

**PROTECTION & REGENERATION OF β -CELLS:
STUDIES USING THE NOD MOUSE**



CHEEN PEEN KHOO

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ABSTRACT

Diabetes is a chronic disease affecting approximately 250 million people. To date, transplantation-based therapy is the therapy of choice; however, its success is hampered by the scarcity of transplantable human material. An alternative strategy is the promotion of regeneration in the pancreas. Endothelial progenitor cells (EPCs), a subpopulation of bone marrow (BM) cells, can contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels. In this thesis, I review and discuss the role and regenerative potential of EPCs in diabetes using non-obese diabetic (NOD) mice, a model of type 1 diabetes (T1D). Flow cytometry analyses of the EPC population in BM and blood of both diabetic and pre-diabetic NOD mice suggested that at the onset of diabetes, BM-derived EPCs are stimulated to enter the systemic circulation in response to signals from the pancreas. To further investigate the contribution of EPCs to β -cell regeneration, whole BM and cultured EPCs were transplanted into pre-diabetic and diabetic NOD mice soon after diabetes was diagnosed. Our data imply that BM cells from wild type mice administered before the onset of diabetes may have an effect in delaying β -cell destruction evidenced by glycaemic control, reduced inflammation and increased number of proliferating Ki-67⁺ Insulin⁺ cells. EPCs transplanted into early diabetic NOD mice had reduced inflammation and a higher survival rate compared to control mice that did not receive EPCs. I therefore believe that both BM and EPCs show great promise in regenerating the damaged pancreas of NOD mice.

In addition to promoting endogenous β -cell regeneration using EPCs, I have investigated a strategy to protect β -cell death via apoptosis, using a protease peptide XG-102 developed by Xigen. Since apoptosis of β -cells is one of the putative mechanisms involved in the cascade of events leading to T1D, we tested XG-102, for prevention of β -cell loss in the NOD mouse. Treated mice had a larger number of islets and inflammation was less prevalent compared to control mice. Additionally, XG-102 treated mice showed better control of their blood glucose.

In conclusion, both therapeutic strategies showed great promise in regenerating the damaged pancreas of NOD mice. While these strategies are still under further investigation, they offer encouragement in the quest for the treatment of early diabetes in the future.

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ABBREVIATIONS

acLDL	Acetylated low-density lipoprotein
Ag	Antigen
APC	Allophycocyanin
APCs	Antigen-presenting cells
β	Beta
bFGF	Basic fibroblast growth factor
BB	BioBreeding
BM	Bone marrow
BMDS	Bone marrow derived Stem cells
BMSCs	Bone marrow stem cells
BSA	Bovine serum albumin
CAD	Coronary artery disease
cEPCs	Circulating EPCs
CFU	Colony forming units
CrmA	Cytokine response modifier A
DAB	3'3'-diaminobenzidine
DISC	Death-inducing signalling complex
DMN	Dimethylnitrosamine
DNA	Deoxyribonucleic acid
E	Embryonic day
EW	Embryonic week
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
Epo	Erythropoietin
ESCs	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluoresceinisothiocyanate
FLICE	Fas-associated death domain-like interleukin 1 β -converting enzyme
FLIP	FLICE-inhibitory protein
FSC	Forward scatter channel
GAD-65	Glutamic acid decarboxylase
Gy	Gray unit
g	Gram
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
HBO	Hyperbaric oxygen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HSCs	Haematopoietic stem cells
HSP60	Heat shock protein 60

HRP	Hydrogen peroxidase
IA-2	Insulinoma antigen
IAA	Insulin autoantibodies
IL-1 β	Interleukin 1 β
IFN- γ	Interferon γ
i.p.	Intraperitoneal
i.v.	Intravenous
JBD	JNK-binding (delta) domain
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
IMDM	Iscove's MDM
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MCT	Monocrotaline
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
min	Minute
MNC	Mononuclear cells
NO	Nitric oxide
NOD	Non obese diabetic
PAH	Pulmonary arterial hypertension
PB	Peripheral blood
PBS	Phosphate-buffered saline
PE-Cy7	Phycoerythrin-Cy7
PH	Partial hepatectomy
PLN	Pancreatic lymph node
SDF	Stromal cell-derived factor
SEC	Sinusoidal endothelial cell
SEM	Standard error mean
SSC	Side scatter channel
STAT-1	Signal transduction and transcription-1
STZ	Streptozotocin
T1D	Type 1 diabetes
TdT	Terminal deoxynucleotidyl transferase
TNF- α	Tumour necrosis factor α
Tregs	Regulatory T-cells
TUNEL	Terminal deoxynucleotidyl uridine nick end labelling
UEA-1	Ulex europaeus agglutinin-1
VECAD	VE-Cadherin
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
vWF	von Willebrand Factor
WT	Wild type
XIAP	X-linked inhibitor of apoptosis protein

All other abbreviations are explained in the text.

PRESENTATIONS

1) Conference: 69th Scientific Sessions American Diabetes Association, New Orleans, USA June 2009 (Accepted for poster presentation)

Title: Intracellular inhibition of JNK kinase reduces lymphocyte homing into endocrine pancreas and glucose levels of non obese diabetic (NOD) mouse

C. P. Khoo^{1,2}, N. Hill¹, S. Carotti³, S. Morini³, I. Barchetta^{2,4}, M. G. Valorani^{1,5}, A. Signore⁶, P. Pozzilli^{1,2}; ¹Centre for Diabetes and Metabolic Medicine, Queen Mary University, London, United Kingdom, ² Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³ Department of Biomedical Research (CIR), Università Campus Bio-Medico, Rome, Italy, ⁴ Dipartimento di Clinica e Terapia Medica, La Sapienza University, Rome, Italy, ⁵ Stem Cell Research, Livio Patrizi Foundation, Research Group BIOS, Rome, Italy, ⁶ 2nd Faculty of Medicine, La Sapienza University, Rome, Italy,

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Title: Intracellular inhibition of JNK kinase reduces lymphocyte homing into endocrine pancreas of non obese diabetic (NOD) mouse

C. P. Khoo^{1,2}, N. Hill¹, M. G. Valorani^{1,3}, A. Signore⁴, P. Pozzilli^{1,2}; ¹Centre for Diabetes and Metabolic Medicine, Queen Mary University of London, London, United Kingdom, ²Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³Stem Cell Research Department, Livio Patrizi Foundation, Research Group BIOS, Rome, Italy, ⁴Medicina Nucleare, University La Sapienza, Rome, Italy.

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Title: Isolation, characterization and prospects for use of endothelial progenitor cells to repair pancreatic damage in diabetes

C. P. Khoo^{1,2}, M. G. Valorani^{1,3}, M. Brittan¹, M. R. Alison¹, U. Johansson⁵, P. Pozzilli^{1,2}; ¹Centre for Diabetes and Metabolic Medicine, Queen Mary University, London, United Kingdom, ²Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³Stem Cell Research, Fondazione Livio Patrizi, Rome, Italy, ⁴Flow Cytometry & Imaging, Queen Mary University, London, United Kingdom, ⁵Centre for Haematology, Queen Mary University, London, United Kingdom.

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Title: Isolation, characterization and prospects for use of endothelial progenitor cells to repair pancreatic damage in diabetes

C. P. Khoo^{1,2}, M. G. Valorani^{1,3}, M. Brittan¹, M. R. Alison¹, U. Johansson⁵, P. Pozzilli^{1,2}; ¹Centre for Diabetes and Metabolic Medicine, Queen Mary University, London, United Kingdom, ²Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³Stem Cell Research, Fondazione Livio Patrizi, Rome, Italy, ⁴Flow Cytometry & Imaging, Queen Mary University, London, United Kingdom, ⁵Centre for Haematology, Queen Mary University, London, United Kingdom.

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Title: Isolation, characterization and prospects of endothelial progenitor cells to aid pancreatic beta cell regeneration

C. P. Khoo^{1,2}, M. G. Valorani^{1,3}, M. Brittan¹, M. R. Alison¹, C. Guglielmi², G. Warnes⁴, U. Johansson⁵, M. I. Hawa¹, P. Pozzilli^{1,2};

¹Centre for Diabetes and Metabolic Medicine, Queen Mary University, London, United Kingdom,

²Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³Stem Cell Research,

Fondazione Livio Patrizi, Rome, Italy, ⁴Flow Cytometry & Imaging, Queen Mary University,

London, United Kingdom, ⁵Centre for Haematology, Queen Mary University, London, United Kingdom.

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Title: Isolation, characterization and prospects for use of endothelial progenitor cells to repair pancreatic damage in diabetes

C. P. Khoo^{1,2}, M. G. Valorani^{1,3}, M. Brittan¹, M. R. Alison¹, U. Johansson⁵, P. Pozzilli^{1,2};

¹Centre for Diabetes and Metabolic Medicine, Queen Mary University, London, United Kingdom,

²Area di Endocrinologia, Università Campus Bio-Medico, Rome, Italy, ³Stem Cell Research,

Fondazione Livio Patrizi, Rome, Italy, ⁴Flow Cytometry & Imaging, Queen Mary University,

London, United Kingdom, ⁵Centre for Haematology, Queen Mary University, London, United Kingdom.

PUBLICATIONS

1) **Khoo CP**, Valorani MG, Brittan M, Alison MR, Guglielmi C *et al.*, (2009) Characterization of endothelial progenitor cells in the NOD mouse as a source for cell therapies. *Diabetes Metab Res Rev.* Jan; 25 (1):89-93.

2) **Khoo CP**, Hill N, Carotti S, Morini, S, Barchetta I *et al.*, (2009) JNK kinase inhibition and delayed beta-cell death in the non-obese diabetic (NOD) mouse (In preparation).

3) Yen T, Valorani MG, **Khoo CP**, Brittan M *et al.*, (2009) Interstitial leukocyte infiltration and tubular cell proliferation in the kidneys of non-obese diabetic mice (Submitted to *International Journal of Experimental Pathology*).

4) Valorani, MG, Germani, A, Biddle A, Otto WR, **Khoo CP** *et al.*, (2009) Hypoxia increases production of pure MSCs from Adipose Tissue, Bone Marrow and Pancreatic Tissue (In preparation).

5) **Khoo CP**, Pozzilli P and Alison MR (2008) Endothelial progenitor cells and their potential therapeutic applications. *Regen Med.* Nov; 3 (6): 863-876.

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CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Diabetes is a debilitating condition where the glucose level in the body is high due to impairment of the body's ability to process it properly. This disease affects nearly 250 million people worldwide and its number is estimated to increase to 380 million in approximately 20 years time (The International Diabetes Federation, 2006)(Table1.1.). Diabetes causes various complications such as kidney failure, heart attacks and stroke which have a significant impact on quality of life.

	2007	2025
Total world population (billions)	6.6	7.9
Adult population (age 20-79, billions)	4.1	5.2
WORLD DIABETES (20-70 age group)		
Comparative prevalence (%)	6.0	7.3
Number of people with diabetes (millions)	246	380

Table 1.1 Estimated statistics for type 1 diabetes prevalence worldwide.
(Modified from Diabetes Atlas third edition, International Diabetes Federation; 2009)

Diabetes is a multigenic disorder that is divided into two groups; type 1 and type 2 diabetes. 'Type 1 diabetes (T1D) is a chronic disease that occurs when the pancreas produces too little insulin to regulate blood sugar levels appropriately' (American Diabetes Association, 2003). This form of diabetes occurs approximately in 5-10% of diabetics. T1D is further subdivided into two types, type 1A (immune-mediated diabetes) which involves autodestruction of β -cells and type 1B (idiopathic diabetes) which causes permanent insulin deficiency and up to now its aetiology is still unknown (American Diabetes Association, 2003). Type 2 diabetes (T2D) is the more common form of diabetes, where the peripheral tissues are resistant to insulin due to the insufficient release of insulin by the pancreatic β -cells. Insulin plays a role in maintaining glucose homeostasis, converting excess glucose to glycogen which is stored, until glucose levels in the body drop drastically, which activates the conversion of glycogen back to glucose.

Transplantation therapies which include whole-organ pancreas and islet transplantation have been in practice since the 1990s as evidenced by the successes of treatments using the Edmonton Protocol developed by Shapiro and colleagues (Shapiro *et al.*, 2000). The Edmonton Protocol consists of techniques for the isolation and preservation of islets with steroid-free immunosuppressive treatment for transplant of pancreatic islet cells in patients with diabetes (Kordella, 2003). However, due to an insufficient supply of donor pancreata and islets and reported immune system rejection, researchers now are looking for alternative treatments.

Embryonic stem cells (ESCs) are pluripotent cells which have the ability to give rise to numerous specialised cells. They are capable of unlimited self-renewal and have recently attracted public attention as a viable source for replacement of insulin-secreting cells. The use of ESCs in clinical application is not viable to date as various issues such as teratoma formation and immune rejection might occur when transplanted into patients. Unlike ESCs, adult stem cells are multipotent cells and can only differentiate to a limited range of cell types. Although ESCs seem to have more potential in producing insulin-secreting cells, there were some successes reported where adult stem cells in the rat liver have been cultured under certain conditions and it was found they were able to differentiate into β -cells (Yang *et al.*, 2002). Other extra-pancreatic cell sources for their transdifferentiation into insulin-secreting cells are bone marrow mesenchymal stem cells (Janus *et al.*, 2003; Moriscot *et al.*, 2005), mouse intestinal epithelium (Suzuki *et al.*, 2003) and monocytes from human peripheral blood (Ruhnke *et al.*, 2005).

Another therapeutic strategy that has gained interest in recent years is the promotion of regeneration in the pancreas without the need to worry about immunorejection. Based on the knowledge that there are still some residual functioning β -cells in patients with diabetes (may vary, depends on severity of disease), studies have tried inducing replication and neogenesis of existing β -cells using glucagon-like peptide (GLP-1) or exendin-4 (Rolin *et al.*, 2002; Xu *et al.*, 1999) and reported a significant increase in β -cell mass, reduced apoptosis and improved glucose tolerance in both diabetic rat and mice models. Excessive β -cell destruction by apoptosis and defective autoimmune regulation have been implicated in the pathogenesis of T1D. The infusion of major histocompatibility complex class-1

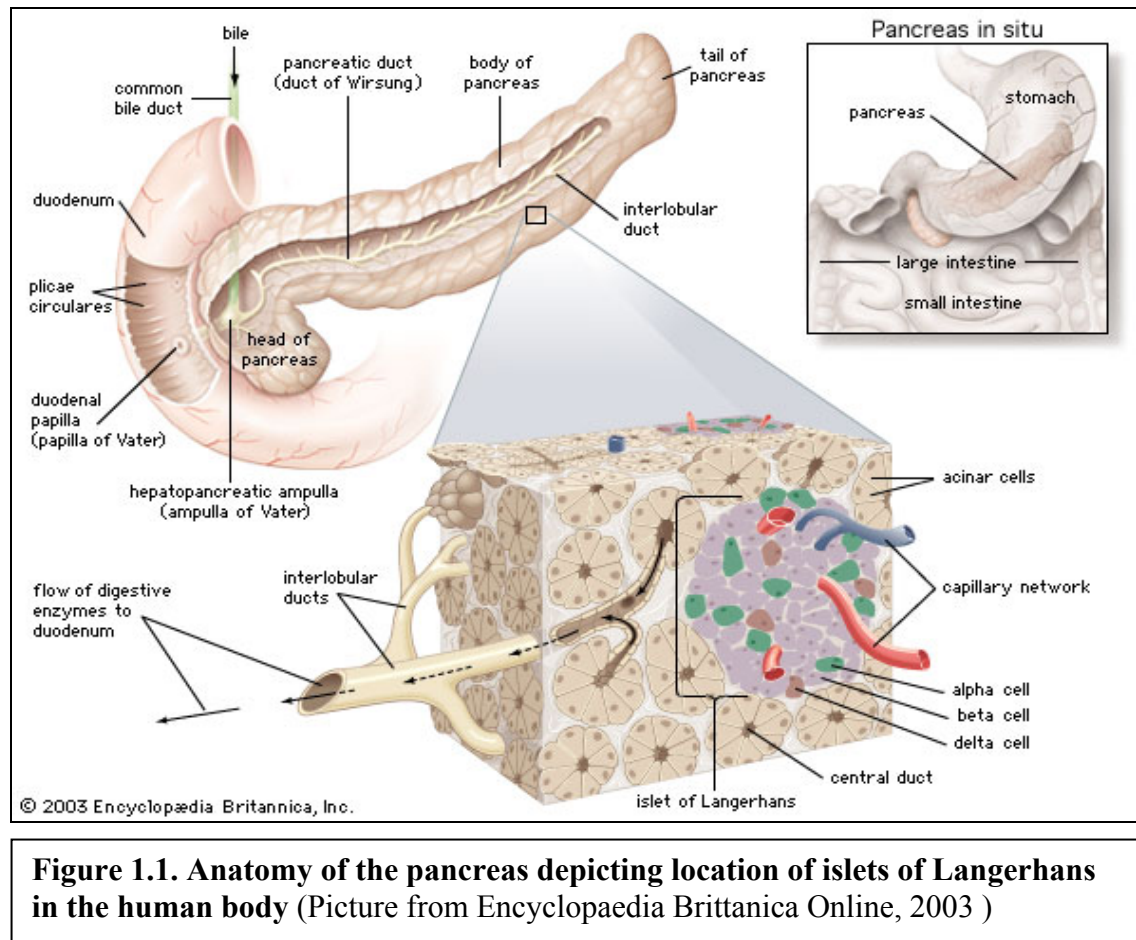
matched spleen cells was shown to attenuate the autoimmune process as well as contribute to islet regeneration, possibly in a paracrine manner (Kodama *et al.*, 2003).

A subset of cells from the bone marrow (BM) called endothelial progenitor cells (EPCs) can contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels i.e. neovascularization (Dome *et al.*, 2006; Hilbe *et al.*, 2004; Mathews *et al.*, 2004). In the STZ-induced model of T1D, several studies have demonstrated that BM-derived EPCs could be stimulated to enter the systemic circulation in response to signals from the pancreas (Hasegawa *et al.*, 2007; Hess *et al.*, 2003; Mathews *et al.*, 2004). Apart from contributing to neovascularization, it is highly likely that BM-derived EPCs have indirectly contributed to islet regeneration by releasing factors that act in a paracrine manner, reducing inflammation and promoting cell survival and proliferation. While this discovery is still under further investigation, it offers hope and brings us a step forward for the treatment of diabetes in the future.

In this thesis, I review and discuss the role and regenerative potential of EPCs in diabetes. Equally important is to understand the mechanisms of β -cell death to prevent excessive β -cell death.

1.2. ANATOMY AND THE EMBRYOLOGY OF THE PANCREAS

The pancreas plays a role in the digestive system. It is divided into two types of tissues; the endocrine and exocrine tissue. Clusters of endocrine cells known as the Islets of Langerhans, are embedded in the exocrine tissue and secrete hormones, including insulin directly into the blood system. The Islets of Langerhans consist of 4 types of cells with β -cells being the majority (Figure 1.1). In contrast, exocrine cells (namely acinar cells) secrete various digestive enzymes. The pancreatic β -cells are important as they secrete insulin which helps maintain blood glucose levels in the body. β -cells also influence the functioning of neighbouring cells *via* autocrine and paracrine interactions (Ishihara *et al.*, 2003).



1.3. STAGES OF PANCREATIC DEVELOPMENT IN MOUSE AND HUMAN

The pancreas is derived from the gut (endodermal germ layer). Pancreatic development in the mouse can be divided into four key stages (Gu *et al.*, 2004): - (i) embryonic day (E) 7.5 where groups of cells envelope the mesoderm and ectoderm forming an ‘unspecified endoderm’; (ii) E10.5 where pancreatic cells start to express the transcription factor *Pdx1*; (iii) E13.5 where endocrine progenitor cells start to express another transcription factor *Ngn3* and (iv) mature islets of Langerhans form (occurs shortly after birth) by aggregation of endocrine cells migrated from the ducts. Pancreatic development in humans however is divided into five distinct stages (Portela-Gomes *et al.*, 1999) :- (i) embryonic day (E) E24 where the dorsal pancreatic bud first appears then the ventral pancreatic bud, a day later (ii) embryonic week (EW) 5-6 where differential growth of the foregut is followed by fusion of dorsal and ventral pancreatic rudiments (iii) EW 10 to EW 15 where endocrine cells start to assemble and form islets; (iv) EW 16 to EW 20 where islets develop into a more vascular form and (v) EW 21 to EW26, period of development of islet innervations.

Golosow and Grobstein (1962) used murine models to propose that the pancreas was developed as a consequence of the interaction between the pancreatic epithelium and mesenchyme and occurs at E10 onwards. It was shown *in vivo* in rodent models that the associated mesenchyme surrounding the pancreatic epithelium modulates the proliferation of immature pancreatic epithelial cells, instructing differentiation into endocrine or exocrine tissue (Polak *et al.*, 2000; Scharfmann, 2000).

Human and mouse pancreatic development follows a general trend; dorsal and ventral bud appear from midgut (Hill, 2005). Pancreatic epithelial cells differentiate into pancreatic β -cells. The continuous development of the pancreas is mainly mediated by active interactions between endoderm and blood vessels (Lammert *et al.*, 2000). The population of insulin-secreting cells rapidly expands and becomes more defined; this indicates the formation of the endocrine pancreas, which eventually leads to the formation of mature islets.

1.4. TYPE 1 DIABETES (T1D)

T1D is an autoimmune disorder with onset occurring mainly in children. Its onset is due to both genetic and external factors where the exposure of a genetically susceptible individual to specific allergens triggers the body's immune response to immunologically destroy pancreatic β -cells (Fig. 1.2). Islet cell autoantibodies form, demonstrating the initial stage of β -cell destruction. The loss of the first phase of insulin secretion follows and β -cell destruction progresses (Imagawa *et al.*, 1999). The immunologic destruction of pancreatic β -cells results in a significant reduction in β -cell mass, preventing the maintenance of normal blood glucose levels due to the inadequate amount of insulin being secreted. Currently, patients with T1D are treated with insulin injections (Zimmet *et al.*, 2001).

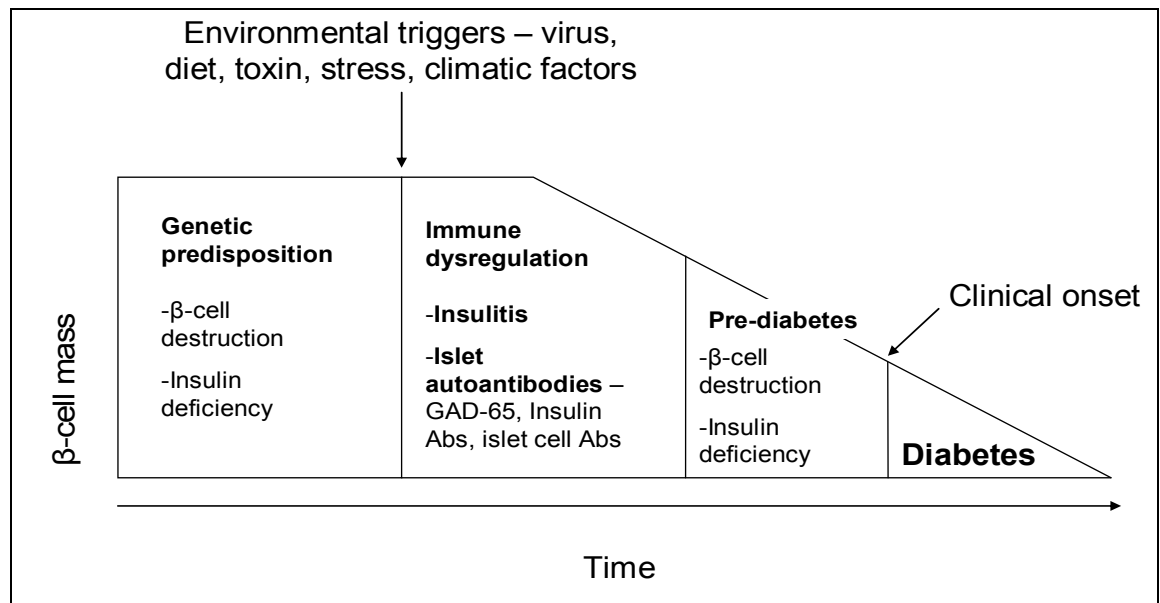


Figure 1.2. Natural history of Type 1 diabetes (adapted from Tisch and McDevitt, 1996)

The pathogenesis of autoimmune T1D is complex and involves the interplay between genetics and environment (Tisch and McDevitt, 1996) (Figure 1.2). Various genes have been implicated in different stages of T1D development including centrally located MHC-HLA class II. In the 1970's, candidate gene studies have identified HLA on chromosome 6 and it was the first locus shown to be associated with T1D (Cudworth and Woodrow, 1975; Nerup *et al.*, 1974), contributing half of the familial basis of T1D (Todd, 1995). The second most important genetic susceptibility factor is the insulin gene on chromosome 11 contributing 10% of susceptibility to T1D (Bell *et al.*, 1984; Gillespie, 2006). In addition, environmental factors play a role in the development of T1D. Some environmental factors that have been implicated include infectious agents (viruses-congenital rubella, enteroviruses), early life factors (cow's milk protein, vitamin D deficiency, rapid growth) and toxins, vaccinations, stress and climatic influences (Dahlquist, 1997; Knip and Akerblom, 1999).

1.5. β -CELL DESTRUCTION

T1D is characterized by selective destruction of β -cells by the immune system, which results in insufficient insulin production to regulate blood glucose level leading to hyperglycaemia. Immunodysregulation in T1D leads to the development of autoantibodies against islet cell components such as glutamic acid decarboxylase antibodies (GAD-65), insulinoma antigen 2 (IA-2), insulin autoantibodies (IAA). The expression of autoantibodies are good markers for prediction of T1D in first degree relatives (Verge *et al.*, 1996), reflecting the magnitude of ongoing β -cell destruction (Lernmark, 1999; Maclaren *et al.*, 2003)

1.5.1. Autoimmunity

T1D occurs in early life due to rapid immune-mediated loss of β -cells. Early studies using spontaneous models of T1D such as the non-obese diabetic (NOD) mouse and the BioBreeding (BB) diabetes-prone rat revealed that T1D is caused by infiltration of T- and B-lymphocytes, a process commonly known as insulinitis (Yang and Santamaria, 2006). This is followed by active destruction of β -cells which leads to diabetes onset. Based on a vast number of studies on the pathogenesis of diabetes, there is consensus that the progression of autoimmune β -cell destruction is a T-lymphocyte-mediated event, where CD8⁺ cells initiate the process while CD4⁺ lymphocytes mediate the islet destructive process (Dotta *et al.*, 2005). Macrophages and/or dendritic cells have also been implicated in the insulinitis stage in the NOD mouse and BB rat (Dotta *et al.*, 2005) (Figure 1.3, Table 1.2).

Characteristics	Human T1D	NOD	STZ- induce (low dose)	BB-rat
Sex predominance	Female = Male	Female >>Male	Female = Male	Female = Male
Insulinitis	Medium severity, mainly T-cells	Destructive, T-cells, macrophages /dendritic cells	Non-specific, T-cells	Destructive, macrophages /dendritic cells
Diabetes onset	Spontaneous	Spontaneous	Induced	Spontaneous
Successful intervention therapies	In progress	Yes	Yes	Yes

Table 1.2. Summary of the characteristics of human T1D and animal models (the NOD mouse model, STZ-induced diabetes (low-dose) and BB-rat.

(Abbreviations: T1D, type 1 diabetes; NOD, non-obese diabetic; STZ, streptozotocin; BB-rat, BioBreeding-rat)

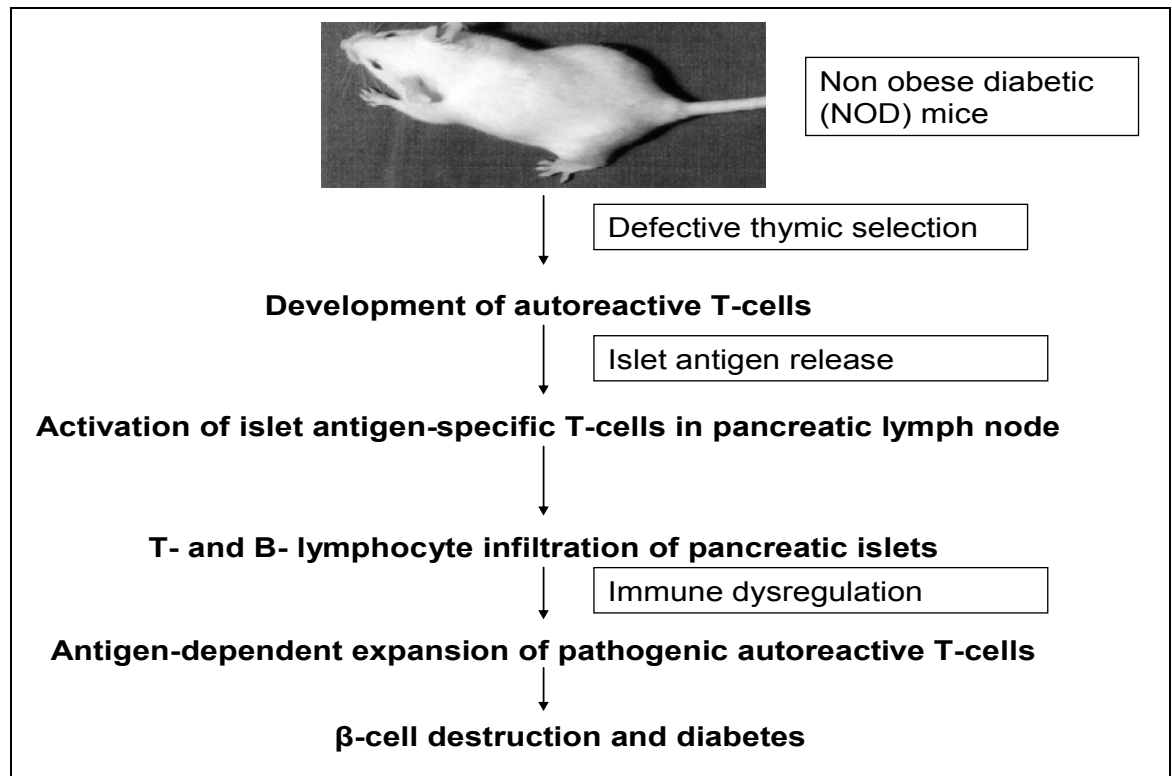


Figure 1.3. Autoimmune response in the NOD mouse (adapted from Yang and Santamaria, 2006)

Environmental factors such as viral infection and stress can trigger the start of β -cell autoimmunity. In the NOD mouse, macrophages and dendritic cells or antigen-presenting cells (APCs) are recruited to the periphery of pancreatic ducts and islets cells around weaning (Homo-Delarche and Drexhage, 2004) (Figure 1.3, 1.4). APCs ingest the β -cell antigens and migrate to the pancreatic lymph node (Mathis *et al.*, 2001). Subsequently, APCs display β -cell antigens to naive β -cell reactive T-cells and activate them. Activated T-cells migrate into the pancreas and re-encounter β -cell antigen marking the initiation of insulinitis. Infiltration of T-cells around islets progresses with age, releasing cytokines and other soluble death mediators.

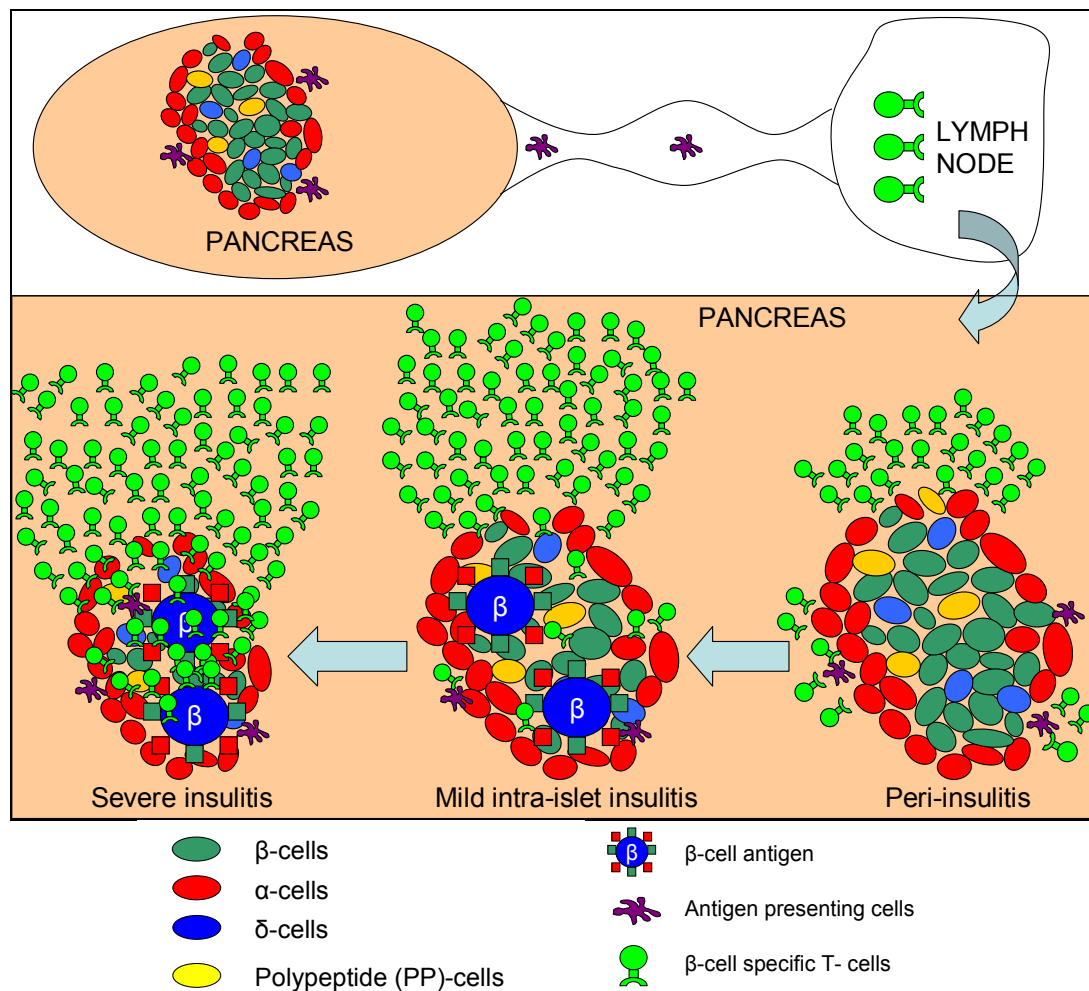


Figure 1.4. Proposed scheme for the initiation of T1D (Adapted from Kaufman, 2003). Environmental factors trigger the start of β -cell autoimmunity in genetically susceptible individuals. β -cell-specific antigens are released and subsequently, T-lymphocytes are mobilized that specifically recognize these antigens resulting in widespread β -cell killing in several stages; peri-insulinitis, intra-islet insulinitis and finally severe insulinitis.

Immunological abnormalities observed in diabetic NOD mice and humans could contribute to the autoimmune process of diabetes, such as dysfunctional natural killer and regulatory T-cells (Brusko *et al.*, 2005; Lindley *et al.*, 2005; Shi *et al.*, 2001). It is further demonstrated in NOD mice that they have a decreased number of helper T-cells (Th1 and Th2 cells), an imbalanced ratio of Th1 effector to Th2 suppressor cells and defective APC mediated co-stimulation that can affect onset and rate of diabetes development (Salomon *et al.*, 2000; Trembleau *et al.*, 2003). In T1D, there is a loss of self-tolerance believed to be

due to defective antigen presentation by APCs to T-lymphocytes resulting in inappropriate T-lymphocyte activation (Kubach *et al.*, 2005).

1.5.2. Apoptosis

Apoptosis is a form of programmed cell death, important in developmental remodelling and/or to maintain homeostasis of the β -cell mass (Trudeau *et al.*, 2000). Many studies to date have suggested that apoptosis is probably the main form of β -cell death in T1D causing gradual β -cell depletion (Reviewed in (Cnop *et al.*, 2005; Eizirik and Mandrup-Poulsen, 2001). β -cell death by apoptosis is easily identifiable, characterized by plasma membrane blebbing, cytoplasmic and organelle contraction and shrinkage, nuclear chromatin condensation and DNA fragmentation/cleavage (Grodzicky and Elkon, 2002). Apoptosis is a multi-step process involving :- (i) induction stage; initiated by extracellular death signals; (ii) effector phase; activation of one or more signal transduction pathways and (iii) degradation phase; where signal transduction pathways converge to a few final death pathways carried out usually by proteases-caspases and caspase-activated DNase (Dotta *et al.*, 2005).

