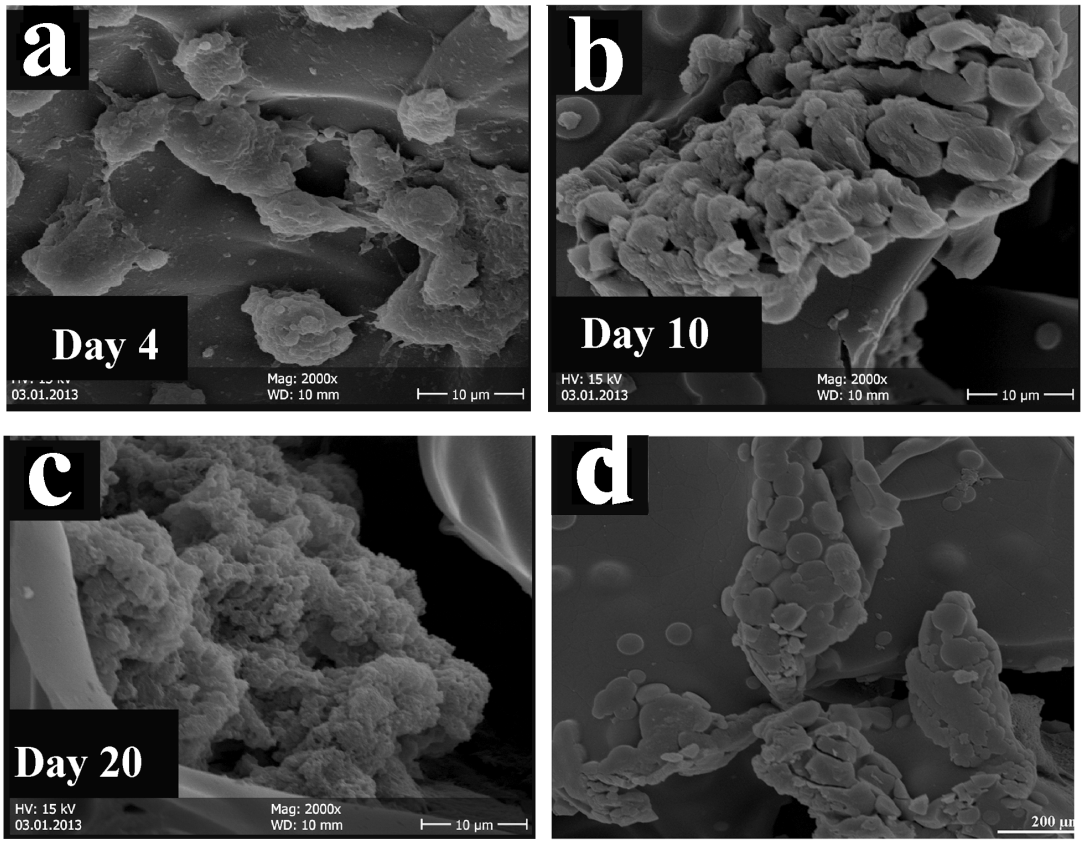

Figure 4.28 Morphology of islet like cell cluster differentiated from rabbit adipose stem cells on DEXGEL scaffold on a) Day 4 b) Day 10 c) Day 20 d) Rabbit islet clusters on DEXGEL scaffold.

Rabbit islet like cells on DEXGEL scaffold also exhibited spherical morphology as evident by SEM micrograph (Figure 4.28). By day 4 cell aggregation was observed and enhanced by day 10. On day 20 typical islet like cluster morphology was observed similar to that of rabbit islets.

4.4.2 Dithizone staining

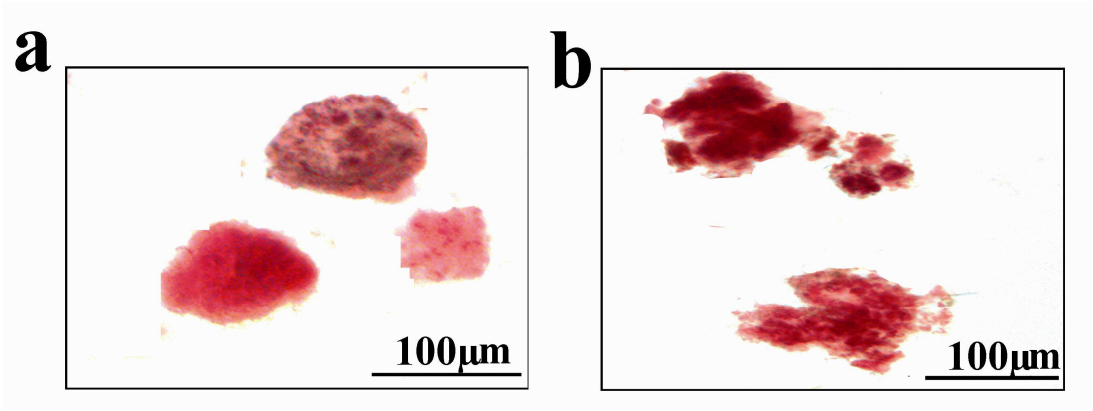

Figure 4.29 Dithizone staining of rabbit islet like cells and rabit islets. a) Islet like cells differentiated from rabbit adipose stem cells and b) rabbit islet cells stained crimson red due to the presence of zinc granules in beta islets.

The differentiated rILC and rabbit islets on 2D culture were positively stained (Figure 4.29) with dithizone indicating the presence of zinc granules in beta islets. The result supports the differentiation of stem cells towards pancreatic β islet like cells.

4.4.3 Viability assay

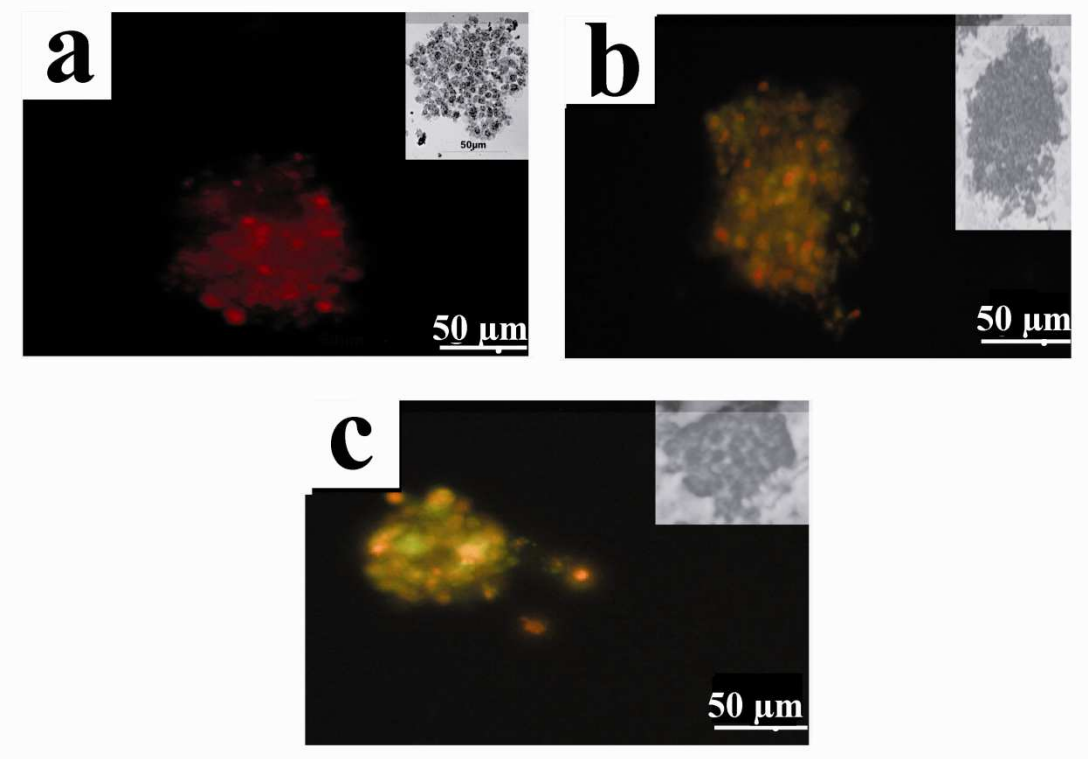

Figure 4.30 Viability of day 30 islet like cells differentiated from rabbit adipose stem cells on a) Tissue culture treated dish- most of the cells were found dead (stained red) b) Ultra low attachment dish- live cells (stained green) and dead cells were observed c) DEXGEL coated dish – live and dead cells were observed. Phase contrast images are shown in inserts.

For assessment of viability of rILC, calcein/ethidium homodimer double stains were used. Calcein (a non-fluorescent, cell permeable compound) cleaved by esterase in living cells yields green fluorescence whereas ethidium homodimer is cell-impermeable hence its red fluorescence is detected only in dead cells.

Figure 4.30 reveals the viability of rILC on 2D cultures and scaffold after 1 month *in vitro* culture. The rILC on tissue culture treated dish entered apoptotic state by day 10 and by day 30 most of the cells were stained red and were non viable. In

ultra low attachment dish and DEXGEL coated dish, cells in the periphery of rILC stained green which implies its live nature but cells in the core were stained red which indicated they were non viable by day 30. Moreover rILC cultured in 2D environment lost their morphology and membrane integrity.

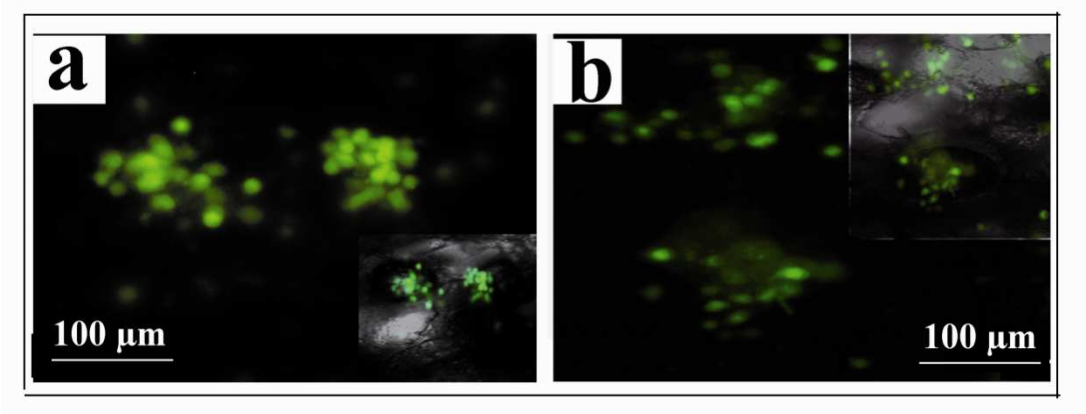

Figure 4.31 Viability of rabbit islet like cells differentiated on DEXGEL scaffold. Cells were found to be live on a) Day 30 and b) Day 60 cultures.

Rabbit islets like cells were viable without any dead cells in the core or periphery with its morphology uncompromised on DEXGEL scaffold (Figure 4.31) throughout the study period of 30 days and 60 days. Live dead ratio was quantified by image analysis software (Leica QWin). Day 30 rILC exhibited a live ratio of 94% ± 4 and in day 60 ILC, 89 % ± 6 were viable. Live and dead cells were counted from minimum 8 different microscopic sections for analyzing live dead ratio.

4.4.4 Reverse Transcription and PCR

Fold expression of insulin gene with respect to reference gene, β actin of rILC on DEXGEL coated dish and on scaffold are illustrated in Figure 4.32a. Insulin

gene expression of rILC on DEXGEL scaffold showed 12.6 fold significant increase when compared to rILC on DEXGEL coated surface. Integrin αv expression was found to be significantly higher in rILC on scaffold in comparison to integrin β1 Figure 4.32b. Here the values are expressed relative to islets cultured on 2D. In comparison to rabbit islet integrin expression results, in rILC integrin αv expression was significantly higher than integrin β1. The results signify that integrin αv are more prominent in contributing to the anti apoptotic signal mediated by binding to extracellular matrix components.