Several signalling pathways have been implicated in apoptosis, mainly divided into (i) extrinsic pathway (death-receptor induced pathway) (ii) intrinsic pathway (reviewed in Grodzicky and Elkon, 2002). The extrinsic pathway is stimulated by death receptor engagement such as Fas and Fas ligand binding which initiates a signalling caspase cascade. Cell surface receptor Fas and Fas ligand (FasL) are expressed on β -cells and CD8+ T-cells respectively (Kawasaki *et al.*, 2004). Once FAS is activated upon binding of FasL, Fas trimerizes and recruits Fas-associated death domain (FADD) to its cytoplasmic part forming a death-inducing signalling complex (DISC). DISC associates with caspase-8 which subsequently gets activated and released, initiating the caspase cascade (Emamaullee and Shapiro, 2006).

The intrinsic pathway occurs when various apoptotic stimuli such as DNA damage or hypoxia activates pro-apoptotic proteins such as Bax and Bad. These translocate to the mitochondria, binding or inactivating anti-apoptotic Bcl-2 proteins (Bcl-2 or Bcl-xl). Bax

and Bad can form pores in the mitochondria membrane, releasing cytochrome c from the mitochondria. Cytochrome c interacts with Apaf-1 and caspase-9 to promote the activation of caspase-3.

1.5.3. Cytokine induced apoptosis

Under basal conditions, apoptosis plays a role in eliminating activated B-and T-cells beyond the course of an infection, to terminate immune responses. Defects in apoptosis regulatory mechanisms have been implicated in T1D pathogenesis, causing excessive apoptosis of β -cells of the pancreas (Mauricio and Mandrup-Poulsen, 1998). Some of the defects that have been reported include defective regulation of apoptosis in lymphoid cells (Watanabe-Fukunaga *et al.*, 1992), and defects in surface expression of the Fas receptor in B-and T-cells (Giordano *et al.*, 1995).

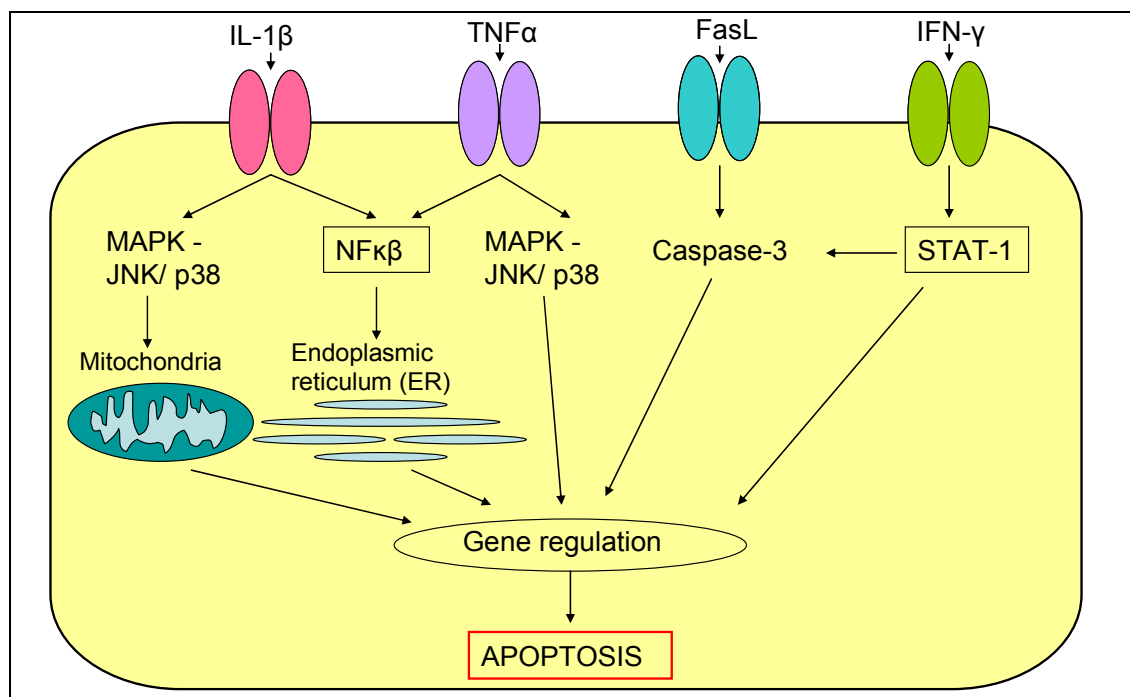


Figure 1.5. Proposed model of cytokine-induced apoptosis. During insulinitis, infiltration of immune cells produces inflammatory cytokines such as IL-1 β , IFN- γ and TNF- α which in turn induce β -cell apoptosis *via* the activation of β -cell gene network under the control of transcription factors such as NF κ B and STAT-1. β -cell death is finally executed by the activation of MAPK (JNK or p38) *via* release of mitochondrial death signals and ER stress trigger (Abbreviations: MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; STAT-1, signal transduction and transcription-1) (adapted from Cnop *et al.*, 2005)

Members of the JNK family, JNK2 and JNK1 have also been implicated in T2D and T1D progression respectively (Jaeschke *et al.*, 2005). Extracellular stimuli such as growth factors, cytokines and radiation activate the JNK pathway (Tawadros *et al.*, 2005). Activated JNK can phosphorylate transcription factors such as c-Jun that play a critical part in controlling the expression of genes involved in apoptosis (Figure 1.5).

1.5.4. β -cell apoptosis and T1D autoimmunity

Apoptosis and autoimmunity in T1D are traditionally thought to initiate destruction of β -cells as two separate entities. However there is increasing evidence of a relationship between apoptosis and autoimmunity.

a) Apoptosis in the initiation or triggering of immune activation

Apoptosis has traditionally been thought to be a non-inflammatory process. However it has been demonstrated that apoptosis can initiate β -cell directed autoimmunity, followed by uptake of β -cell antigens by islet APCs, inducing the formation of autoantibodies against such as GAD-65, a known target in young NOD mice (Tian *et al.*, 2001). In NOD mice, apoptosis of β -cells initiates antigen uptake by APCs, activating T-cells in pancreatic lymph node. Immune cells accumulate around islets, invade islets and spread, destroying β -cells (Mathis *et al.*, 2001). Trudeau *et al.* demonstrated that a neonatal wave of β -cell apoptosis occurring around 2-3 weeks of age in BB rats and NOD mice causes these apoptotic cells to display autoreactive antigens in their surface blebs eventually leading to more β -cell apoptosis and insulinitis (Trudeau *et al.*, 2000). A higher incidence of β -cell apoptosis was observed in both BB rats and NOD mice compared to different strains that do not develop diabetes.

b) Massive destruction of the islets

Apoptosis is also involved in the direct destruction of islet β -cells through contact dependent CD8 T-cells, Fas/FasL and cytokine mediated pathways. In the NOD mouse and BB rat, macrophages and/or dendritic cells interact with T-cells and infiltrate the

pancreatic islets leading to an inflammatory response releasing proinflammatory cytokines – interleukin 1 β (IL-1 β), tumour necrosis factor α (TNF- α) and interferon γ (IFN- γ). The high concentration of cytokines in the islets leads to the induction of apoptotic signalling cascades such as perforin/granzyme and Fas-mediated apoptosis which cause massive destruction of β -cells (Cnop *et al.*, 2005; Dotta *et al.*, 2005)

Defects in apoptosis regulatory mechanisms have been implicated in T1D pathogenesis, causing excessive apoptosis of β -islet cells of the pancreas. Activation of native T-cells results in massive expansion of Ag-specific T-cells, the majority of which die by natural apoptosis and the remaining cells may survive and differentiate into memory cells. The excessive apoptosis due to a defect in the regulation of apoptosis may trigger an autoimmune reaction through presentation of autoantigens to the lymphocytes and production of autoreactive antibodies (Božič and Rozman, 2006). The failure of β -cells to clear apoptotic fragments is another key pathogenic factor leading to diabetic autoimmunity (Savill, 2000).

1.5.5. Current strategies to block β -cell destruction

a) Strategy 1: Direct inhibition of death receptor interaction

Studies have shown that the Fas-mediated cytotoxicity pathway is one of the common inducers of β -cell apoptosis in humans and the NOD mouse model (Grewal *et al.*, 1996; Itoh *et al.*, 1997). Fas-Fas ligand interaction initiates a signalling cascade mediated by caspase-8 or Fas-associated death domain–like interleukin 1 β -converting enzyme (FLICE) activation. In 1990s, cellular FLICE-inhibitory protein (FLIP) was developed which functions by blocking protein-protein interaction between caspase-8 and FADD, thus preventing the activation of caspase-8 and downstream caspases (Irmeler *et al.*, 1997). Newly diagnosed T1D patients have increased surface expression of Fas (CD95) on β -cells where the apoptotic signal is delivered *via* FasL (CD95L) present on infiltrating CD8+T-cells. Neutralizing antibodies to Fas (CD95) successfully block apoptosis and preserve β -cells function (Allison *et al.*, 2005).

The overexpression of Bcl-2 in human islets results in functioning insulin secretion and full protection from IL-1 β , TNF- α , IFN- γ cytokine induced β -cell destruction (Rabinovitch *et al.*, 1999). Another important gene target is cytokine response modifier A (CrmA) where NOD transgenic mice with target expression of CrmA have reduced spontaneous development of autoimmune diabetes, and pancreatic β -cells were protected from cytokine-mediated cytotoxicity. In addition, β -cells from NOD CrmA mice were significantly protected from the destruction by diabetogenic T-cells after adoptive transfer. During apoptosis, anti-apoptotic Bcl-2 proteins such as Bcl-X_L are inactivated by Bax. Klein *et al.* showed that Bcl-X_L is effective in preventing apoptosis triggered by IL-1 β , staurosporine and serum withdrawal *in vitro* (Klein *et al.*, 2004).

b) Strategy 3: Combination of inhibiting extrinsic and intrinsic pathways

The majority of inhibitors developed so far, specifically target either the extrinsic or intrinsic pathway, but do not inhibit both pathways due to difficulties in efficiently and reproducibly delivering and regulating multiple genes at the same time. Recently, a potent inhibitor of apoptosis was developed known as X-linked inhibitor of apoptosis protein (XIAP), and it was demonstrated that its overexpression could block cell apoptosis beyond the convergence point of most apoptotic pathways. XIAP acts by binding to the active site of all the main effector caspases (caspase 3, 7, and 9), thus preventing apoptosis (Schimmer, 2004; Schratzberger *et al.*, 2001).

c) Strategy 4: Modulation of cytokine signalling

Studies have shown that two groups of cytokines exist where one group enhances β -cell destruction such as the Th1 subset of CD4 T-cells and Th1 cytokines; IFN- γ , TNF- α , IL-1, IL-12 and IL-18, whereas the other group inhibits β -cell destruction consisting of Th2 and Th3 cytokines; IL-4, IL-5, IL-10 and TGF- β . Administration of antigen-specific regulatory T-cells (Tregs) can induce tolerance by releasing cytokines that inhibits β -cell destruction (Cernea and Pozzilli, 2008). Candidate autoantigens include insulin, glutamic acid decarboxylase (GAD65) and heat shock protein 60 (HSP60).

e) Strategy 5: Inhibition of JNK-c-Jun interaction

JNK inhibitors have been discovered by high throughput screening of compound libraries and subsequent structure-activity studies and cell culture testing (reviewed by (Bogoyevitch and Arthur, 2008). JNK peptide inhibitors are preferable to the JNK knockout model, due to their reported high specificity in disrupting only the interaction between their target and a small subset of potential binding partners (Waetzig and Herdegen, 2005). JNK inhibitors are divided into two groups (i) ATP-competitive JNK inhibitors which directly interfere and target highly conserved ATP binding sites and, (ii) ATP-non-competitive JNK inhibitory peptides which function by competing with protein substrate specific docking sites. Although these JNK inhibitors are highly specific, their cell permeability and rapid breakdown are problematic. To address these issues, Holzberg *et al.* discovered and developed a cell-permeable peptide that contains the JNK-binding (delta) domain (JBD) of human c-Jun, which selectively inhibits the JNK-c-Jun interaction, thus influencing some of the transcriptional effects of JNKs (Holzberg *et al.*, 2003).

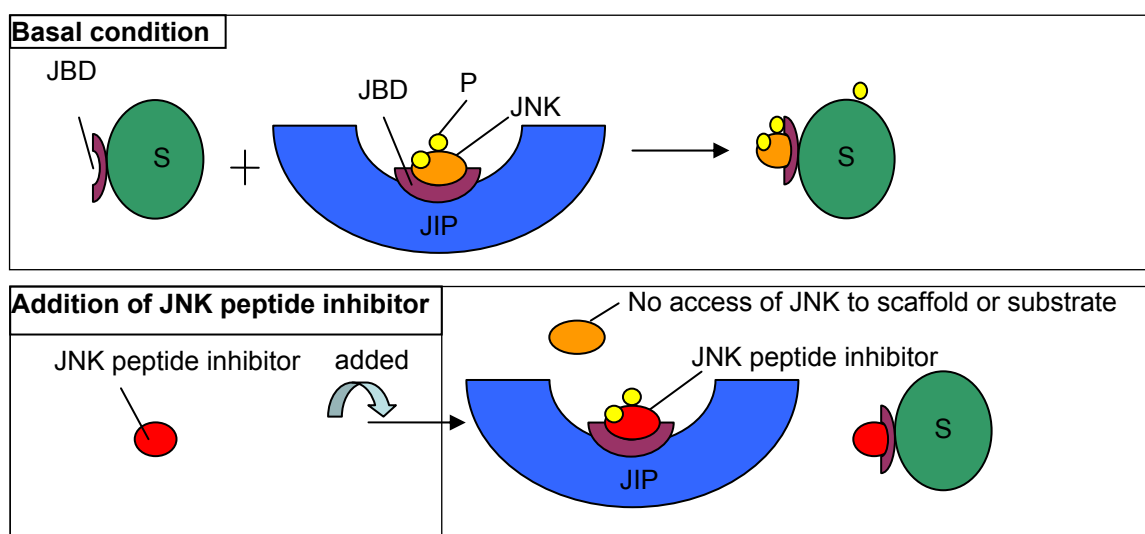


Figure 1..6. Overview of the JNK peptide inhibitor process

- (i) **Basal condition:** Substrate with JBD interacts with activated JIP signal complex causing phosphorylation of substrates.
- (ii) **Addition of JNK peptide inhibitor:** JNK inhibitor enters the cells and competes with JNK preventing JNK to bind to JIP scaffold, thus inhibiting activation of JNK signalling pathway. Simultaneously JNK inhibitor binds to JBD of substrates or upstream kinase preventing phosphorylation of substrates by JNK. Abbreviations: JNK, c-Jun terminal kinase; S, substrate (e.g. c-Jun); P, phosphate; JBD, JNK binding (delta) domain; JIP, JNK-interacting protein (Adapted from (Borsello *et al.*, 2003; Waetzig and Herdegen, 2005)

Extracellular stimuli such as growth factors and cytokines activate JNK, which induces the phosphorylation of transcription factors and cellular substrates involved in cell survival and proliferation, insulin receptor signalling and mRNA stabilization. JNKs bind to and are activated in signal complexes such as JNK-interacting protein (JIP) and subsequently phosphorylate their substrates using the JNK binding domain of the substrate (Figure 1.6). When the cell-permeable JNK peptide inhibitor is added, the binding of JNKs to both their substrates and their scaffolds is blocked. Activation of JNK and the phosphorylation of substrates by JNK are impaired.

1.6. ENDOTHELIAL PROGENITOR CELLS

1.6.1. INTRODUCTION

The successful isolation of endothelial progenitor cells (EPCs) in 1997 by Asahara *et al.* (Asahara *et al.*, 1997) has led to many studies documenting how EPCs derived from the bone marrow (BM) and peripheral blood (PB) contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels i.e. vasculogenesis. EPCs are believed to originate from the BM and are often mobilized into the circulation in response to growth factors and cytokines released following a variety of stimuli that include vascular trauma (Gill *et al.*, 2001), ischaemia (Garcia-Barros *et al.*, 2003; Takahashi *et al.*, 1999), wounding and cancer (Lyden *et al.*, 2001). EPCs home to and incorporate into sites of damage and mediate repair by inducing neovascularization. In addition to the BM and PB, EPCs can also be isolated from umbilical cord blood (Murohara *et al.*, 2000), foetal liver (Peichev *et al.*, 2000) and skeletal muscle (Majka *et al.*, 2003). Traditionally, EPCs were thought to only contribute to neovascularization during embryonic development but recent observations show that EPCs are also involved in postnatal neovascularization through two mechanisms: vasculogenesis, the recruitment of BM-derived EPCs which migrate and differentiate in response to signals to form entirely new blood vessels and angiogenesis, the sprouting of pre-existing capillaries (Taniguchi *et al.*, 2006). EPCs exist in very small numbers especially in circulating blood in adults where they only account for 0.01% of all cells, probably due to a very low endothelial cell turnover rate (Zammaretti and Zisch, 2005).

1.6.2. EPC ORIGIN

EPCs are derived from haemangioblasts, the same precursor cells of haematopoietic stem cells (HSC). During embryogenesis, the differentiation of the vascular endothelium is closely associated with the appearance of HSCs (reviewed in Rumpold *et al.*, 2004). Additionally, HSCs and EPCs share common markers (CD31, CD34, VE-Cad, Tie-2; Table 1.2). In 1997, Asahara isolated EPCs from human peripheral blood using CD34 and other endothelial cell markers (Asahara *et al.*, 1997), suggesting that haemangioblasts are present in blood.

Haemangioblasts differentiate into angioblasts (in blood islands in the yolk sac during embryogenesis) or EPCs (in the adult BM) and haematopoietic precursors. In postnatal neovascularization, EPCs derived from BM can be mobilized into the blood circulation in response to appropriate stimuli. EPCs circulate in the blood (at this stage, known as cEPCs) and either contribute to new blood vessels within pre-existing but expanding capillary networks, or are directed to form entirely new blood vessels that eventually connect to the extant capillary system.

1.6.3. EPC PHENOTYPE

Debate ensues as to the true phenotype of adult EPCs, which is often clouded by the overlap of the phenotype of EPCs with HSCs and endothelial cells, and thus, a universally-accepted EPC phenotype remains to be achieved (Table 1.2). EPCs express Flk-1, von Willebrand factor (vWF), vascular endothelial cadherin (VE-Cadherin), CD31, Tie-2/TEK (angiopoietin-1 receptor precursor or tunica intima EC kinase), Tie-1, c-Kit, CD34, CD133, Sca-1 and Flt-1 (Asahara *et al.*, 1997; Drake and Fleming, 2000; Grant *et al.*, 2002; Hess *et al.*, 2003; Jackson *et al.*, 2001; Loomans *et al.*, 2006; Peichev *et al.*, 2000; Sato *et al.*, 1993; Sato *et al.*, 1995; Wu *et al.*, 2005). EPCs overlap in phenotype with a variety of vascular cell stages extending from primitive haemangioblasts to fully differentiated endothelial cells.

Haematopoietic stem cells (HSCs)	Mature endothelial cells
VEGFR-2, Flk-1 (H,M) (Haruta <i>et al.</i> , 2001; Ziegler <i>et al.</i> , 1999)	VEGFR-2, Flk-1 (M,H) (McCloskey <i>et al.</i> , 2006; Nguyen <i>et al.</i> , 2008)
CD34 (M) (Baldwin <i>et al.</i> , 1994)	CD34 (H) (Peichev <i>et al.</i> , 2000)
CD45 (M) (Shaw <i>et al.</i> , 2004)	CD45 (H) (Rogers <i>et al.</i> , 2007)
Tie-1 (M) (Puri and Bernstein, 2003)	Tie-1 (M,H) (Nguyen <i>et al.</i> , 2008; Sato <i>et al.</i> , 1993)
Tie-2 (M) (Hsu <i>et al.</i> , 2000)	Tie-2 (M,H) (Nguyen <i>et al.</i> , 2008; Sato <i>et al.</i> , 1995)
vWF (H) (Hill <i>et al.</i> , 2003; Peichev <i>et al.</i> , 2000)	vWF (S, H) (Kaushal <i>et al.</i> , 2001; Quirici <i>et al.</i> , 2001)
AC133/CD133 (H) (Gallacher <i>et al.</i> , 2000; Yin <i>et al.</i> , 1997)	AC133/ CD133 (S, H) (Kaushal <i>et al.</i> , 2001; Quirici <i>et al.</i> , 2001)
c-kit (M) (Simmons <i>et al.</i> , 1994)	VE-Cadherin (H) (Peichev <i>et al.</i> , 2000)
Sca-1 (M) (Grant <i>et al.</i> , 2002; Spangrude and Scollay, 1990)	Sialomucin CD146 (H)(S-endo, P1H12Ag)(Kaplan <i>et al.</i> , 2005)
Flt-1/VEGFR-1 (M) (Solovey <i>et al.</i> , 2001)	CD31/PECAM-1(S, H) (Kaushal <i>et al.</i> , 2001; Quirici <i>et al.</i> , 2001)
Endothelial progenitor cells (EPCs)	
VEGFR-2, Flk-1 (M, H)	(Balasubramaniam <i>et al.</i> , 2007; Peichev <i>et al.</i> , 2000)
Tie-1 (M)	(Sato <i>et al.</i> , 1993; Sharpe <i>et al.</i> , 2006)
CD34 (H, M)	(Asahara <i>et al.</i> , 1997; Nguyen <i>et al.</i> , 2008)
VE-Cadherin (M)	(Drake and Fleming, 2000; Sharpe <i>et al.</i> , 2006)
CD133 (H, M)	(Asahara <i>et al.</i> , 1997; Canizo <i>et al.</i> , 2007)
c-kit (M)	(Loomans <i>et al.</i> , 2006)
Sca-1 (M)	(Balasubramaniam <i>et al.</i> , 2007)
VEGFR-1 (M, H)	(Loomans <i>et al.</i> , 2006)
CD31/PECAM-1 (M, H)	(Hess <i>et al.</i> , 2003; Ye <i>et al.</i> , 2007)
vWF (M)	(Wang <i>et al.</i> , 2008)
Tie-2 (M,H)	(Sato <i>et al.</i> , 1993; Sharpe <i>et al.</i> , 2006)
CD45 (M, H)	(Balasubramaniam <i>et al.</i> , 2007; Case <i>et al.</i> , 2007)

Table 1.3. Cell surface markers of mature endothelial cells, HSCs and EPCs
(Abbreviations: H, human; M, mouse; S, sheep; HSCs, Haematopoietic stem cells; EPCs, Endothelial progenitor cells)

EPCs have also been characterised by their ability to form colonies (colony-forming units) in endothelial specific medium, endocytose acetylated low-density lipoprotein (acLDL), bind Ulex Europaeus-1 (UEA-1) lectin, form tubules and migrate in cell culture (Hill *et al.*, 2003; Kalka *et al.*, 2000; Mukai *et al.*, 2008; Peichev *et al.*, 2000). Another approach commonly used to isolate EPCs in culture is by initial enrichment of cells for a progenitor stem cell marker such as CD133, which is not expressed by mature endothelial cells (Mizrak *et al.*, 2008).

Some studies isolate EPCs based on the outgrowth derived from human PB in culture, known as early and late EPCs (Hur *et al.*, 2004; Lin *et al.*, 2000). These two populations of EPCs share the same endothelial phenotype (CD31, vWF and VEGFR-2) but have a different morphology, growth pattern and survival rate. The early EPCs are spindle-shaped and can be seen after 5-7 days culture whereas late EPCs adopt a cobblestone morphology after 12-21 days in culture (Hur *et al.*, 2004). Mukai *et al.* tested the tubule forming potential of early EPCs from human PB-MNCs and late EPCs from umbilical cord blood-MNCs using an ear vessel occlusion model; the late EPCs were able to form new blood vessels after transplantation into occlusion tissue whereas early EPCs could not, suggesting that late EPCs are more suitable for use in EPC transplantation to promote neovasculogenesis (Mukai *et al.*, 2008).

EPCs can be characterized based on their size and antigen expression (Yamamoto *et al.*, 2008). Using 2D-dot plots and fluorescent activated cell sorting (FACS) of MNCs differentiating towards the endothelial lineage, two populations were defined as small and large EPCs; small EPCs were seen after 1 day of culture and were CD34⁺CD14⁻ whereas large EPCs were only noted between 3-7 days of culture with a CD34⁻CD14⁺ phenotype. Unfortunately in this study, the ability of the two populations to contribute towards neovascularization was not tested.

To date, the lack of defined techniques to characterise human and mouse EPCs has led to confusion as to the true EPC phenotype and the isolation of functionally suboptimal EPC populations, most likely attributable to differing culture conditions, characterization techniques and sources (Bischoff *et al.*, 1997; Kraling *et al.*, 1996; Nishiwaki *et al.*, 2007). It is therefore vital to set a 'gold standard' to universally define EPCs in order to utilise them for the advancement of future clinical therapies.

1.6.4. EPC MOBILIZATION

EPCs are mobilized by various stimuli such as tissue injury, ischaemia and hypoxia. These conditions can lead to tissue necrosis in which the dying cells release stimulatory factors such as FGF-2 to promote blood-vessel growth. Apart from contributing to neovascularization, it is highly likely that BM-derived EPCs contribute to the regeneration of the damaged tissue by releasing factors that act in a paracrine manner, reducing inflammation and promoting cell survival and proliferation of tissue-residing progenitor cells (Hess *et al.*, 2003; Ye *et al.*, 2007).

Upon tissue injury, an ischaemic environment is created. The ischaemic tissue upregulates hypoxia inducible factor-1 α , which in turn stimulates the production of VEGF and SDF-1, important EPC chemoattractants (Asahara *et al.*, 1999; Heissig *et al.*, 2002; Takahashi *et al.*, 1999). These growth factors act synergistically to mobilize EPCs into the circulation by first activating matrix metalloproteinase (MMP)-9 in the BM (Figure 1.7) (Heissig *et al.*, 2002; Rafii *et al.*, 2002). Activated MMP-9 cleaves membrane-bound Kit ligand on c-Kit⁺ cells and releases soluble kit ligand. c-Kit⁺ progenitor stem cells move from the quiescent to the proliferative niche (Aicher *et al.*, 2005).

At the site of injury, recruited macrophages and fibroblasts release VEGF, which induces phosphorylation and activation of endothelial nitric oxide synthase (eNOS), expressed by osteoblasts and endothelial cells (Aicher *et al.*, 2003). eNOS is important in the BM microenvironment, especially in the maintenance and regulation of EPC mobilization in response to signals such as VEGF. eNOS elevates nitric oxide (NO) levels in the BM (Brem and Tomic-Canic, 2007), facilitating the release of EPCs from BM into the bloodstream (Figure 1.7). Once in the circulation, EPCs are recruited to the site of injury in response to injury-induced chemokines, such as SDF-1, released from the damaged organ (Kucia *et al.*, 2004). In diabetes, this compensatory response of increased EPC mobilization is inadequate and fails to promote therapeutic EPC-mediated neovascularization. Hyperoxia *via* hyperbaric oxygen therapy (HBO) can reverse this EPC mobilization defect by increasing BM nitric oxide levels (Gallagher *et al.*, 2007). The combination of exogenous administration of an EPC mobilization factor such as SDF-1 α

and HBO therapy in streptozotocin (STZ)-induced diabetic mice synergistically increased EPC number, and promoted homing into the wounded area (Gallagher *et al.*, 2007).

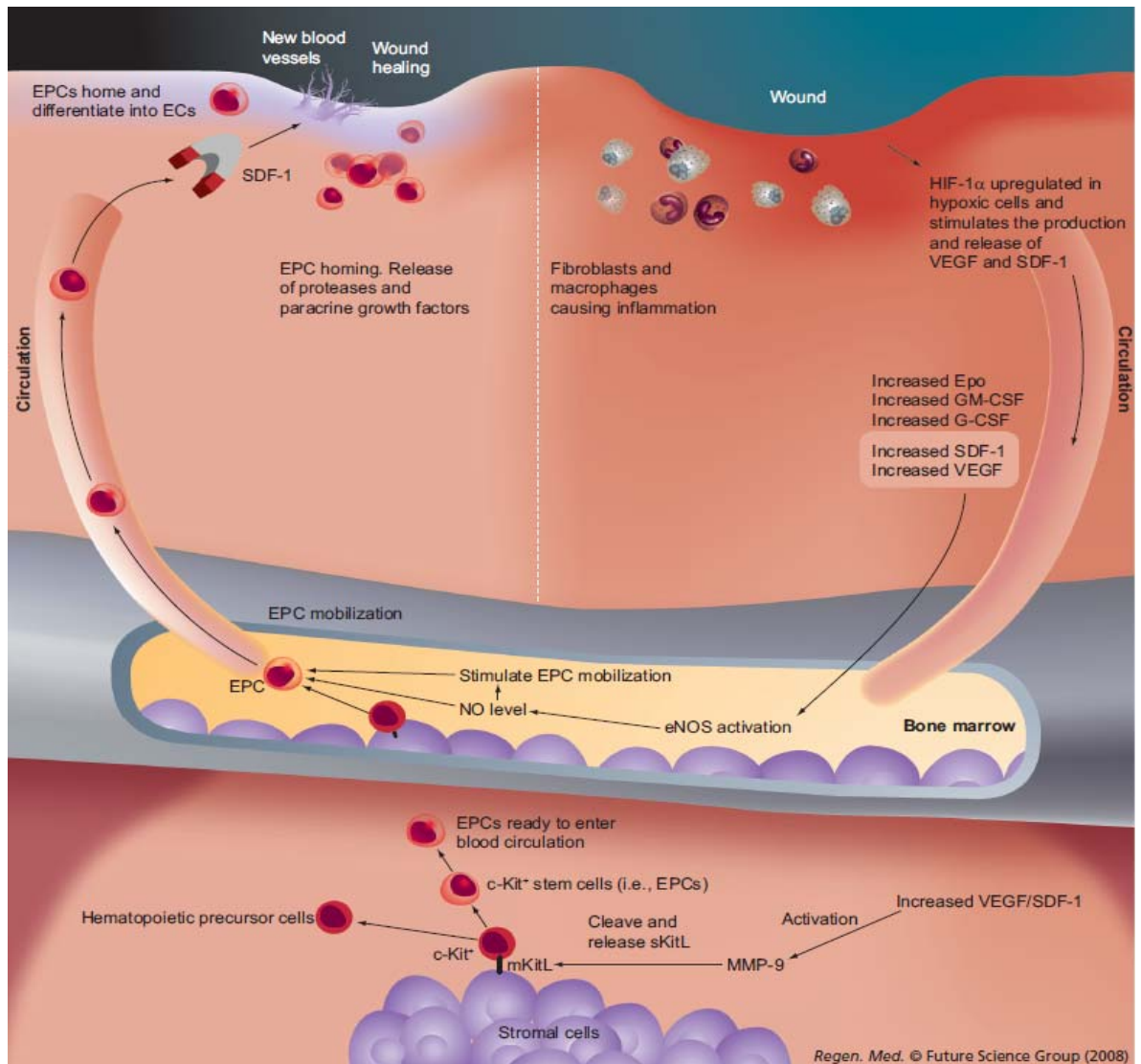


Figure 1.7. EPC mobilization and recruitment during wounding. Wounding attracts fibroblasts, neutrophils and macrophages and causes inflammation. Hypoxic signals in the wound upregulate mobilizing growth factors. These growth factors activate eNOS, increasing NO levels which in turn regulate the release of EPCs into the circulation. Mobilized EPCs home to the wound site. In the wound site, EPCs differentiate to endothelial cells in response to signals and can form entirely new blood vessels as well as contributing to sprouting capillaries (angiogenesis). In addition, EPCs may contribute to the regeneration of the wound by secreting factors that act in a paracrine manner, reducing inflammation and promoting cell survival and proliferation. EC: Endothelial cell; eNOS: Endothelial nitric oxide synthase; EPC: Endothelial progenitor cell; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte macrophage colony-stimulating factor; HIF: Hypoxia-inducible factor; MMP: Matrix metalloproteinase; NO: Nitric oxide; SDF: Stromal cell-derived factor (Picture from Khoo CP *et al.*, 2008).

1.6.5. EPC TRANSPLANTATION

EPCs can be mobilized through exposure to a variety of growth factors, including VEGF, granulocyte macrophage colony-stimulating factor (GM-CSF), SDF-1, PLGF and erythropoietin (Epo). Epo, a factor known to promote mature endothelial cell proliferation and migration, has been used to stimulate HSC proliferation and enhance mobilization of EPCs from the BM of ischaemic limb mice (Heeschen *et al.*, 2003). Numbers of cEPCs were increased after Epo treatment and were mainly early outgrowth EPCs. Owing to their potential to promote mobilization *in vivo*, growth factors such as PLGF and HGF have been administered concurrently with the transplantation of BM- or PB-derived EPCs to augment neovascularization in models of emphysema and hindlimb ischaemia (Table 1.3) (Ishizawa *et al.*, 2004; Tamarat *et al.*, 2004).

BM- and PB-derived EPCs have the potential to augment neovascularization when transplanted into disease models (Table 1.3). EPCs differentiate into endothelial cells and incorporate into growing vessels (angiogenesis) and can create new vessels (vasculogenesis). There is an ongoing debate as to whether EPCs can actually create new blood vessels on their own, as there is evidence of cooperation between endothelial cells and perivascular cells in during vascular development (Darland and D'Amore, 1999). Recently Au *et al.* (Au *et al.*, 2008) and Melero- Martin *et al.* (Melero-Martin *et al.*, 2008) noted that co-implantation of perivascular cells and cultured EPCs resulted in robust vascular networks *in vivo* (Au *et al.*, 2008; Melero-Martin *et al.*, 2008). When EPCs were transplanted alone, both adult PB and CB-derived EPCs were still capable of vasculogenesis, but the process was less pronounced and the vessels rapidly regressed.

Therapeutic neovasculogenesis represents a new approach to treat patients with vascular disease. Many studies have demonstrated successful EPC-derived cell engraftment and a contribution to neovasculogenesis in adult tissues, a process enhanced by increasing the regenerative demand within a diseased or damaged tissue (Table 1.3).

Treatment strategy	Animal /Model used	Result	Reference
Cell mobilization <i>in vivo</i>			
HMG-CoA inhibitor (simvastatin)	Corneal injury after BM transplantation (M)	Augmented corneal neovascularization.	(Llevadot <i>et al.</i> , 2001)
Transplantation of <i>ex vivo</i> cultured EPCs			
CD31 ⁺ CD105 ⁺ CD45 ⁻ (CB, H)	Flap ischaemia model (M)	Increased neovasculogenesis and tissue repair.	(Nagano <i>et al.</i> , 2007)
7 day cultured MNCs (spleen, M)	Carotid artery injury model (M)	Re-endothelialization of denuded carotid artery inhibition of neointimal hyperplasia.	(Zhao <i>et al.</i> , 2007)
10 day cultured MNCs (BM, R)	Dimethylnitrosamine-induced liver fibrosis model (R)	Hepatic fibrogenesis was suppressed. Normal liver function regained and increased proliferation of hepatocytes.	(Ueno <i>et al.</i> , 2006)
10 day cultured MNCs (PB, H & M)	Carbon tetrachloride-induced acute liver injury (M)	Improved survival of mice following liver injury. Greater proliferation of hepatocytes.	(Taniguchi <i>et al.</i> , 2006)
Transplantation of <i>ex vivo</i> cultured EPCs supplemented with growth factor			
Fresh MNCs with placental growth factor (BM, M)	Hindlimb ischaemic model (using diabetic mice, streptozotocin-induced) (M)	Improved postischaemic neovascularization	(Tamarat <i>et al.</i> , 2004)
Transplantation of <i>ex vivo</i> cultured EPCs pre-treated with growth factor			
Sca-1 ⁺ Flk-1 ⁺ c-Kit ⁺ cells (BM, M) treated with G-CSF	Emphysema (M)	Significant reduction in emphysema. Augmented number of BM-derived EPCs in regenerated alveoli	(Ishizawa <i>et al.</i> , 2004)
Freshly isolated CD34 ⁺ MNCs (PB, H) pre-treated with G-CSF	Myocardial ischaemia (R)	Decreased apoptosis of hypertrophied myocytes in the peri-infarct region. Long-term salvage and survival of viable myocardium.	(Kocher <i>et al.</i> , 2001)
Transplantation of gene-modified EPCs			
EPC transduced with VEGF (PB, H)	Hindlimb ischaemic model (M)	Increased neovascularization & blood flow recovery	(Iwaguro <i>et al.</i> , 2002)
EPC transduced with eNOS (PB, Ra)	Neointimal hyperplasia (Ra)	Augmented endothelialization & reduced neointima size	(Kong <i>et al.</i> , 2004)
EPC transduced with human telomerase reverse transcriptase (PB,H)	Hindlimb ischaemic model (M)	Augmented neovascularization in terms of limb salvage and increase capillary density.	(Murasawa <i>et al.</i> , 2002)

Table 1.4. Examples of endothelial progenitor-derived cells to vascular lineages in various models of disease. (Abbreviations: MNCs, mononuclear cells; EPCs, endothelial progenitor cells; BM, bone marrow; M, mouse; R, rat; Ra, rabbit; PB, peripheral blood; H, human).

The relative scarcity of EPCs in the peripheral circulation has led to the *ex vivo* expansion of PB-EPCs prior to their transplantation. The common method to isolate EPCs is by seeding mononuclear cells on fibronectin-coated plates and to be grown in endothelial-specific media for several days (Fadini *et al.*, 2008). These cells then undergo FACS analysis and immunostaining to confirm phenotype. The administration of *ex vivo* expanded EPCs in rodent models of hindlimb and myocardial ischaemia was shown to augment capillary density as well as successfully revascularize the ischaemic target region (Kocher *et al.*, 2001; Tamarat *et al.*, 2004). In addition to the transplantation of *ex vivo* EPCs to potentiate neovascularization, GM-CSF has been used to induce angiogenesis *in vivo* in a rabbit ischaemic hindlimb model (Takahashi *et al.*, 1999).

Myocardial gene transfer of VEGF has been used for patients with symptomatic myocardial ischaemia with reduction of ischaemia evidenced by perfusion imaging and angina symptoms (Losordo *et al.*, 1998). Although many favourable outcomes have been reported after transplantation of EPCs into humans and animal models, there is a possibility that some undesirable side effects may occur, such as uncontrolled neovascularization favouring the development of cancer (Rafii and Lyden, 2008) and atherosclerotic plaque destabilization (Moreno *et al.*, 2004).

As native EPCs from patients with diabetes (Tepper *et al.*, 2002), arteriosclerosis (Landmesser and Drexler, 2005) or Alzheimer's disease (Vagnucci and Li, 2003) have endothelial dysfunction, it becomes important to use either healthy EPCs or genetically modified EPCs for successful therapy. One gene commonly used for genetic modification of EPCs is VEGF, the protein having an important role as an endothelial motogen (Iwaguro *et al.*, 2002; Schratzberger *et al.*, 2001). Another gene transfected has been SDF-1, SDF-1 being important in EPC mobilization into the peripheral circulation as shown in a mouse hindlimb ischaemia model, where there was augmented recovery of blood perfusion to the ischaemic limb and increased capillary density (Hiasa *et al.*, 2004). Genes encoding HMG-CoA reductase inhibitors, in particular simvastatin, increase numbers of cEPCs and improve neovascularization in a murine model of corneal injury (Llevadot *et al.*, 2001), while eNOS inhibited neointimal hyperplasia in rabbits, augmenting endothelialization and reducing neointima size with no incidence of thrombosis (Kong *et al.*, 2004). Fully

differentiated endothelial cells have also been used to deliver a therapeutic gene, for example, healthy liver sinusoidal endothelial cells were transplanted to correct factor VIII deficiency in haemophilia A mice (Follenzi *et al.*, 2008).

To date, no EPC cell line has been generated, as most studies use fresh isolated BM-or PB-derived EPCs, although Qiu *et al.* have successfully immortalized EPCs from human CB by transfecting with the SV40 large T antigen (Qiu *et al.*, 2006); the transplanted immortalized EPCs improved neovascularization and augmented blood flow in a model of ischaemic lung injury.

In the context of tissue engineering, EPCs can also be used to improve the biocompatibility of vascular grafts. When autologous CD34⁺ cells (containing EPCs) were seeded onto artificial vascular grafts (Dacron grafts) and implanted into the canine thoracic aorta, both improved surface endothelialization and vascularization were noted (Bhattacharya *et al.*, 2000). In a similar study, CD34⁺ cells were seeded onto the graft with addition of granulocyte CSF (G-CSF), resulting in increased mobilization of leukocytes (including CD34⁺ cells), thus enhancing vascularization of the vascular graft (Kaushal *et al.*, 2001). This strategy may be useful in islet transplantations where, during harvest from the donor pancreas, islets lose their vascular connections. The augmentation of neovascularization might thus increase the success rate of islet transplantations. Common treatment strategies for EPC transplantation are summarized in Table 1.3.

1.6.6. EPCs and diseases

1.6.6.1. Myocardial infarction

Preclinical studies have shown that transplantation of human PB-derived EPCs can reduce left ventricular (LV) remodelling in a nude rat model of myocardial infarction (Losordo *et al.*, 1998). This strategy not only promoted neovascularization, but also decreased apoptosis of hypertrophied myocytes in the periinfarct region, resulting in an improvement in cardiac function; EPCs are probably acting in a paracrine manner, releasing anti-inflammatory and prosurvival factors into the infarct region, as they do in the regenerating pancreatic islet (Mathews *et al.*, 2004). With respect to future clinical therapy, Kawamoto

et al. were the first group to investigate the potential of autologous EPC (CD31⁺ MNCs) transplantation using the swine chronic myocardial ischaemia model (Kawamoto *et al.*, 2002). Adopting a novel model of real-time ischaemia mapping to directly deliver cells to the ischaemic site, they successfully reduced the size of the ischaemic area as well as improving swine LV function. Clinical trials of autologous transplantation, such as the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI), involve the intracoronary infusion of EPCs into patients with acute myocardial infarction. Preliminary results suggest a significant improvement of myocardial function after 4 months of treatment (Assmus *et al.*, 2002; Schachinger *et al.*, 2006). Other trials have adopted a therapeutic strategy of infusing cultured EPCs with the mobilization factor GM-CSF into patients with acute myocardial infarction (Ellis *et al.*, 2006; Zohnhofer *et al.*, 2007). Despite notable successes in animal studies, there was no significant improvement in myocardial function in these clinical trials, although there was an increased number of cEPCs.

1.6.6.2. Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a vascular disease characterized by severe endothelial dysfunction. These patients produce insufficient NO, a vasodilator important in vessel growth in the pulmonary vasculature. Initial studies of transplantation of autologous EPCs into the lung resulted in attenuation of monocrotaline (MCT)-induced PAH in dogs (Takahashi *et al.*, 2004). Nagaya *et al.* used human CB-derived EPCs transduced with adrenomedullin, a potent vasodilator peptide and transplanted them into MCT-treated rats (Nagaya *et al.*, 2003; Takahashi *et al.*, 2004). PAH was markedly ameliorated in these rats, causing a 39% decrease in pulmonary vascular resistance compared with 16% in rats receiving unaltered EPCs. Similar results were reported by Zhao *et al.* using human cord blood-derived EPCs transfected with eNOS, the enzyme involved in the production of NO (Zhao *et al.*, 2005). These encouraging results using genetically modified EPCs led to the initiation of an early Phase IIa clinical trial, the Pulmonary Hypertension Assessment of Cell Therapy (PHACeT), in 2006, which was the world's first human gene therapy trial for fatal lung disease, pioneered by scientists at Northern Therapeutics Inc., (Toronto, Canada), combining both cell and gene therapy (St. Michael's Hospital, 2006). This

innovative approach involved the injection of eNOS-transduced EPCs derived from the patient's own blood into the lung circulation, with the aim of restoring lung vascular function in severe PAH; results are awaited.

1.6.6.3. Liver regeneration & repair

Fujii *et al.* investigated the contribution of BM cells to liver regeneration after partial hepatectomy (Fujii *et al.*, 2002) of mice transplanted with GFP⁺ BM. After liver injury, increased numbers of GFP⁺ cells were observed, with the majority of these cells coexpressing CD31, suggesting they were sinusoidal endothelial cells. In the carbon tetrachloride and dimethylnitrosamine- induced models of liver injury, fresh EPCs from BM and PB were used, respectively (Taniguchi *et al.*, 2006; Ueno *et al.*, 2006). Both studies observed a suppression of liver fibrosis and an increased survival rate; EPCs may have contributed to liver regeneration by engraftment into hepatic sinusoids, promoting hepatocyte proliferation through increased levels of growth factors such as EGF, HGF, TGF- α and VEGF in the regenerating liver. The contribution of BM-progenitor cells to neovascularization may depend on the type of injury. Using models of partial hepatectomy (PH) and allyl alcohol (AA)-induced injury, Kienstra *et al.* found LacZ-labeled HSCs engrafted within the vascular endothelium of the AA-damaged liver, with the majority present in highly injured areas (Kienstra *et al.*, 2008). AA injury induces ischaemia and causes damage to periportal areas where local endogenous stem and progenitor cells reside (Alison *et al.*, 2007). Ablated stem cells may provide space for donor HSCs (which will contain EPCs) to engraft, resulting in the expansion of microvessels and larger vessels. By contrast, transplanted cells were not found in the vasculature of regenerated liver following PH; here, existing endothelial cells may simply expand in numbers. In a clinical setting, Yannaki *et al.* performed three mobilization courses of G-CSF into two patients with end-stage cirrhotic liver disease to increase the number of circulating stem cells such as cEPCs (Yannaki *et al.*, 2006). Increased numbers of CD34⁺ cells and raised serum VEGF levels were noted in the two patients, suggesting again the possible contribution of EPCs to liver repair; the increased mobilization of stem cells and their homing to the damaged region resulted in lasting amelioration during 30 months of follow-up.

1.6.6.4. Inflammatory bowel disease

There is little evidence for a contribution of BM cells to the direct regeneration of the mucosa in inflammatory bowel disease. In a trinitrobenzene sulfonic acid-induced colitis model, BM cells engraft not only as myofibroblasts, but also as endothelial cells, vascular smooth muscle cells and pericytes in regions of inflammation (Brittan *et al.*, 2005). A similar fate of BM-progenitor cells may underlie the beneficial effects of autologous BM therapy in patients with refractory Crohn's disease (Oyama *et al.*, 2005).

1.6.6.5. Diabetes

Diabetic patients have reduced cEPCs and deregulated EPC function (reviewed in (Loomans *et al.*, 2006)). Islet transplantations have been carried out since the publication of the Edmonton Protocol (Shapiro *et al.*, 2000), however a shortage of cadaveric pancreata and a return to insulin dependence in the majority of recipients within 5 years means alternative treatments are required. Several studies have suggested that BM transplanted after STZ-induced diabetes in mice may contribute to islet neovasculation and promote islet β -cell regeneration. Hess *et al.* intravenously transplanted GFP⁺ BM cells into Streptozotocin (STZ)-induced diabetic mice 5 days after the last STZ administration (Hess *et al.*, 2003). Blood glucose levels were reduced, with serum insulin levels restored to almost normal levels within 7 days. Contribution to vasculature was noted, with 9.2% of the engrafted donor cells at ductal and islet regions being endothelial cells (GFP⁺ PECAM-1⁺) suggestive of the contribution of EPCs to the engraftment. Two other murine studies obtained similar results following host BM ablation and subsequent BM transplantation into STZ-diabetic mice (Hasegawa *et al.*, 2007; Mathews *et al.*, 2004). These studies suggested that BM, presumably containing EPCs, differentiated into endothelial cells and contributed to neovasculation in the injured islet, possibly also aiding islet regeneration. In addition, it should be remembered that BM may contain other cells that aid in the neovascular response. In this scenario, vascular basement membrane proteins, such as laminin and collagen IV, can act as endothelial signals, promoting β -cell differentiation and influencing their function (Nikolova *et al.*, 2006).

1.6.6.6. Prognostic implications

Patients with cardiovascular risk states such as arteriosclerosis (Vagnucci and Li, 2003), diabetes (Hill *et al.*, 2003; Hink *et al.*, 2001) and Alzheimer's disease (Shintani *et al.*, 2001; Vagnucci and Li, 2003) have endothelial dysfunction. EPC numbers are reduced, and are also impeded in function, with a reduced ability to migrate and form tubules in these patients - this correlates with poor clinical prognosis. For example, BM-derived EPCs are mobilized by eNOS activation in the BM; a process that might be impaired in diabetics, thus preventing these cells from reaching the damaged islets in significant numbers (Brem and Tomic-Canic, 2007; Schmidt-Lucke *et al.*, 2005).

EPCs from non-diabetic patients cultured under hyperglycaemic conditions show diminished proliferative capacity. These differences in proliferation are inversely correlated with glycaemic control, there is a 48% decrease in cEPCs (dual-stained cells for acLDL and lectin) in T2D patients compared with non-diabetic control subjects (Tepper *et al.*, 2002). Likewise a 44% reduction in cEPCs in T1D patients (Loomans *et al.*, 2006) was noted. Linear regression analysis revealed that EPC numbers in both studies were inversely correlated with haemoglobin A1c levels (Loomans *et al.*, 2006; Tepper *et al.*, 2002). As T2D patients have reduced cEPC numbers and have a three- to four-fold increase in cardiovascular risk (Garber, 1998; Haffner *et al.*, 1998), cEPC numbers in diabetes can be used as a barometer of cardiovascular risk (Fadini *et al.*, 2006).

The number of EPC-CFUs derived from the PB can predict multivessel coronary artery disease (CAD), independent of traditional risk factors such as hypertension, family history and hyperlipidaemia (Kunz *et al.*, 2006). Patients with multivessel CAD had significantly lower EPC counts and also had a lower CFU count. This may relate to a general failure of the body's EPC-driven repair mechanisms owing to either failure of EPC release from the BM or overall depletion of EPC numbers caused by the overwhelming demand for repair. Levels of cEPCs can thus be used to identify patients having a high risk of vascular accidents such as myocardial infarction and stroke (Werner *et al.*, 2005). Patients with lower numbers of cEPCs have a higher Framingham risk score (Hink *et al.*, 2001). Other pathological conditions associated with a decrease in cEPCs include chronic renal failure,

which contributes to accelerated atherosclerosis and impaired angiogenesis (Choi *et al.*, 2004), and hypercholesterolemia (Chen *et al.*, 2004), promoting atherosclerosis and CAD. Thus the measurement of cEPCs as a predictive clinical parameter is useful for evaluating disease risk and prognosis, as well as response to treatment.

1.6.7. EPCs and neoplasia

EPCs contribute to angiogenesis in various tumours, including hepatocellular carcinoma (Spring *et al.*, 2005) and non-small-cell lung cancer (NSCLC)(Dome *et al.*, 2006; Hilbe *et al.*, 2004). Tumours secrete growth factors such as VEGF that promote mobilization of EPCs to sites of vasculogenesis. The number of EPCs that home to perivascular sites increases to compensate for the increasing demands for oxygen and nutrients to support tumour growth. The early stage of tumour neovascularization is characterized by vessel dilation, followed by extensive vessel sprouting into a tumour, which is temporally mediated by the release of VEGF by the tumour and mobilization of EPCs (Hoeben *et al.*, 2004; Natori *et al.*, 2002; Spring *et al.*, 2005).

It has been suggested that more advanced tumours have a higher propensity to recruit BM cells to the developing vasculature as shown in a study of murine liver and pancreatic tumours (Hammerling and Ganss, 2006). Some studies of BM-EPCs in supporting tumour vasculature have reported that less than 1% of blood vessels have BM-derived endothelial cells in a B6RV2 lymphoma model (Larrivee *et al.*, 2005) or even a nonexistent contribution to vessels in a Lewis lung carcinoma model (Shinde Patil *et al.*, 2005). Both studies, however, allowed only 10–14 days for tumour development before animal sacrifice. In a similar vein, BM-derived endothelial cells were only found in approximately 1% of vessels in mice engineered to develop mammary tumours (Dwenger *et al.*, 2004).

In a tumour-resistant mouse with an angiogenic defect (impaired VEGF mobilization), the Id knockout mouse (Id1⁺/Id3^{-/-}), tumour angiogenesis and growth were restored with the transplantation of β -galactosidase marked wild-type (WT) BM (Lyden *et al.*, 2001). When BM cells from Id mutant mice were transplanted into WT mice followed by inoculation of B6RV2 lymphoma, tumour growth retardation was seen. Gao *et al.* has developed a

lentiviral-based Id synthetic microRNA (miR-30)-based short hairpin RNA lentivirus whose activity can be induced by doxycycline to target Id expression *in vivo*, allowing the selective suppression of Id during metastasis progression without compromising the contribution of BM-derived endothelial cells to the growth of the primary tumour (Gao *et al.*, 2008). The blocking of the transcription factor Id expression in the mammary tumour-bearing mice significantly inhibited angiogenesis and reduced numbers of cEPCs (c-Kit⁺ VEGFR-2⁺ CD11b⁻), importantly impairing formation of lethal lung macrometastases and increasing mice survival. This study illustrated that EPCs are important mediators of the angiogenic switch, aiding the progression of metastatic lesions.

Not all studies concur that PB-EPCs and cEPCs play a significant role in tumour development (Purhonen *et al.*, 2008), failing to find GFP-tagged endothelial cells in WT mice that had received a GFP⁺ BM transplant and subsequent inoculation of syngenic B16 melanoma. Moreover, no GFP⁺ endothelial cells were found in intestinal adenomas when GFP⁺ mice were parabiotically paired with young Apc^{Min/+} mice. These studies provoked a vehement response from advocates of the importance of EPCs to tumour neovascularization and an equally strident rebuttal from the authors of the negative paper (Kerbel *et al.*, 2008).

Unlike mouse studies, there are very few studies that have investigated the contribution of BM-derived EPCs to human tumour neovascularization. In a clinical setting, Peters *et al.* analyzed several tumour types from six individuals who developed cancer after receiving a sex-mis-matched BM transplant, finding that BM-derived cells contributed to the tumour endothelium, albeit at a low level averaging only 4.9% (Peters *et al.*, 2005). In addition, they eliminated the possibility of cell fusion between BM-derived cells and existing endothelial cells in the tumour by using FISH probes for X and Y chromosome detection.

1.6.7.1. Prognostic implications

A prerequisite for cancer progression is the formation of new blood vessels either from pre-existing blood vessels or *via* the recruitment of EPCs from the BM to form entirely new blood vessels. Thus, levels of cEPCs, potentially recruitable to the growing vascular

infrastructure, may serve as a marker for disease prognosis and predict the risk of cancer relapse after dormancy.

In patients with NSCLC, a high level of cEPCs (>1000/ml) in the PB is associated with poor prognosis (Dome *et al.*, 2006). Post-treatment levels of cEPCs were significantly lower in patients who had achieved a partial/complete remission compared with patients with stable or progressive disease, therefore suggesting that EPC levels correlate with tumour burden. EPCs have been grown from the PB of patients with hepatocellular carcinoma and compared with healthy patients and patients with liver cirrhosis (Ho *et al.*, 2006); higher levels of cEPCs (defined by CFU counts) were found in patients with liver cancer compared with the other patient groups. In addition, higher levels of cEPCs were noted in patients with unresectable and recurrent cancer compared with respectable and disease-free patients enrolled a year before. The levels of CD133 mRNA (cEPC numbers) in the PB of colorectal cancer patients has been advocated as a useful screen for recurrence (Lin *et al.*, 2007); cEPC numbers may also be useful in the prediction of the patient's response to chemotherapy (Bertolini *et al.*, 2003). These studies show that cEPCs could represent a class of biomarkers to gauge the response to anti-angiogenic treatment or chemotherapy. In rectal cancer patients, a decrease in viable PB-EPCs was observed after receiving a single infusion of bevacizumab (a VEGF-specific antibody) (Willett *et al.*, 2004). This strategy had a direct anti-vascular effect in the patients and resulted in decreased tumour perfusion, vascular volume, microvascular density and interstitial fluid. Similar results were seen following infusion of VEGFR-2-targeted anti-angiogenic agents into murine models of lung and breast cancer (Shaked *et al.*, 2005). In late-stage breast cancer patients receiving chemotherapy, a 28% decrease in the number of EPCs suggested this measurement could be a useful biomarker or even a therapeutic target (Naik *et al.*, 2008).

1.7. AIMS OF PROJECT

Endothelial progenitor cells (EPCs) in the bone marrow (BM) and peripheral blood (PB) contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels, and thus have a degree of stem cell potential. Previous studies indicate that diabetic patients have reduced EPC number and deregulated EPC function, although the regenerative properties of EPCs in diabetes are unknown. I wish to characterise and compare EPCs from pre-diabetic and diabetic non-obese diabetic (NOD) mice, a model of T1D, in order to delineate the role of these cells in the pathogenesis of autoimmune diabetes. As EPC numbers are low in number from BM and blood, I aim to isolate these EPCs from BM, expand and characterize them. The purified EPCs will be transplanted into diabetic NOD mice in the hope of promoting the restoration of pancreatic function following the onset of autoimmune diabetes, possibly by contributing to tissue regeneration *via* the mechanism of neovascularogenesis.

In addition to promoting endogenous β -cell regeneration using EPCs, I intend to investigate the potential of a protease peptide XG-102 developed by Xigen, to block β -cell death *via* apoptosis. It was previously shown that XG-102, has efficiently protected neurons against cell death in animal models of stroke. Since apoptosis of β -cells is one of the putative mechanisms involved in the cascade of events leading to T1D, I aim to test XG-102 for β -cell protection and prevention of β -cell loss in the NOD mouse.

In general both experimental strategies will be evaluated for their ability to prevent or delay diabetes in the NOD mouse.

CHAPTER 2: METHODS

2.1. INTRODUCTION

Two strains of mice were used in this study. The first strain, NOD mice came from the NOD/Ba colony established in 1987 at St. Bartholomew's Medical College, London, UK, originally derived from Dr E. Leiter's laboratory (Bar Harbor, ME, USA). There is a stable cumulative incidence of diabetes of approximately 60% in female and 15% in male mice at 30 weeks of age (Mansfield *et al.*, 1992). The colony is housed in a purpose-built area and maintained strictly according to international (N.I.H., 1985) and UK HMSO (Her Majesty's Stationary Office), 1986 guidelines for animal care.

The diabetic status of the mice was initially diagnosed by monitoring urinary glucose levels (Diabur Test 5000, Mannheim, Germany) and then confirmed by measuring blood glucose levels (≥ 11.5 mmol/l) using Accu-chek AVIVA Blood Monitor (Roche Diagnostics GmbH, Mannheim, Germany). Balb/c mice were obtained from Charles River Laboratories (Charles River Laboratories, Maine, USA). Both strains of mice are maintained at the biological animal unit of Queen Mary, University of London. Overall health of mice enrolled is checked every day and urinary glucose level checked once every week at a fixed time. Mice that were unhealthy during the duration of study were sacrificed by cervical dislocation. Blood glucose levels of mice were taken before culling. All mice were sacrificed by cervical dislocation.

2.2. BONE MARROW AND ORGAN COLLECTION

After cervical dislocation, the femurs, tibias and iliac crests were dissected out using a disposable scalpel (Swann-Morton, Sheffield, UK), cleaned and collected in Bijou tubes containing sterile x 1 phosphate buffer saline (PBS).

Pancreata from these groups of mice were also acquired and divided, where half were fixed in 10% neutral buffered formalin (BDH Laboratory Supplies Inc., Hertfordshire, UK) for 24 hours before storing in 70% alcohol and the remaining pancreata snap frozen in liquid nitrogen and kept at -80°C . The pancreata were then processed and paraffin-embedded for immunohistochemical studies.

2.3. ISOLATION OF WHOLE BONE MARROW CELLS

Bones (femurs, tibiae and iliac crests) from a single mouse were immersed in sterile PBS and kept on ice until use for experiments. The bones were cleaned with sterile vernaid dressing towel (Vernon-Carus Limited, Lancashire, UK) and the ends of these bones trimmed with a pair of sterile scissors. BM cells were collected by flushing the bones with Iscove's Modified Dulbecco's Medium (Iscove's MDM) with 2% Foetal Bovine Serum (FBS)(Stem Cell Technologies, Vancouver, Canada) using a 23G needle (Neolus Terumo Corporation, Madrid, Spain). After each flushing of a part of the bone, the cells were collected in a 5ml Falcon polystyrene tube and were mixed using a 21G needle (BD Microlance 3, Becton-Dickinson) several times to prevent coagulation. Next the cells were filtered with a 70 µm cell strainer (Becton-Dickinson) into another 5ml Falcon polystyrene tube and centrifuged (Hettich Zenrifugen Microcentrifuge, Jensions-Pls, Bedfordshire, UK) at 1500 rpm for 6 mins at 4⁰C. The resulting pellet was resuspended in 1ml of cold 1% FBS PBS and kept on ice. Cells (10 µl) were counted using a haemocytometer and diluted with x 1 PBS to obtain 1 x 10⁶ cells.

2.4. *IN VIVO* ANIMAL EXPERIMENTS

2.4.1. *Gender mis-matched cell transplantation*

In order to investigate the contribution of BM cells to adult cell lineages in the pancreas, sex-mis-matched, male to female, BM transplants were performed where the donor cells and their differentiated progeny could be identified by the presence of the Y chromosome in the female recipient tissues. Breeding and isolation of BM were carried out under UK Home Office procedural and ethical guidelines (Home Office Project License NO. 70/5962) at Biological Services Unit, Charterhouse Square. Administration of BM and stem cells and maintenance of chimaeric mice were carried out in the Biological Resource Unit, Cancer Research UK, London Research Institute. BM cells were collected from femurs, tibiae and iliac crest of donor male Balb/c and female NOD mice aged 10 weeks of age on the day of transplantation.

2.4.2. Preparation of cell transplant

Cells for transplant were resuspended in 200 μ l sterile 2% PBS FBS for intravenous (i.v.) tail vein injection into the recipient female mice on the same day. The number of nucleated cells was counted using a haemocytometer by the dye exclusion test using trypan blue (Stem Cell Technologies). Cells were stored on ice for no more than 1 hour before administration. Following administration, the mice were housed in sterile conditions and specific protocols were performed in accordance to each defined study.

2.4.3. Monitoring diabetes development

During the duration of transplantation, blood glucose levels will be evaluated once every 7 days using an Accu-chek AVIVA Blood Monitor (Roche). To monitor diabetes development, mice were classified diabetic if blood glucose was confirmed as ≥ 11.5 mmol/l. At the end point, all mice were killed by cervical ‘dislocation’ and/or CO₂ inhalation dependent on the type of tissue collected. Pancreas, spleen, kidney and liver were fixed in 10% neutral buffered formalin (BDH Laboratory Supplies Inc.) for 24 hours at room temperature (RT) before being embedded in paraffin wax. Mice transplanted with BM or EPCs were monitored for 14 weeks and 3-4 weeks respectively. The number of animals used for each treatment group is detailed in Table 2.1.

Cell fraction	Number of animals	Animal model	Number of cells transplanted
Whole BM cells	20	Lethally irradiated pre-diabetic mice (12week)	2×10^6 cells
EPCs	13	Early diabetic mice (0-3 days diabetic)	5×10^4 cells

Table 2.1. Details of cell transplantation.

Experimental details for BM and EPC transplant into prediabetic and early diabetic mice (Abbreviations: EPCs, endothelial progenitor cells; BM, bone marrow)

2.5. HISTOCHEMISTRY

2.5.1. Tissue fixation, embedding and tissue sectioning

All mice were killed by 'dislocation' and/or CO₂ inhalation, dependent on type of tissue collected. Half of the organs (pancreas, spleen, kidney and liver) were fixed in 10% v/v neutral buffered formalin (BDH Laboratory Supplies Inc.) for 24 hours at RT and the remaining snap frozen in liquid nitrogen. Formalin-fixed tissues were transferred to 70% v/v ethanol and then embedded in paraffin wax. Paraffin embedded tissues were sectioned at a thickness of 5 µm and placed on ThermoShandon plus slides (Thermo Fisher Scientific Inc., Waltham, USA) and allowed to dry in an incubator for 40 °C overnight. Slides were then stored at RT until required. Pancreata snap frozen in liquid nitrogen were serially sectioned at 7µm and kept at -80 °C.

2.5.2. Dewaxing, blocking endogenous peroxidase and rehydration

Paraffin embedded slides first were deparaffinized with xylene for 5 mins twice and then rehydrated for 1 min each through a descending series of ethanol (100, 95 and 70% v/v ethanol) and then blocked with 0.3% v/v hydrogen peroxidase in methanol (DakoCytomation) for 10 mins to block endogenous peroxidase activity when using peroxidase-dependent immunostains.

2.5.3. Heat-mediated antigen retrieval

Heat-mediated antigen retrieval was used in some of our immunohistochemistry protocols to restore the immunoreactivity of the antigen. After dewaxing, blocking of endogenous peroxidase and rehydration steps, slides were subjected to antigen retrieval by treatment in pre-heated 10mM sodium citrate buffer (pH 6.0) in a microwave (100-V, 800-W microwave) for between 5-20 mins depending on the type of antibody used.

2.5.4. Haematoxylin and eosin (H&E) staining

H&E staining is used to examine the morphological structure of the tissue section. Paraffin embedded sections first were deparaffinized with xylene as outlined in method section 2.5.2. Next, sections were stained with haematoxylin for 2 mins before washing the excess

in tap water for 1 min. Sections were then dipped in acid water before counterstaining with eosin. After counterstaining, sections were dehydrated for 2 mins in 70%, 90% and 100% v/v ethanol for 5 mins followed by clearing in xylene twice for 1 min before mounting with DEPEX (BDH Laboratory Supplies Inc.) and coverslipping.

2.5.5. General immunohistochemistry protocol

For specific immunostaining, the following antibodies were used at a dilution as specified. Paraffin embedded slides were first deparaffinized with xylene, blocked with 0.3% v/v hydrogen peroxidase in methanol for 10 mins and then rehydrated in a descending series of ethanol (100, 95 and 70% v/v ethanol). After washing in distilled water for 1 min, slides were subjected to heat-mediated antigen retrieval for between 5-20 mins depending on the type of antibody used. To block non-specific binding, slides were next incubated with serum from the same species from which the secondary antibody was raised in at a dilution of 1:25 for 15 mins at RT. Primary antibody was next added to the sections for 35 mins at RT. Sections were then washed in PBS for 5 mins twice, and next incubated with the biotinylated secondary antibody for 35 minutes at RT. The sections were then washed in PBS and incubated with streptavidin: horseradish peroxidase (Dako) for 35 mins at RT. After washing in PBS, DAB (3, 3'-diaminobenzidine) chromogen (1:4)(Impact DAB diluent, Vector Laboratories) was applied to sections and the substrate-reaction allowed to take place for 1-3 mins. This reaction (brown colour) was stopped by washing in distilled water for 1 min and followed by counterstaining in haematoxylin for 1 min, rinsing in tap water, acid water, lithium carbonate, tap water, 70%, 90% and 100% v/v alcohol, xylene, and finally mounted with DEPEX and coverslipped.

2.5.6. CD31 microvessel staining

To evaluate blood vessel density, an anti-CD31 antibody was used to stain for endothelial cells lining blood vessels. Both primary and secondary antibodies were diluted in blocking buffer, made up of goat serum (Dako) diluted in PBS (1:25). Frozen pancreata (half) were serial sectioned at 7 µm thickness and three sections from positions 500 µm apart for each block were used for microvessel staining. The sections were then brought to temperature before fixation in ice cold acetone (BDH) for 2 mins. Next, sections were air dried for 1 hour and rehydrated in PBS for 10 mins. Sections were blocked with blocking buffer for 30

mins before adding rat anti-mouse CD31 (BD Pharmigen) (1:100) for 1 hour. After 3 washes in PBS for 5 mins, goat anti-rat Alexa 488 (Dako) (1:600) and DAPI (Invitrogen) (1:2000) was added to the sections for 1 hour. Sections were washed with PBS three times before coverslipping with vectashield mounting medium (Vector Laboratories). For scoring of blood vessel density, see section 2.6.2.

2.5.7. Combined endomucin immunohistochemistry and Y chromosome detection

Immunohistochemistry for various antigens was used to establish cell phenotype. In tissues of female recipient mice that received a male BM transplant, *in situ* hybridisation for the Y chromosome was used to distinguish the transplanted cells. Therefore combined immunohistochemistry and *in situ* hybridisation permitted both lineage tracing and phenotypical analyses. For all immunohistochemical and *in situ* hybridisation protocols, appropriate positive and negative controls were used. Prior to use of any newly acquired antiserum, a serial dilution was tested on positive control sections of fixed tissue in order to establish the optimal dilution of each antibody.

To differentiate between the host- and donor-derived endothelial cells, *in situ* hybridisation for the Y chromosome was combined with immunohistochemical characterisation of endomucin, an endothelial marker. Paraffin sections were dewaxed, blocked with 0.3% v/v hydrogen peroxidase in methanol and rehydrated through a descending series of alcohol in PBS (100%, 95% and 70% v/v ethanol), before incubation in 20% v/v acetic acid/methanol for 10 mins. After incubation, slides were rinsed with PBS before addition of rabbit serum (Dako) for 30 mins. Rat monoclonal anti-mouse Endomucin (V.7C7) (Santa Cruz). Antibody was added (1:250) for 40 mins and subsequently washed twice with PBS for 5 mins. Secondary antibody, rabbit biotinylated anti-rat IgG (1:100) (Vector Laboratories) was added to sections and incubated for 35 mins. Sections were washed twice for 5 mins before addition of alkaline phosphatase streptavidin (1:50) (Vector Laboratories) for 25 mins. After washing sections twice for 5 mins, sections were developed according to manufacturer's instructions using alkaline phosphatase substrate kit 1 (Vector Laboratories). Sections were washed in PBS twice for 5 mins.

Subsequently sections were incubated in 1 mol/l sodium thiocyanate for 10 mins at 80 °C, washed in PBS, and then digested in 1.2% w/v pepsin in 0.1 mol/L HCl at 37 °C for varying times, depending on the pre-treatment used for immunohistochemistry. The protease was quenched in 0.2% v/v glycine in double-concentration PBS and the sections were then rinsed in PBS, post fixed in 4% w/v paraformaldehyde in PBS for 2 mins, dehydrated through graded alcohols, and air dried. A fluorescein isothiocyanate-labelled Y chromosome paint (Cambio Ltd) was added to the sections, sealed under a glass coverslip, heated to 80 °C for 10 mins, and incubated overnight at 37 °C. The next day, slides were washed with SSC to remove unbound DNA sequences. Slides were mounted with hardset mounting 4'6-diamidino-2-phenylindole (DAPI) medium (Vector Laboratories). A Leica microscope, with x 40 objective lens, was used to detect the signal and BM engraftment was calculated by counting the number of Y-chromosome⁺ nuclei (donor origin) and total number of DAPI-stained nuclei in 10 random fields and was expressed as a percentage +/- SEM.

2.5.8. Combined Ki-67 and insulin immunohistochemistry

To quantify β -cell proliferation and regeneration in the pancreata after BM transplantation, pancreatic sections were stained with anti-insulin and anti-Ki-67 antibodies. For insulin/Ki-67 double labelling, alkaline phosphatase staining with a guinea pig anti-insulin antibody (Dako) was followed by a rabbit anti-Ki-67 antibody (NCL-Ki-67-P, Novocastra), which was developed with immunoperoxidase staining.

Two paraffin embedded sections from positions 100 μ m apart (5 μ m thick) were used for double immunostaining using Ki-67 and insulin antibodies. The sections were routinely dewaxed and blocked. For development of alkaline phosphatase, sections were then incubated with ice cold 20% v/v acetic acid in methanol for 5 mins. Antigen retrieval was performed by microwaving sections (700W) in 0.01M citrate buffer at pH 6 for 10-20 mins. After cooling sections for 5 mins in PBS, sections were pre-incubated with goat serum (Dako) at a dilution of 1:25 for 15 mins to reduce non-specific background staining. The sections were then incubated with a guinea pig anti-insulin antibody (1:400) for 35 mins. Following two washes in PBS, sections were incubated with a biotinylated goat anti-guinea pig antibody at 1:200 (Dako) for 35 mins. For the third layer following PBS

washing, alkaline phosphatase (Vector Laboratories) at a 1:50 dilution was applied to sections for 35 mins. Slides were developed using alkaline phosphatase substrate kit according to the manufacturer's instructions (Vector Laboratories) for 10 mins. Sections were next washed in PBS before staining for Ki-67. Sections were now blocked with goat serum (Dako) at a dilution of 1:25 for 15 mins followed by incubation with a rabbit anti-Ki-67 polyclonal antibody at a 1:200 dilution for 35 mins. For the second layer following PBS washing (twice for 5 mins each), sections were incubated with a biotinylated swine anti-rabbit antibody (Dako) at a 1:500 dilution for 35 mins. Following PBS washing, goat anti-rabbit (Invitrogen) at a 1:150 dilution were applied to the sections for 35 mins. After PBS washing, slides were mounted using Vectashield (Vector Laboratories).