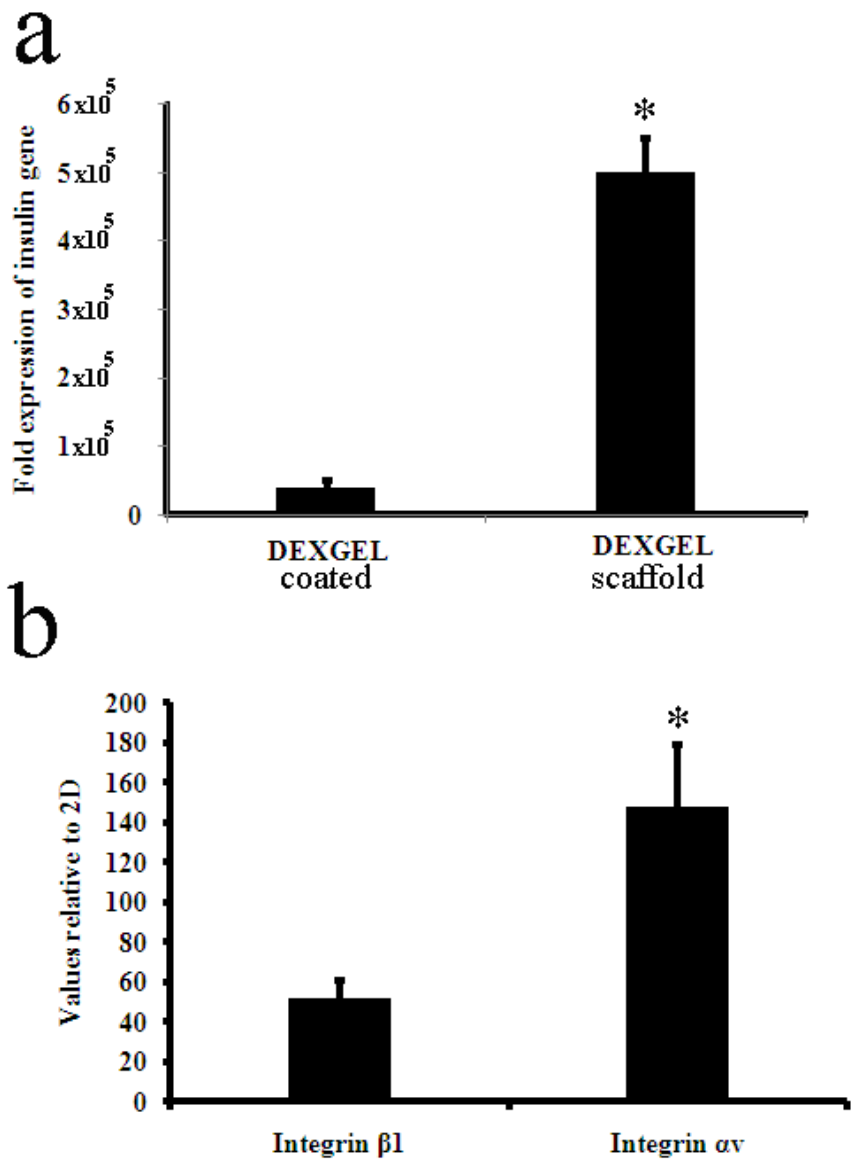

Figure 4.32 Expression of pancreatic beta islet specific genes on DEXGEL coated dish and DEXGEL scaffold studied using Quantitative Real Time Polymerase Chain Reaction. a) Insulin gene expressed by rabbit stem cell differentiated islet like cells (ILC) was significantly higher on DEXGEL scaffold in comparison to DEXGEL coated dish cultured ILC. b) Expression of integrin αv by ILC on DEXGEL scaffold was found to be significantly higher than integrin β1. The values are expressed relative to ILC cultured on DEXGEL coated dish. (* p value < 0.05).

4.4.5 Immunophenotype

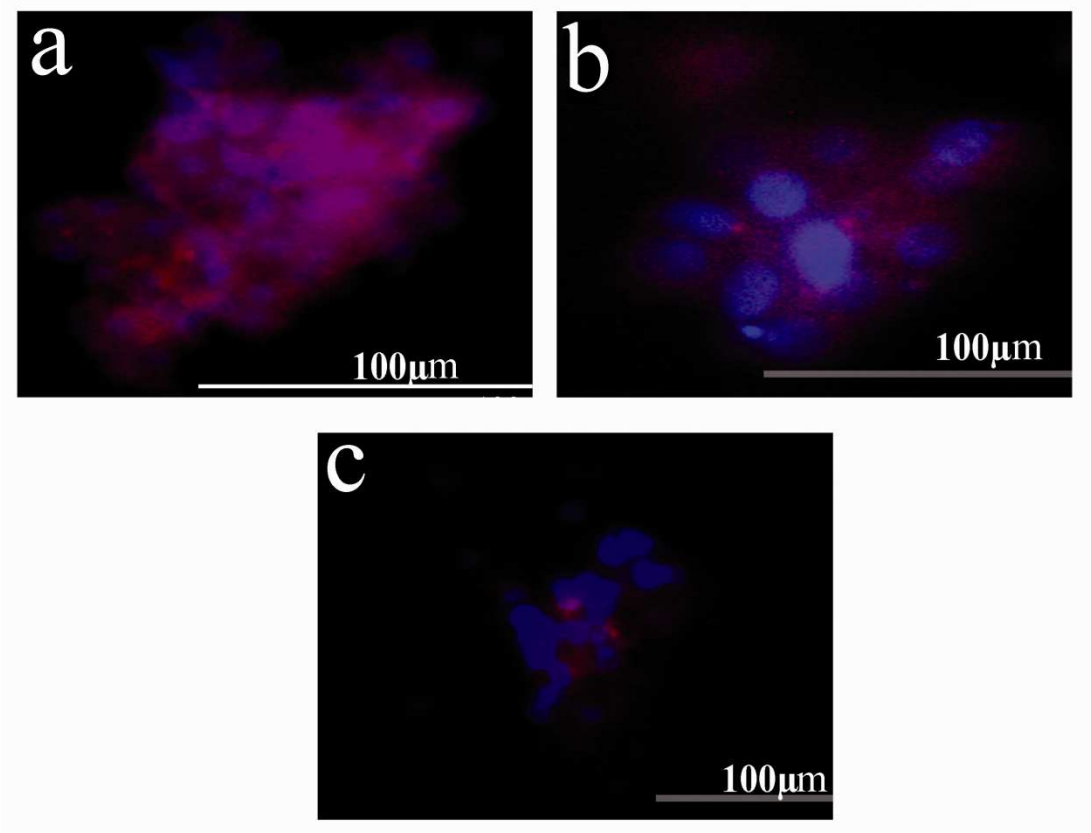

Figure 4.33 Immunophenotype characterization of rabbit islet like cells (rILC) cultured on DEXGEL coated dish. The cells expressed pancreatic endocrine specific markers a) Insulin b) Glucagon c) Somatostatin.

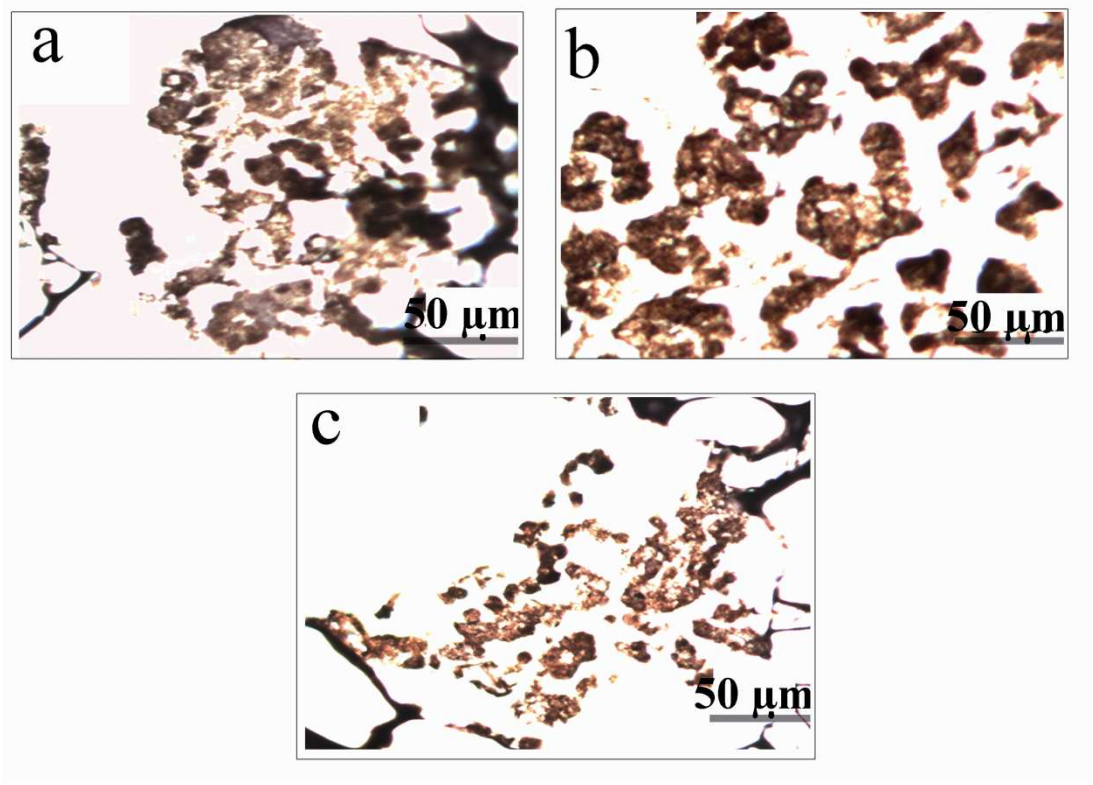

Figure 4.34 Immunohistochemical staining of rabbit stem cell differentiated islet like cells (rILC) on DEXGEL scaffold. The rILC expressed pancreatic islet specific markers a) Insulin b) Glucagon and c) Somatostatin.

Immunocytochemistry (Figure 4.33) of rILC and immunohistochemical analysis of paraffin embedded sections of rILC on DEXGEL constructs (Figure 4.34) confirmed the presence of insulin glucagon and somatostatin. The result suggests that the three stage protocol also directed the differentiation of few stem cells to functional alpha and delta cells which are found in the native islet cluster imparting paracrine effects on maintaining islet function.

4.4.6 Insulin quantification

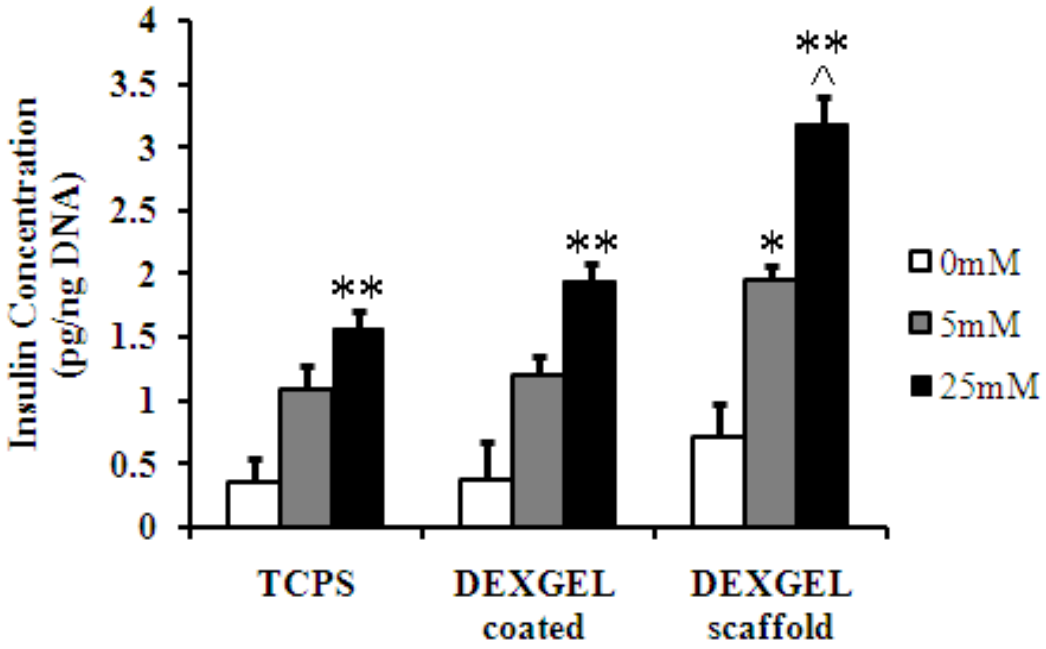

Figure 4.35 Glucose challenge based insulin secretion assay. Rabbit islet like cells cultured on tissue culture polystyrene (TCPS) dish, DEXGEL coated dish and DEXGEL scaffold were challenged with various glucose concentrations (0mM, 5mM and 25mM). The amount of insulin secreted was quantified by Enzyme Linked Immunosorbent Assay (ELISA). Rabbit islet like cells from the three groups secreted significantly higher amount of insulin at high glucose concentration (** p value <0.05). Rabbit islet like cells on DEXGEL scaffold secreted significantly greater amount of insulin at 5mM and 25mM glucose concentrations in comparison to the other two groups (*and ^ p value <0.05).