The stained sections were examined at 400 x magnification. For the relative proportion of Ki-67⁺- β -cells, total numbers of β -cells indicated by positive insulin staining with Ki-67⁺ nuclei were counted and expressed as a percentage (see section 2.6.3).

2.6. TISSUE ANALYSIS

2.6.1. Scoring of insulinitis

In order to investigate the effect of XG-102 or BM-derived cells on the homing of antigen driven T-cells in the endocrine pancreas, insulinitis was evaluated by H&E staining of islets from two pancreatic sections (200 μ m difference) of each group after XG-102 or PBS injections. Sections were examined in a "blinded fashion" and islet infiltration was scored as follows: no infiltration (grade 0); peri-insulinitis (grade I), where about 10% of the islet area is infiltrated by a peripheral ring of lymphocytes; moderate (grade II), where 25% to 50% of the islet shows mononuclear infiltration and severe (grade III), where over 50% of the islet area was infiltrated by lymphocytes.

2.6.2. Microvessel density calculation

CD31-positive vessels were used for assessment of vascular density. Islets were identified and viewed at 200 x magnification using a Nikon Eclipse TE2000-S microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) with the observer blinded to

pathology data. Images were taken using single channel colours and merged using Adobe Photoshop CS2.

To calculate the volume fraction of stained regions, a picture was taken of the islet and surrounding tissue. Each positive endothelial cell or group of cells in contact with a spot was counted as an individual vessel. Endothelial staining in large vessels with tunica media, and non-specific staining of non-endothelial structures, was disregarded in vessel counts. Measure of vessel density was estimated by the number of blood vessels in the islet in the total field of view. Vessel density was quantified in all islets from 3 stained sections (obtained at 500 μm apart). Blood vessel density in each of these sections was then averaged to give a final measurement for each pancreatic block.

2.6.3. Ki-67/Insulin calculation

Cells double stained for Ki-67 and insulin antibody were used for assessment of islet regeneration. Islets were identified and viewed at 400 x magnification using a Nikon Eclipse TE2000-S microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) with the observer blinded to pathology data.

To calculate the % of insulin-positive cells that were also Ki-67 positive, a picture was taken of the islet at 400 x magnification. Ki-67⁺ Insulin⁺ cells were quantified in all islets from two stained sections (obtained 100 μm apart) and was then averaged to give a final percentage for each pancreatic block.

2.7. STATISTICS

Results are expressed as means \pm SEM. Statistical significance of differences was assessed using Student's unpaired t-tests.

2.8. BUFFERS AND SOLUTIONS

Medium	Medium constituents	Company
EPC medium	EGM-2 bullet kit	Lonza
	20% FBS	PAA
	Pen/Strep	Invitrogen
MACS buffer	2mM EDTA	Sigma
	PBS	PAA
	0.5% BSA	Sigma
4% Paraformaldehyde		
Paraformaldehyde	20 g	Sigma
PBS	500 ml	PAA
20 X SSC (pH 7.5)		CRUK
NaCl	175.5 g	Sigma
Na citrate	88 g	Sigma
Distilled water made to 1L total volume		
Sodium citrate buffer (pH 6.0)		CRUK
NaCl	175.5 g	Sigma
Na citrate	88 g	Sigma
Distilled water made to 1L total volume		

Table 2.2. Buffers and solutions used in experiments.

2.9. SUPPLIERS AND DISTRIBUTORS

BDH Laboratory Supplies Inc.	Hertfordshire, UK.
BD Biosciences	New Jersey, USA.
BD Pharmingen	San Diego, USA.
Becton-Dickinson	Bedford, USA.
Cambio Ltd.	Cambridge, UK.
Cedarlane Ltd.	East Sussex, UK.
Charles River Laboratories	Maine, USA
Dako	Cambridge, UK.
Diabur	Mannheim, Germany.
Gibco	Paisley, UK.
Invitrogen	Paisley, UK.
Jensons-Pls	Bedfordshire, UK.
Lonza	Wokingham, UK.
Millipore	Watford, UK.
Molecular Probes	Leiden, Netherlands.
Neolus Terumo Corporation	Madrid, Spain.
Nikon Instruments Europe B.V.	Amstelveen, Netherlands.
Novocastra	Newcastle, UK.
Roche Diagnostics GmbH	Mannheim, Germany.
Santa Cruz Biotechnology	Santa Cruz , USA.
Sarstedt	Numbrecht, Germany.
Scientific Laboratory Supplies Ltd	Nottingham, UK.
Sigma Chemical Co.	Oxford, UK.
Stem Cell Technologies	Vancouver, Canada.
Swann-Morton	Sheffield, UK.
Thermo Fisher Scientific Inc.	Waltham, USA.
Weldtite Products Ltd.	Barton-on-Humber, UK.
Vector Laboratories Inc.	Burlingame, USA.
Vernon-Carus Ltd.	Lancashire, UK.
Zymed Laboratories, Inc.	San Francisco, USA.

Table 2.3. List of suppliers and distributors.

CHAPTER 3: STUDIES ON BLOOD AND BONE MARROW EPC-LIKE CELLS

3.1. INTRODUCTION

The successful isolation of EPCs in 1997 by Asahara *et al.* (Asahara *et al.*, 1997) has led to many studies of EPCs to date, and it is now well known that EPCs derived from the BM and PB contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels, i.e. neovasculogenesis. EPCs are believed to originate from the BM and are often mobilized into the circulation in response to growth factors and cytokines released following a variety of stimuli which include vascular trauma (Gill *et al.*, 2001), ischaemia, wounding or cancer (Takahashi *et al.*, 1999). EPCs home-to and incorporate into sites of damage and mediate repair by inducing neovascularization.

Recent studies on angiogenesis have revealed a significant impairment of angiogenesis in diabetic patients (Caballero *et al.*, 2007; Loomans *et al.*, 2004). Additionally, these patients have a reduced number of PB EPCs (Fadini *et al.*, 2005; Loomans *et al.*, 2004). Loomans *et al.* showed that this endothelial dysfunction does not occur due to increased apoptosis but perhaps is associated with alterations in BM mobilization and hyperglycaemic stress (Loomans *et al.*, 2004). Parallel studies have been carried out in ischaemic (Emanueli *et al.*, 2007) and induced diabetic animal models (Caballero *et al.*, 2007; Thum *et al.*, 2007a), but has yet to be carried on the most valuable spontaneous model of T1D such as the NOD mouse.

Accordingly, the aim of this study was to characterize and compare BM and PB EPCs from pre-diabetic and diabetic NOD mice, in order to delineate their fate in the natural history of autoimmune diabetes.

3.2. METHODS

3.2.1. Mice

Three groups of mice were used in this study, pre-diabetic NOD, diabetic NOD and wild type (Balb/c) mice. These mice were maintained in the animal unit at Charterhouse Square. Diabetes status of the mice was monitored and confirmed by measuring blood glucose levels (≥ 11.5 mmol/l) using an Accu-chek AVIVA Blood Monitor as outlined in Method 2.1.

3.2.2. Blood collection

A single blood aliquot was collected by cardiac puncture from the ventricle under terminal isoflurane anaesthesia using appropriate size needles and collected into heparin coated tubes (Sarstedt, Numbrecht, Germany). Fresh blood collected in heparin coated tubes was mixed thoroughly to prevent coagulation and spun down briefly. Blood was stored on ice and used within two hours of collection.

3.2.3. Bone marrow collection and isolation

Outlined in Method 2.2 and 2.3.

3.2.4. Isolation of peripheral blood

Fresh blood collected in heparin coated tubes was mixed thoroughly and spun down briefly. A volume of 100 μ l of blood was aliquoted into separate 15 ml Falcon tubes (Scientific Laboratory Supplies Ltd, Nottingham, UK). Next, 2 ml of FACS lysing solution (Becton-Dickinson, Bedford, USA) were added into each tube, vortexed and left to incubate for 15 mins in the dark. After incubation, 2 ml of x 1 PBS were added into each tube to dilute the FACS lysing solution and spun down at 2200 rpm for 7 mins. The resultant supernatant was removed and pellet resuspended in 2 ml of x 1 PBS. The mixture was centrifuged at 2500 rpm for 5 mins and resuspended in x 1 PBS for FACS analysis.

3.2.5. Direct staining of cells for fluorescence-activated cell sorting (FACS) analysis

FACS analysis was carried out to quantify the numbers of EPC and cEPCs from BM and PB respectively.

3.2.5.1. Staining: Whole bone marrow cells

Cells that do not stain with CD23 (platelets, natural killer cells), CD45 (leukocytes), CD11b (monocytes, macrophages) and TER119 (erythrocytes) antibodies were defined as lin^- . EPCs were defined by positive staining for c-Kit (BD Pharmingen, San Diego, USA), stem cell antigen 1 (Sca-1; BD Pharmingen) and CD31 (BD Pharmingen). Appropriate fluorochrome-conjugated isotype controls were used for each staining procedure.

3.2.5.2. Staining: Whole blood

A volume of 100 μ l PB was incubated for 15 mins in the dark with an FITC-conjugated monoclonal antibody against mouse CD45 (lin^-) (BD Pharmingen) in combination with an APC-conjugated antibody against mouse c-Kit (BD Pharmingen), PE-Cy7-conjugated antibody against mouse stem cell antigen 1 (Sca-1; BD Pharmingen) and PE-conjugated antibody against CD31 (BD Pharmingen). Isotype-identical antibodies served as controls (BD Pharmingen). After incubation, erythrocytes in the PB were lysed and washed with PBS before analysis. Each analysis included approximately 10,000 events.

3.2.6. FACS analysis

3.2.6.1. Analysis gating strategies: Whole bone marrow cells

Characterization of EPCs from whole BM cells (approximately 1×10^6 cells) was analyzed with the fluorescence-activated cell sorter (FACS) Aria (Becton Dickinson). Single colour and negative selection antibodies with isotype controls were analysed first (Table. 3.1). This was followed by the analysis of cells labelled with lineage positive antibodies ($c\text{-Kit}^+ CD31^+ Sca1^+$) but not antibodies: TER119, CD23, CD11b, CD45, i.e. lin^- . Compensation and gating strategies were performed by Dr. Gary Warnes (blind manner). $c\text{-Kit}^+ lin^-$ cells were first isolated. These cells are then gated to include only $CD31^+ Sca1^+$ cells. Only

single cells of c-Kit⁺ CD31⁺ Sca1⁺ lin⁻ were obtained and located in the mononuclear region of a FSC vs. SSC graph.

3.2.6.2. Analysis gating strategies: Whole blood

Characterization of EPCs (c-Kit⁺ CD31⁺ Sca1⁺ lin⁻) from lysed blood was performed (approximately 0.5 x10⁶ cells) as carried out previously using bone marrow cells. Cells were analysed on BD LSR II (Becton Dickinson). Single colour, negative selection antibody with isotype controls followed by combination of positive (c-Kit⁺ CD31⁺ Sca1⁺) and a negative selection antibody, CD45 i.e. lin⁻ were analysed. Gating strategies was performed similar to the gating strategies to obtain BM-EPCs, as explained previously in which c-Kit⁺ lin⁻ cells were first obtained and further gated to obtain CD31⁺ Sca1⁺ cells. Doublet discrimination was performed to obtain an EPC niche consisting c-Kit⁺ CD31⁺ Sca1⁺lin⁻ cells.

Antibodies	Isotype	Fluorochrome	
CD45 (Ly-5)	Rat (LOU/Ws1/M) IgG2b, κ	FITC	Lineage negative Abs
TER-119 (Ly-76)	Rat (WI) IgG2b, κ	FITC	
CD23	Rat (LOU/M) IgG2a, κ	FITC	
CD11b	Rat (DA) IgG2b, κ	FITC	
Antibodies			
CD31 (PECAM-1)	Rat (LEW/Cr1BR) IgG2a, κ	R-PE	Lineage positive Abs
c-Kit (CD117)	Rat (Wistar) IgG2b, κ	APC	
Sca-1 (Ly-6A/E)	Rat (LEW) IgG2a, κ	PE-Cy7	

Table 3.1. Details of FACS antibodies used. All antibodies were purchased from BD Pharmingen, San Diego, USA. (Abbreviations: FITC, Fluorescein Isothiocyanate; R-PE, R-Phycoerythrin; APC, Allophycocyanin)

3.3 RESULTS

3.3.1. Information on batches of bone marrow and blood used in the experiments

Matching BM and blood samples from 3 groups of mice were used in this study: Wild type (Balb/c) mice; pre-diabetic NOD mice and diabetic NOD mice (> 11.5mmol/l glucose).

3.3.2. Characterization and numbers of EPCs in peripheral blood and bone marrow of NOD mice

Debate ensues as to the true phenotype of adult EPCs, which is often clouded by the overlap of the phenotype of EPCs with HSCs and endothelial cells, and thus, a universally-accepted EPC phenotype remains to be achieved. Based on recent EPC studies in mice, we used flow cytometry and determined the numbers of c-Kit⁺ CD31⁺, Sca-1⁺ lin⁻ cells in the PB and BM.

FACS analysis was first carried out on BM and PB of wild type (Balb/c) and non-diabetic NOD mice (Figure 3.1). To obtain a single cell population of EPCs, c-Kit⁺ lin⁻ cells were first gated and subsequently gated for CD31⁺ and Sca-1⁺ cells. FACS analyses revealed that there was no significant difference in EPC number (%) (n=5) (c-Kit⁺ CD31⁺ Sca-1⁺ lin⁻ cells) in BM and blood from non-diabetic NOD mice compared to WT (Balb/c) mice (Figure 3.1). In the following experiments, BM and blood from non-diabetic NOD mice served as controls and the same gating strategies were used.

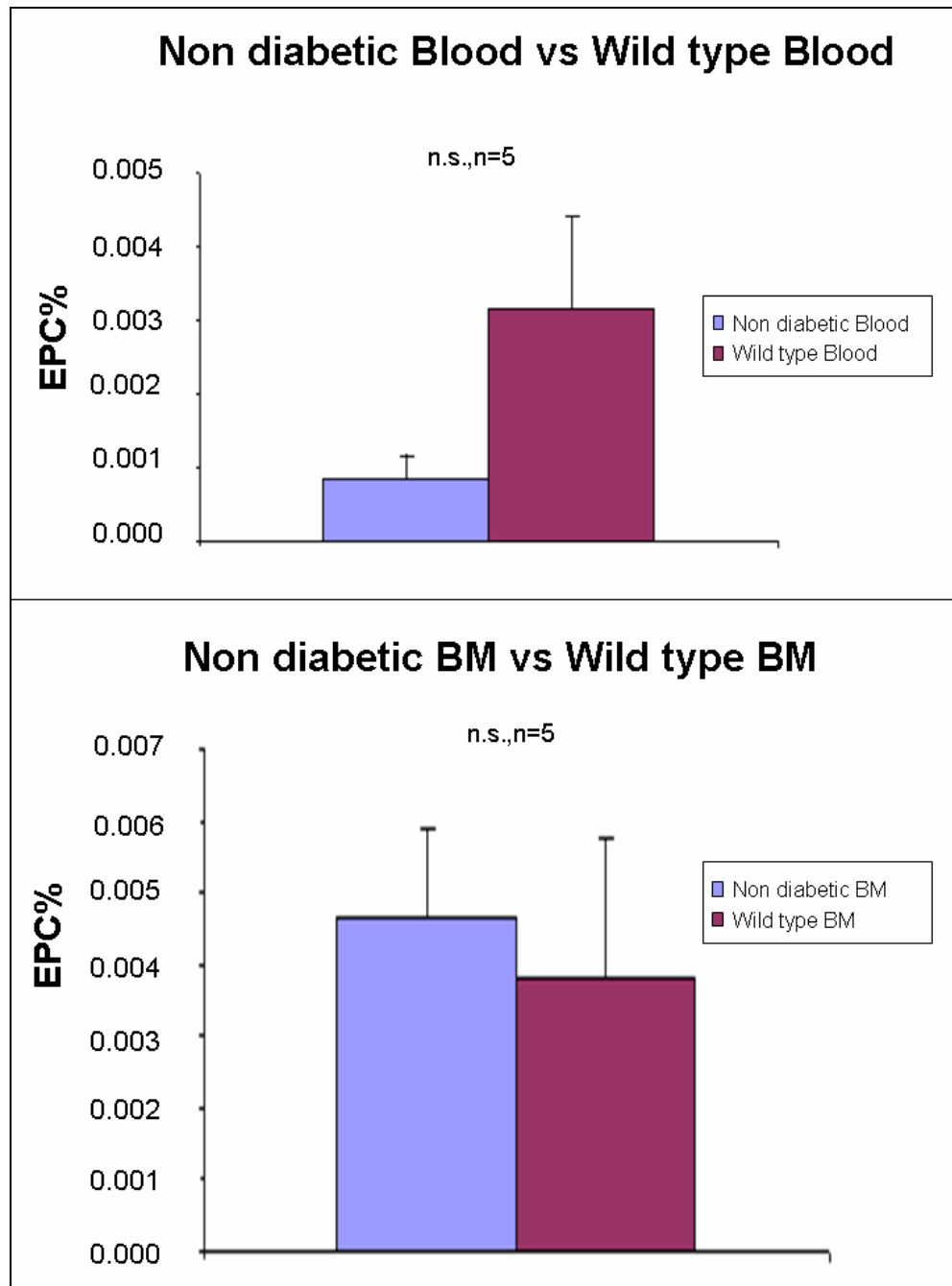


Figure 3.1. EPC numbers (%) comparison (bone marrow and blood) between non-diabetic NOD and WT (Balb/c) mice. EPCs ($c\text{-Kit}^+$ $CD31^+$ $Sca1^+$ lin^-) were obtained using flow cytometry. All experimental data are expressed as means \pm SEM of respective number of experiments, using two types of mice ($n = 5$) (non-diabetic NOD mice *versus* Balb/c mice).

Using the same gating strategies, FACS analyses were carried out to compare the number of BM and PB EPCs in the diabetic and non-diabetic NOD mice. Figure 3.2 and Figure 3.3 show the representative FACS gating strategies to obtain BM and blood EPCs ($c\text{-Kit}^+$ CD31^+ Sca-1^+ lin^-) respectively.

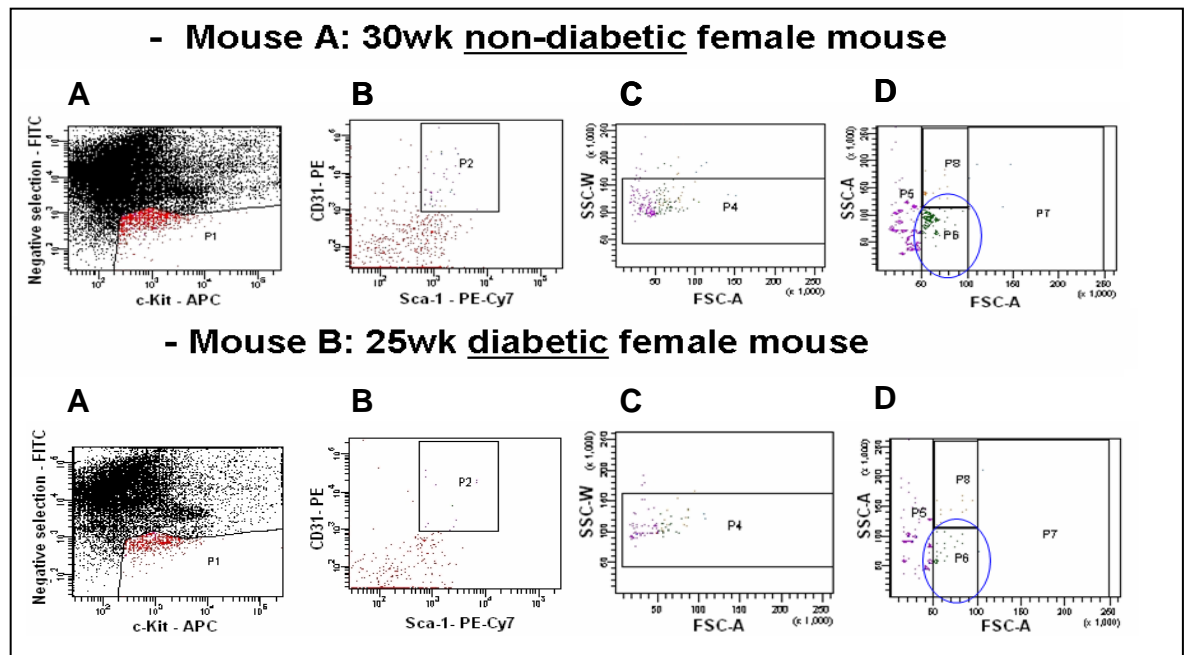


Figure 3.2. Characterization of EPCs ($c\text{-Kit}^+$ CD31^+ Sca-1^+ Lin^-) from whole bone marrow cells (1.0×10^6 cells). Cells were analysed on a BD FACSARIA cell sorter. Single colour and negative selection antibodies with isotype controls were also analysed. (A) Compensation and gating strategies were performed by firstly gating $c\text{-Kit}^+$ lin^- (APC +ve and FITC -ve cells presented as histogram, figure not shown), (B) CD31^+ Sca1^+ cells (PE and PE-Cy7+ve cells) were then gated from $c\text{-Kit}^+$ lin^- cells, (C) A band of uniform expressing APC +ve cells were then gated against forward side scatter (FSC) and doublet discrimination achieved by use of side scatter width (SSC-W) parameter against FSC, (D) These events were then back-gated against FSC versus SSC to obtain single cells of $c\text{-Kit}^+$ CD31^+ Sca1^+ lin^- which are located in the mononuclear region of the FSC versus SSC graph.

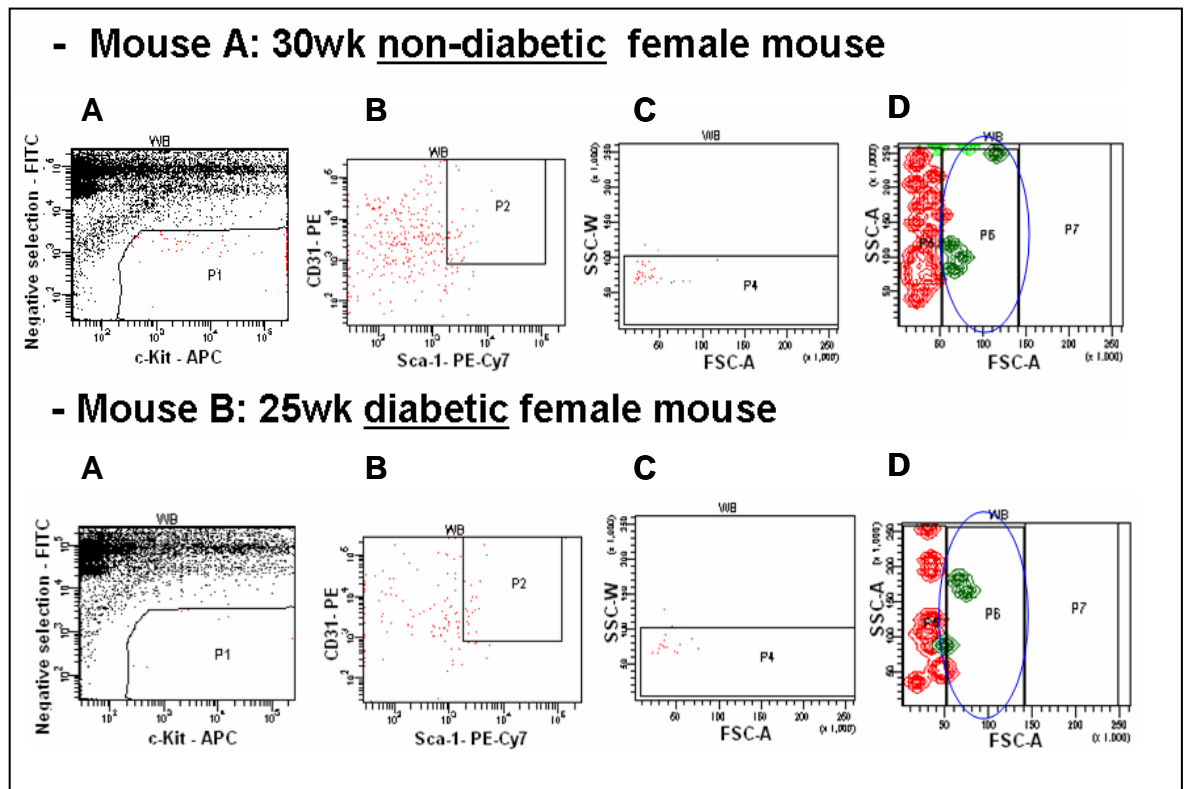


Figure 3.3. Characterization of EPCs ($c\text{-Kit}^+ \text{CD31}^+ \text{Sca-1}^+ \text{Lin}^-$) from peripheral blood cells ($< 0.5 \times 10^6$ cells). Heparinized and lysed PB cells were analysed on a BD LSRII cell sorter. Single colour and negative selection antibodies with isotype controls were also analysed. (A) Compensation and gating strategies were performed by firstly gating $c\text{-Kit}^+ \text{lin}^-$ (APC +ve and FITC -ve cells presented as histogram, figure not shown), (B) $\text{CD31}^+ \text{Sca1}^+$ cells (PE and PE-Cy7 +ve cells) were then gated from $c\text{-Kit}^+ \text{lin}^-$ cells, (C) A band of uniformly expressing APC +ve cells were then gated against FSC and doublet discrimination achieved by use of SSC-W parameter against FSC, (D) These events were then back-gated against FSC *versus* SSC to obtain single cells of $c\text{-Kit}^+ \text{CD31}^+ \text{Sca1}^+ \text{lin}^-$ which are located in the mononuclear region of the FSC *versus* SSC graph.

The results demonstrated a significant decrease in EPC number ($\text{CD31}^+ c\text{-Kit}^+ \text{Sca-1}^+ \text{lin}^-$) in BM from diabetic compared to non-diabetic NOD mice ($P=0.021$) (Figure 3.4.). Conversely, EPC number (%) was significantly increased in PB from diabetic (compared to non-diabetic NOD mice ($P=0.015$)).

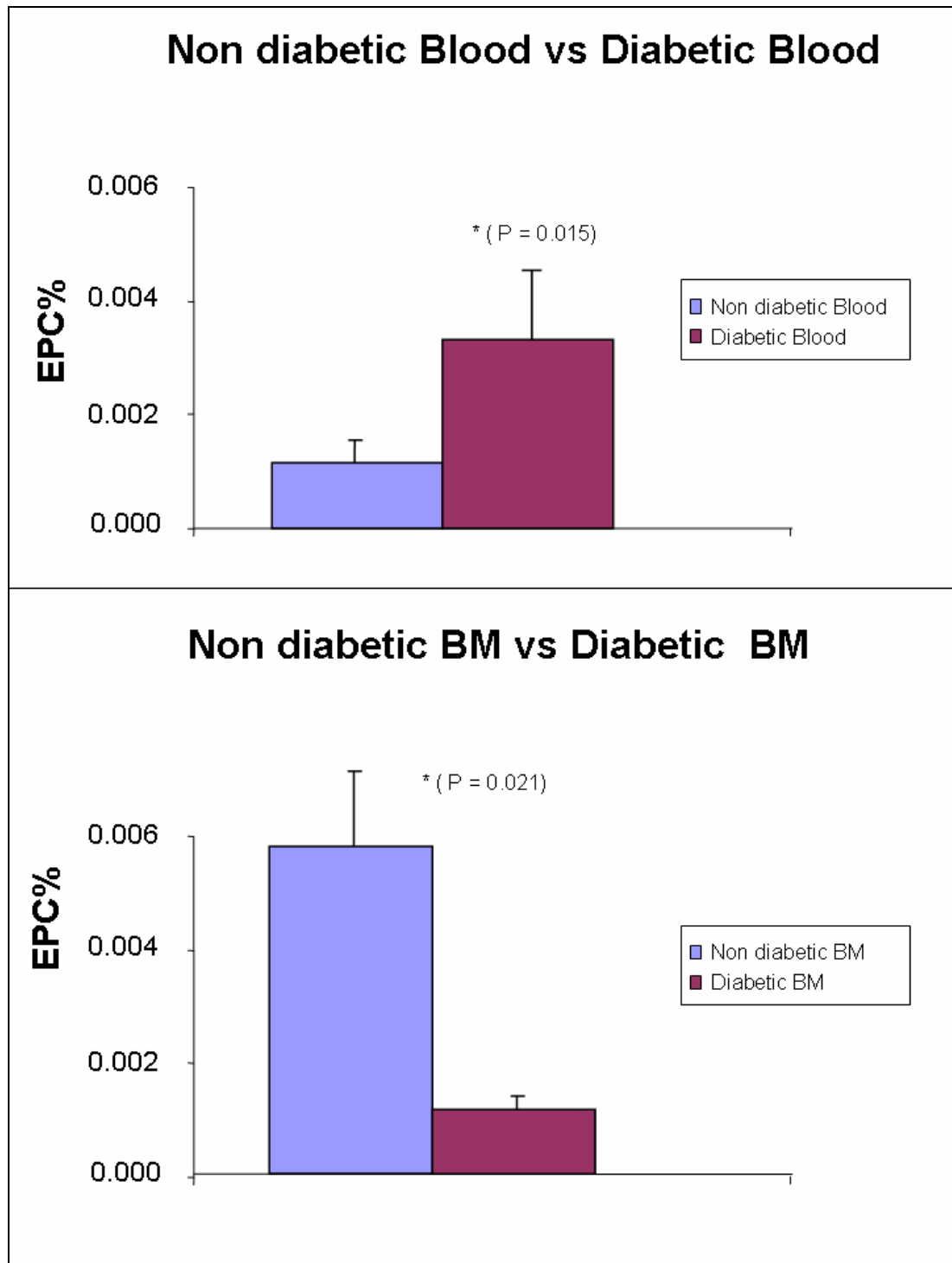


Figure 3.4. EPC numbers (%) are plotted based on the average EPC% obtained from FACS analyses from five non-diabetic and five diabetic mice. All experimental data are expressed as means \pm SEM of respective number of experiments, using two types of mice (diabetic versus non-diabetic mice). Statistical significance was tested by Student's unpaired t-test, * = significant.

As demonstrated in many studies, EPCs are present in very low numbers in the BM and circulating blood, in accordance with the above results, we demonstrated using FACS sorted BM, EPCs are reduced in diabetic mice compared to non-diabetic mice (Figure 3.5). The FACS sorting was carried out using pooled BM from two mice and we obtained approximately 1217 viable (DAPI⁺) EPCs from non-diabetic mice, more so than in diabetic female mice (~ 140 cells).

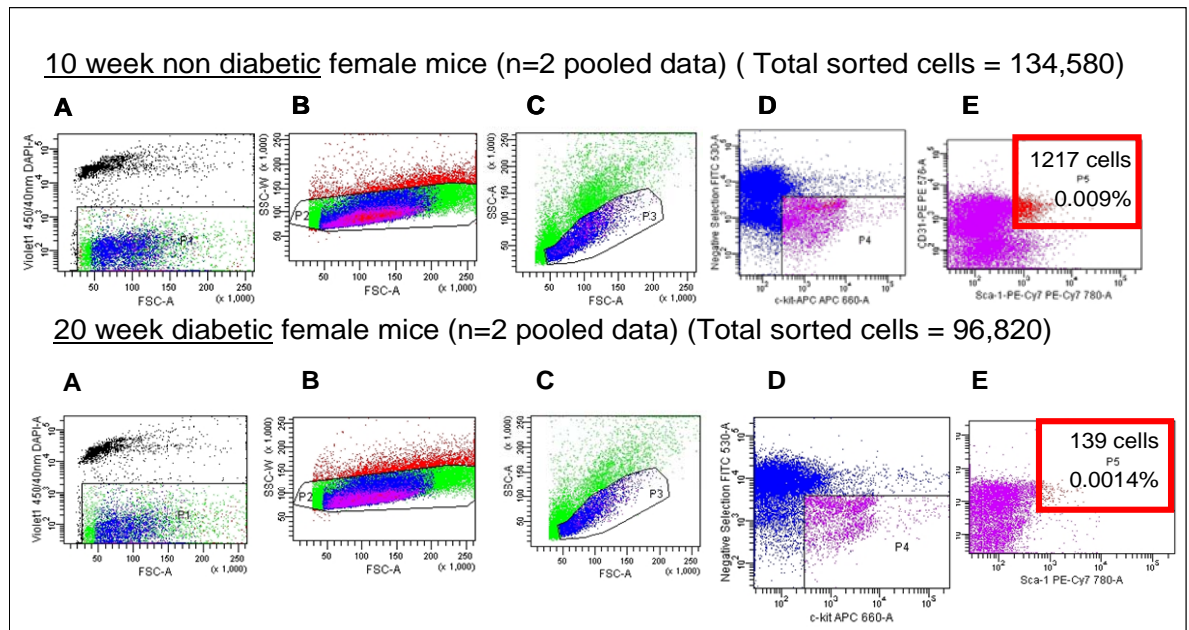


Figure 3.5. Characterization and sorting of viable bone marrow-derived EPCs from non-diabetic and diabetic NOD mice (n=2) respectively. (A) Double discrimination, (B) Selection of viable cells using DAPI, (C) Gating for mononuclear cells, (D) Gating for c-Kit⁺ Lin⁻ cells (APC +ve and FITC -ve cells presented as histogram, figure not shown), (E) Further gating for CD31⁺ Sca-1⁺ cells to get obtain c-kit⁺ CD31⁺ Sca-1⁺ cells.

3.4. DISCUSSION

In the present study, we have characterized and isolated EPCs from the BM and PB of Balb/c and NOD mice. To characterize EPCs from the BM and blood, c-Kit, CD31 and Sca-1 markers were used. c-Kit is a primordial stem marker and is important in EPC mobilization (Jackson *et al.*, 2001). Sca-1 (stem cell antigen-1) is a well known progenitor stem cell marker and is important in the regulation of c-Kit. The third marker used in this study was CD31/PECAM-1, suggested to be a marker for early angiogenesis (Rongish *et al.*, 1996) as well as the earliest marker of endothelial cell differentiation (Baldwin *et al.*,

1994). To isolate a purified population of EPC cells, lineage antibodies (CD23, CD11b, TER119 and CD45) were used to eliminate platelets, natural killer cells, macrophages and leukocytes. In our study, fresh cells were isolated from BM and PB. In contrast to other studies that used enriched mononuclear fractions (Loomans *et al.*, 2004; Zhang *et al.*, 2006), we have demonstrated that EPC or EPC-like cells can be isolated from freshly prepared whole BM and PB, although EPC populations are small. A low number of circulating EPCs was similarly observed by Thum *et al.* where they reported the number of circulating EPCs (CD133⁺ VEGFR2⁺) in human is in the range of 0.003%-0.009% (Thum *et al.*, 2007b). When cells are pooled, analysed and sorted using flow cytometry, a six-fold difference between diabetic and non-diabetic BM EPCs (Figure 3.5) was noted, which is in line with our FACS analysis data in which we demonstrated a reduction of BM EPCs in the diabetic mice and an increase of circulating EPCs in the blood (Figure 3.4).

Based on these data, BM-derived EPCs may have been stimulated to enter the systemic circulation in response to signals from the pancreas, although this hypothesis requires more evidence. Further studies need to be carried out to investigate whether EPCs can home to the damaged pancreas and contribute to β -cell regeneration. Mathews *et al.*, have demonstrated that BM-derived EPCs could be stimulated to enter the systemic circulation in response to signals from the pancreas (Mathews *et al.*, 2004). This latter observation is supported by several studies of BM-derived stem cell engraftment and contribution to neovasculogenesis in adult tissues (Brittan *et al.*, 2005; Fujii *et al.*, 2002; Hatzopoulos *et al.*, 1998; Ishizawa *et al.*, 2004; Kocher *et al.*, 2001; Taniguchi *et al.*, 2006), which is enhanced by increasing regenerative demand within a diseased or damaged tissue. The exact signaling pathways and molecular mechanism for this EPC recruitment are as yet unknown.