The insulin secreted by rILC upon glucose challenge was quantified by ELISA. Figure 4.35 illustrates the response of rILC to various glucose concentrations on 2D cultures and on DEXGEL scaffold. The rILC on 2D and 3D cultures exhibited similar insulin release pattern at 0mM glucose concentration. rILC on DEXGEL scaffold showed significantly higher insulin release when challenged with 5mM (*p value <0.05) and 25mM (^p value <0.05) glucose concentrations, in comparison to

rILC on DEXGEL coated dish and TCPS. At a concentration of 5 mM glucose, rILC on DEXGEL scaffold secreted insulin which was 1.6 and 1.8 times higher compared to rILC on DEXGEL coated dish and TCPS respectively. Further at 25mM glucose concentration, there were 1.64 and 2.03 times significant increase of insulin on DEXGEL scaffold group than on DEXGEL coated dish and TCPS respectively. Within the TCPS, DEXGEL coated dish and DEXGEL scaffold groups there were significant increase in insulin secretion in response to high glucose concentration(25mM) when compared to that of low glucose concentrations (0mM and 5mM) (**p value <0.05). When the functionality of rILC is compared with rabbit islets, the quantity of insulin secreted at 5mM and 25mM glucose concentrations were found to be higher on rabbit islets (Figure 4.23).

4.4.7 Analysis of ECM formation

ECM formation in rILC-DEXGEL constructs was evident by faint collagen expression on day 30 and increased expression was observed on day 60 construct (Figure 4.36). Collagen is an important extracellular matrix component of pancreatic islet cells whose role is to serve as an intra islet capsule as well as serve as a basement membrane for enhancing islet survival and function

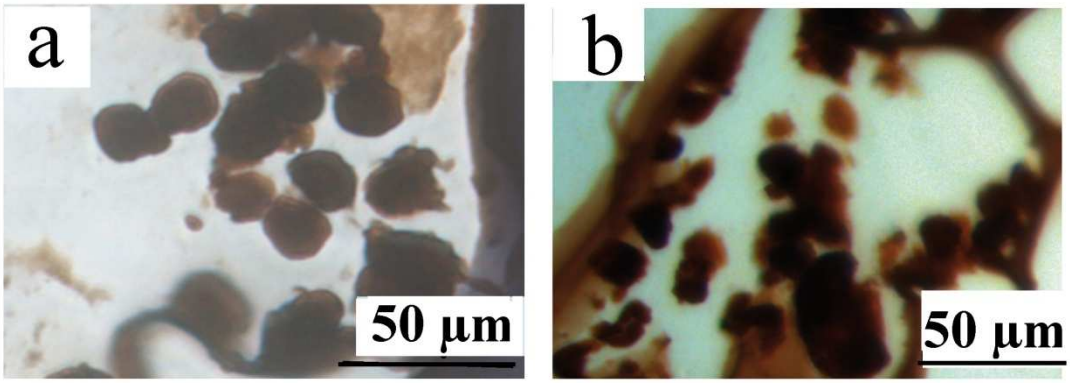

Figure 4.36 Immunohistochemical staining of rabbit islet like cell-DEXGEL scaffold constructs. The cell seeded scaffold constructs showed presence of collagen on a) Day 30 and b) Day 60 cultures. Collagen is a major extracellular matrix component of islet cells required for its proper function and survival**.**

4.5 Differentiation of human adipose stem cells to islet like cells

4.5.1 Differentiation of stem cells to islet like cells *in vitro* on DEXGEL coated dish and DEXGEL scaffold

Human adipose stem cells were differentiated on culture plate coated with DEXGEL and on DEXGEL scaffold. Phase contrast image (Figure 4.37) reveals the changes in morphology occurring during differentiation of stem cells to hILC on 2D culture at different time points. Cell aggregation was observed within 24 hours under the influence of SFM A. The size of cell clusters ranged from 80-150µm which resembled pancreatic islet cells. The 'SFM A' mediated the differentiation of stem cells to endodermal lineage under the influence of activin A. The 'SFM B' promoted the differentiation towards pancreatic endodermal lineage. The 'SFM C' contains nicotinamide which results in maturation of islets. Betacellulin and activin are potent stimulators in mediating differentiation towards pancreatic endocrine cells as well as enhance β cell's insulin secretion function.

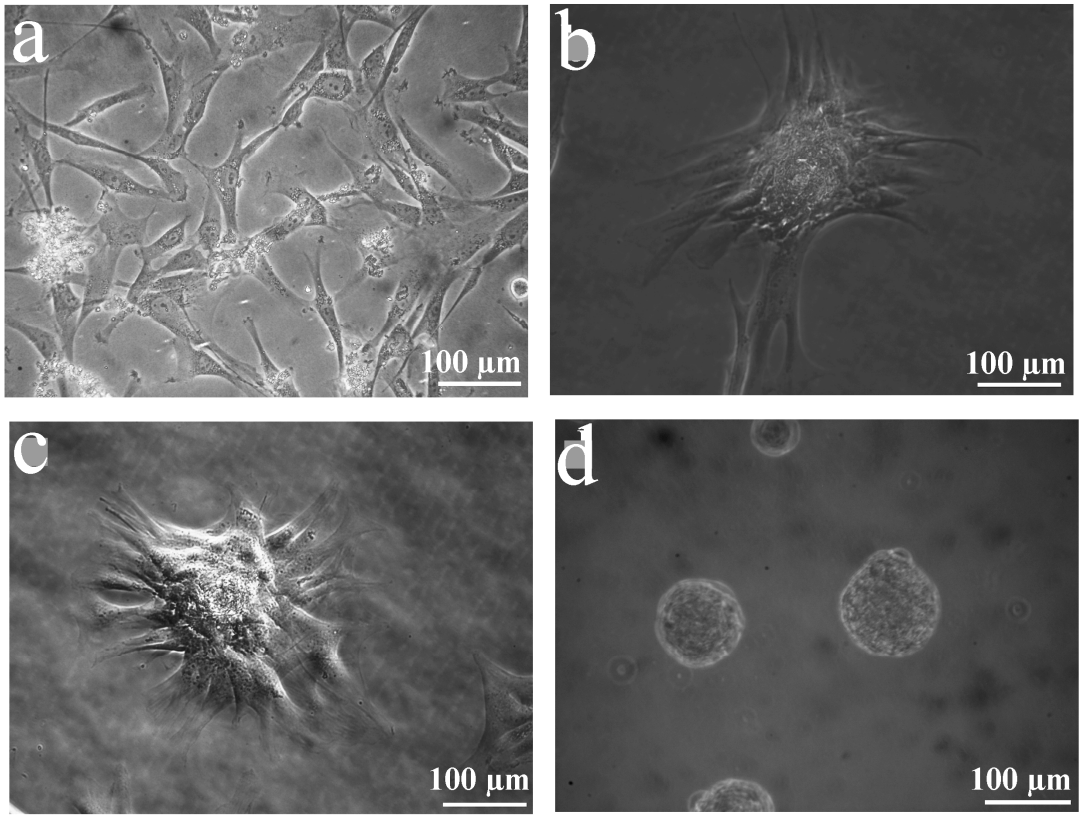

Figure 4.37 Morphology of islet like cells differentiated from human adipose stem cells on DEXGEL coated dish. a) Day 1, cells attached onto the culture dish b) Day 4- clustering pattern observed c) Day 10 d) Day 20- typical islet like morphology observed.

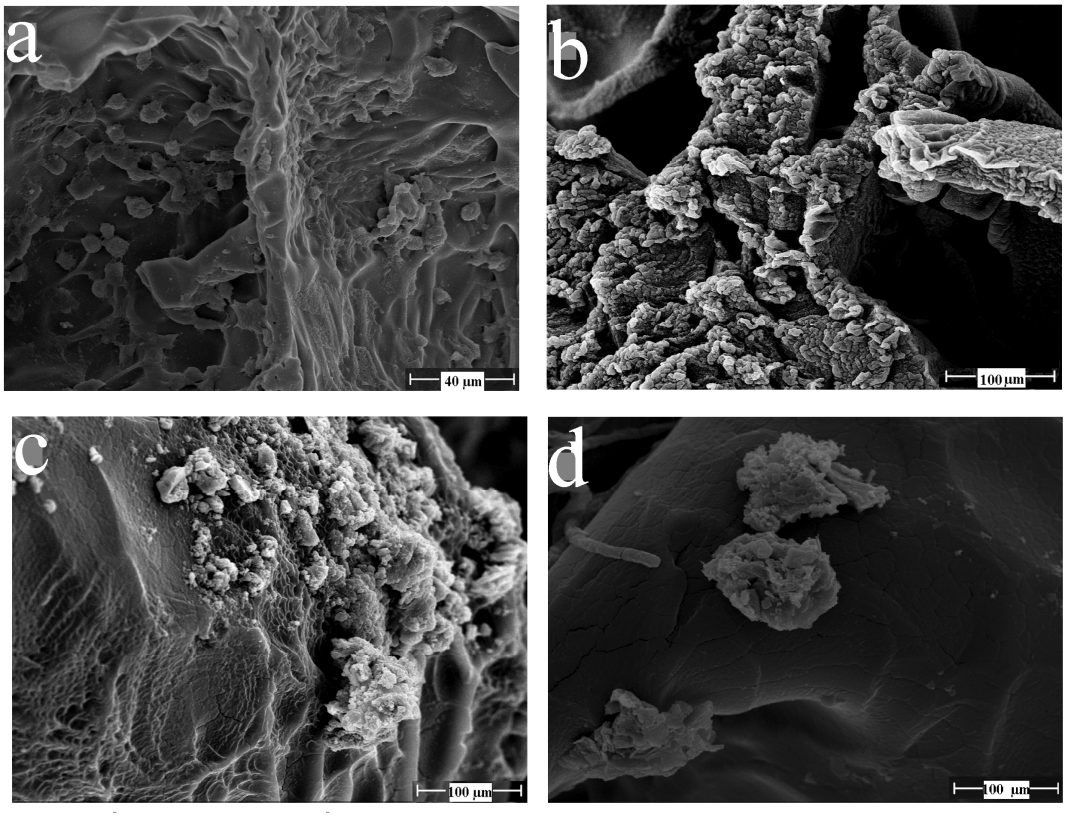

Figure 4.38 Differentiation of human adipose derived stem cells to islet like cells on DEXGEL scaffold. a) Day 1- cells attached on the scaffold b) Day 4- clustering phenotype observed. c) Day 10 – cluster size increased d) Day 20- islet like cells observed attached onto scaffold.

Scanning electron microscopy (Figure 4.38) was utilized for analyzing the morphology of hILC on scaffold during the differentiation period. More number of clusters was observed on day 10 hILC-scaffold in comparison to 2D cultures. The size of cell clusters was increased on day 20. The cell clusters were found to adhere onto the scaffold and maintained the typical islet like morphology.

4.5.2 Gene expression analysis

The upregulation of pancreatic endoderm specific genes like Pdx1 and Ngn3 on scaffold cultures were observed in comparison to 2D culture (Figure 4.39a). The

results show the importance of scaffold matrix in the significant expression of early pancreatic endocrine markers for differentiation of stem cells towards pancreatic lineage. Gene expression analysis of day 20 hILC on scaffold confirmed the significant higher expression of islet specific genes such as insulin, glucagon, somatostatin and ghlerin on scaffold cultures in comparison to 2D culture (Figure 4.39b). However there was no significant difference in pancreatic polypeptide gene expression on 2D and 3D cultures. The hILC comprised of gamma and epsilon cells in addition to alpha, beta and delta cells. This could favor the proper functioning of hILC mediated by cell-cell signaling and paracrine effects. Insulin gene expression was found to be 10 fold greater on scaffold than on 2D. The result shows that more functional β islets were differentiated on 3D constructs. The more expression of insulin highlights the functionality of hILC in glucose response.

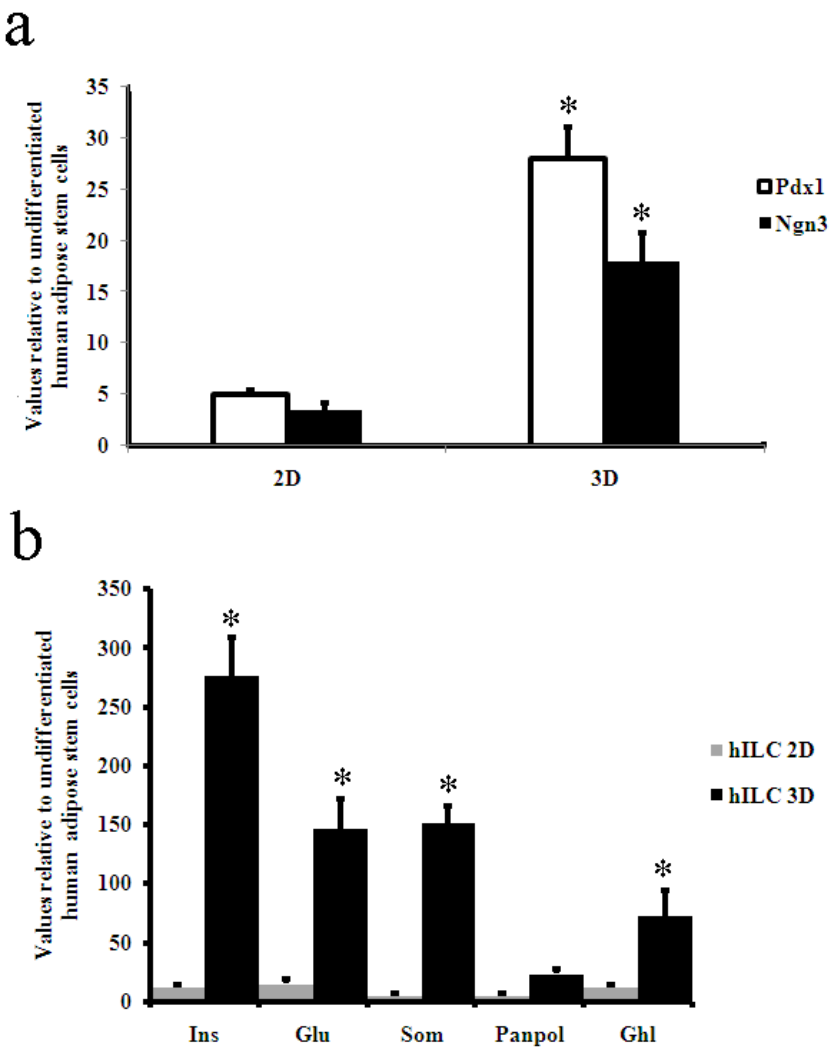

Figure 4.39 Quantitative analysis of pancreatic islet specific genes on human adipose stem cell differentiated islet like cells (hILC) on DEXGEL coated dish (2D) and DEXGEL scaffold (3D) using Real Time Polymerase Chain Reaction. a) Expression of early pancreatic endodermal markers (Pdx1 and Ngn3) was found to be significantly higher on scaffold groups in comparison to 2D culture. b) Expression of pancreatic islet specific markers insulin (Ins), glucagon (Glu), somatostatin (Som) and ghlerin (Ghl) was found to be significantly higher on scaffold group hILC in comparison to 2D culture. There was no significant difference in the expression of pancreatic polypeptide (Panpol) on 2D and 3D cultures.

4.5.3 Immunophenotype

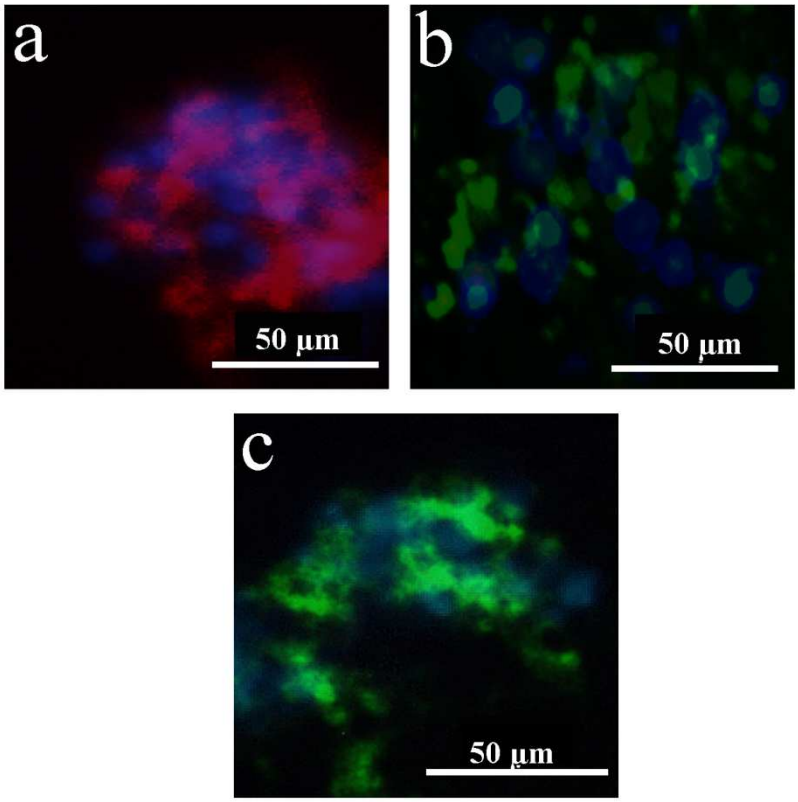

Figure 4.40 Immunophenotype characterization of human adipose stem cell differentiated islet like cells (hILC). The hILC expressed pancreatic islet specific markers a) Insulin b) Glucagon c) Somatostatin.

Immunocytochemical (Figure 4.40) and immunohistochemical analysis (Figure 4.41) confirmed the protein level expression of insulin, glucagon and somatostatin in hILC on 2D and 3D culture. The results show that the differentiated hILC comprises of functional alpha, beta and gamma cells capable of producing glucagon, insulin and somatostatin respectively.

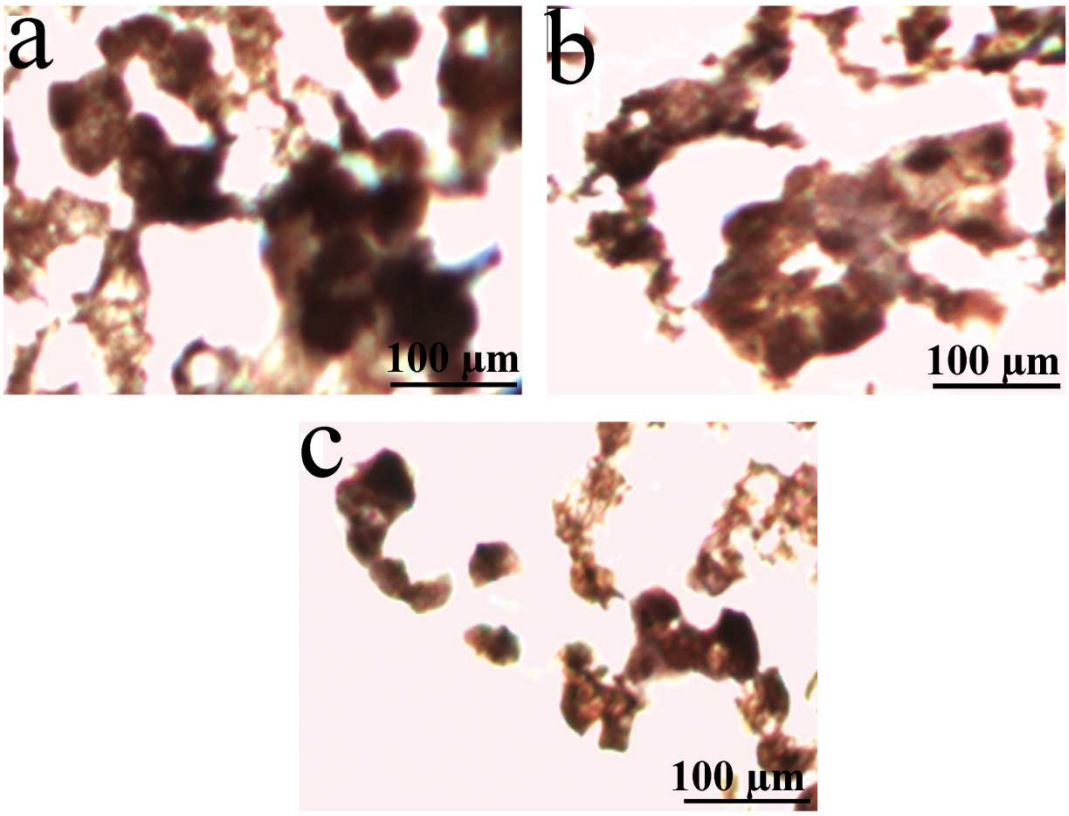

Figure 4.41 Immunohistochemical staining of human adipose stem cell differentiated islet like cells (ILC) on DEXGEL scaffold. The ILC expressed pancreatic islet specific proteins a) Insulin b) Glucagon c) Somatostatin.

4.5.4 Viability assay

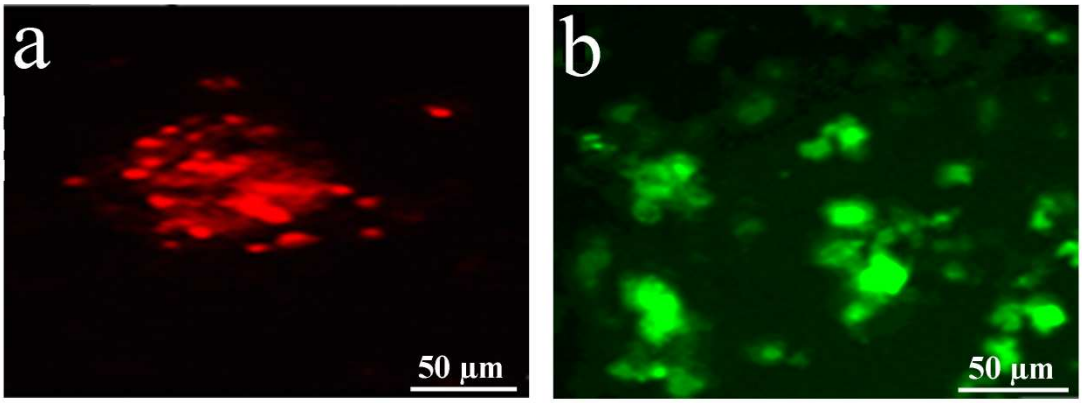

Figure 4.42 Viability of human adipose stem cell differentiated islet like cells (hILC) on DEXGEL coated dish (2D) and DEXGEL scaffold (3D) assessed by calcein-ethidium bromide staining. a) Dead cells were observed on day 30 hILC cultured on 2D whereas b) The hILC on 3D were found to be live.

The viability of hILC was better on scaffold constructs when compared to 2D cultures. The hILC on scaffold were found to be viable on day 30 (Figure 4.42) whereas dead cells were observed on 2D culture. The results show the importance of scaffold in providing a favorable matrix for cell attachment and the signals mediated by them favor the cell survival. Islet cluster morphology was also maintained on scaffold culture which implies the role of scaffold in mimicking a 3D environment for the spatial orientation of hILC.

4.5.5 Glucose challenge study

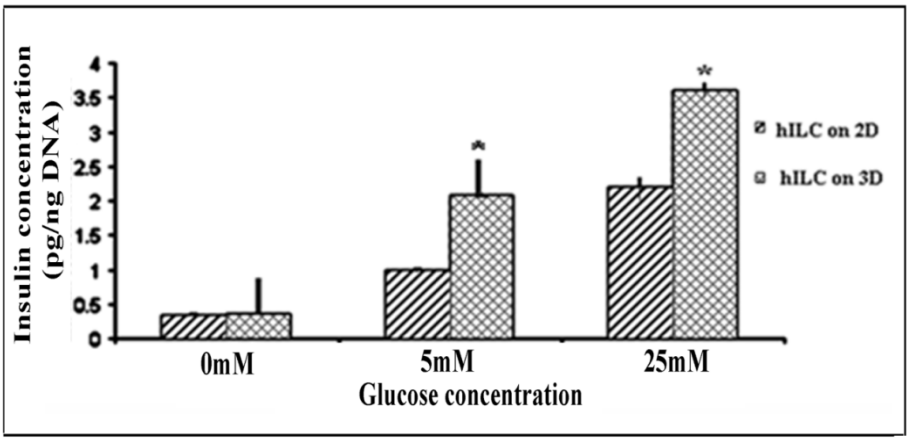

Figure 4.43 The amount of insulin secreted by human adipose stem cell differentiated islet like cells (hILC) on DEXGEL coated dish (2D) and DEXGEL scaffold (3D) in response to various glucose concentrations (0, 5 and 25mM) quantified by Enzyme Linked Immunosorbent Assay (ELISA). At 5 and 25 mM glucose hILC on 3D culture secreted significantly higher amount of insulin when compared to 2D culture.

The hILC and hILC- scaffold constructs responded differently when challenged with various glucose concentrations. (Figure 4.43) The values are expressed relative to DNA concentration. At 5 and 25mM glucose, hILC on scaffold secreted 2.2 and 3.4 pg insulin/ng DNA respectively At 5mM and 25mM glucose concentrations insulin secretion of hILC on scaffold were significantly higher in comparison to 2D cultures. Both hILC on 2D and 3D responded in a similar pattern in the control (0mM) with no significant difference in insulin secretion.