Human studies have demonstrated a reduction of EPCs in the PB of diabetics (Loomans *et al.*, 2004). However we observed that there is an increase of circulating EPCs in diabetic mice compared to non-diabetic NOD mice. This could be attributed to the extent of pancreatic damage of this disease model in which the mice used in the study have been diabetic for two weeks. Besides their ability to migrate and differentiate into endothelial cells when recruited, there is existing evidence suggesting that EPCs have paracrine effects in which EPCs release cytokines or growth factors to contribute to tissue regeneration at a

damaged site. There is a possibility that the EPCs were recruited to the injury site during the pre-diabetic stages and successfully engraft with the help of the cytokines released to aid regeneration of the pancreas. However as shown in humans, the EPCs in diabetic mice may be dysfunctional, and thus, they would be unable to completely vascularize or regenerate the damaged site.

The progression of autoimmune T1D in mouse and humans is characterized by infiltration of inflammatory cells and increasing blood glucose levels. Cytokines such as IL-1 β can be released during the inflammation process in the islets. Amano *et al.*, have shown that IL-1 β can increase vascular cell adhesion molecule (VCAM-1) expression on endothelial cells (Amano *et al.*, 2004), and thus may play a role in mobilization and recruitment and homing of EPCs in response to the signals from the pancreas. Further studies are thus warranted to investigate the relationship between insulinitis and EPC recruitment.

Various methods have been used to characterize and isolate EPCs from blood or BM which includes their ability to proliferate, adhere, form tubules and migrate in cell culture. However, debate still ensues as to the true phenotype of adult EPCs, which is often clouded by the overlap of the phenotype of EPC cells with haematopoietic stem cells and endothelial cells, and thus, a universally-accepted EPC phenotype remains to be achieved. These approaches are further hampered by the fact that only a small number of cells can be isolated from blood or BM (Lin *et al.*, 2000).

In conclusion, our data have illustrated that in the most appropriate model of autoimmune diabetes, the decrease in EPC of the BM and the increase in circulatory EPC's may reflect increased mobilization of BM cells. The role of these cells in β -cell regeneration therapy should be investigated in the future.

CHAPTER 4: XG-102 STUDIES ON DELAYING β -CELL DEATH USING THE NOD MOUSE

4.1. INTRODUCTION

In type 1 and type 2 diabetes, JNK (c-Jun N-terminal protein kinase), a member of the mitogen-activated protein kinase (MAPK) family plays an important role in apoptosis. Extracellular stimuli such as growth factors, cytokines and radiation activates the JNK pathway (Tawadros *et al.*, 2005). JNK activity is controlled by IB-1, a scaffold protein which interacts with upstream signalling components such as cell surface receptors, receptor-like proteins and upstream G proteins that can activate kinase substrates such as JNK. Activated JNK can phosphorylate transcription factors such as c-Jun that play a critical part in controlling expression of genes involved in apoptosis. The JNK protein kinase is a member of the MAPK group that is activated in response to dual phosphorylation on threonine and tyrosine. Ten JNK isoforms were identified in human brain by molecular cloning. These protein kinases correspond to alternatively spliced isoforms derived from the *JNK1*, *JNK2* and *JNK3* genes.

JNK signalling is important in normal development and pathogenesis of several diseases, thus JNK represents a valuable target in development of therapies. In recent years, JNK inhibitors were developed and found to have a therapeutic effect in models of rheumatoid arthritis (Gaillard *et al.*, 2005), cardiac ischaemia (Ferrandi *et al.*, 2004) and diabetes (Bonny *et al.*, 2000). To date, two main strategies to study the biological function of JNK are by using JNK knockout models and using JNK peptide inhibitors. Ten JNK isoforms have since been identified, where each JNK protein kinase corresponds to alternatively spliced isoforms derived from *JNK1*, *JNK2* and *JNK3* genes. The loss of a single isoform in the JNK1 knockout mouse was shown to alter T-cell differentiation, where they differentiated preferentially into T helper cells and exhibited reduced activation-induced cell death in T-cells, in which activation through the T-cell receptor results in apoptosis (Dong *et al.*, 1998).

IB-1 is highly expressed in pancreatic β -cells and is important in the regulation of JNK activity (Haefliger *et al.*, 2003). Cytokines such as IL-1 β and TNF- α activate the JNK pathway, contributing to cell signalling towards apoptosis. Dickens *et al.*, used JNK binding domain (JBD) of IB-1/JIP-1 to block JNK activation, successfully preventing apoptosis >90% (Dickens *et al.*, 1997). The importance of IB-1 in JNK signalling is further highlighted in a study where IB-1 was overexpressed in a mouse insulin-secreting cell line β TC-3. JNK mediated activation of transcription factors, c-Jun, ATF2 and Elk-1 was prevented with IL-1 β mediated apoptosis decreased by 80% (Bonny *et al.*, 2000). A similar study used adenovirus-mediated gene transfer of IB1 to increase IB-1 content in primary pancreatic islets and INS-1 cells; they showed a decrease in JNK activity and cells were protected from cytokine-induced apoptosis (Haefliger *et al.*, 2003).

As with pancreatic β -cell death, JNK signalling has been shown to have a part in neuronal death (Borsello and Forloni, 2007; Wu *et al.*, 2000). To investigate the role of this kinase in cell death, a protease-resistant peptide derivative of IB-1 was developed, D-JNK11 (trade name XG-102) and administered in rat models of cerebral ischaemia (Borsello *et al.*, 2003; Hirt *et al.*, 2004). XG-102 is made up exclusively of D-amino acids, in which this D-retroinverso form renders them protease resistant and additionally expand its half-life in vivo (Borsello *et al.*, 2003). Borsello *et al.*, first showed a significant reduction in brain lesion volume in rat models of middle cerebral artery occlusion (transient) and permanent occlusion after 6-12 hours of ischaemia (Borsello *et al.*, 2003). Hirt *et al.*, observed a neuronal protective effect as little as 48 hours after drug administration and demonstrated a significant reduction in infarct volume 3 hours after the onset of ischaemia, in severe cerebral ischaemia mouse model (Hirt *et al.*, 2004). This compound has thus shown striking efficacy in protecting neurons against cell death in both studies by selectively blocking the access of JNK to c-Jun and other substrates by a competitive mechanism. XG-102 competitively blocks the propagation of the pro-apoptotic events by blocking protein-protein interaction between IB1 and its partner JNK.

Members of JNK family, JNK2 and JNK1 have often been implicated in T1D and T2D progression respectively (Hirosumi *et al.*, 2002; Jaeschke *et al.*, 2005). The role of JNK1 in T1D however is not known. Since apoptosis of β -cells is one of the putative mechanisms

involved in the cascade of events leading to T1D, we intend to test XG-102 that might be useful for β -cell protection and prevention of β -cell loss in the NOD mouse. Our study using the NOD mouse is the first of its kind to investigate the potential role of JNK1 in T1D progression.

4.2. METHODS

4.2.1. Mice

Early diabetic female NOD mice were used in this study and maintained in animal unit of Charterhouse Square. Diabetes status of the mice was monitored and confirmed by measuring blood glucose levels (≥ 11.5 mmol/l) using an Accu-chek AVIVA Blood Monitor as outlined in Method 2.1.

4.2.2. XG-102 administration into diabetic NOD mice

4.2.2.1 Pilot study

NOD mice enrolled for this study (0-7 days diabetic and having blood glucose in the range of 11.5 mmol/l to 30 mmol/l) were divided into 3 groups (Figure 4.1):

- a) **Group A:** 5 female animals (15 to 25 weeks old) have been injected intraperitoneally (i.p.) with XG-102 (0.1 mg/kg) once a week for 4 weeks. Mice that were unhealthy during the study were sacrificed (around week 3, 4, 5).
- b) **Group B:** 6 female animals (15 to 25 weeks old) have been injected i.p. with XG-102 (0.1 mg/kg) once a week for 4 weeks with intended follow-up for 4 weeks, before re-administration with the same dose at the end of the 4th week. Following the 2nd treatment, mice to be followed-up until week 13; mice that were unhealthy during the study were sacrificed (around week 3, 4, 5 and onwards).
- c) **Group C:** 5 female animals (15 to 25 weeks old) as control (PBS i.p. weekly for 4 weeks) mice that were unhealthy during the study were sacrificed (around week 3, 4, 5).

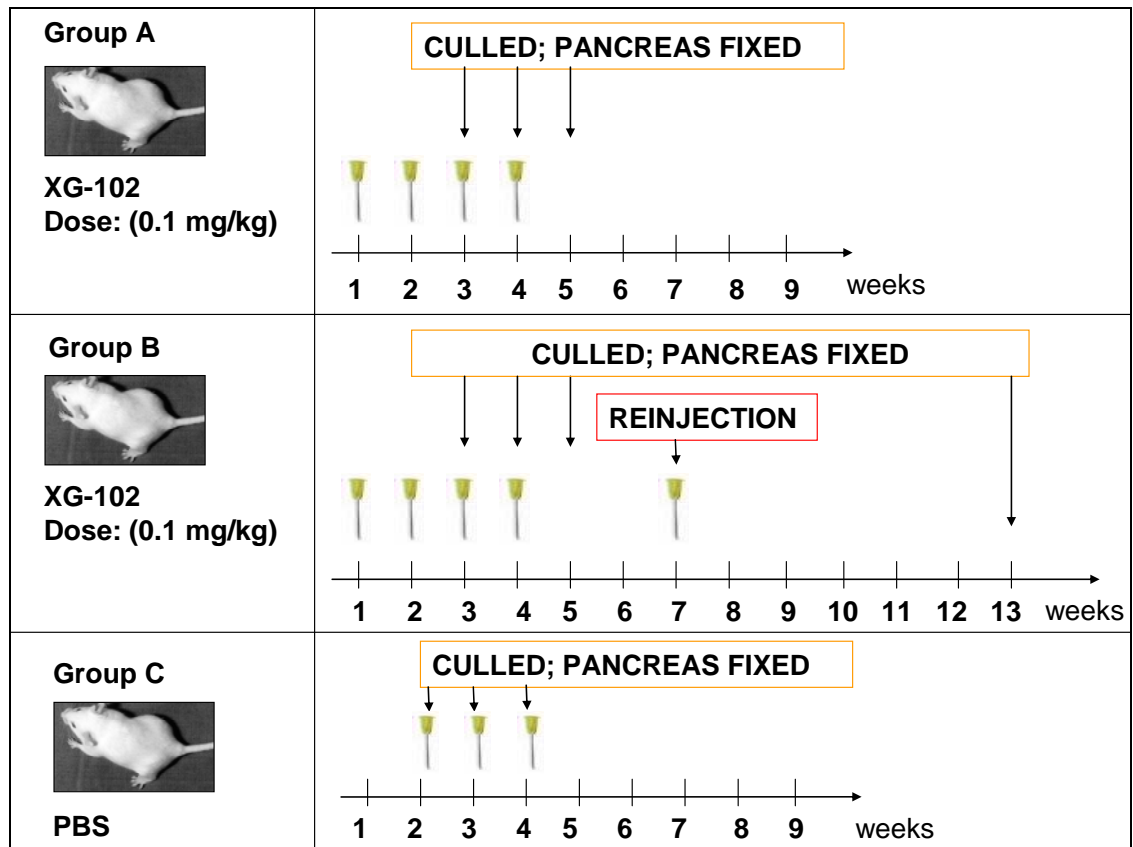


Figure 4.1. Initial experimental strategy.

Group A and B mice were injected i.p. with XG-102 (0.1 mg/kg) once a week for 4 weeks. Mice that survived past 4 weeks were to be re-administered with the same dose at the end of the 4th week of follow-up to further inhibit apoptosis (Group B). Group C, control mice were injected i.p. with PBS once a week for 4 weeks (Abbreviations: PBS, phosphate buffer saline; i.p., intraperitoneal)

4.2.2.2 Second study

Mice enrolled in this study (0-3 days diabetic with blood glucose in the range of 11.5 mmol/l to 25 mmol/l) were divided into 2 groups (Figure 4.2): a) **Group A:** 6 female animals (15 to 25 weeks old) were injected intraperitoneally (i.p.) with XG-102 (0.1 mg/kg) once a week for 4 weeks. **Group B:** 6 female animals (15 to 25 weeks old) acted as control (PBS i.p. weekly for 4 weeks). All mice that were unhealthy during the study were sacrificed by cervical dislocation (approximately at week 3, 4, 5).

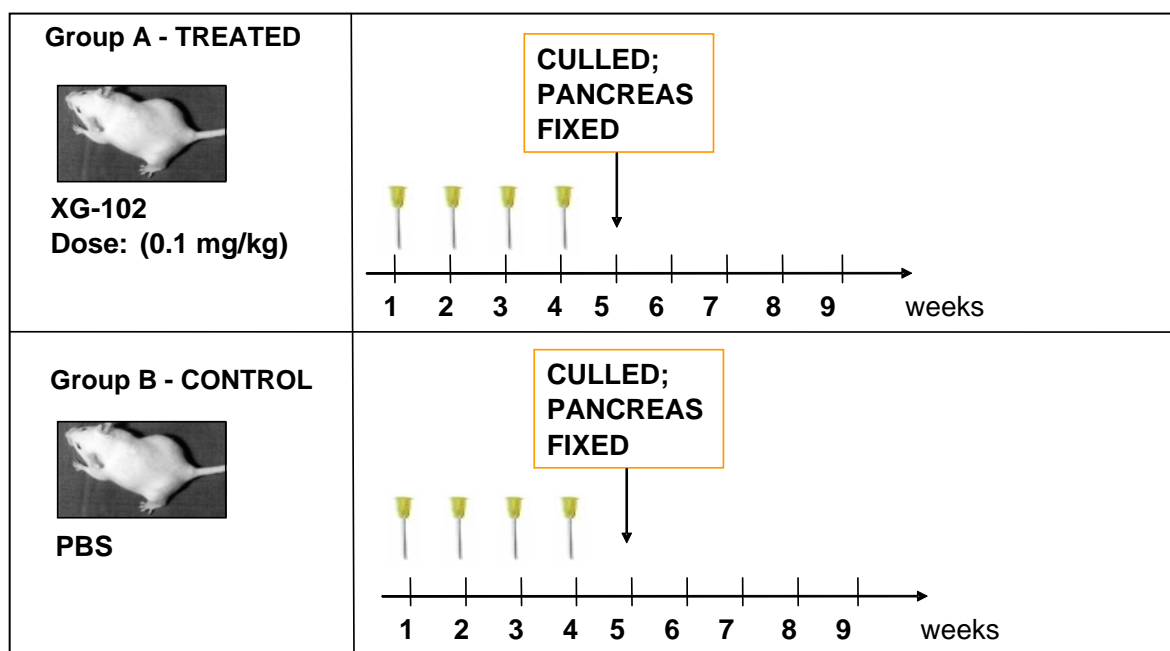


Figure 4.2. Chosen experimental strategy. Group A mice were injected i.p. with XG-102 (0.1 mg/kg) once a week for 4 weeks. Group B, control mice were injected i.p. with PBS once a week for 4 weeks (Abbreviation: PBS, phosphate buffer saline; i.p., intraperitoneal)

4.2.3. Insulin and glucagon immunohistochemistry

Paraffin embedded sections previously fixed in 10% v/v neutral buffered formalin were used (Method 2.2). For specific immunostaining the following antibodies were used at a dilution as specified. Guinea-pig anti-insulin (DakoCytomation, Carpinteria, USA) was used at a dilution 1:100 to identify β -cells. Rabbit anti-glucagon (Zymed Laboratories, Inc, San Francisco, USA) was used at a dilution 1:50 to visualise α -cells.

Sections were incubated with CAS Block (Zymed Laboratories) to block non-specific binding for 10 mins at RT after dewaxing and blocked with 0.3% v/v hydrogen peroxidase. Primary antibody was next added to the sections for 1 hour at RT. Sections were washed in PBS for 5 mins thrice and next incubated with the secondary antibody using LSAB+ System-HRP system (DakoCytomation) (at the dilution supplied by the vendor) for 30 mins at RT. Sections were then washed in PBS and incubated with Streptavidin peroxidase complex (DakoCytomation), for 30 mins at RT. After washing in PBS, DAB (3, 3'-diaminobenzidine) chromogen (Vector Laboratories) was applied to sections and the

substrate-reaction allowed to take place for 1-3 mins. This reaction (brown colour) was stopped by washing in distilled water for 1 min and followed by counterstaining in haematoxylin for 1 min, rinsed in tap water, 70%, 95% and 100% v/v alcohol, xylene, before mounting with hardset mounting medium (Vector Laboratories) and coverslipped. Controls were performed to exclude non-specific staining. As positive control for insulin and glucagon, murine pancreas sections were stained.

4.2.4. TUNEL staining for detecting apoptotic β -cells

The presence of apoptosis in the pancreas was confirmed by staining tissue sections using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling method (TUNEL), which allows the identification of apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dNTP to the DNA strand breaks caused by internucleosomal cleavage. The TUNEL method was performed using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, USA) according to the manufacturer's instructions. Briefly, the sections were deparaffinized in xylene and then rehydrated in a descending series of ethanol (100, 95 and 70% v/v ethanol) before incubating in proteinase K solution (20 μ g/ml, Dako) for 15 mins. Sections were next washed in two changes of distilled water for 2 mins each and next blocked with 0.3% v/v hydrogen peroxidase in methanol for 10 mins. After two changes in PBS for 5 mins each, sections were incubated with a mixture of terminal deoxynucleotidyl transferase and reaction buffer containing digoxigenin-dUTP (Oncor) in a humidified chamber for 1 hour at 37°C, washed in wash buffer (Oncor) for 10 mins and washed in PBS thrice for 5 mins each. Sections were incubated in anti-digoxigenin-peroxidase (Oncor) for 1 hour at RT, washed in PBS for 5 mins twice before developing in DAB for 1-3 mins. This reaction was stopped by washing in distilled water for 1 min and followed by counterstaining in haematoxylin for 1 min, rinsing in tap water, acid water, lithium carbonate, tap water, 70%, 90% and 100% v/v alcohol, xylene, and finally mounted with DEPEX and coverslipped. Controls were performed to exclude non-specific staining. Intestinal villus sections were used as a positive control for TUNEL.

4.2.5. Scoring of insulinitis

Insulinitis was evaluated using stained H&E sections as outlined in Method 2.6.1.

4.2.6. Quantification of apoptosis

As apoptosis is a major mechanism for β -cell death, we aimed to investigate whether treatment with XG-102 reduces the rate of programmed cell death by calculating the number of apoptotic cells in the islets of each group of treated and control mice. Apoptosis was evaluated by first staining with H&E. Apoptotic cells are detected by the presence of morphological characteristics such as condensed nuclei, membrane blebbing and cellular fragmentation.

The presence of apoptosis in the pancreas was confirmed by staining tissue sections using the TUNEL method, which allows the identification of apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dNTP to the DNA strand breaks caused by internucleosomal cleavage (Figure 4.3). The cleavage site can be detected with the reaction of horseradish peroxidase (HRP) conjugated streptavidin and DAB chromogen, giving a brown colour.

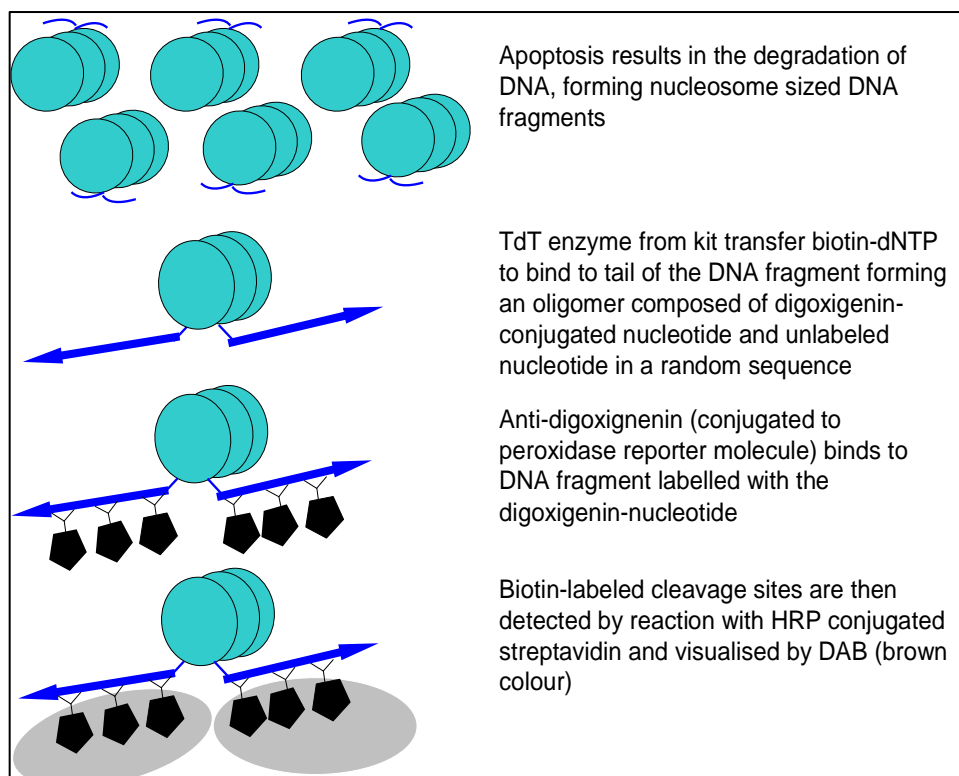


Figure 4.3. TUNEL ApopTag® method. Diagram showing the detection principle of the TUNEL method (Abbreviations: TdT, terminal deoxynucleotidyl transferase; HRP, hydrogen peroxidase; DAB, 3,3'-diaminobenzidine)(Modified from ApopTag® Plus, Oncor)

TUNEL-positive cells were viewed under a 40 x objective (total magnification = x 400) in all islets present in the pancreas to provide an index of apoptotic β -cell death. The field of view where islets reside were taken using Nikon Eclipse TE2000-S microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) with the observer blinded to the origin of data. Islet area was calibrated and measured using ImageJ version 1.38x software (<http://rsbweb.nih.gov/ij/>). Cells showing typical features such as condensed nuclei, absence of cytoplasmic staining and cellular fragmentation were counted as TUNEL-positive cells. Necrotic cells with weak diffuse cytoplasmic staining without nuclear condensation were not included. Index of β -cell death was expressed as the number of islet TUNEL-positive cells/mm² islet area.

4.3 RESULTS

4.3.1. Information on batches of mice used in experiments

Mice enrolled for this study were pre-diabetic mice confirmed by measuring blood glucose levels. Mice enrolled in Study 1 (Initial study) had blood glucose levels in the range from 11.5 mmol/l to 30 mmol/l and were 0-7 days diabetic prior to diagnosis whereas mice from Study 2 (Modified study) had blood glucose levels in the range from 11.5mmol/l to 25 mmol/l and were 0-3 days diabetic.

4.3.2. Initial study

A pilot study was first carried out to estimate the possible survival rate of these diabetic mice with treatment of XG-102 and PBS. During the course of the study, mice started to become sick after 3 weeks on XG-102 and PBS. Some mice from Group A survived past 5 weeks after drug administration (Figure 4.4). Group B mice also received XG-102 once/week for 4 weeks. The majority of Group B mice did not survive long enough to receive the 2nd administration of XG-102 at the 7th week, so they are grouped together to calculate their survival rate. At approximately 4 weeks after the 1st treatment, the treated mice (i.e. Group A & B) mice had an average survival rate of 55% compared to 20% survival rate for non-treated mice (Group C) (Figure 4.4). One mouse from Group B managed to survive past the 7th week, received a 2nd dose of XG-102 and survived until week 13.

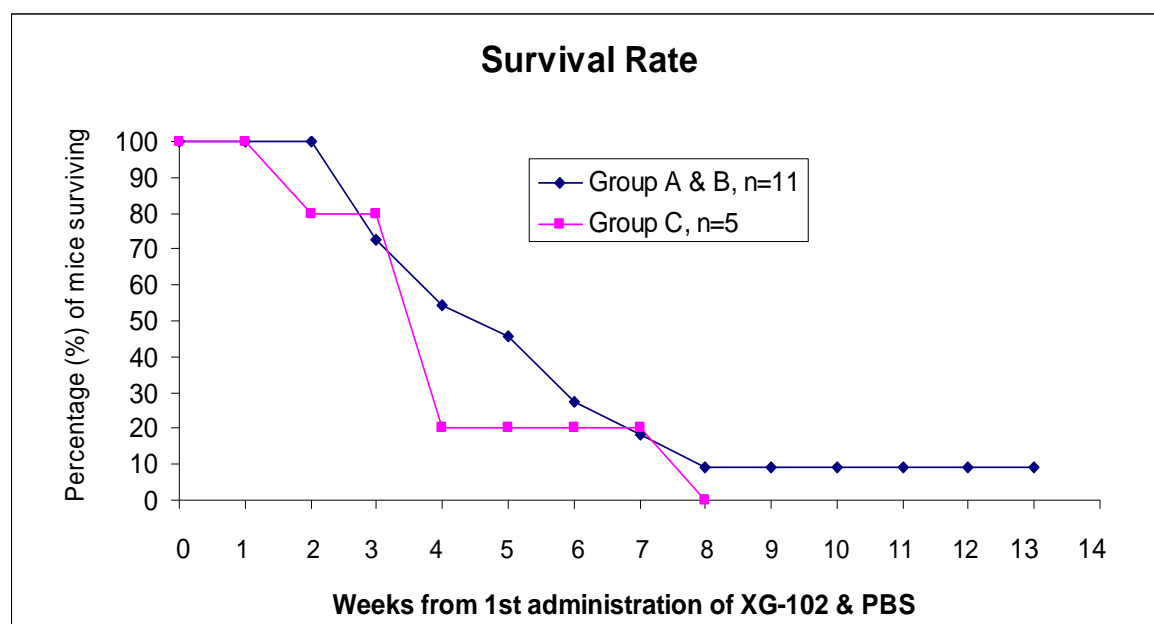


Figure 4.4. Survival rate of mice (%) after 1st administration of XG-102 and PBS
 Group A & B; mice treated with XG-102. Group C; mice treated with PBS
 The survival rate of Group A & B mice; treated with XG-102 and Group C mice; treated with PBS was plotted (n=16).

4.3.3. Modified study

The pilot data as outlined above was used as a basis for a modified study to limit factors that may affect the outcome of the study. Mice enrolled in the modified study were 0-3 days diabetic; separated into two groups, had narrower blood glucose levels ranging of 11.5 mmol/l to 25 mmol/l and kept for a set period of time (3-4 weeks).

The successful incorporation of XG-102 by i.p. injection was confirmed by staining of anti-XG-102 (performed by Xigen partners) in several pancreatic sections of treated animals (Figure 4.5A.). Rat kidney section (treated *via* i.v. with 11 mg of XG-102/kg/day) was used as a control for XG-102 (Figure 4.5B.). XG-102 was not observed in the untreated mice (not shown).

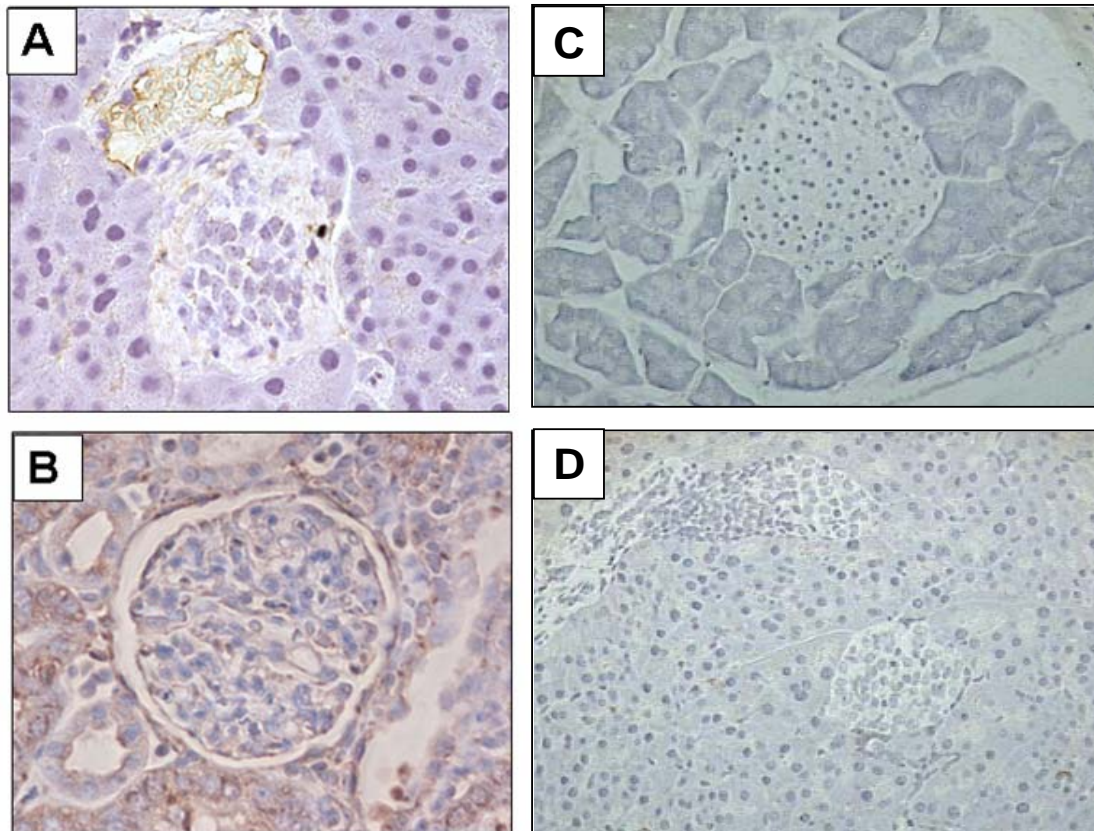


Figure 4.5. Pancreas immunoreactivity to anti XG-102 antibody staining in treated animal (A) XG-102 peptide is present in the islet after administration for 4 weeks (brown colour staining). This is evident because XG-102 is cell-permeable **(B)** Postive control: Rat kidney section (treated i.v. with 11 mg of XG-102/kg/day) was used as a control for the XG-102 antibody **(C)** Negative control: Mouse pancreas section (CD-1 mouse) (no XG-102 treatment) was used as a control for the XG-102 antibody (x 400 magnification). **(D)** Mouse pancreas section (treated i.p. with PBS) was used as a control for the XG-102 antibody (x 200 magnification).

4.3.4. Reduced destructive insulitis in treated mice

Insulitis was gauged using H&E stained sections and carried out by an observer blinded to the treatment group. The following grading standard was used (Fig. 4.6).

To study the effect of XG-102 on lymphocyte homing into islets, insulitis were scored in pancreatic sections from 6 mice each in both group A and group B (Figure 4.6). Insulitis was quantified in all islets present in two H&E-stained sections from each animal, each

section being separated from the next by at least 200 μm . These mice had been on XG-102 or PBS for the same duration of time of 4 weeks.

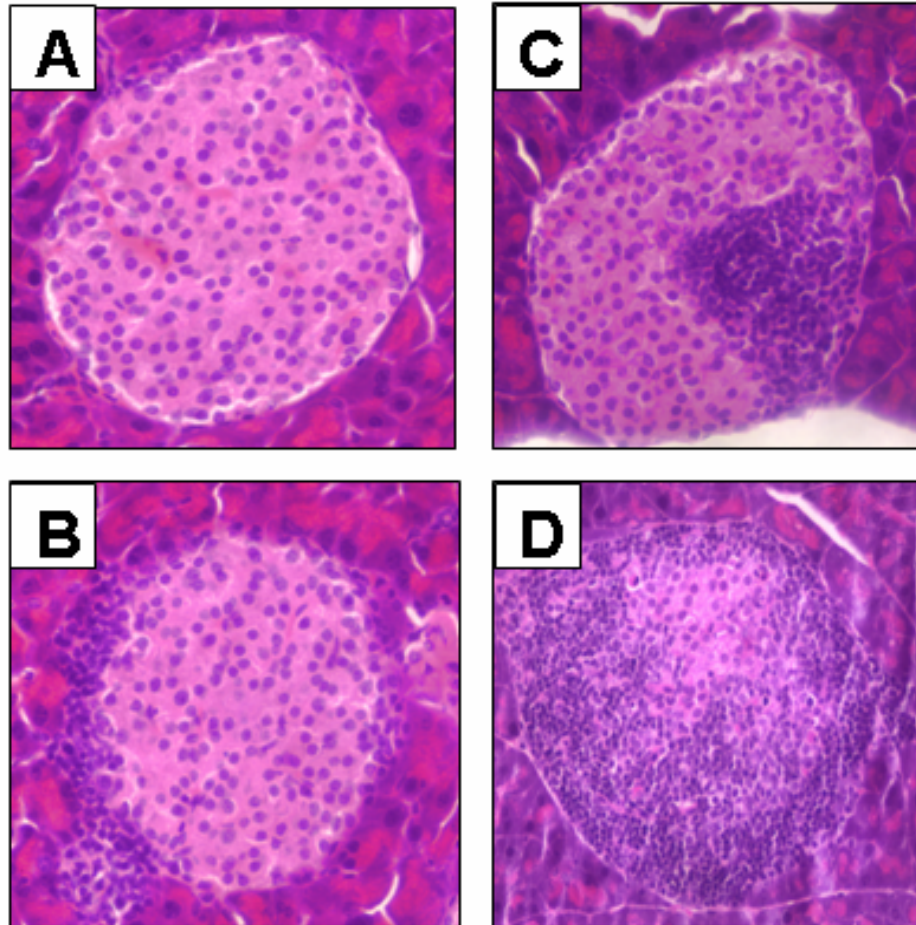


Figure 4.6. Standard insulitis grading

(A) Grade 0, normal islet without any infiltration ; (B) Grade I, peri-insulitis, where 10% of islet area is infiltrated by a peripheral ring of lymphocytes; (C) Grade II, moderate insulitis, where 25% to 50% of the islet shows mononuclear infiltration; (D) Grade III, severe insulitis, where over 50% of the islet area was infiltrated by lymphocytes.

After 4 weeks of injection with XG-102, insulitis in group A mice was less prevalent compared to the control mice. The majority (36 %) of islets from Group A mice showed little infiltration and destruction of pancreatic β -cells (grade 0 insulitis) whereas only 7 % of islets from Group B mice had no insulitis with the majority of islets heavily infiltrated (*P-value: 0.04) (Figure 4.7).