4.6 Co-culture of human islet like cells and endothelial cells on DEXGEL coated dish and DEXGEL scaffold

4.6.1 Co-culture study

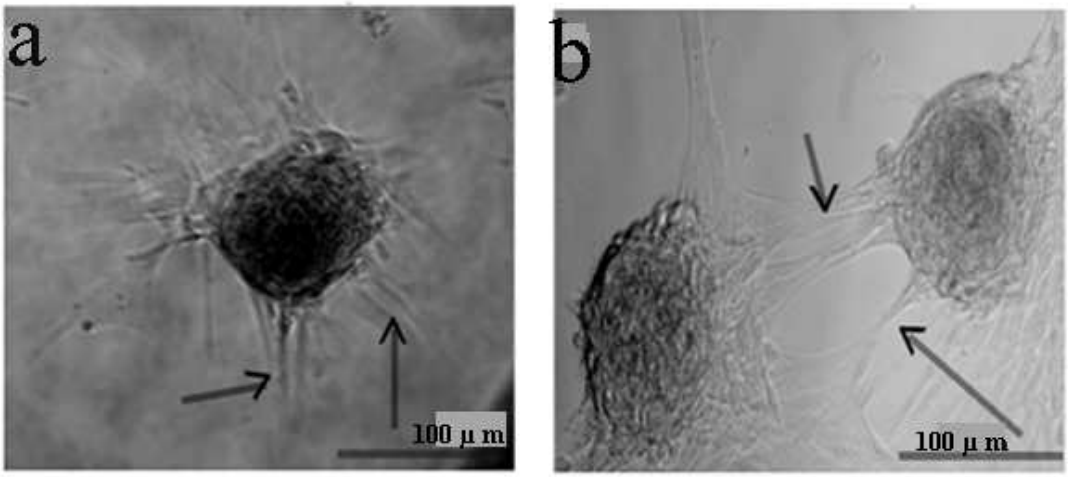

Figure 4.44 Co-culture of human adipose stem cell differentiated islet like cells (hILC) and human umbilical vein endothelial cells (EC) on DEXGEL coated dish (2D). a) hILC and EC, day 15, 2D culture small sprouts were observed. b) On day 20, the numbers of sprouts were increased indicated by arrows.

Human islet like cells were cocultured with EC on DEXGEL coated dish and DEXGEL scaffold. Sprouting was observed on hILC-EC constructs by day 15 and was found to increase by day 20, (Figure 4.44) in contrast to hILC cultured alone (Figure 4.37).

4.6.2 Immunophenotype

In the co-culture 3D constructs hILC were positive for insulin and EC positive for vWF. Nuclei were stained blue with DAPI (Figure 4.45 a). The hILC-EC scaffold construct showed significant collagen type IV expression (Figure 4.45 b), since endothelial cells are responsible for vascular basement membrane

formation in islets comprising of mainly collagen type IV and laminin. Integrin β1 are receptors for collagen in basement membrane. The ILC-EC on scaffold showed significant integrins β1 expression (Figure 4.45 c) which could bind to the basement membrane proteins thereby mediating bidirectional signaling.

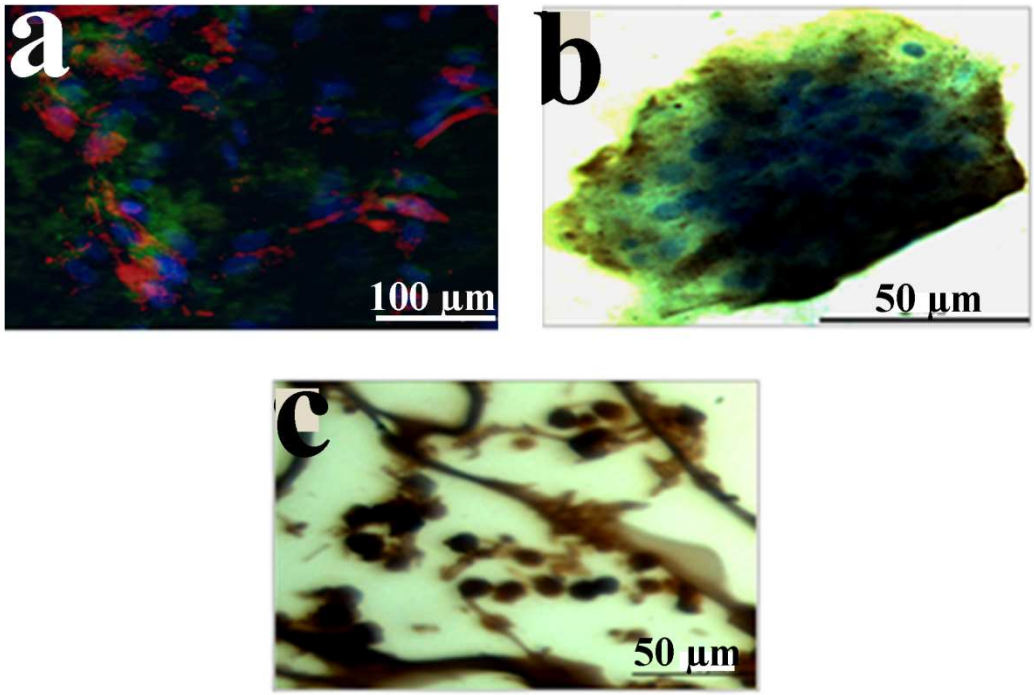

Figure 4.45 Imunophenotype characterization of cocultured islet like cells (hILC) and endothelial cells (EC) on DEXGEL scaffold (3D). a) Double immunofluorescence showing the presence of insulin (green) in hILC and von willebrand factor (vWf) (red) in EC. Nucleus stained blue by DAPI. Immunoistochemical staining showing expression of b) Collagen IV and c) Integrin β1.

4.6.3 Viability assay

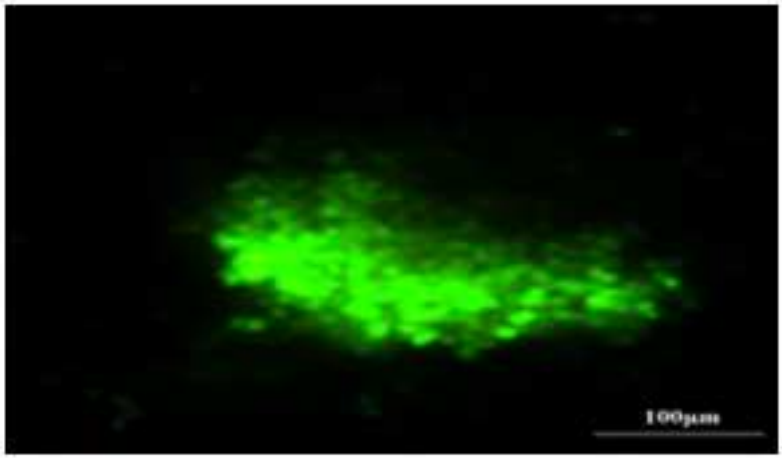

Figure 4.46 Viability of co-cultured islet like cells and endothelial cells on DEXGEL scaffold on day 30. The cells were found to be viable.

The viability of hILC was better on scaffold constructs when compared to 2D cultures. The hILC-EC culture alone on scaffold was viable on day 30 (Figure 4.46). The result reflects that the scaffold architecture and pore size are important factors in determining islet phenotype and survival.

4.6.4 Gene expression analysis

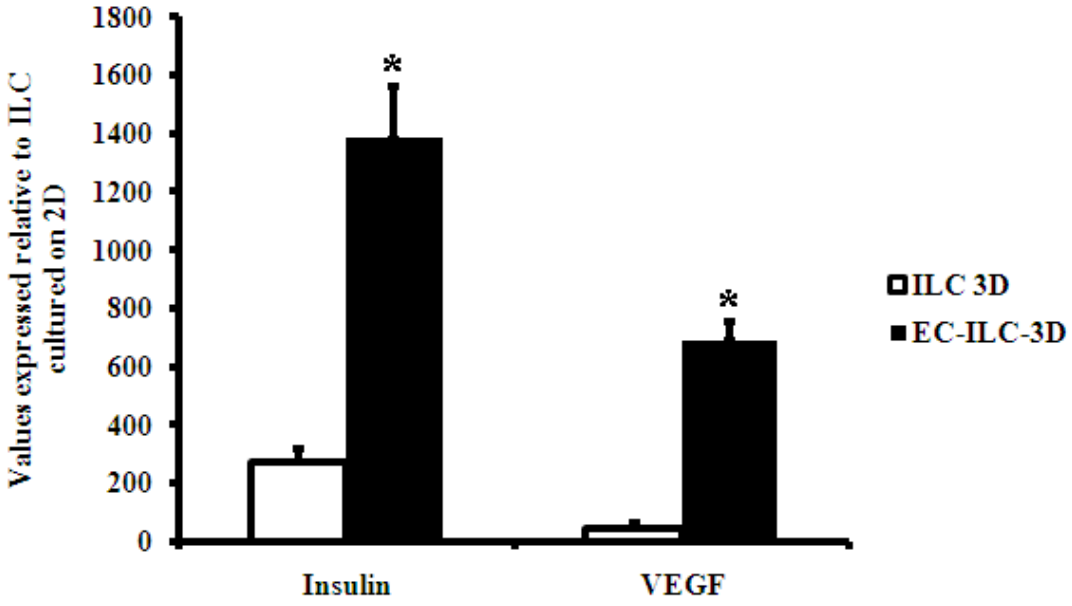

Figure 4.47 Quantitative analysis of insulin and VEGF A genes on co-cultured islet like cells (hILC) and endothelial cells cultured on DEXGEL scaffold (3D) in comparison to hILC cultured alone on 3D studied by Real Time Polymerase Chain Reaction. The values are expressed relative to hILC cultured on DEXGEL coated dish (2D). The expression of insulin and VEGF A was found to be significantly higher on 3D coculture constructs when compared to ILC 3D constructs. (*p value < 0.05).

The insulin gene expression pattern was analyzed in hILC-EC-scaffold constructs when compared to hILC-scaffold culture. There was a significant fold increase in the expression of insulin gene in co-culture-scaffold constructs. Insulin gene expression was found to increase by 9 fold in hILC-EC scaffold constructs in comparison to ILC-scaffold culture (Figure 4.47). The values were expressed relative to 2D cultures. Early islet marker expression was also significantly increased in co-culture-scaffold construct. The VEGF A gene expression was also significantly greater on co-culture constructs on scaffold. Significant changes in islet

gene expression mainly insulin and VEGF A was observed when EC were added onto day 10 hILC culture.

4.6.5 Glucose challenge study

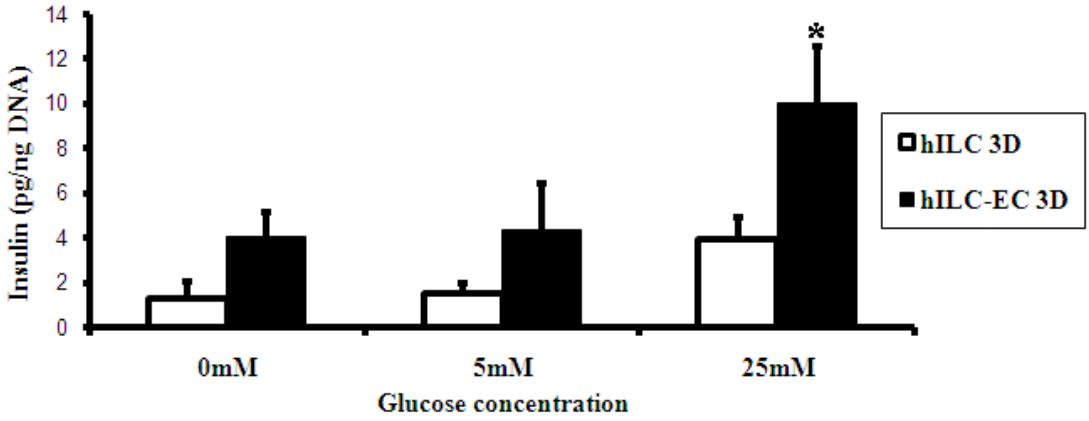

Figure 4.48 Glucose challenged insulin secretion by endothelial cell cocultured human islet like cells (hILC) on DEXGEL scaffold (3D) quantified by Enzyme Linked Immunosorbent Assay (ELISA). The insulin secreted was found to be significantly higher on cocultured 3D constructs in comparison to hILC cultured alone. The values are expressed relative to DNA concentration. (* p value <0.05).