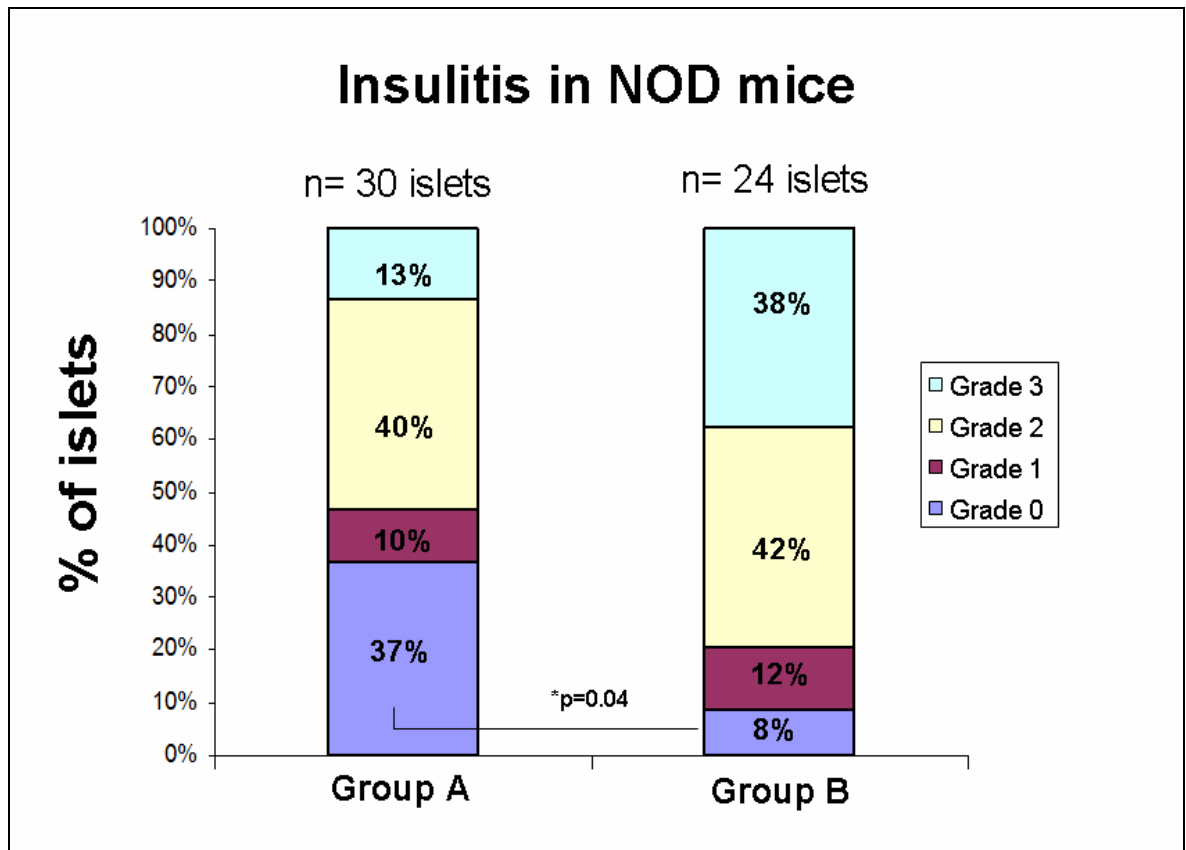


Figure 4.7. Grades of insulinitis observed in Group A and Group B early diabetic NOD mice. Grade 0, normal islet ; Grade 1, peri-insulitis ; Grade 2, moderate insulitis; Grade 3, severe insulitis. The data are the sum of islets from six mice for each group. On average, 5 islets commonly located at the “tail” part of the pancreas, were scored from each mouse. (Abbreviation: n= number of islets scored)

4.3.5. Significant change in blood glucose level before and after XG-102

After 3-4 weeks of XG-102 administration, Group A had an average blood glucose level increase of 7% compared to the significant increase of 73% in Group B mice (*P-value: 0.04) (Figure 4.8). These data suggest that Group A mice have a better control of blood glucose level compared to control mice (Group B). The significant increase in blood glucose levels is expected as they did not receive any XG-102 drug.

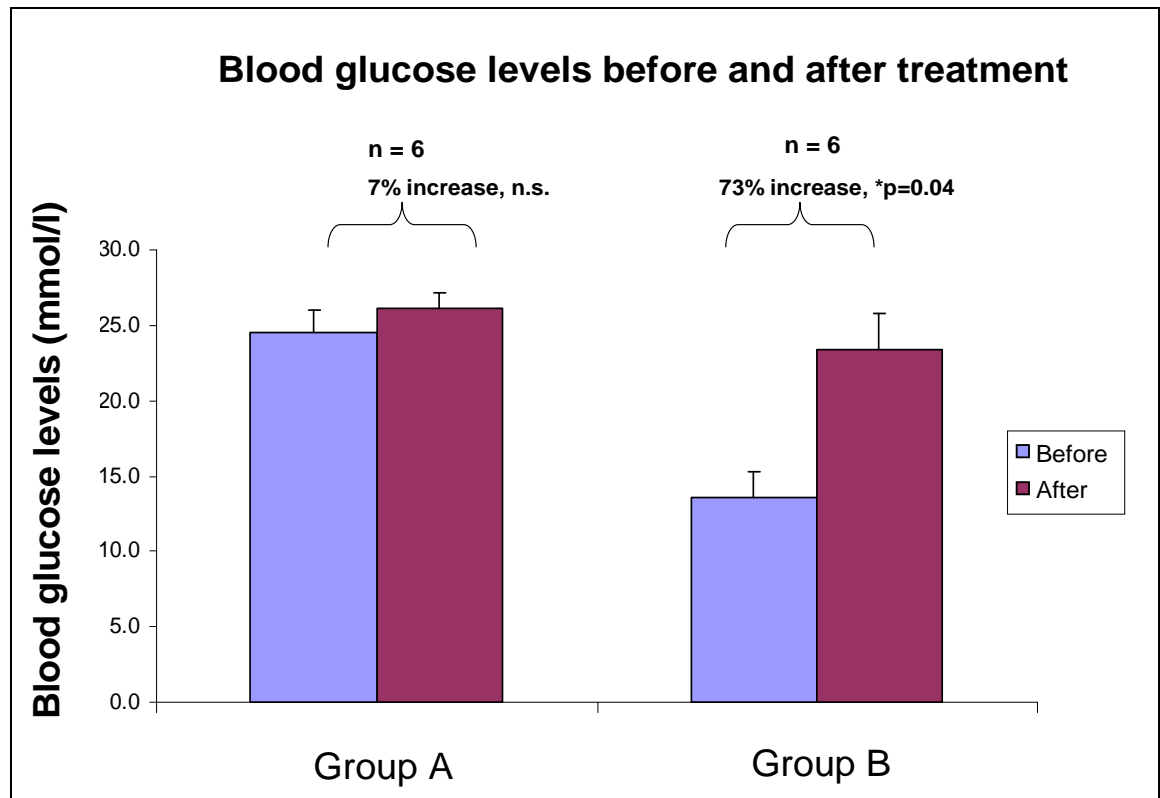


Figure 4.8. Average blood glucose levels (random, non-fasting blood glucose levels) in Group A (n=6) and Group B (n=6) diabetic NOD mice before and after treatment. Statistical significance was tested by Student's t-test (Abbreviation: n= number of mice)

4.3.6. Analysis of cell death

Following H&E staining, the apoptotic index for islets cells in each group was determined by TUNEL staining (Figure 4.9). TUNEL-positive cells matched the H&E stained cells showing characteristics of condensation of nuclear chromatin, membrane blebbing and presence of apoptotic bodies. Since TUNEL is a measure of apoptotic cell death, apoptotic cell death was observed in insulin-negative area, mainly in the intra-islet infiltrate.

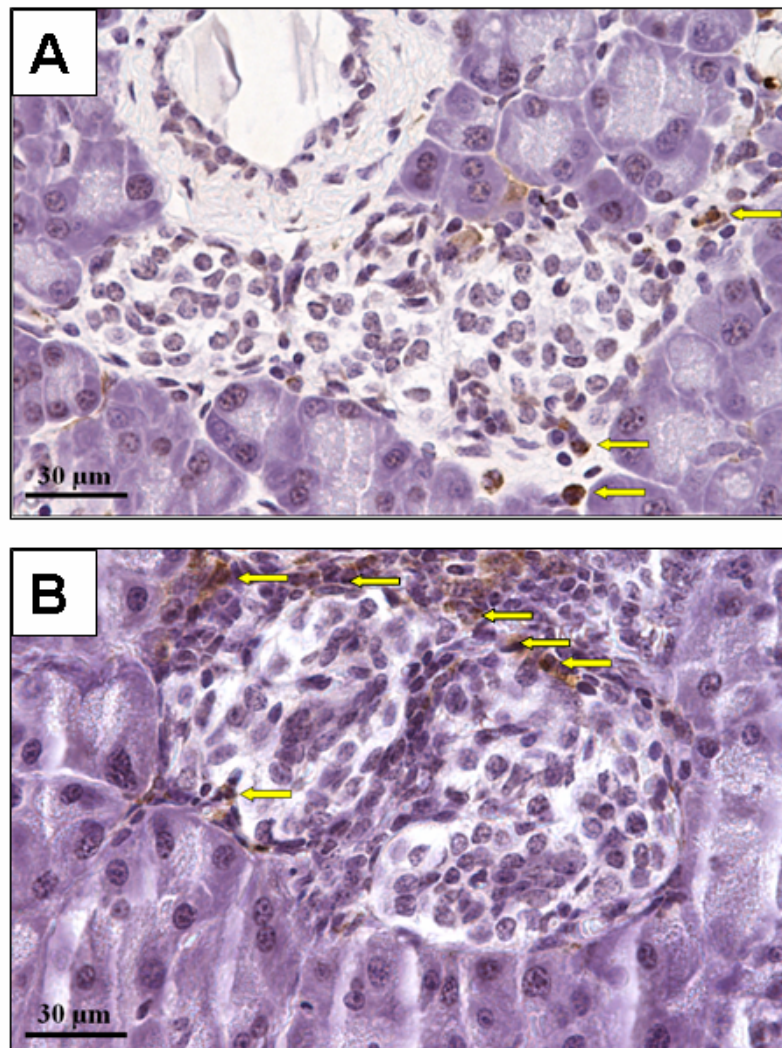


Figure 4.9. Representative islets stained using TUNEL method.

The brown staining represents TUNEL+ve cells; mainly localized at the periphery of the islet (x 400 magnification) (arrows) (A) treated mice; (B) control mice.

Although, there are very few TUNEL⁺ cells observed in the islets, there is a higher frequency of islets containing TUNEL-positive cells in control mice than treated mice (Figure 4.10)

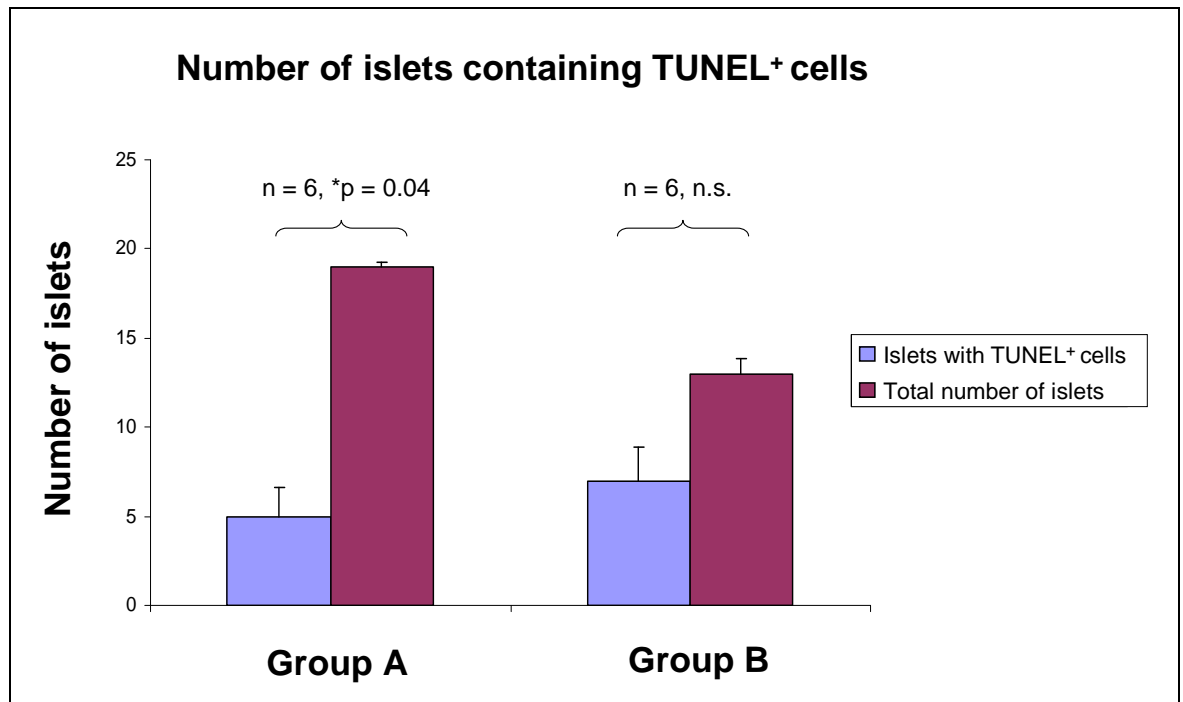


Figure 4.10. Number of islets which contained more than one TUNEL-positive cell. Statistical significance was tested by Student's t-test (Abbreviation: n= number of mice)

4.3.7. β -cell function

To investigate whether the treatment could have affected insulin production in β -cells, insulin staining was carried out (Figure 4.11A). Very few insulin-positive cells were present in the islets and thus we were unable to carry out a quantitative analysis. However, the majority of cells remaining in the islets were alpha cells as confirmed by glucagon staining (Figure 4.11C).

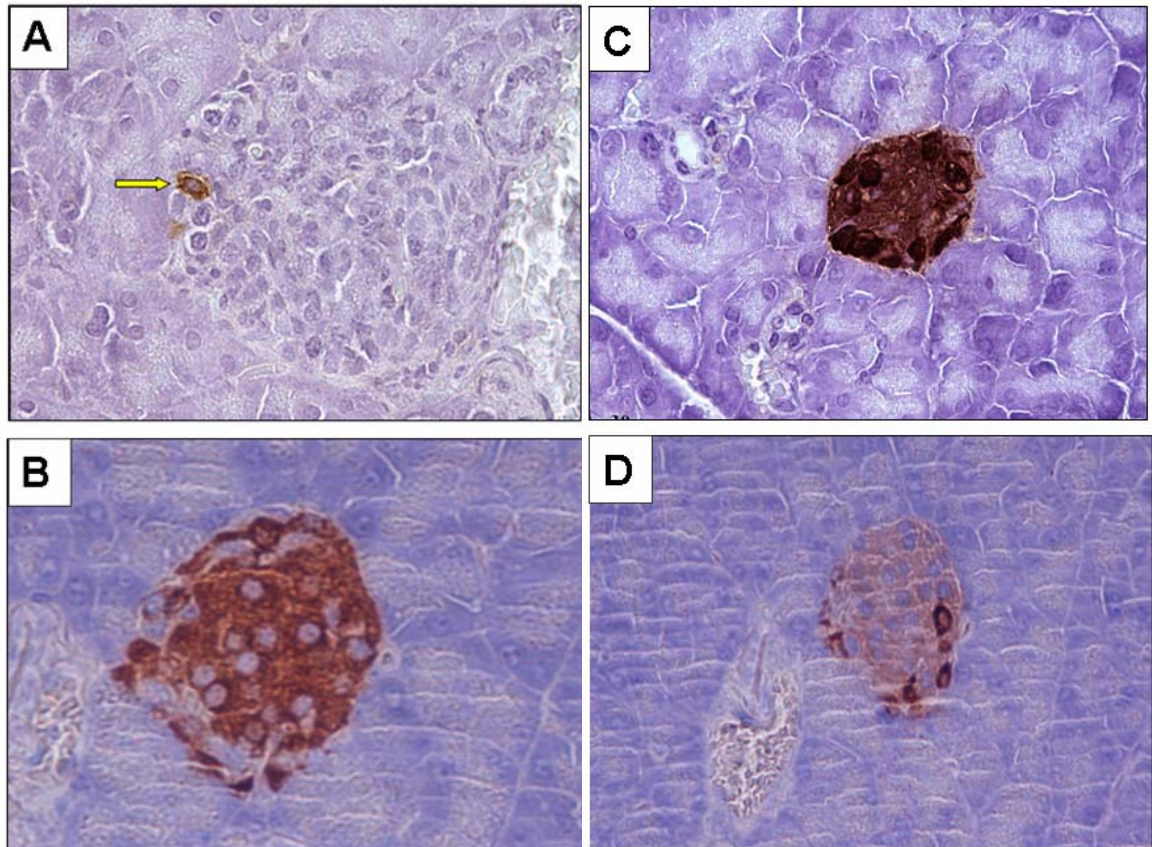


Figure 4.11. Insulin and Glucagon immunohistochemistry. (A) Representative insulin staining on pancreatic section from treated animal (B) Wild type pancreatic section was used as a control for the insulin antibody. The brown staining represents insulin immunoreactivity; all positive staining was localized within the islets (x 400 magnification) (C) Representative glucagon staining on pancreatic section from treated animal (D) Wild type pancreatic section was used as a control for the glucagon antibody. The brown staining represents glucagon immunoreactivity; all positive staining was localized within the islets (x 200 magnification).

4.4. DISCUSSION

In the present study, we have administered a JNK inhibitor, XG-102 to female diabetic NOD mice to test whether it reduces the rate of programmed β -cell death. Female mice were chosen for this study as a higher incidence of diabetes is reported, 60% compared to 15% in males. Before administration of XG-102 or PBS, blood glucose levels of mice were determined to confirm chosen mice were diabetic. This method of blood glucose measurement however may be suboptimal owing to the fact that mice may have just eaten before blood glucose measurement. To account for non-fasting glucose measurement, mice were subjected to weekly measurements during the duration of the study.

The blood glucose range used in this study was 11.5 mmol/l to 30 mmol/l. Thus, the severity of the diabetes in the mice used maybe underestimated and this may explain their low survival rate even with the presence of XG-102. In the first experimental study, we observed that mice treated with XG-102 (Group A and B) survived longer than non-treated mice (Group C). One mouse from Group B however managed to survive past 10 weeks and was finally culled at week 13. The blood glucose level of that particular mouse increased marginally from 12 mmol/l (start of dosing) to 14.83 mmol/l (after 13 weeks). There is a possibility therefore that the mouse treated with XG-102 was protected from extensive β -cell destruction and thus extending its survival. More mice were enrolled in the 2nd study to further investigate the role of XG-102 in β -cell death.

T1D is an autoimmune disease, commonly characterized by insulinitis and subsequent destruction of the insulin-secreting β -cells of the pancreas. To investigate the influence of JNK inhibition on the infiltration of mononuclear cells in the endocrine pancreas, we graded the incidence of insulinitis based on severity. We observed less severe insulinitis in treated mice compared to non-treated mice.

Immunohistochemical staining of pancreatic specimens from diabetic NOD mice 4 weeks after treatment with XG-102 showed very few insulin-positive cells. These β -cells may have become undifferentiated or discharged their insulin content in order to normalise blood glucose levels in the animal. The majority of islet cells were positive for glucagon, indicating the selective destruction of β -cells. This suggests that in the absence of β -cells

that normally make up the large, inner part of the islet, other pancreatic cells, notably alpha cells form small dense conglomerates (Zorina *et al.*, 2003).

It is well established now that JNK plays a major role in apoptosis, the main mode of death in β -cells. Jaeschke *et al.*, showed the importance of JNK2 in T1D where the disruption of the gene encoding JNK2 in NOD mice decreased destructive insulinitis and reduced disease progression (Jaeschke *et al.*, 2005). Additionally, they have demonstrated that JNK2 deficient NOD mice have lower numbers of CD4 and CD8 T cells which correlates with the reduced production of the pro-inflammatory cytokine IFN- γ (secreted by Th1 cells). JNK2 therefore may have a role in regulating T-cell differentiation to T helper cells (Th1), implicated in immune responses. Thus, when JNK signalling is inhibited such as in our study, regulation of T-cell differentiation as well as autoimmunity may be affected.

As apoptosis is a major mechanism for β -cell death, we aimed to measure the number of apoptotic cells in the islets of each group of treated mice. In mice treated with XG-102, we observed fewer numbers of islets containing apoptotic cells compared to control mice although the difference was not statistically significant. Staining also showed that apoptotic cells were found only in islets infiltrated by mononuclear cells, as similarly observed by Augstein *et al.* in the NOD mouse (Augstein *et al.*, 1998). These immuno-inflammatory cells may therefore be the major source of apoptotic events.

In some conditions, apoptosis could cause autoimmunity. Apoptosis has traditionally been thought to be a non-inflammatory process. However it has been demonstrated that apoptosis can initiate β -cell directed autoimmunity following the discovery that a neonatal wave of β -cell apoptotic cells can provide auto-antigens by allowing apoptotic cells to display autoreactive antigens in their surface blebs (Trudeau *et al.*, 2000). Apoptotic cells can also activate dendritic cells and induce formation of auto-antibodies after exposure of apoptotic thymocytes in a mouse model of systemic lupus erythematosus (SLE), triggering autoimmunity (Mevorach *et al.*, 1998). Based on our study, β -cell destruction by apoptosis may have been delayed which consequently affects autoimmune islet destruction.

Our data imply that XG-102 may be acting by reducing lymphocyte homing into the islets, which could explain the reduction in blood glucose levels, however the competitive blocking of JNK1 induced by XG-102 had little effect on β -cell apoptosis. These data support our assumption that when the process of β -cell destruction is well advanced and with few residual β -cells left, it is difficult to reverse the disease. Attempts should be made to intervene earlier in the natural history of β -cell destruction when more β -cells are present.

CHAPTER 5: EPC EXPANSION & CHARACTERIZATION

5.1. INTRODUCTION

EPCs are derived from the BM and PB, contributing to tissue repair in various pathological conditions *via* the formation of new blood vessels, that is, neovascularization. In 1997, Asahara *et al.* successfully isolated EPCs from human PB and demonstrated the ability of these cells to differentiate into mature endothelial cells (Asahara *et al.*, 1997). These cells were thought to be only involved in vasculogenesis during embryogenesis, but are now recognized to have a significant bearing upon disease outcome through their contribution to neovascularization in a variety of pathological states in adulthood. Additionally, there is existing evidence suggesting that EPCs have a paracrine role in tissue regeneration (Di Santo *et al.*, 2009).

Presently, the two main techniques to obtain EPCs are flow cytometry and/or cell culture. Flow cytometry is useful to enumerate EPCs from fresh PB or BM and quantifying cells expressing a number of markers (Fadini *et al.*, 2008). Additionally, the analysis by FACS can be variable owing to technical factors such as washing and centrifugation steps, number of cells, buffers, antibodies and gating technique used (Ozdogu *et al.*, 2007).

EPCs exist in very small numbers, especially in circulating blood in adults where they only account for 0.01% of all cells. Owing to the low frequency of these cells, cell culture is often used to expand them. Generally, the mononuclear fraction of BM or blood is isolated by centrifugation, seeded on fibronectin coated plates and cultured in endothelial growth medium. Cell culture using this technique results in two distinct EPC populations, which are early outgrowth cells and late outgrowth cells. These populations differ in timing of their emergence during culture, tubule formation and paracrine properties (Table 5.1) (Hur *et al.*, 2004; Shantsila *et al.*, 2008; Yoder *et al.*, 2007). EPCs have also been characterized by their ability to form colonies known as colony-forming units (CFUs) which involves the pre-plating of non-adherent cells after 2 days of plating MNCs (Hill *et al.*, 2003). These CFU colonies have been shown to be haematopoietic in origin, the majority possessing myeloid progenitor cell activity (Yoder *et al.*, 2007). Although isolation of EPCs *via* culture is useful to expand their numbers, these early progenitor cells compared to

circulating cells may lose their angiogenic and phenotypic properties as they mature in culture (Timmermans *et al.*, 2008).

Early EPCs	Late EPCs
Appear after 3-5 days of culture	Appear after 7-14 days of culture
Round to spindle shaped appearance	Typical cobblestone morphology
Express endothelial markers	Express endothelial markers
Express haematopoietic markers	Does not express haematopoietic markers – CD133, CD14 or CD45
Bind UEA-1 lectin and uptake Dil-ac-LDL	Bind UEA-1 lectin and uptake Dil-ac-LDL
Some proliferative potential	Robust proliferative potential
Do not form vascular tubes <i>in vitro/in vivo</i> in matrigel	Form vascular tubes <i>in vitro/in vivo</i> in matrigel
Augment vascular network in a paracrine fashion/ secrete angiogenic cytokines	Does not exert paracrine angiogenic effects
Improve neovascularization <i>in vivo</i>	Improve neovascularization <i>in vivo</i>

Table 5.1. Comparison of properties between early and late EPCs (adapted from Timmermans *et al.*, 2008) (Abbreviations: UEA-1 lectin, Ulex europaeus agglutinin-1; Dil-ac-LDL, Dil-labelled acetylated low density lipoprotein)

In addition to phenotyping EPCs using FACS or immunocytochemistry, it is important to test the functional properties of these newly derived cells. The most common *in vitro* assays are the uptake of Dil-labelled acetylated low-density lipoprotein (Dil-ac-LDL), binding of the ulex lectin, and chemokine and tubule formation assays. Many studies have also tested the angiogenic properties of these cells in *in vivo* models such as flap ischaemia and induced liver fibrosis model (Nagano *et al.*, 2007; Ueno *et al.*, 2006).

Due to different morphologies and biological properties used in earlier studies, the phenotypical and functional properties of newly derived EPCs should be carefully re-examined and taken into consideration when future studies are performed. Additionally, the continual maturation of these cells needs to be taken into account as it results in an antigenic marker shift, and thus markers used for initial isolation of cells using FACS may not be suitable to isolate cultured EPCs (Shantsila *et al.*, 2008).

This study aimed to characterize and isolate BM-derived EPCs using two methods – (i) using selective media and (ii) magnetic cell sorting for c-Kit⁺ cells. The EPC population that demonstrates both phenotypic and functional properties of endothelial cells will then be transplanted into NOD mice, to test their ability to form new blood vessels or contribute to pancreatic regeneration *via* release of paracrine factors.

5.2. METHODS

5.2.1. Foetal bovine serum batch testing

To ensure optimal EPC growth conditions, six batches of defined-FBS from Sigma, PAA and Lonza were tested (Sigma Lot# 036K3395, 076K3395, #085K3395 and 105K3396; PAA Lot# A15207-1979 and Lonza Lot# 75B0018). An equal number of whole BM cells (approximately 1×10^7 cells) (n=3) were plated onto human fibronectin-coated (Sigma, St. Louis, USA) 6-well culture plates at a density of $1-2 \times 10^6$ cells/well with endothelial growth media and the respective batch of FBS (Table 5.2). Cells were cultured in the 6 constituted medium in parallel for 12 days, in triplicate wells and the number of colonies was counted on day 6 and day 12 of culture. Overall growth was also evaluated by morphological evaluation for a set period of 2 weeks to define the optimal EPC growth.

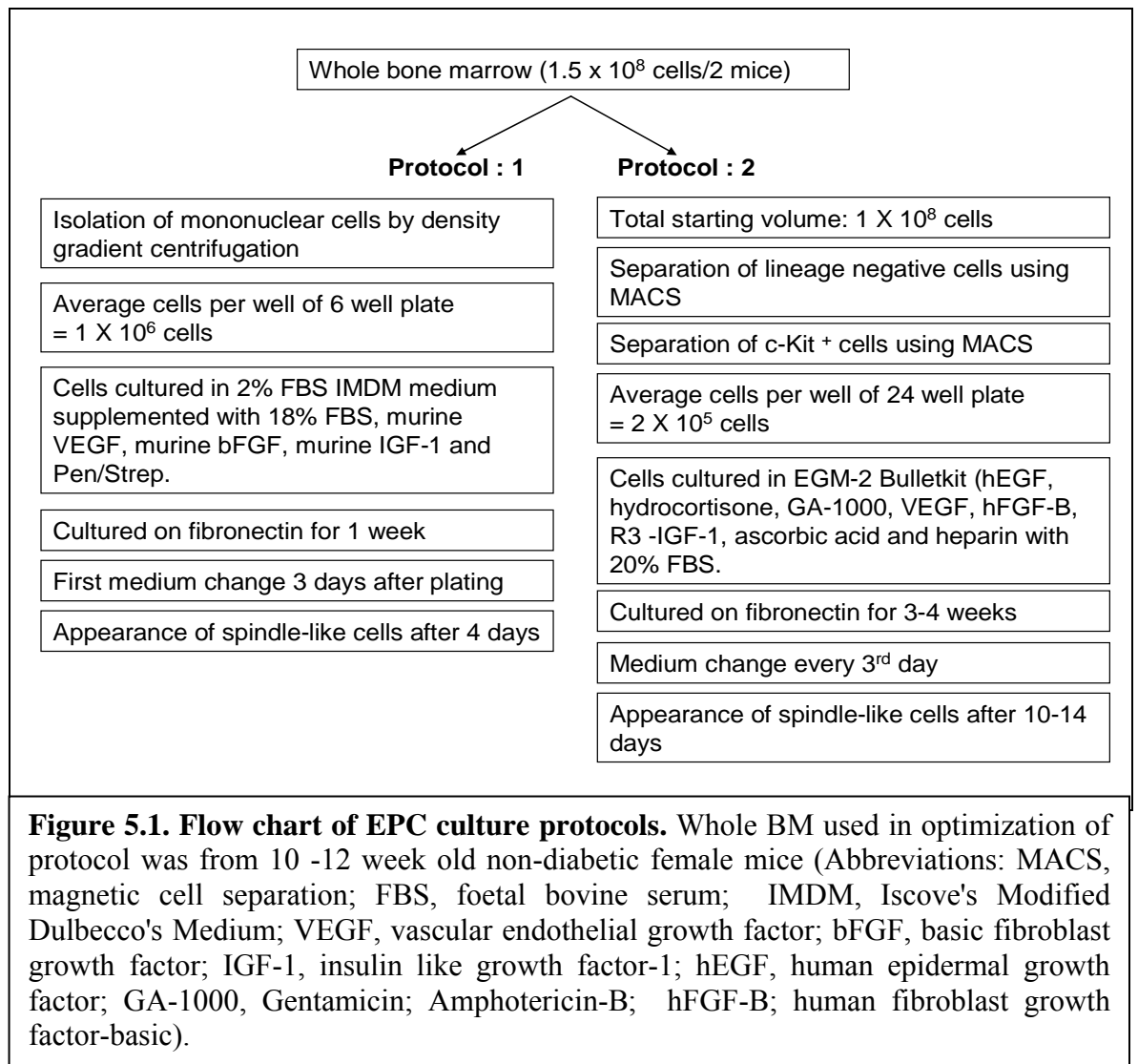
FBS Name	Catalogue No	Lot No	Company	n of colonies (Day 6)	n of colonies (Day 12)
FBS A	F7524	#036K3395	Sigma	1	1
FBS B	F7524	#076K3395	Sigma	1	1
FBS C	F0804	#105K3396	Sigma	3	5
FBS D	A11-152	#A15207-1979	PAA	1	5
FBS E	F9665	#085K3395	Sigma	1	14
FBS F	DE14-870C	#76B0018	Lonza	0	6

Table 5.2. Foetal bovine serum batch details and number of colonies obtained after day 6 and day 12 of culture (Abbreviation: FBS, foetal bovine serum)

5.2.2. Protocol 1: Isolation of EPCs using selective media

Whole BM cells from non-diabetic NOD mice (control) were first isolated as previously described. Density gradient centrifugation (Cederlane) was performed on the BM cells to obtain mononuclear cells (MNCs). Whole BM and MNCs were suspended in 2% FBS

IMDM medium (Stem cell technologies) supplemented with 18% FBS (Autogen Bioclear), murine VEGF (Peprotech EC Ltd, London, U.K.), murine bFGF (Peprotech), murine IGF-1 (Peprotech) and Pen/Strep (Gibco) and plated onto separate human fibronectin-coated (Sigma, St. Louis, USA) 6-well culture plates, incubated in a 5% CO₂ incubator at 37 °C. First media change was performed 3 days after plating and maintained for 12 days. Each cluster or colony was followed-up every day (Figure 5.1).



5.2.3. Protocol 2: Isolation of EPCs using magnetic cell separation system (MACS)**5.2.3.1. Magnetic labelling with lineage negative microbeads**

Whole BM cells were isolated as previously described and washed in cold EPC medium. Cells were passed through a 30 µm filter (Miltenyi Biotec Ltd., Surrey, U.K.) and then centrifuged at 1500 rpm for 5 mins at 4 °C. Supernatant was removed and cells resuspended in 1 ml of MACS buffer. A fraction of 100 million cells was aliquoted into a new 5ml Falcon polystyrene tube and incubated with the lineage negative cocktail according to the manufacturer's instructions (Mouse Lineage Cell Depletion Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, cells were resuspended in 400 µl MACS buffer. Next, 100 µl biotin antibody cocktail was added, mixed well and incubated for 10 mins in the fridge. After incubation, a further 300 µl MACS buffer was added and incubated with 200 µl anti biotin cocktail for 15 mins in the fridge. Cells were subsequently washed with 1ml MACS buffer and centrifuged at 300 x g (1300 rpm) for 10 mins at 4°C. Pelleted cells were resuspended in 500 µl MACS buffer and kept on ice ready for cell separation.

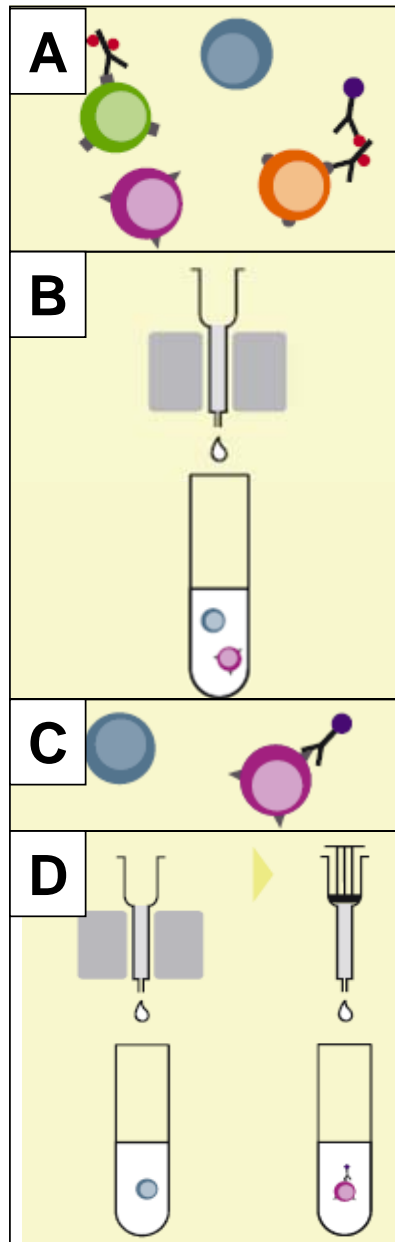


Figure 5.2. Isolation for enriched c-Kit⁺lin⁻ EPCs using MACS cell separation system. (A) 1st magnetic labelling: Washed whole bone marrow cells were magnetically labelled with biotinylated lineage negative antibody cocktail and subsequently, anti-Biotin MicroBeads (B) 1st magnetic separation: Undesired cells such as natural killer cells and erythrocytes are retained in a MACS® Column placed in a MACS Separator while the unlabeled cells (lineage negative cells, lin⁻) pass through (C) 2nd magnetic labelling: Lin⁻ were then magnetically labelled with c-Kit⁺ MicroBeads (D) 2nd magnetic separation: Cells were then passed through another prepared column and c-Kit⁺lin⁻ cells were retained in the column while unlabelled cells pass through. After the column is removed from the separator, the target cells are eluted as the enriched, positively selected c-Kit⁺ cell fraction (adapted from Miltenyi Biotec, <http://www.miltenyibiotec.com>).

5.2.3.2. Magnetic separation

LS columns (Miltenyi Biotec Ltd.) were used to magnetically separate labelled positive lineage cells from the lineage negative cells. LS column was placed on a MidiMACS separator unit (Miltenyi Biotec Ltd.). Column was rinsed with 1000 µl MACS buffer (3 times). Cell suspension was then applied onto the column into a 5ml Falcon polystyrene tube. This was followed by washing of the column with 1000 µl MACS buffer (3 times) into the same tube which collects all unlabelled cells (i.e. negative fraction of cells). To

obtain the positive fraction of cells, 1000 μ l MACS buffer was added onto the column and subsequently flushed out into a new 5 ml polystyrene tube using the supplied plunger. Positive labeled cells collected were spun at 300 x g for 10mins at 4 $^{\circ}$ C, resuspended in appropriate volume and kept on ice.

5.2.3.3. Positive selection of c-Kit⁺ cells by magnetic cell sorting

Lineage negative cells (Lin⁻) were enriched for c-Kit⁺ cells using a c-Kit Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, lineage negative cells collected were spun down at 300 x g for 10 mins at 4 $^{\circ}$ C and resuspended in 800 μ l MACS buffer. Next, 100 μ l CD117 microbeads (Miltenyi Biotec Ltd.) were added and incubated for 15 mins in the fridge. Cells were subsequently washed with an additional 1ml MACS buffer and spun down at 300 x g for 10 mins at 4 $^{\circ}$ C. Pelleted cells were resuspended in 500 μ l MACS buffer and loaded onto a pre-washed column to obtain c-Kit⁺ Lin⁻ cells.

5.2.3.4. Analysis of c-Kit⁺ purity using flow cytometry

An aliquot of the c-Kit⁺ cell fraction was analyzed to assess purity. The purity of c-Kit⁺ selected cells was determined after isolation. Isotype-matched mouse immunoglobulin served as controls. All incubations were performed at RT. After each incubation, cells were washed in PBS containing 2% FBS. Cells were incubated with a c-Kit antibody for 15 mins. Each analysis included at least 5000 events. The percentage of c-Kit⁺ cells was assessed after correction for the percentage of cells reactive with an isotype control.

5.2.3.5. c-Kit⁺ Lin⁻ cell culture

Freshly isolated c-Kit⁺ Lin⁻ cells were spun down at 300 x g for 10 mins at 4 $^{\circ}$ C. Cells obtained were resuspended in 300 μ l MACS separation buffer and manually counted by using a haemocytometer. c-Kit⁺ Lin⁻ cells were cultured at a density of $2 \times 10^5 - 3 \times 10^5$ cells in 24-well plates coated with fibronectin (Sigma) and maintained using EPC medium EGM-2 Bulletkit (Lonza Wokingham Ltd, London, U.K.) which includes EGM basal media and growth factor supplements containing hEGF, hydrocortisone, GA-1000

(Gentamicin, Amphotericin-B), VEGF, hFGF-B, R3-IGF-1, ascorbic acid and heparin (Lonza Wokingham Ltd.) and additionally supplemented with 20% FBS (PAA Laboratories Ltd, Yeovil Somerset, U.K.) and Pen/Strep (Invitrogen Ltd, Renfrew, U.K.), at 37 °C in a 5% CO₂ atmosphere. For immunostaining, c-Kit⁺ Lin⁻ cells were plated at a density of 8 x 10⁴ – 12x 10⁴ cells in 8-well chamber slides (BD) coated with fibronectin. Half media change was performed every two days and maintained for 3-4 weeks.

5.2.4. Characterization of EPCs

5.2.4.1. FACS analysis of bone marrow-EPCs

For analysis of cultured EPCs, cells were washed with PBS twice before adding 0.25% trypsin EDTA (Invitrogen). After 5 mins, cells were neutralised with 2% v/v PBS FBS and spun down at 1500 rpm for 5 mins. Resuspended cells were then incubated with c-Kit antibody (APC, BD Biosciences) (1:100 dilution) and analysed using BD FACSAria (BD Biosciences). Analysis was performed with isotype-matched IgG controls.

5.2.4.2. Immunocytochemistry of EPCs

Cells cultured on 8-well chamber slides for 3-4 weeks were washed twice with PBS and fixed for 15 mins with 4% w/v paraformaldehyde. Specimens were then incubated for 45 mins with a primary antibody. The following primary antibodies were used: anti-CD31 (BD), anti-CD34 (eBioscience, San Diego, USA), anti-von Willebrand factor (Dako), anti-CD133 (Abcam) and anti-VEGFR-2 (BD) (Table 5.3). In brief, we used a fluorescent direct immunostaining method and this was performed as follows: fixed cells were washed twice with PBS, then permeabilised with 0.2% v/v Triton-100 for 10 mins, and washed twice with PBS. After blocking with 2% v/v goat serum for 30mins, cells were incubated with primary antibody at RT for 45 mins. Then, cells were washed with PBS, incubated with appropriate fluorescent label (with the addition of DAPI, Molecular Probes) for 45 mins, and washed with PBS before mounting with Vectashield (Vector Laboratories Ltd). Negative controls were performed by omitting the primary antibodies. Fluorescent images were taken using a fluorescence microscope (Nikon, Japan).

Primary antibodies	Dilution	Vendor	Secondary antibodies	Dilution	Vendor
Rat anti-CD31	1:100	BD Biosciences	Goat anti rat-488	1:500	Molecular Probe
Rabbit anti-vWF	1:50	Dako	Goat anti rabbit-488	1:1000	Molecular Probe
Rabbit anti-CD133	1:100	Abcam	Goat anti rabbit-488	1:1000	Molecular Probe
Rat anti-CD34	1:100	eBioscience	Goat anti rat-488	1:500	Molecular Probe
Rat anti-VEGFR-2	1:30	BD Biosciences	Goat anti rat-488	1:500	Molecular Probe

Table 5.3. Details of primary and secondary antibodies used for immunostaining of cultured cells

5.2.4.3. Dil-acLDL uptake assay and lectin staining of cultured EPCs

The phenotype of culture BM-EPCs was examined by uptake of Dil-labelled acetylated LDL (Dil-acLDL) and staining for lectin. Briefly, attached cells (after 5 days of culture) were incubated with Texas-Red Dil-ac-LDL (Invitrogen). After one hour incubation, cells were fixed with 4% w/v paraformaldehyde for 10 mins. Cells were then stained with FITC-UEA-1-lectin (Sigma) and viewed under the fluorescent microscope. Cells double positive for Dil-ac-LDL and UEA-1 lectin were deemed EPCs and quantified by examining random microscopic fields (Figure 5.3). Human carcinoma cell line (Caco2) cells were used as negative control for Dil-ac-LDL and UEA-1 lectin to confirm that only EPCs are stained positive.

5.2.4.4. Tubule formation assay

Cells were starved with 1% v/v FBS EPC medium overnight. Growth factor reduced matrigel (BD) was coated on 96 well culture plates and allowed to polymerize for 1 hr at 37 °C. Approximately 3×10^4 cells were seeded on the matrigel in 0.5% w/v BSA RPMI medium supplemented with and without 50ng/VEGF and incubated for 5 hr. Images were captured at x 200 magnification in 5 random fields of view. Number of cell clusters and branching were counted, and expressed as mean folds of branching compared to control group.

5.2.4.5. Migration assay

Migration assays were performed in Transwell tissue culture wells containing 8 µm pore size inserts (Nunc). Cells were starved with 1% FBS EPC medium overnight. Gelatin (Sigma) was coated on 24 well culture plates and allowed to set overnight at 4 °C. Approximately 1×10^3 cells were seeded on the gelatin in 0.5% BSA RPMI medium supplemented with and without 50ng/VEGF (Peprotech) and incubated for 4 hrs. After incubation period, filter was removed from the respective wells and non-migrated cells on the upper side of the filter were scraped-off gently with cotton bud. Next, insert were fixed in 4% PFA at RT for 15 mins and stained with crystal violet. Filters were washed with PBS several times before viewing under the microscope. Numbers of cells migrating through the membrane were counted and image captured at 200× magnification in 8 high power fields on the underside of each insert membrane.

5.3 RESULTS

5.3.1. Foetal bovine serum batch testing

Overall, EPCs grew well in all different batches of FBS. However, PAA serum was chosen as the FBS of choice, as it favours the formation of cell colonies. Additionally, the cells were morphologically similar each time new cells were plated using the same FBS supplement. PAA FBS was therefore used in further EPC expansion cultures.

5.3.2. Isolation of EPCs using selective media

Whole BM cells and MNC cells were isolated and grown in selective media as used by Zhang *et al.* (Zhang *et al.*, 2006). Briefly, BM cells and MNC cells were isolated and seeded onto fibronectin-coated tissue plates in media, which contained 20% FBS, VEGF, b-FGF, IGF and IMDM basal media to promote EPC outgrowth. Cells changed from round shaped (Figure 5.3.A) to thin and flat (Figure 5.3. B&C), then pebble-like shaped and arranged in a linear manner after 12 days of culture (Figure 5.3.D). Both BM and MNCs differentiated in the same manner and morphology in culture during the duration of 12 days.

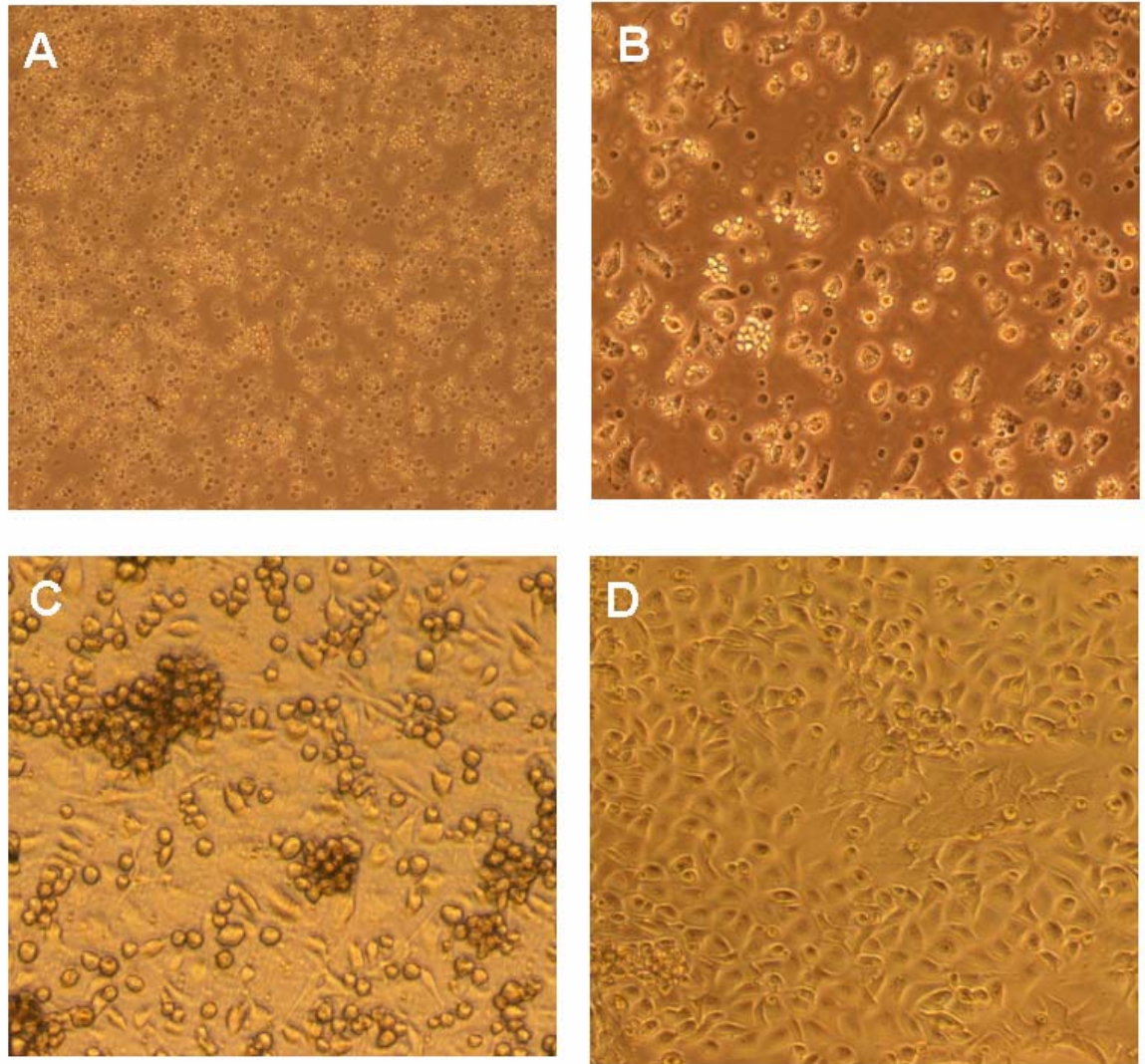


Figure 5.3. Morphological changes in murine bone marrow cells cultured in selective media. (A) day 1 (x 100 magnification), (B) day 3, (C) day 5 and (D) day 12 of culture (x 200 magnification)

5.3.3. Isolation of EPCs using MACS

The isolation of EPCs by enriching for c-Kit⁺ lin⁻ cells was performed as previously described by Zhang *et al.* with some modifications (Zhang *et al.*, 2006). c-Kit was chosen as the initial isolation marker as it is an early stem cell progenitor marker and receptor for the cytokine, stem cell factor. Briefly, isolated BM cells were first incubated with lineage negative antibodies to remove non-target cells such as natural killer cells, macrophages and then positively selecting c-Kit⁺ cells using sequential sorting developed by MACS. Total number of whole BM cells used per isolation was 1×10^8 cells and approximately $561,829 \pm 93,744$ (n=7) c-Kit⁺ cells were obtained, aliquoted into separate wells and grown for 3-5 weeks.

The purity of MACS-isolated c-Kit⁺ cells was analyzed using flow cytometry. Approximately 60-70% of cells were positively stained for c-Kit (Figure 5.4).

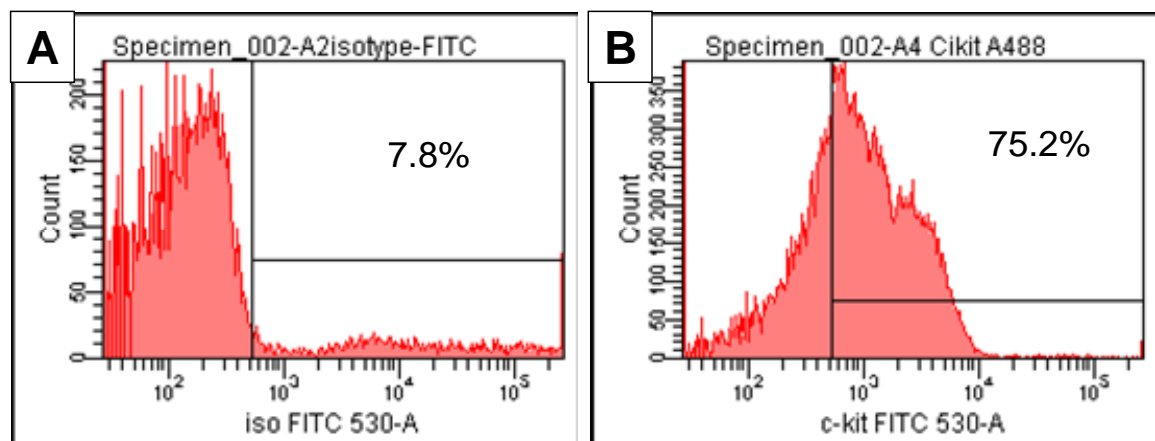


Figure 5.4. Representative FACS analysis of c-Kit⁺ cells isolated after MACS.
(A) Cells incubated with isotype antibody (B) Cells expressing c-Kit marker.

Freshly isolated c-Kit⁺ cells were plated within 1-2 hours of culture. Once cells began to adhere, cells formed a monolayer, consisting predominantly of small-sized cells. Single, large cells with endothelial morphology were also observed. No significant proliferation was noted between 1- 7 days of culture. From day 7 onwards, a proliferating population of round cells occurred. Morphological analysis of the adherent cells around 10 days in culture revealed a heterogeneous cell population, comprising small-sized round cells, large-sized round cells with cytoplasmic granules, and large flat cells with an endothelial

morphology in some areas of the coated plates (Figure 5.5A). At 14 days of culture, round and spindle shaped cells formed circular structures (Figure 5.5B) which eventually expanded into colonies of cells (Figure 5.5C). The majority of cells were capable of forming cords in culture (Figure 5.5D).

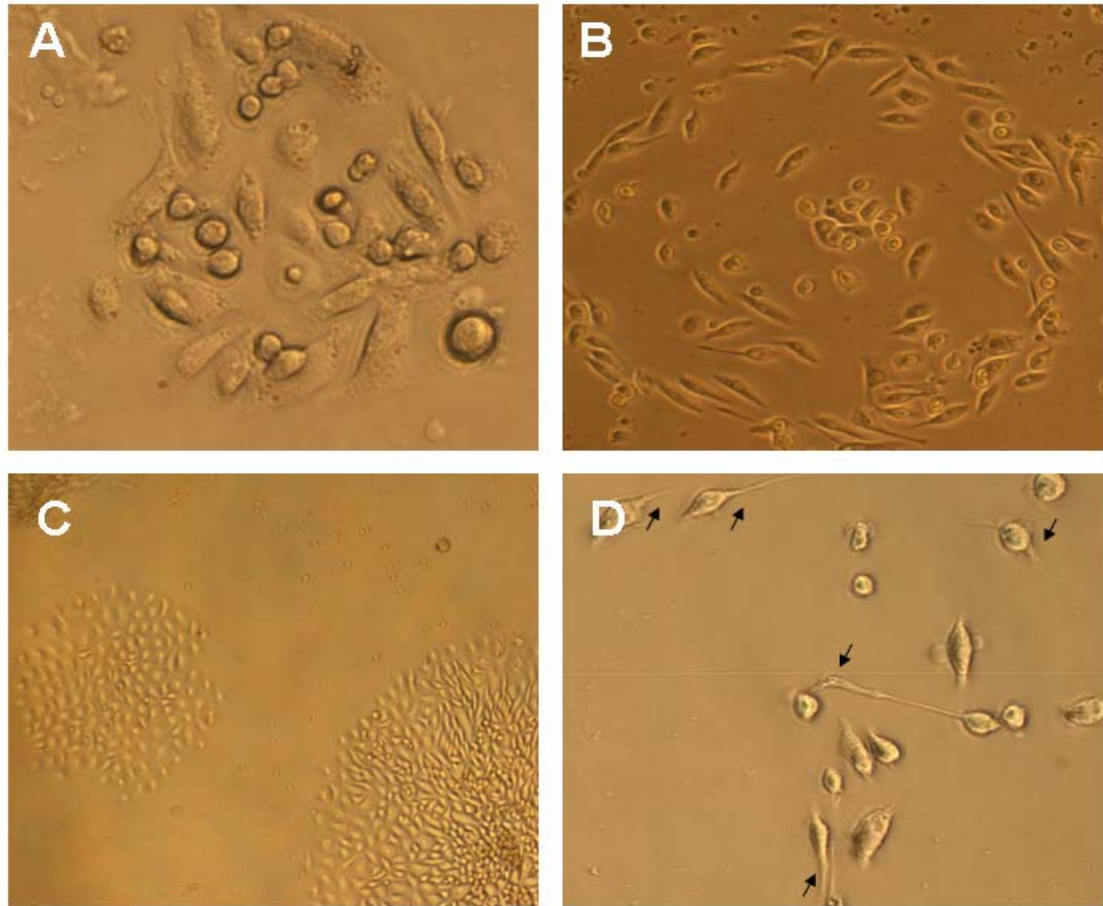


Figure 5.5. Representative phase contrast pictures of cells cultured from c-Kit⁺ cells isolated from MACS (A) A mixture of round and spindle shape cells appears after 10 days of culture (x 400 magnification) (B) Spindle shaped cells start to form circular colonies around 14 days of culture (x 200 magnification) (C) Two typical extended EPC colonies after 2-3 weeks (x 100 magnification) (D) Formation of cord-like structures (arrows) in culture after 3 weeks culture on fibronectin coated plate (x 200 magnification)

5.3.4. Characterization of EPCs

EPC-derived cells from method 1 and 2 incorporated Dil-ac-LDL and bind with UEA-1, which are functional phenotypes of endothelial cells (Figure 5.6).

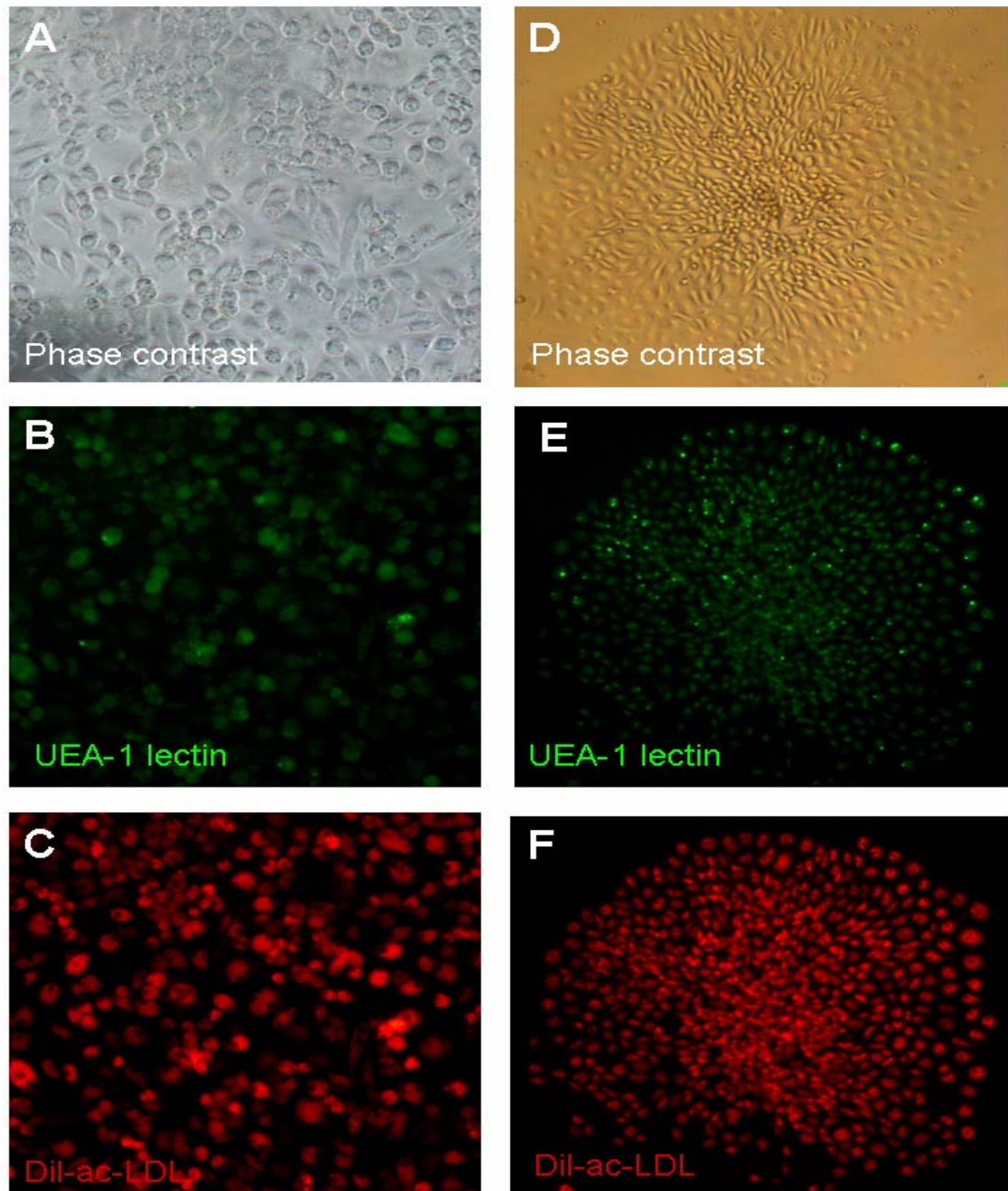


Figure 5.6. c-Kit⁺ EPCs binding and uptake of Dil-ac-LDL. (A-C) Phase contrast and binding of UEA-1 lectin and uptake of Dil-ac-LDL in cells isolated using selective media. (D-F) Phase contrast and binding of UEA-1 lectin and uptake of Dil-ac-LDL in cells isolated using the MACS method (x 200 magnification).

EPC-derived cells using method 1 and 2 expressed numerous cell surface antigens including CD31, CD34, vWF, CD133, VEGFR-2 (Figure 5.7).

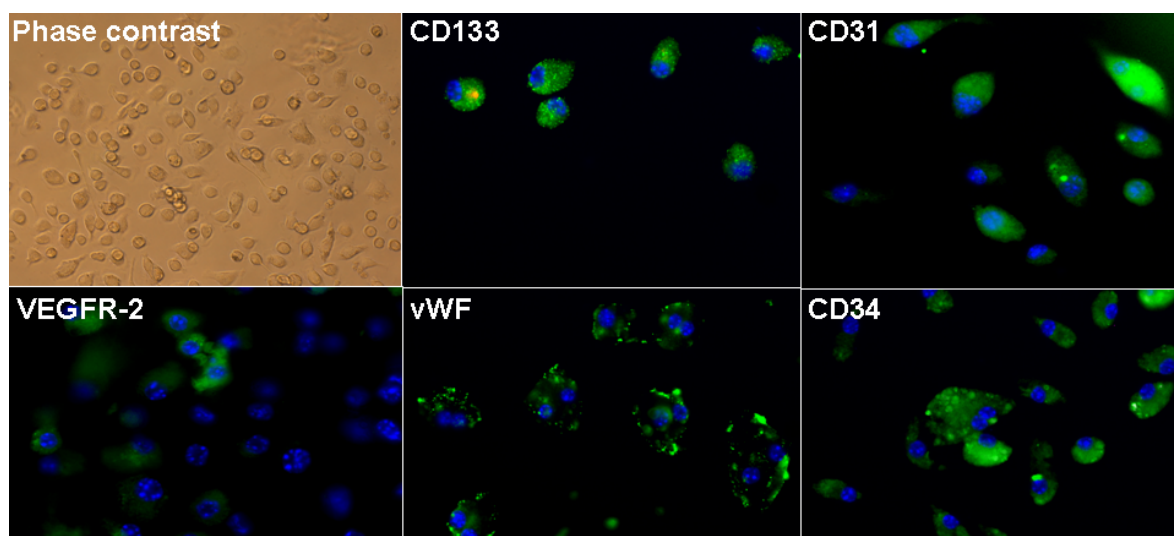


Figure 5.7. Fluorescence microscopic demonstration of markers of bone marrow-derived EPCs. The antibodies were labelled with FITC (x 400 magnification)

Similar results were observed in 3 independent experiments. Antigen staining was corroborated by FACS analysis where c-Kit⁺ lin⁻ cells isolated using method 2 highly express CD31, VEGFR-2, CD34 and vWF which are characteristic of late EPCs (Table 5.4). BM-derived EPCs grown in selective endothelial growth media only highly expressed VEGFR-2 and vWF.

Antigen	Method 1	Method 2
CD31	7.1 ± 3.8	16.5± 2.7
VEGFR-2	27.7 ± 3.5	16.2± 2.0
CD34	0.55 ±0.27	19.5± 3.2
CD133	4.87 ±3.34	2.7± 0.5
vWF	36.5 ± 10.3	22.9± 1.6

Table 5.4. Cell surface antigen expression using FACS analysis of EPCs isolated using Methods 1 and 2.

In addition to FACS analysis and immunocytochemistry, tubule formation assays and migration studies were carried out to confirm the functional endothelial properties of the BM-derived EPCs. BM-derived EPCs using method 1 were able to form tubules in matrigel and also able to migrate towards 50ng/ml VEGF growth factor (25 ± 2 cells) compared to basal conditions (3 ± 2 cells) (Figure 5.8 A&B). c-Kit⁺ EPCs cultured for 28 days were also able to form tubules and migrate, but due to lack of cell numbers isolated, we were unable to perform a statistical analysis.

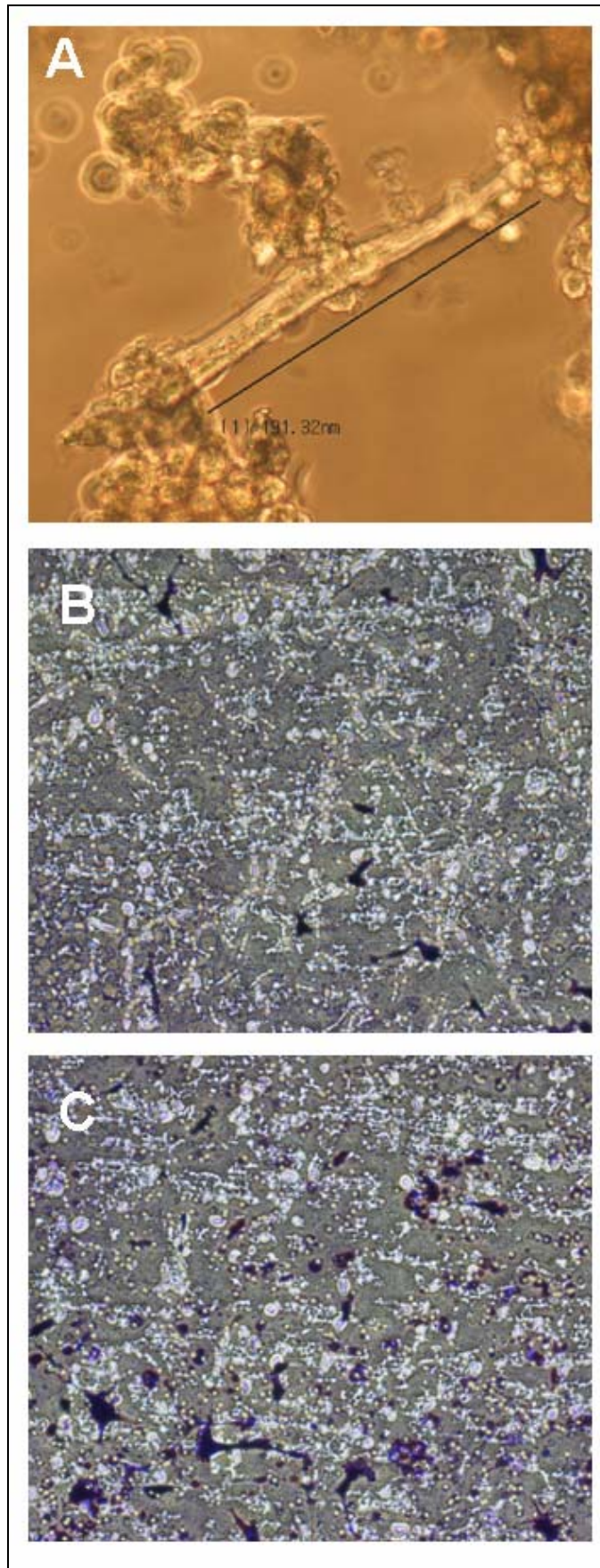


Figure 5.8. *In vitro* assays testing endothelial function

(A) Tubule formation of BM-derived cells (c-Kit⁺ EPCs) on reduced growth factor matrigel after 18 hours incubation (x 400 magnification)

(B&C) Migration assay tests the ability of BM-derived EPC using selective media to migrate towards a stimulus (50ng/ml VEGF) (Picture C) compared to basal condition (Picture B). Cells were fixed with 4% PFA, stained with crystal violet (purple) and image captured at x 200 magnification.

5.5. DISCUSSION

EPCs have received a lot of attention over the past few years due to their ability to form new postnatal blood vessels, as demonstrated in many injury models. The phenotypic properties of these cells however are debatable, with many studies using various combinations of cell surface markers to identify EPCs.

Human EPCs are commonly isolated from peripheral blood and normally characterised by the expression of CD133⁺ CD34⁺ (Asahara *et al.*, 1999; Nguyen *et al.*, 2008). In the present study, whole BM cells were used to obtain c-Kit⁺ cells using magnetic cell sorting and grown in endothelial growth medium. In contrast to other studies, we used whole BM instead of an enriched mononuclear fraction to avoid the loss of cells from centrifugation and vigorous cell-washing procedures (Ozdogu *et al.*, 2007). Total BM cells were then incubated with lineage negative antibodies to remove platelets, natural killer cells, macrophages and leukocytes and subsequently selected for c-Kit⁺ cells. BM cells were enriched for c-Kit as c-Kit is reported to be an early mouse stem cell progenitor marker and these cells can differentiate towards the endothelial lineage (Zhang *et al.*, 2006). Additionally, Li *et al.*, demonstrated that c-Kit⁺ cells can contribute to neovascularization when transplanted into ischaemic mice (Li *et al.*, 2003).

Various methods to isolate and culture EPCs have been used to date. Some studies have used peripheral blood mononuclear cells as their starting cells, and re-plate non-adherent cells after 24 hours in culture (Hill *et al.*, 2003). In our study, we use whole BM cells and did not re-plate the cells, but instead non-adherent cells such as monocytes, macrophages, and any circulating mature endothelial cells were removed to prevent contamination. We compared two methods of isolation of BM-derived EPCs: (i) method 1, the culture of BM cells in selective endothelial growth media and (ii) method 2, the culture of enriched c-Kit⁺ cells in commercial endothelial growth medium. Based on previous studies, we have generated early EPCs using method 1 as they start to produce colonies at 4-7 days of culture. These cells also bind to UEA-1 lectin and demonstrate uptake of Dil-ac-LDL, form vascular tubes in matrigel and have some proliferative potential in culture, characteristics reported by many studies (Hur *et al.*, 2004; Lin *et al.*, 2000). EPCs grown

for more than 21 days using method 2, demonstrated similar characteristics as cells isolated from method 1, but have very limited proliferative potential. This could be due to the low density of c-Kit⁺lin⁻ cells (5×10^5 cells/well) initially plated compared to number of cells plated for method 1 (2×10^6 cells/well).

To date, there is no defined panel of markers to identify mouse EPCs. Based on several papers, we have identified six markers of importance, namely VEGFR-2, CD34, CD133, CD31 and vWF for quantification using FACS analysis (Balasubramaniam *et al.*, 2007; Matsumoto *et al.*, 2008; Nguyen *et al.*, 2008; Wang *et al.*, 2008; Ye *et al.*, 2007). FACS analysis shows that cells isolated from method 1, highly express vWF and VEGFR-2, but have low expression of CD34 and CD133. c-Kit⁺lin⁻ cultured cells show similar expression of CD31, VEGFR-2, CD34 and vWF. Based on FACS and immunocytochemistry data, our c-Kit⁺lin⁻ cultured cells express the same markers as late EPCs, i.e. CD133⁻, vWF⁺, CD34⁺, VEGFR-2⁺, CD31⁺ (reviewed by Hristov *et al.*, 2004). We therefore have demonstrated the potential of c-Kit⁺lin⁻ cells, of being functional EPCs in culture as shown by the ability of these cells to express a combination of endothelial and early progenitor markers, as well as the ability to form tubules and migrate towards a stimulus.

CHAPTER 6: BONE MARROW TRANSPLANTATION INTO NOD MICE

6.1. INTRODUCTION

Islet transplantations have been carried out since the publication of the Edmonton Protocol (Shapiro *et al.*, 2000) as a treatment for T1D. However, a shortage of cadaveric pancreata and a return to insulin dependence in the majority of recipients within 5 years means alternative treatments are required.

Some studies have reported improvement of hyperglycaemia when whole BM was transplanted into diabetic animal models such as streptozocin (STZ)- induced diabetic mice (Ianus *et al.*, 2003), E2f1/E2f2 mutant mice (Li *et al.*, 2003) and NOD mice (Lee *et al.*, 2006). This is not the case for some studies where they fail to show normalization of blood glucose levels as well as low rates of haematopoietic chimaerism (Lechner *et al.*, 2004; Mathews *et al.*, 2004). These discrepancies could be due to several factors such as non efficient irradiation, different mouse strains, extent of diabetic injury and lineage tracing methods.

Several studies have suggested that BM transplanted after STZ-induced diabetes in mice may contribute to islet neovasculature and promote islet β -cell regeneration (Hasegawa *et al.*, 2007; Hess *et al.*, 2003; Mathews *et al.*, 2004). In particular, Hess *et al.* intravenously transplanted GFP⁺ BM cells into STZ-induced diabetic mice 5 days after the last STZ administration (Hess *et al.*, 2003). They reported reduction in blood glucose levels and serum insulin levels within 7 days after transplantation of GFP⁺ BM cells. Additionally, the authors observed that 9.2% of engrafted cells were endothelial cells (GFP⁺PECAM-1⁺) suggesting that BM, presumably containing EPCs, may have differentiated into endothelial cells and contributed to neovasculature in the injured islet, possibly also aiding islet regeneration.

BM contains a number of stem cell populations including mesenchymal stem cells (MSCs) and HSCs, which have the ability to differentiate into other cell types. Ianus *et al.*

suggested BM cells transplanted into diabetic animal can transdifferentiate into β -cells in an attempt to regenerate the pancreas (Ianus *et al.*, 2003). Other studies followed attempting to reproduce the results, but no donor-derived insulin-expressing cells were found (Choi *et al.*, 2003; Lechner *et al.*, 2004; Taneera *et al.*, 2006). It is clear that BM cells may play a role in β -cell regeneration by other mechanisms rather than repopulating the islets with transdifferentiated β -cells. Some suggest that β -cell regeneration could be due to replication of existing β -cells (Dor *et al.*, 2004; Gao *et al.*, 2008) and β -cell neogenesis from pancreatic ducts (Bonner-Weir *et al.*, 2004). Other mechanisms which may occur at a lesser extent include cell fusion and dedifferentiation (Herzog *et al.*, 2003; Terada *et al.*, 2002).

As diabetes is an autoimmune disease, some studies used purified MSCs and HSCs derived from the bone marrow as immunomodulators, in an attempt to arrest the autoimmune reaction against β -cell auto-antigens in the pancreas. Administration of purified HSCs into diabetic NOD mice has been shown to restore glucose homeostasis by altering thymic and peripheral T-cell responses against auto- and allo-antigens, thus augmenting regeneration of remaining endogenous β -cells (Beilhack *et al.*, 2003).

Accordingly, the aim of this study was to investigate the potential of whole BM from wild type (Balb/c) and NOD mice in delaying diabetes using non diabetic NOD mice. Additionally, I studied the effect of whole BM cells on blood glucose levels, insulinitis and β -cell replication.