The hILC and hILC-EC scaffold constructs responded differently when challenged with various glucose concentrations. (Figure 4.48) The values are expressed as fold increase relative to values on 2D culture. The hILC-EC scaffold construct secreted significantly 10 times fold higher insulin when compared to hILC cultured alone at 25mM glucose concentration.

CHAPTER 5

DISCUSSION

Clinical islet transplantation is a potential therapeutic option for treatment of diabetes mellitus. Normal glucose homeostasis could be achieved with the transplantation of islets of Langerhans which involves the isolation of pancreatic islets from cadaveric donors, followed by their respective infusion into the patient's portal vein. Previous studies have shown that transplanted islets cultured *in vitro* can decrease the immunogenicity of grafts and immunological rejection by recipients (Rutzky *et al*., 2002). Islet transplantation therapy is hampered by decreased function and low survival rate of islets as well as limited donor organs. The isolation step that removes islets from their native environment not only disrupts interactions between blood vessels and endocrine cells, but also dramatically changes islet cell interaction with the ECM, which leads to decreased cell survival. Extracellular matrix is a mixture of a variety of proteins which serves as a substrate for cell attachment and plays a role in the growth and maturation of cells. Native islets on *in vitro* culture have been reported to undergo apoptosis by 7th day (Chun *et al*., 2008). Thus there is a need to find a suitable substrate that provides favorable biological support to preserve islets for long term culture.

Scaffolds composed of natural and synthetic polymeric biomaterials have been explored for culturing islets. The scaffolds could mimic a 3D niche or growth environment *in vitro* for prolonging the survival of islets. Development of a 3D

microenvironment with adequate characteristics is essential to maintain islet viability and function (Beattie *et al*., 2002).

The requirement of large number of islets for transplantation is limited by donor scarcity. One approach to overcome the present situation is to generate insulin producing ILC *in vitro* from stem cells. The present study aims towards fabricating a 3D scaffold for culturing islet cells as well as deriving insulin producing ILC from stem cells under *in vitro* conditions using appropriate growth factors. The functionality of native and differentiated islet cells is compared in 2D culture system as well as on 3D scaffold.

5.1 Isolation and characterization of cells

Dithizone staining is recognized as a valuable method to specifically identify the islet cell mass harvested from cadavers (Miyamoto *et al*., 1998). Isolated islets from rabbit pancreas stained with DTZ appeared crimson red. Dithizone is a zinc-chelating agent, and pancreatic islets from animal species as mouse, dog, pig, and human are known to be stained crimson red by its treatment, because of the higher zinc granules in β islets compared with other tissues (Clark *et al*., 1994). The isolated rabbit cells were also stained positive for insulin, glucagon and somatostatin indicating the presence of β, α and δ cells in islets.

Adult stem cells with their high potential of proliferation and differentiation offer great promise for generating β islets. The use of somatic or adult stem cells, found in adult tissues, escapes the controversy associated with the use of stem cells derived from embryos and fetal tissues. Also, adult stem cells have the potential to

elude immunological rejection and disease contraction when transplanted back into the donor (Merok and Sherley, 2001). The concept of plasticity in stem cells suggests its ability to dedifferentiate from their source cell type and then differentiate into new cell types depending on the culture conditions (Guilak *et al*., 2006).

The isolated cells from rabbit and human adipose tissue in culture displayed an elongated, spindle-like fibroblastic morphology. The cells isolated from rabbit and human were plated at a density of 6,000 cells/cm^2 and 10^4 cells/cm^2 respectively. Lower seeding density did not exhibit colony formation, suggesting that the cells prefer to have adjacent cells within a closer range. The cells on reaching confluence stage have their proliferation/growth arrested due to contact inhibition.

Growth characteristics of rabbit adipose stem cells revealed that cells completed one cell cycle approximately every 2.1 days and after passage 7 doubling time was significantly increased. Previous reports suggests that human adipose tissue harbor approximately 2–6 x10 ^{6} cells per 300 ml of processed lipoaspirate which can be maintained in culture through passage 13 (Zuk *et al*., 2001). Rabbit and human adipose stem cells underwent a change in morphology after passage 6 hence passage 4 stem cells were used throughout the study. Adipose stem cells have been reported to express cell surface markers CD9, CD10,CD13, CD29, CD49 d , CD49 e , CD44, CD54, CD55, CD59,CD105, CD106, CD146 and CD166 (Gronthos *et al*., 2001). Our experimental procedure for isolation yielded multipotent CD44+/CD105+ cells from rabbit and human adipose tissue, confirmed by flow

cytometry analysis and immunofluorescence assays. Flow cytometry analysis confirmed that more than 80% of the isolated cells from rabbit adipose tissue were positive for mesenchymal marker vimentin and stem cell marker CD44. The rabbit and human adipose tissue derived cells obtained after isolation were cultured and non adherent cells were removed from culture after 1 day. The adherent cells on reaching confluency was subcultured using Trypsin EDTA and passage 1 cells were used for Magnetic activated cell sorting/Flow cytometry.We expect the hematopoetic cells (non adherent) were removed after day 1 on medium change. Hence this is the reason for the absence of negative markers CD45/CD34.The perecentage of cells for stem cell markers before and after magnetic separation has been described in (Figure 4.10) .The percentage of stem cell markers CD105 and CD44 cells was increased from 63.3% to 99% , and 63.5% to 83% respectively after Magnetic activated cell sorting. The fraction of cells positive for stem cell markers in the crude preparataion was not estimated. We confirmed the multi-lineage capacity of stem cells isolated from rabbit/human adipose tissue. Adipose stem cells subjected to adipogenic and osteogenic differentiation conditions were all stained positive.

Endothelial cells cultured from umbilical veins grew as a homogeneous monolayer of large polygonal cells with cobblestone morphology expressing endothelial specific markers like vWF and CD31 (Jaffe *et al*., 1973). Human CD31/platelet endothelial cell adhesion molecule (PECAM)-1 is a 130-kd cell surface molecule belonging to the immunoglobulin superfamily expressed by endothelial cells (DeLisser *et al*., 1994). Von Willebrand factor is a multimeric

plasma glycoprotein constitutively secreted by EC and stored in the Weibel-Palade bodies (Sporn *et al*., 1986). The vWF is required for platelet binding to the subendothelium during hemostasis and serves as a carrier for coagulation factor VIII (Sakariassen *et al*., 1979). Endothelial cells cultured after passage 3 underwent change in cobblestone morphology hence passage 2 and 3 cells were used for co-culture study.

5.2 Characterization of scaffold

Non-cytotoxicity, pore size, adequate cross-linkage, biodegradability and swelling behavior parameters of the scaffold should be considered in islet culture. Furthermore, cell attachment to a biomaterial scaffold is an important early step in the generation of *in vitro*-engineered tissue substitutes. Integrins are heterodimeric transmembrane receptors that bind to proteins within the ECM including fibronectin, laminin, various collagens, and many other molecules. Integrins are composed of α and β subunit and the pairing of these subunits dictate specificity for ligand (Hynes, 2002).

Natural polymers such as gelatin and dextran were chosen for the present study due to its wide use for tissue engineering applications. The most important features of the DEXGEL scaffold is its biocompatible nature; the scaffold and its degradation products are non-cytotoxic. The MTT assay exhibited an increased percentage of cell survival on test extract treated cells in comparison to the untreated controls but there was no statistical difference between the results. The degradation products of DEXGEL are expected to be amino acids from gelatin and glucose units

from dextran. Additionally, glucose is a potent stimulator for islet neogenesis and amino acids are also beneficial to the cell which adds on the benefit of DEXGEL scaffold.

Gelatin, derivative of collagen which is a major component of islet ECM could be a better option for mimicking the role of ECM. Gelatin has been well utilized in tissue engineering applications on account of its biocompatible nature. Gelatin is considered less immunogenic than collagen due to the absence of aromatic groups (Tyrosine and Tryptophan) and very less amount of Phenylalanine. A gelatin matrix has also been reported to restore sufficient cell–ECM interactions to induce glucose dependent insulin production in dissociated β cells (Del Guerra *et al*., 2001). Gelatin based scaffold have been exploited in islet cell culture in regard that it is a partially degradable form of collagen which constitutes the major part of ECM in native islets. The short peptide fragments or RGD sequence in gelatin plays major role in binding of integrins mainly, $\alpha5\beta1$ and $\alpha5\beta3$ which helps native islets in maintaining its intact architecture (Muthyala *et al*., 2010). Dextran is also known to enhance angiogenic effects which could be exploited to promote tissue neovascularization post transplantation in future *in vivo* experiments (Sun *et al*., 2011). In addition, gelatin and oxidized dextran has the property of self crosslinking without the aid of any external crosslinking agents. The DEXGEL scaffold exhibited a hydrophilic-hydrophobic balance important for cell culture, evident by contact angle measurement owing to the efficient crosslinking of gelatin and dextran dialdehyde.

The time required for partial oxidation of dextran was 6 h at 37 ^{0}C. However at lower temperatures more time was required to achieve 40-50% oxidized dextran. Pore diameter and pore interconnectivity also plays a major role in maintaining islet phenotype and function. In the present study, we have optimized pore size ranging from 100-300 µm in our initial standardization experiments based on cell viability and function for culturing ILC whose mean size is around 80-150 µm. Optimal pore size is required for multiple islet-islet interactions and spatial orientation of islets and ILC. Smaller pores were generated by increasing the stirring speed during DEXGEL formation which was found to be suboptimal for ILC. Parameters like stirring speed, cross-linking temperature were optimized to reach the most favorable pore size in DEXGEL scaffold for ILC culture. Higher stirring speed resulted in small pores which was not favorable for culturing large islet clusters. Lower temperature for crosslinking resulted in generation of small pores less than 100 µm which was not considered for the present study. Pore interconnectivity is essential for efficient cellular ingrowth, nutrient transport and vascularization.

The rate of biodegradation of DEXGEL was found to be less than 20% during the period of 30 days under *in vitro* condition and further study has to be done to investigate degradation pattern *in vivo*. The FT-IR data of DEXGEL cultured for 60 days confirmed the presence of aldehyde and amino groups which are expected to be from oxidized dextran and gelatin respectively as biodegradation occurs. Since the degradation rate was slow, significant aldehyde and amino groups were not observed in DEXGEL cultured for 7 days and 30 days. The data confirmed the slow degradation profile of DEXGEL owing to the efficient cross linking

between amino group of gelatin and aldehyde group of dextran dialdehyde. Schiff base is a covalent bond whose stability is highly dependent on pH of the surrounding media and favoured by neutral pH. The cross-linking mechanism occurs due to the attack of nucleophilic nitrogen of the amino group of gelatin on aldehyde carbon of dextran dialdehyde resulting in loss of one water molecule, thereby forming C=N bond (Schiff's base). In the present study, the pH of culture medium is neutral which prevents transition of $-NH_2$ to protonated state thereby enhancing the stability of Schiff base in DEXGEL. The presence of hydrophilic gelatin adds to the increased swelling ratio of DEXGEL which facilitates cells to gain maximum internal surface area of the scaffold.

5.3 Rabbit islets on scaffold

Islet cell viability and function compromised by islet isolation techniques have shown to result in high levels of islet apoptosis. Islet cells cultured using traditional cell culture approaches have been reported to undergo cell death after 7 days (Chun *et al*., 2008).

Rabbit islets isolated by collagenase digestion were cultured on 2D (TCPS) and 3D (DEXGEL scaffold) culture conditions. The disrupted morphology of islets on 2D may be due to the lack of extracellular matrix signaling for adherence and maintaining its phenotype. Meanwhile, islets on scaffold grew in a 3D pattern by adhering to it and formed cell clusters with characteristic cluster phenotype and in general formed the 3D islet tissue. The positive influence of the scaffold matrix appears to be related to its ability to promote islet cell spreading. Apparently, this

3D architecture of the scaffold served as a framework, favoring the proper orientation and configuration of islets to maintain its phenotype. The gene expression analysis (Figure 4.21) highlights the fact that proper maintenance of the islet architecture in turn results in enhanced insulin gene expression.

Various ECM components have been shown to improve the maintenance and function of islet cells *in vitro* (Daoud *et al*., 2010). In the present study, we show that the viability of islet cells is increased and the morphology is maintained when cultured on the scaffold. The destruction of islet microenvironment that occurs during isolation or culture subjects them to cellular stress that leads to structural and functional abnormalities. The ECM constitutes the major component of islet microenvironment serving as a cellular scaffold as well as regulating cell survival and function (Wang and Rosenberg, 1999).

The cell-matrix relationship is characterized by interaction between cell surface integrin receptors and matrix molecules of the ECM. The viability is attributed to the RGD sequence of the gelatin scaffold. The anti- apoptosis signal, generated by ECM molecules, can be mimicked by RGD peptides on binding to the integrins present on islet cells. The macroporous structure of the scaffold and the RGD peptide sequences may be responsible for promoting the enhanced functionality of islets (Pinkse *et al*., 2006).

The evidence of ECM formation typically collagen IV as shown in Figure 4.24 highlights the importance of scaffold in serving as a supporting framework for islets to restore its own ECM. The major component of islet ECM constituting the vascular basement membrane proteins are contributed by endothelial cells but there

is also a non vascular source of collagen (collagen IV) in islets forming the islet capsule (Stendahl *et al*., 2009).

5.4 Stem cell differentiated islet like cells

Several reports have confirmed the differentiation ability of adipose stem cells to ILC *in vitro*. In our study we differentiated rabbit and human adipose stem cells to ILC on an in-house fabricated biodegradable 3D scaffold composed of natural polymers (dextran and gelatin) and the results were compared with 2D cultures (tissue culture polystyrene and DEXGEL coated surface).

We adopted a three stage protocol with growth factor concoction in serum free high glucose medium for the differentiation procedure. Serum free condition and high glucose medium are known to enhance differentiation of stem cells to insulin producing cells. Glucose at 20-30mM concentration is a potent stimulator for β cells by escalating its replication and insulin secretion *in vitro* (Swenne, 1982). But high glucose alone could not induce the differentiation of stem cells to ILC. We used 4nM activin along with beta mercaptoethanol in SFM A for effective differentiation of stem cells towards endodermal lineage.

Growth supplements such as fibroblast growth factor, epidermal growth factor, glutamine and amino acids in SFM B enhanced the differentiation of endodermal cells to pancreatic lineage. Fibroblast growth factor promotes cell proliferation and terminal differentiation at the level of Notch activation (Xu *et al*., 2011). Various research groups have reported the role of epidermal growth factor in inducing β cell neogenesis (Suarez-Pinzon *et al*., 2005, Guo *et al*., 2011). In SFM C,

nicotinamide, along with activin and betacellulin enhanced the differentiation towards pancreatic endocrine lineage. Nicotinamide play an important role in maturation of ILC in addition to preserving islet viability and promoting insulin secretion through poly (ADP-ribose) polymerase (PARP) (Kolb and Burkart, 1999). The combined effects of activin and betacellulin support islet differentiation, maturation and insulin secretion (Sulzbacher *et al*., 2009, Demeterco *et al*., 2000).

The three stage protocol adopted in the study could derive pancreatic β islet like cells *in vitro* by using appropriate growth factors at correct time point. The size and morphology of ILC improved and differentiated cells resembled islet like morphology by day 20. The mean size of ILC ranged from 80-150 µm. Small islet clusters are superior to larger islets in function and transplantation procedures with regard to higher oxygen consumption and reduced necrosis (Lehmann *et al*., 2007).

5.5 Rabbit islet like cells

Dithizone stained rILC appeared crimson similar to that of rabbit islets indicating the presence of zinc granules. Zinc plays an important role in synthesis, storage and secretion of insulin as well as in maintaining the conformational integrity of insulin in hexameric form (Chausmer, 1998). In the rILC differentiated from rabbit adipose stem cells alpha and beta cells were interspersed throughout the ILC and native islets. Very few delta cells were found in rILC and rabbit islets. Furthermore, differentiated rILC expressed other pancreatic endocrine hormones like glucagon and somatostatin in addition to insulin resembling native islet architecture so that paracrine interactions among alpha, beta and delta cells

contribute to better functioning of ILC. Islet morphology is an important criterion in determining its function (Wang and Rosenberg, 1999). The rILC on TCPS, ultralow attachment dish and DEXGEL coated dish lost their morphology and membrane integrity before day 30. On TCPS plates the rILC had adherent nature but spherical cluster morphology was maintained for a short period on the ultralow attachment and DEXGEL coated dish. The lack of optimal microenvironment for spatial orientation and cell-cell interactions in ultralow attachment and DEXGEL coated dish caused the rILC to enter apoptotic state by day 30. On the other hand, rILC maintained viability on DEXGEL scaffold for over 60 days of the study period due to the favorable microenvironment supporting its phenotype provided by the biomimetic scaffold. The comparison of DEXGEL scaffold and coated surface in terms of viability and function clearly indicates that rILC require an optimum microenvironment for maintaining its spherical architecture and spatial orientation which could be provided by a favorable scaffold.

The purpose of biodegradable scaffold was to provide a supporting framework for rILC to maintain its structural integrity for the time required by the cells to re-create its own ECM thereby prolonging its longevity *in vitro*. In our study we observed faint expression of collagen on the periphery of rILC on day 30 which increased by day 60 thereby confirming that rILC are actively functioning within DEXGEL. Differentiated rILC responded to low and high concentrations of glucose similar to that of control rabbit islets; however total insulin secretion from rabbit islets was greater. In 2D culture rabbit islets exhibited decreased insulin release due to the disruption of ECM during isolation procedure but 3D condition of DEXGEL

scaffold suitably mimicked the role of ECM by providing a favorable milieu, hence the yield, insulin content and viability of differentiated rILC on scaffold were significantly greater in comparison to 2D cultures. Earlier reports have implied role of extracellular matrix (ECM) in islet survival and activation of MAP kinase-ERK signaling pathway induced by extracellular matrix is required for survival of islets (Weber *et al*., 2008, Hammar *et al*., 2004).

Surface modification of scaffolds with various ECM proteins could stimulate various islet responses and integrin mediated behaviour (Daoud *et al*., 2010). The PLGA-collagen hybrid scaffolds coated with laminin and fibronectin were used to maintain rat islet survival for a period of 6 days (Kawazoe *et al*., 2009). In the present study, no such coating was used and yet the period of retention of viable islets on scaffolds was significantly greater. The result implies that the scaffold effectively mimics the role of extracellular matrix by providing a beneficial environment for rILC. It is also hypothesized that the dextran based scaffold is more conducive for the growth and maintenance of rILC. The positive impact of biodegradable poly (lactic-co-glycolic acid) scaffold coated with matrigel on endoderm commitment from human embryonic stem cells over traditional monolayer cultures and reversal of hyperglycemia in diabetic mice using islets derived from embryonic stem cells on PLGA scaffolds were reported (Mao *et al*., 2009).

The macroporous nature with pore size in the range of 100-300µm of the scaffold provide a spatial environment for maintaining the morphology thereby sustaining the survival and function of rILC.

5.6 Human islet like cells

Adipose tissue has been recognized as a safe and abundant source for large quantities of adult stem cells with minimal risk (Fraser *et al*., 2006). Several reports have verified the ability of MSC to transdifferentiated into functional insulin producing cells (IPC) however their ability to respond to varying glucose concentrations is challenging.

Adopting the three stage differentiation protocol definitive pancreatic endocrine cells was successfully derived from hASC on 2D culture and 3D scaffolds. The hILC on 2D and 3D culture exhibited islet like cluster morphology by day 10 and size of the clusters were increased by day 20. Differences in the pancreatic endocrine gene expression profile between cells differentiated in 2D or 3D cultures were observed. The expressions of early islet markers Pdx-1 and Ngn3 were upregulated during the differentiation process, but higher expression was observed on 3D cultures. Late pancreatic endocrine markers insulin, glucagon, somatostatin, pancreatic polypeptide and ghlerin expressions were significantly higher on 3D cultures in comparison to 2D culture. The results illustrate the impact of 3D scaffolds on early stem cell differentiation towards the pancreatic lineage. The Pdx1 also known as insulin promoter factor 1, is a transcription factor required for pancreatic development and β-cell maturation. The fact that Pdx1 is a master gene for early pancreatic development has been demonstrated by the pancreatic agenesis occurring after bud formation in mice lacking functional Pdx1 (Jonsson *et al*., 1994). The Ngn3 which belongs to a family of basic helix-loop-helix (bHLH) transcription factors is expressed in endocrine progenitor cells. Neurogenin 3 play an important

role in the formation of pancreatic endocrine precursors (Kubo *et al.*, 2011). In a study on mice lacking Ngn3, the four pancreatic endocrine cells, which produce insulin, glucagon, somatostatin and pancreatic polypeptide were absent (Gradwohl *et al.*, 2000). The results demonstrate that hASC differentiated hILC adopted a pancreatic endocrine phenotype morphologically and functionally under a defined three-stage induction protocol *in vitro* without any genetic manipulation.

Protein level analysis of hILC on 2D and 3D cultures by immunophenotype confirmed the expression of pancreatic endocrine hormones insulin, glucagon and somatostatin. The viability of hILC on 2D cultures and DEXGEL scaffold were analyzed with live dead calcein AM/ethidium homodimer-1 stains. The detection of live and dead islet cells by calcein AM and ethidium homodimer-1 staining showed that 3D cultures had significantly lower numbers of dead cells, compared with those in 2D culture. Calcein AM(excitation 495nm, emission 515nm) are retained within live cells and ethidium homodimer (excitation 495nm, emission 635nm) are excluded by the intact plasma membrane of live cells. The viability of hILC on 2D cultures was analyzed using fluorescent microscope (Leica, Germany, Calcein-Bandpass (BP) 450-490, Longpass (LP) 515 and Ethidium homodimer-BP 515-560, LP 590) and on scaffold using Confocal microscope (Carl Zeiss 510 Meta, Germany) (Cacien excited at 488nm using Argon laser and ethidium homodimer was excited at 543nm using HeNe laser). The viability and glucose stimulated insulin response of hILC were enhanced on 3D cultures in comparison to 2D cultures. Adipose stem cells were seeded at a density of 10^5 cells /cm^2 and 10^7 cells/cm^3 on DEXGEL coated dish (2D) and DEXGEL scaffold (3D) respectively.

On 2D culture cell number is expressed per area of the dish and on 3D culture cell number is expressed per volume of the scaffold. Since cell number is relative per area and volume of matrix, the amount of insulin is described in relative to DNA concentration.

Formation of islet amyloid toxic protein aggregates and loss of islet ECM are two important non immune factors that contribute to β cell dysfunction and death both during pre transplant islet culture and in islet grafts. The 3D scaffolds have been reported to reduce amyloid formation and improve viability and function of human islets *in vitro*. Further collagen matrices and fibroblasts populated collagen matrices have been demonstrated to have additive effects in enhancing islet function and reducing amyloid formation (Zhang *et al*., 2012).

Adult human islets are surrounded by extracellular matrix, which is closely associated with a capsule consisting of a single layer of fibroblasts and collagen fibers produced by these cells (Stendahl *et al*., 2009). Several studies have shown that islet morphology, function, and viability are heavily influenced by the islet extracellular matrix (Beattie *et al*., 2002). The results of the study are based on multiple human samples [lipectomy(n=6-8) and liposuction (n=4)]. Each sample was run on triplicates for the analysis. The results of glucose stimulated insulin response shows significant increase on scaffold culture in comparison to 2D culture, further highlighting the importance of scaffold in enhancing islet function and viability.

5.7 Co-culture study

The influence of EC co-culture on hILC was studied on 2D and 3D cultures. The results reveal the importance of EC in inducing the differentiation of pancreatic endodermal cells towards pancreatic endocrine islets. Morphological analysis of these cultures showed that the cells aligned with one another, indicating interactions between the cells. Endothelial cells are adherent cells that interact with ECM. Here EC adhered on DEXGEL coated matrix on 2D culture and by 15^{th} day sprouting was observed on co-culture constructs in contrast to hILC cultured alone. The sprouts further increased by day 20 revealing the interactions among hILC and EC. On 3D cultures, interaction of EC and hILC was evident by immunofluorescence staining for vWF as well as insulin. The VEGF A gene expression was also significantly greater on coculture constructs on scaffold. This is mainly due to the interaction of VEGF A and VEGF A receptor mediated signaling between islets and EC.

Significant changes in islet gene expression mainly insulin and VEGF A was observed when endothelial cells were added onto day 10 hILC culture. This is because in the pancreas, upregulation of VEGF A occurs during islet formation when they start to secrete hormones. Islets express VEGF A at later stages of their development to attract capillaries. The islets are known to produce angiogenic signals mainly VEGF A which interacts with the VEGF A receptor present on endothelial cells (Reinert *et al*., 2014). This will induce fenestrate formation within islets which result in enhanced sprouting and vessel formation of endothelial cells (Lammert 2003). The VEGF A is essential for revascularization of islets (Brissova 2006).

Islet endothelium is important in fine-tuning of sensing blood glucose and secretion of insulin. Endothelium is responsible for vascular basement membrane formation of islets. The signals from basement membrane are responsible for insulin secretion, function and proliferation of β cells (Nikolova 2006). Signaling is mediated by integrins of which β1 and αv play an important role. Integrin mediated signaling is responsible for upregulation of insulin gene expression and enhanced insulin secretion function in ILC. Immunohistochemical analysis also confirmed the presence of collagen IV and integrin β1 on coculture 3D constructs. Cell-cell and cell-ECM interactions are important for promoting viability of islets. The scaffold serves as a supporting framework in maintaining islet phenotype thereby enhancing cell-cell interactions.

The RGD sequence of gelatin is a known factor in signaling islet survival and function. The ILC could bind to RGD sequences through integrin receptors which favor its enhanced survival on scaffold. The RGD peptides have been reported to confer survival to islets. The anti -apoptotic signals are generated by the ECM molecules which could be mimicked by the RGD peptides by interacting with integrin receptors thereby initiating signaling process. Islets enter into apoptotic state unless cultured on ECM components or RGD peptides to mimic ECM ligation (Pinske 2006). Preventing the islets to enter apoptotic state may help to improve islet viability and function. The sprouting mechanism by ECs enhances the islet-islet interactions thereby contributing to cellular function. The extracellular matrix formation in ILC contributed by signals from endothelial cells as well as by scaffold matrix resulted in enhanced function and viability of ILC.

Integrin β1 are receptors for collagen in basement membrane. The ILC-EC on scaffold showed significant increase in integrin β1 expression which could bind to the basement membrane proteins thereby mediating bidirectional signaling. The β1 integrin has been reported in mediating islet survival by adhering to matrices collagen I and collagen IV (Wang 2005). The long-term survival of rat adult β cells has been demonstrated due to the activation of MAPK/ERK through β1-integrin/focal adhesion kinase signaling mechanism (Hammar 2004).

Johansson *et al*., 2008 reported that coculturing islets with EC and MSC improve islet viability and function. In this study we used adipose derived mesenchymal stem cells for the differentiation procedure. Chandra *et al*., 2011 reported that there was a decline in the stem cell marker expression during differentiation to pancreatic endocrine lineage. However stem cell properties are not totally shut down. This property may enhance in the attachment of EC on ILC which in turn progresses the interaction between EC and ILC. Furthermore the insulin secretory function of ILC in response to different glucose concentrations was significantly increased on coculture 3D constructs highlighting the importance of EC and scaffold on islet viability and function.

CHAPTER 6

SUMMARY AND CONCLUSION

Diabetes mellitus, the chronic metabolic disease caused either due to insulin insufficiency or its resistance, resulting in lifelong dependency on insulin has become one of the most significant non-communicable diseases globally. The complications associated with the disease include diabetic neuropathy, nephropathy, retinopathy, cardiovascular disease.

Diabetes is mainly treated by administration of oral hypoglycemic drugs or insulin injection. However these approaches cannot mimic the physiological oscillating pattern of insulin release to achieve normoglycemia thereby cannot prevent the long term complication associated with diabetes and hypoglycemic shock. Transplantation of pancreatic islets aims to achieve euglycemia by following the insulin release pattern as in physiological manner. But islet cells need to be cultured *in vitro* prior to transplantation and long term culture without compromising cell viability and function is challenging.

The need to obtain multiple donor pancreases for each patient and the uncertainty regarding long term side effects from immunosuppression limits the benefits of islet transplantation to patients with highly uncontrolled diabetes. Isolated pancreatic islets *in vitro* culture require a solid matrix to serve as a supporting matrix to promote its survival and substitute for the absence of native

ECM. It is also known that pancreatic endocrine cells strictly require endothelial signals for their differentiation and function.

In order to address these concerns, the present study on “Co-culture of stem cell derived islets & endothelial cells on a three dimensional biomaterial towards a tissue engineered bio-hybrid pancreas” has explored the first hypothesis that adipose stem cells could be a better source to derive pancreatic endocrine lineage cells *in vitro* which could overcome the severe scarcity of donor islets for transplantation purpose. Recognizing the importance of scaffold in tissue engineering islets the second hypothesis is that a biodegradable and biomimetic 3D scaffold with adequate pore sizes and mechanical strength is a critical requirement to support islet cell survival. Since endothelial cells play an important role in islet neogenesis and function, third hypothesis is that culturing stem cell differentiated islets and endothelial cells together as a co-culture model on the scaffold matrix may improve islet cell survival and function.

The overall results obtained from the current study, emphasize on the significance of microenvironment in islet survival, maintenance of spherical morphology of ILC as well as insulin secretion. The present work demonstrates the importance of endothelial cells and feasibility of scaffold in promoting islet viability and insulin secretion which could serve as a carrier vehicle in islet transplantation, a curative therapy for diabetes mellitus.

The findings in the present study suggest that cell-cell interaction in an artificial three dimensional matrix is essential for islet viability. The favorable environment offered by the scaffold of our study is attributed to the presence of

peptide fragments, the balance of hydrophobic and hydrophilic domains and the lack of toxic crosslinking agents.

The present study has demonstrated the potential of adipose stem cells to differentiate into mature islet- like clusters on 2D culture and 3D scaffold using a three stage protocol. The use of autologous adipose stem cells will be a better option in case of severe scarcity for donor organs. Since adipose stem cells do not undergo uncontrolled proliferation as tumor cells it could be used as substitute in transplantation set up. The ILC on DEXGEL scaffold exhibited elevated levels of secreted insulin in response to glucose concentration compared to 2D cultures. The DEXGEL scaffold reported in this study exhibited positive impact on islet survival, its integrity and function *in vitro* for a significantly longer period. The overall findings signify that adipose stem cells cultured on DEXGEL scaffold in the presence of appropriate growth factors could be a preferred way to generate viable ILC thereby a promising strategy for long term islet culture for pancreatic tissue engineering. In contrast to the scaffold-free control group, islets or ILC cultured on scaffold exhibited improved morphology, less cell death, and prolonged survival time.

The use of ILC-EC scaffold composite had beneficial effects on improvement of islet function as well as viability. Endothelial cells may contribute to microvasculature formation within islets which can improve the survival of graft after implantation. Moreover EC contribute to the formation of vascular basement membrane proteins important for fine tuning of glucose stimulated insulin secretion. This could be a better approach for islet transplantation since it requires lesser time

for graft vessels to integrate with the host vasculature. This could be an alternative therapeutic approach for diabetes mellitus in the future.

FUTURE PROSPECTS

At present, only collagen expression was studied and further investigations should be conducted for analyzing the expression of other ECM components like laminin and vitronectin. The present report deals with only *in vitro* studies and more detailed investigations about the signaling cues from the scaffold should be made in future through *in vivo* experiments.

The *in vivo* studies of the effectiveness of tissue engineered construct comprising ILC and EC on scaffold for reversal of diabetes in animal models need to be explored, before it can be translated as a therapeutic approach of the future.

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LIST OF PUBLICATIONS

- Aloysious, Neena, and Prabha D. Nair. "Enhanced survival and function of islet-like clusters differentiated from adipose stem cells on a three-dimensional natural polymeric scaffold: an in vitro study." *Tissue Engineering Part A* 20.9-10 (2014): 1508-1522. (Impact factor 4.448)
- Book Chapter titled “Perspectives of Islet Cell Transplantation as a Therapeutic Approach for Diabetes Mellitus” Regenerative Medicine and Tissue Engineering; From Cells to Organs / Book 2. Authors. Prabha D. Nair and Neena Aloysious
- Indian Patent filed (Application Number- 4867/CHE/2013)- Porous biodegradable polymeric scaffold for tissue engineering. Prabha Damodaran Nair and Neena Aloysious

PAPERS PRESENTED AT CONFERENCES

- Neena Aloysious & Prabha D. Nair .Tissue engineered biohybrid pancreas from autologous adipose stem cell derived pancreatic islets :A promising therapy approach for Diabetes mellitus" at the International Symposium on '"Type 2 Diabetes : From Molecular Insights to Therapeutic Interventions" organized by Institute of Life Sciences, University of Hyderabad and Merck & Co Inc. held at Novotel Hyderabad during 18-09-2009 to 20-09-2009 (Oral presentation)
- Neena Aloysious & Prabha D. Nair. Presented a poster titled "Autologous Adipose Stem Cell Derived Pancreatic Islets Delivery On A 3D Scaffold – A Promising Regenerative Medicine Approach For Diabetes Mellitus"at International Conference On Biomaterials, Artificial Organs & Tissue Engineering (ICBAT),March 1st , 2010, at,Cochin, Kerala. (Poster presentation)
- Neena Aloysious & Prabha D. Nair. The positive impact of scaffold in differentiation of stem cells to isle like clusters for islet transplantation' at ICNT 2013, Kottayam, Kerala. (Oral presentation)
- Neena Aloysious & Prabha D. Nair. Co-culture of endothelial cells and stem cell differentiated islet like clusters on a natural biodegradable scaffold in the development of a tissue engineered pancreas' at IUMRS 2013, Bangalore.(Oral presentation)
- Neena Aloysious & Prabha D. Nair. The role of scaffold in mimicking the biological matrix of islets: implications for islet transplantation in treatment of diabetes mellitus in 26th Kerala Science Congress held on 28th-31st January, at Wayanad. Kerala.(Oral presentation)

CURRICULUM VITAE

Education

2009-present	PhD Scholar at Sree Chitra Tirunal Institute for Medical Sciences and Technology. Biomedical Technology Wing, Trivandrum, Kerala, India. Advisor: Dr. Prabha D Nair, Ph.D.
2004-2006	Master of Science in Biotechnology, Anna University, Tamil Nadu
2001-2004	Bachelor of Science in Biotechnology, Periyar University, Tamil Nadu

Awards and Honors

2014	Best Paper Award in Health Science at 26th Kerala Science Congress for the paper titled “The role of scaffold in mimicking the biological matrix of islets: implications for islet transplantation in treatment of diabetes mellitus”
2009	First Prize for oral presentation at the International Symposium on '"Type 2 Diabetes: From Molecular Insights to Therapeutic Interventions”, Hyderabad for the paper titled “Tissue engineered biohybrid pancreas from autologous adipose stem cell derived pancreatic islets: A promising therapy approach for Diabetes mellitus”.
2008	Junior Research Fellowship, Council for Scientific and Industrial Research, Government of India.

APPENDIX

A-1

PREPARATION OF KREB'S RINGER BICARBONATE HEPES (KRBH) BUFFER

Sodium chloride-	114mM
Sodium bicarbonate-	29.5mM
Potassium chloride-	4.4mM
Magnesium sulphate-	1mM
Calcium chloride-	1.28mM
HEPES-	10mM
Bovine serum albumin-	0.1%

Adjust the pH to 7.4

A-2

TITRATION FOR CALCULATING THE DEGREE OF OXIDATION

a) Standardization of sodium thiosulphate

0.1N sodium thiosulphate, 0.1N potassium iodate (KIO3), 2% (w/v) starch and 10% (w/v) potassium iodide (KI) solutions were prepared. In a conical flask take 20 mL of KIO3 solution, 5ml of KI and 1 ml of concentrated hydrochloric acid (HCl) (solution turns dark brown) and was titrated against sodium thiosulphate solution (taken in burette). Drop by drop sodium thiosulphate solution was added till the solution turned pale yellow. To this add 20ml of distilled water and 2ml starch; solutions turn dark violet color. Further titrate against sodium thiosulphate solution till the solution turns colorless. The procedure was continued for three concordant values.

b) Normality of oxidized dextran

The burette was filled with sodium thiosulphate solution. To the conical flask add 4ml oxidized dextran, 1ml KI, and 1 drop HCl. Titrate the solution in conical flask against sodium thiosulphate. When the solution in conical flask turns pale yellow add 10mL distilled water and 1ml starch solution, thereafter titrate against sodium thiosulphate till the solution turns colorless. The endpoint was noted till three concordant values.

c) Calculation

i) Normality of oxidized dextran

N1 x V1= N2 X V2

N1 = Normality of oxidized dextran (?)

V1=Volume of oxidized dextran

N2= Normality of sodium thiosulphate

V2= Volume of sodium thiosulphate (endpoint value after titration)

ii) Weight of unreacted sodium metaperiodate (NaIO3) =

Normality of NaIO3 X Molecular weight of NaIO3

iii)Weight of reacted NaIO3= Total weight of NaIO3 added – Weight of unreacted NaIO3

iv)Number of moles of NaIO3 reacted =

Weight of reacted NaIO3/ Molecular weight of NaIO3

v) Actual percentage of oxidized dextran = (50 x Moles of NaIO3)/0.03

A-3

PREPARATION OF 0.1M SODIUM BORATE BUFFER

Boric acid	-	0.0618g
Disodium tetraborate	-	0.0954g
Dis H_2O	-	65ml

Adjust pH to 9.4 using 1M Sodium hydroxide

Make up the final volume to 100ml