6.2. METHODS

6.2.1. Mice

Two groups of mice were used in this study, pre-diabetic NOD and wild type (Balb/c) mice. Wild type mice were purchased from Charles River Laboratories and housed in the animal facility at Charterhouse Square. NOD mice were bred and housed in the same facility. At 10-11 weeks of age, NOD mice were transferred to Biological Resource Unit, Cancer Research UK, London Research Institute to receive BM transplantation.

6.2.2. Bone marrow transplantation into pre-diabetic and early diabetic NOD mice

6.2.2.1. First study

Recipient NOD mice were 10-11 weeks old non diabetic female NOD mice with a blood glucose level in the range of 5 mmol/l to 6 mmol/l (Figure 6.1):

- a) **Group A:** 10 female animals were irradiated with a split dose (2 x 500 cGy, 2-3 hours apart) of total body irradiation (TBI) and were reconstituted within 3 hours *via* intravenous (i.v.) injection of freshly isolated male WT BM (2×10^6 cells). Mice were kept for 14 weeks unless unwell due to diabetes.
- b) **Group B:** 10 female animals were irradiated with a split dose (2 x 500 cGy, 2-3 hours apart) of TBI and were reconstituted within 3 hours *via* i.v. injection of freshly isolated female non diabetic NOD BM (2×10^6 cells). Mice were kept for 14 weeks unless unwell due to diabetes.
- c) **Group C:** 6 female non diabetic NOD mice served as a control. These mice did not receive any irradiation nor BM transplantation, and were kept together with Group A and B mice.

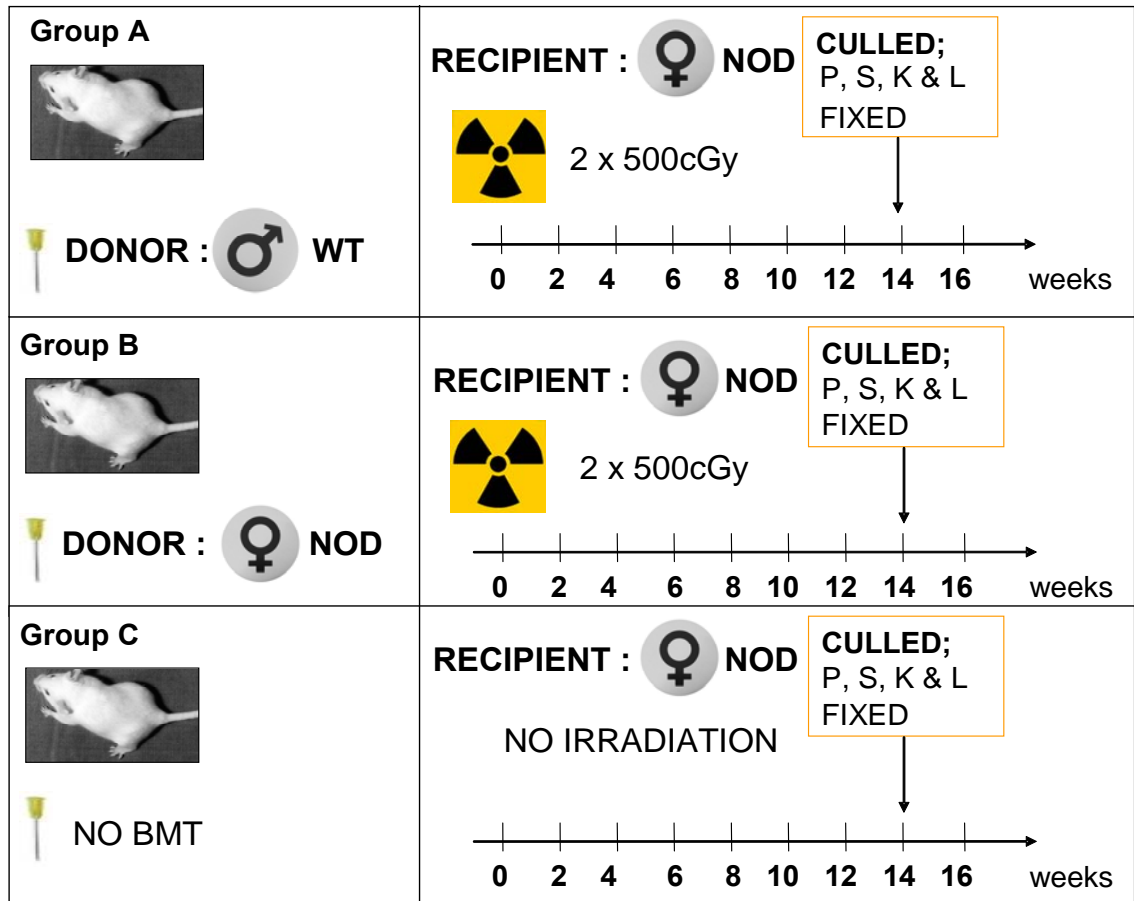


Figure 6.1. Initial experimental strategy.

Group A and B mice (10-11 weeks old) were injected i.v. with 2 million freshly isolated BM cells after 2 doses of irradiation (1000 cGy). Group C, control mice did not receive a lethal dose of irradiation nor BMT (Abbreviations: BMT, bone marrow transplantation; Gy, gray; i.v., intravenous, P, pancreas; S, spleen; K, kidney; L, liver)

6.2.2.2. Second study

This second study was carried out to investigate whether BM cells can engraft after transplantation of 2 million BM cells into early diabetic and non-diabetic NOD mice, measured at 14 days after transplantation. Mice enrolled in this study were either diabetic (0-4 days diabetic with blood glucose in the range of 11.5 mmol/l to 25 mmol/l) or non diabetic (<11.5 mmol/l):

a) **Group A:** 3 female animals (early diabetic NOD, 20-24 weeks old) were irradiated with a split dose (2x 500cGy, 2-3 hours apart) of TBI and were reconstituted within 3 hour *via* i.v. injection of freshly isolated male NOD BM (2×10^6 cells). Mice were kept for 2 weeks.

b) **Group B:** 3 female animals (non diabetic NOD, 10 weeks old) were irradiated with a split dose (2x 500cGy, 2-3 hours apart) of TBI and were reconstituted within 3 hour *via* i.v. injection of freshly isolated male NOD BM (2×10^6 cells). Mice were kept for 2 weeks.

6.2.3. Gender mis-matched bone marrow transplantation into NOD mice

Breeding and isolation of BM were carried out under UK Home Office procedural and ethical guidelines (Home Office Project License NO. 70/5962) at the Biological Services Unit, Charterhouse Square. Administration of BM and maintenance of chimaeric mice were carried out in the Biological Resource Unit, Cancer Research UK, London Research Institute (refer to Method 2.4.1 for more details).

6.2.4. Preparation of bone marrow transplant

Bone marrow transplantation procedures were carried out in sterile conditions. Female pre-diabetic NOD recipient mice (10-11 weeks of age) underwent whole body, lethal gamma irradiation with 1000 cGy in a divided dose, 2-3 hours apart, to ablate their BM using an IBL 637 gamma irradiator equipped with a caesium 137 source (Cis-Bio International). This was followed by a tail vein injection with whole bone marrow from the donor at a dose of 2×10^6 cells per female recipient.

The cells for bone marrow transplantation were obtained on the same day from 10 weeks old wild type (Balb/c) and NOD male mice. Femur, tibia and iliac crest from the male donors were removed and bone marrow was flushed with 2% v/v FBS PBS using a 5 ml needle syringe. Cells were filtered through a 70-micron filter and centrifuged at 1500 rpm for 5 mins to separate the supernatant from the cells. The pellet was then resuspended in 200 μ l sterile PBS for i.v. tail vein injection into the recipient female mice on the same day (refer to Method 2.4.2. for more details).

6.2.5. Monitoring of diabetes development

During the duration after transplantation, blood glucose levels were evaluated once every 7 days using an Accu-chek AVIVA Blood Monitor (Roche). To monitor diabetes, mice were classified diabetic if blood glucose was confirmed as ≥ 11.5 mmol/l. At the end point, all mice were killed by cervical 'dislocation' and/or CO₂ inhalation, dependent on the type of tissue collected. Pancreas, spleen, kidney and liver were fixed in neutral buffered formalin for 24 hours at room temperature before being embedded in paraffin wax. Mice transplanted with BM were monitored for 14 weeks (First study).

6.3 RESULTS

6.3.1. Mice that received a bone marrow transplant prior to onset of hyperglycaemia

In order to study the possible contribution of BM in delaying hyperglycaemia, sex-mismatched BM from wild type (Group A) and non-diabetic NOD mice (Group B) were transplanted prior to the onset of diabetes (First study). Of the 10 female mice transplanted with WT BM cells at 10-11 weeks of age prior to the onset of diabetes, 9 mice survived and 1 mouse died 6 days post transplant of unknown cause (Group A). The survival rate of Group B mice were the same with Group A with 8 out of 9 mice surviving. In control group mice, 5 mice out of 6 survived, but 1 mouse became very sick due to diabetes and was culled before the end-point.

6.3.2. Little evidence of bone marrow contribution in pre-diabetic recipient NOD mice

To characterise BM engraftment, Y-Fluorescent In Situ Hybridization (Y-FISH) analysis was performed on paraffin embedded sections containing pancreata, kidney, liver and spleen. Y-FISH was performed as previously described by our group (Brittan *et al.*, 2005). All staining on test sections were carried out in parallel with male control sections (Figure 6.2.A). The success of detecting Y-chromosome in these sections required vigorous optimization of parameters such as duration and concentration of pepsin digestion and the denaturing temperature. To rule out the over digestion of tissue by pepsin, pancentromeric hybridization was performed using the same conditions. DNA of the cells was intact and

not completely digested (Figure 6.2.B). After repeated experiments, only a few donor-derived Y chromosome-positive cells were observed in the spleen, islets and in the exocrine pancreas (Figure 6.2.C).

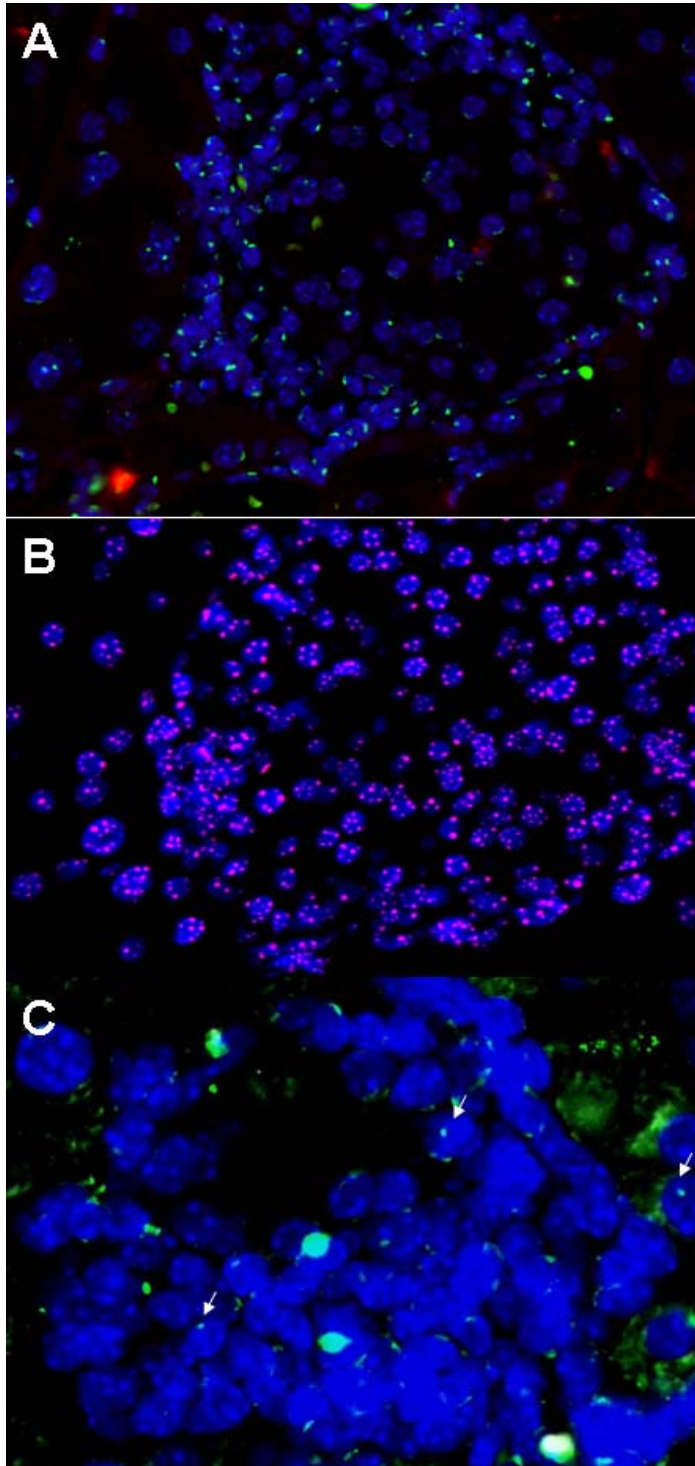


Figure 6.2. Y-Fluorescent In Situ Hybridization (Y-FISH) analysis

(A) Y chromosomes present in pancreas of male control (x 200 magnification)

(B) Pancentromeric hybridization on female mouse pancreas transplanted with NOD BM (x 200 magnification)

(C) Female mouse pancreas that received WT BM demonstrating FITC-labelled Y-chromosome probe (Y-chromosomes – white arrows) (x 400 magnification)

Due to a low number of Y- chromosomes present in pancreata, double staining for endomucin (endothelial marker) was not performed, and thus I was unable to confirm that donor cells were of the endothelial lineage.

6.3.3. Proof of principle: Bone marrow engraftment in diabetic recipient NOD mice

Due to low number of Y-chromosomes observed in recipient mice from the first BM study, another batch of mice were enrolled to investigate whether BM cells can engraft after transplantation of 2 million BM cells into early diabetic and non-diabetic NOD mice. All staining on test sections were carried out in parallel with male control sections and parameters optimized for each section such as duration and concentration of pepsin digestion and the denaturing temperature. After repeated experiments, Y-chromosomes were present in all early diabetic mice and mainly present in the spleen, islets (Figure 6.3) and in the exocrine pancreas.

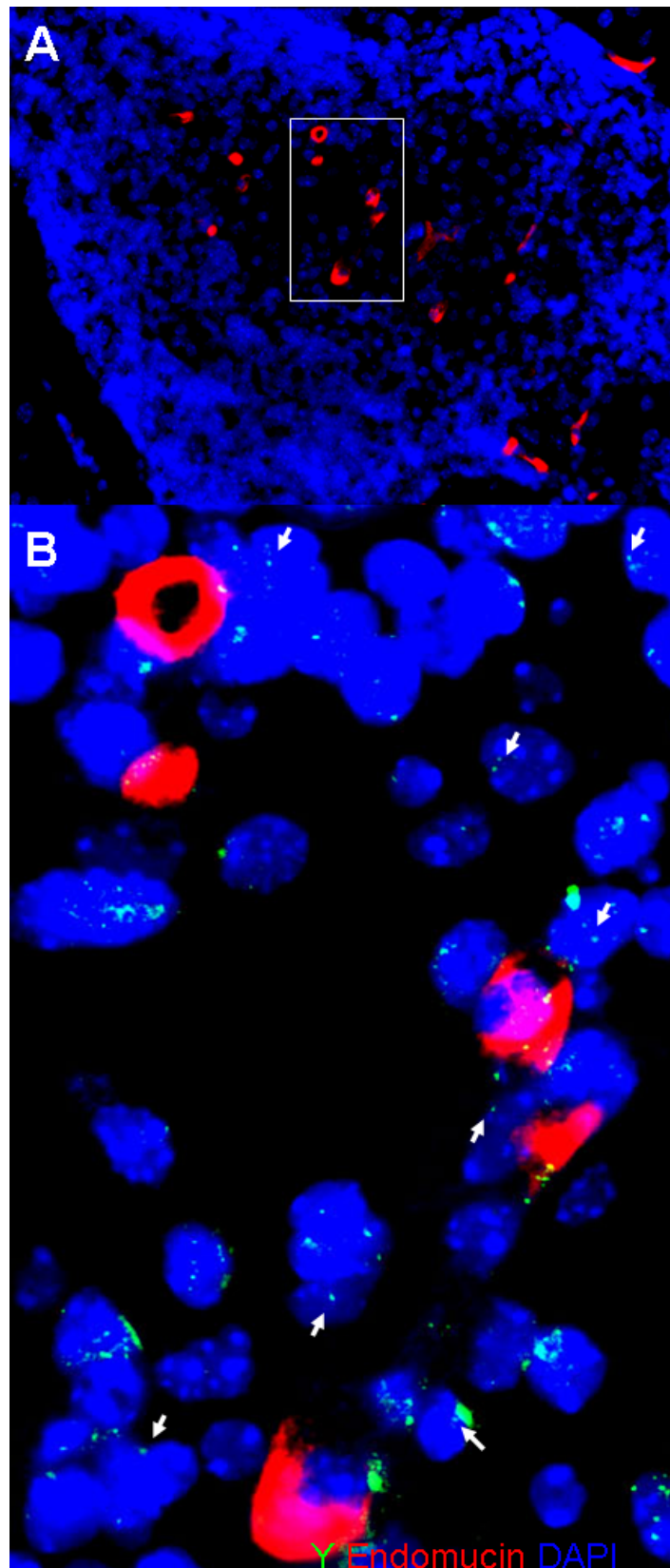


Figure 6.3. Y-chromosomes present in the pancreas of female diabetic recipient transplanted with NOD BM cells (A) Image of an islet stained with endomucin antibody (red) (x 200 magnification) (B) Close up image of Y-chromosomes (arrows).

6.3.4. Large number of Grade 0 islets in bone marrow transplanted animals

Insulinitis was gauged using H&E stained sections and carried out by an observer blinded to the treatment group.

To study the effect of whole BM transplantation on lymphocyte homing into islets, insulinitis was scored in pancreatic sections from 8-9 mice each in both group A and group B (using grading standard shown on Figure 4.6). Insulinitis was quantified in all islets present in two H&E-stained sections from each animal, each section being separated from the next by at least 100µm. These mice have been irradiated and transplanted with BM either from WT or NOD for the same duration of time of 14 weeks.

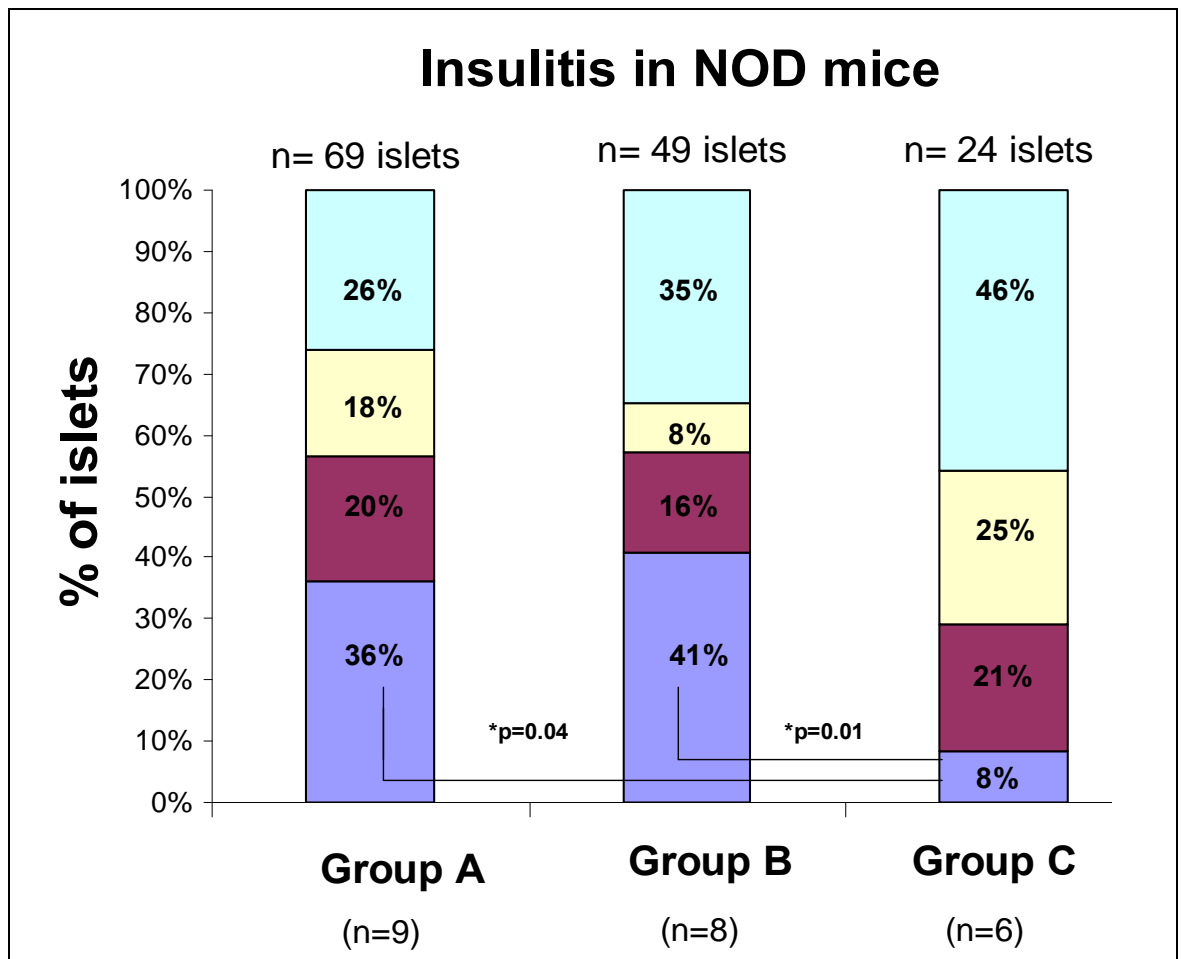


Figure 6.4. Grades of insulinitis observed in Group A, B and C mice. Grade 0, normal islet; Grade 1, peri-insulinitis; Grade 2, moderate insulinitis; Grade 3, severe insulinitis. The data are the sum of islets from nine mice for each group (Abbreviation: n= number of islets scored)

The majority of islets (36-41%) of islets from Group A and B showed little infiltration and destruction of pancreatic β -cells (grade 0 insulinitis) whereas only 10% of islets from control mice were free of insulinitis with the majority of islets heavily infiltrated (*P-value: 0.01-0.04) (Figure 6.4.). The % of islets with grades 1-3 insulinitis were almost the same in Group A & B mice, thus indicating that BM effects on the NOD mice is the same regardless of the strain of donor mice.

6.3.5. Significant change in blood glucose level before and after bone marrow transplantation

Blood glucose levels of Group A and Group B mice were in the range of 5-6 mmol/l before BMT transplantation. Average blood glucose levels of control mice began rising at 8 weeks after BMT (18-20 weeks of age) steadily increasing over the next 6 weeks, the blood until end point, whereas blood glucose level of Group A and Group B mice did not change significantly (Figure 6.5A). At 14 weeks after BMT, Group A and B had an average blood glucose level increase of 1% and 43% respectively (Figure 6.5B). Control mice had an average increase of 84% in blood glucose levels (*P-value=0.05), which is expected as they did not receive any lethal irradiation and subsequent BM transplantation (Figure 6.5B). These data suggest that mice transplanted with BM (Group A and B) have a better control of blood glucose level compared to Group C mice.

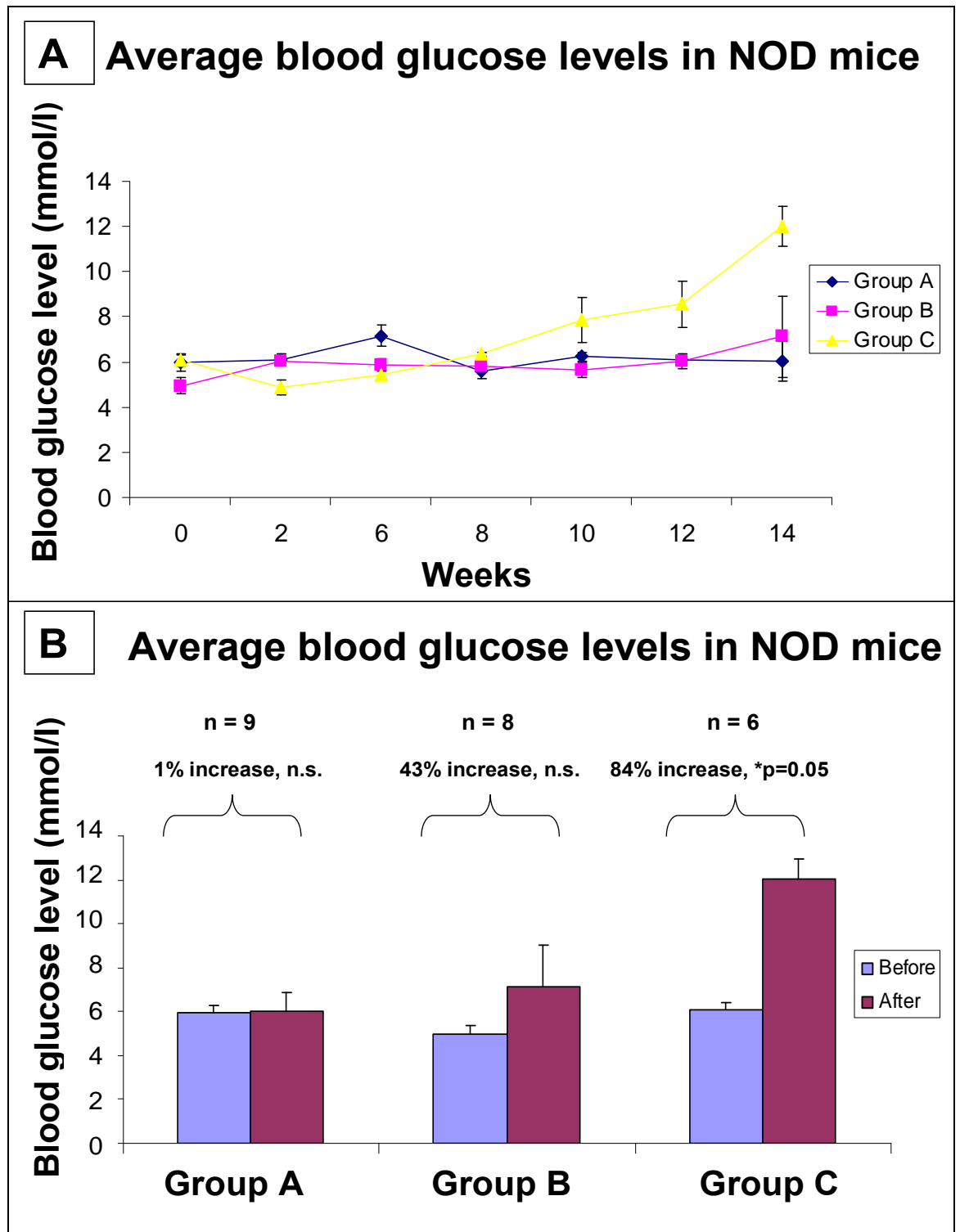


Figure 6.5. Blood glucose levels (random, non-fasting blood glucose levels) in Group A, B and C mice (A) Average blood glucose levels of Group A, Group B and Group C (control) mice over 14 weeks since BMT (B) Average blood glucose levels of Group A and B mice before and after BMT. Statistical significance was tested by Student's t-test (Abbreviations: n= average number of mice).

6.3.6. Evidence of islet regeneration by bone marrow transplantation once disease is established.

To quantify β -cell proliferation and regeneration in the pancreata after BM transplantation, pancreatic sections were stained with anti-Ki-67 and anti-insulin antibodies. Wild type mouse gut and pancreas were used as positive control for the anti-Ki-67 and anti-insulin antibodies (Figure 6.6A,B).

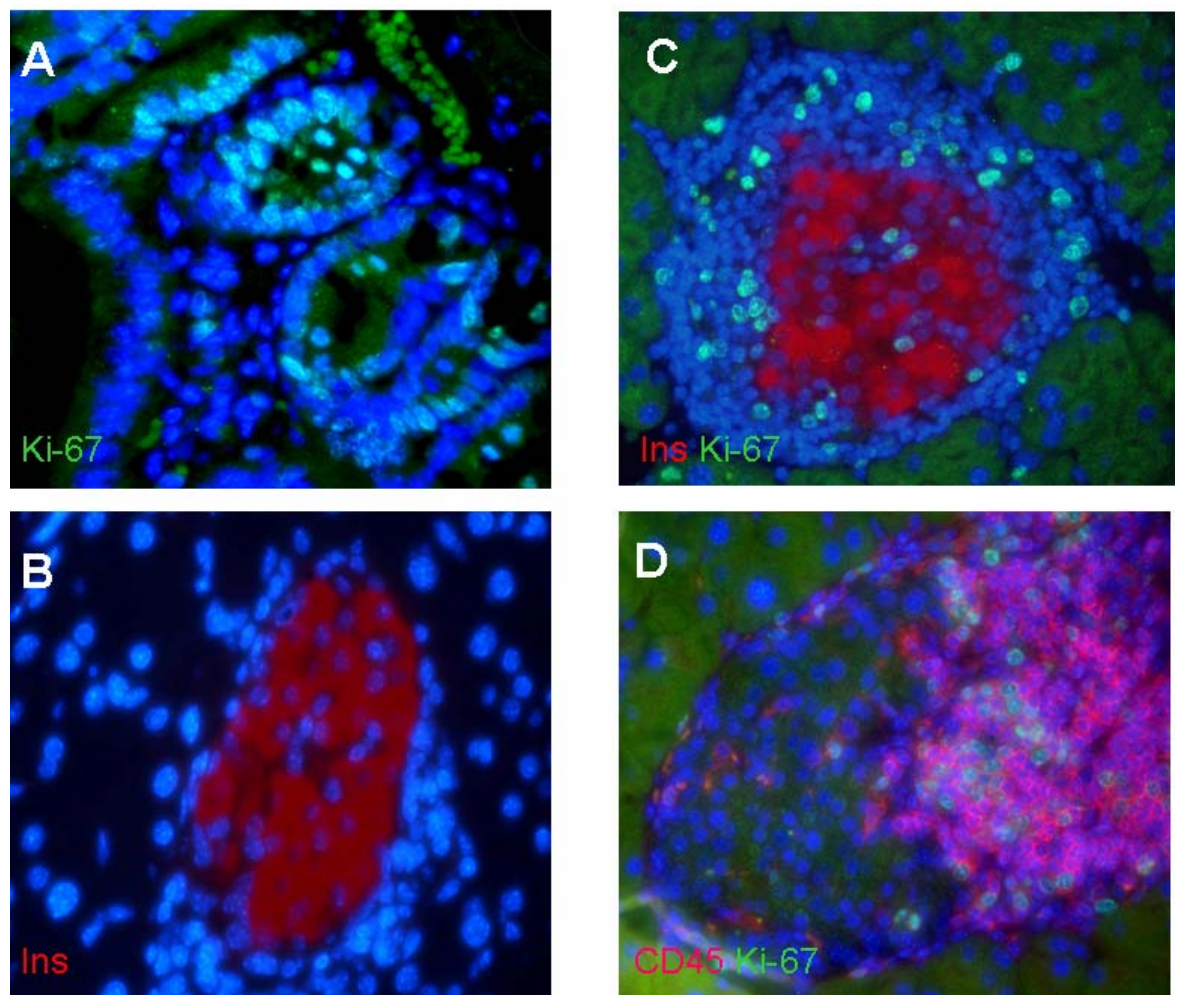


Figure 6.6. Insulin, Ki-67 and CD45 immunohistochemistry. (A) Wild type small intestine section was used as a control for the Ki-67 antibody (B) Wild type pancreas section was used as a control for the insulin antibody. The red staining represents insulin immunoreactivity; all positive staining was localized within the islets (C) Representative insulin and Ki-67 staining on pancreatic section from pre-diabetic WT BM transplanted animal. (D) Representative CD45 and Ki-67 staining on pancreatic section from pre-diabetic NOD BM transplanted animal (x 400 magnification)

Most Ki-67⁺ Insulin⁺ cells (Figure 6.6C) surrounding the islets stained positive for the pan-haematopoietic marker (CD45) (Figure 6.6D).

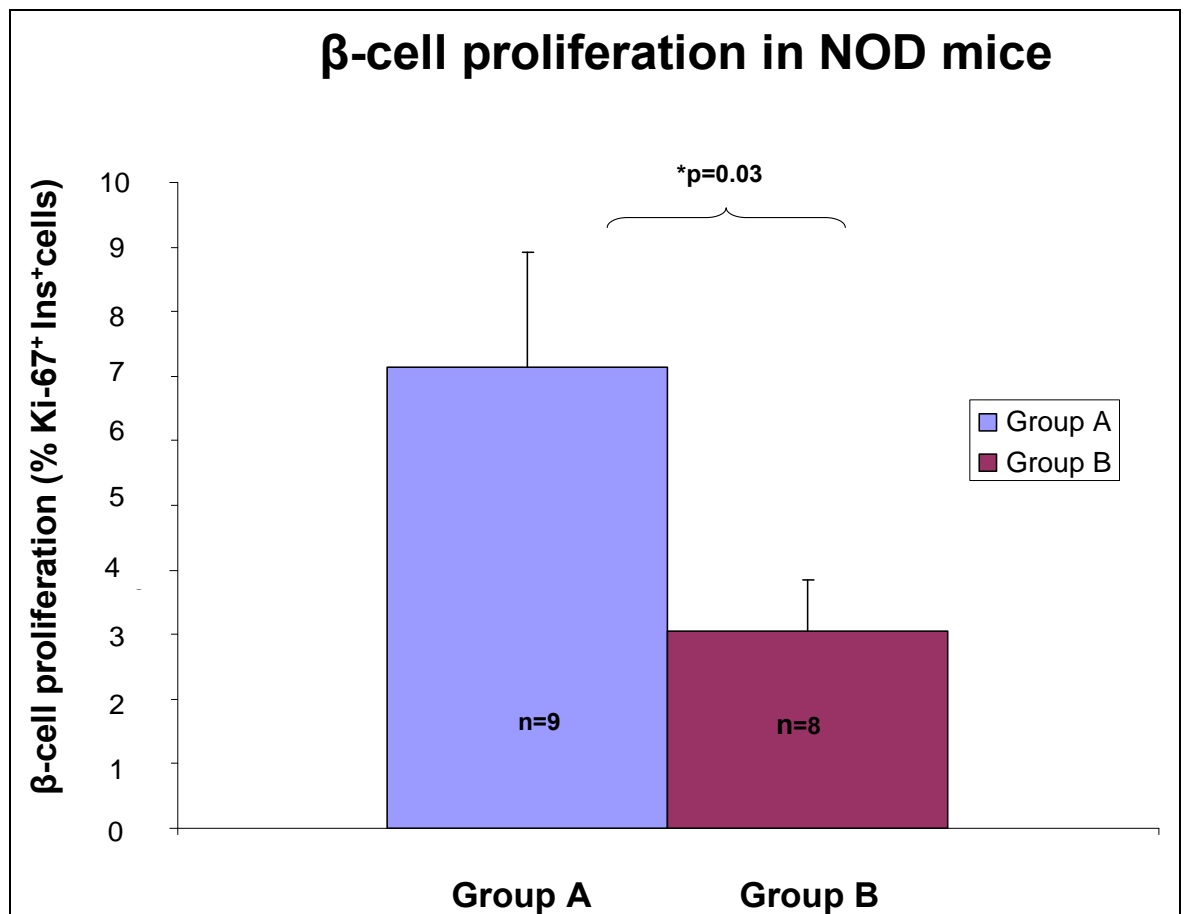


Figure 6.7. Ki-67 labelling index of Insulin-positive cells of Group A and Group B mice. Statistical significance was tested by Student's t-test (Abbreviations: n= average number of mice, Ins= insulin)

Staining for Ki-67 and insulin revealed a higher rate of proliferation in the islets of WT BM transplanted mice compared to NOD BM transplanted mice (Figure 6.7).

6.3.7. Increased islet neovascularization in both groups of mice.

To evaluate the contribution of BM-derived endothelial cells in β -cell regeneration by neovascularization, blood vessel density of pancreatic sections was assessed using a CD31 antibody as a marker to stain for endothelial cells lining blood vessels (Figure 6.8A).

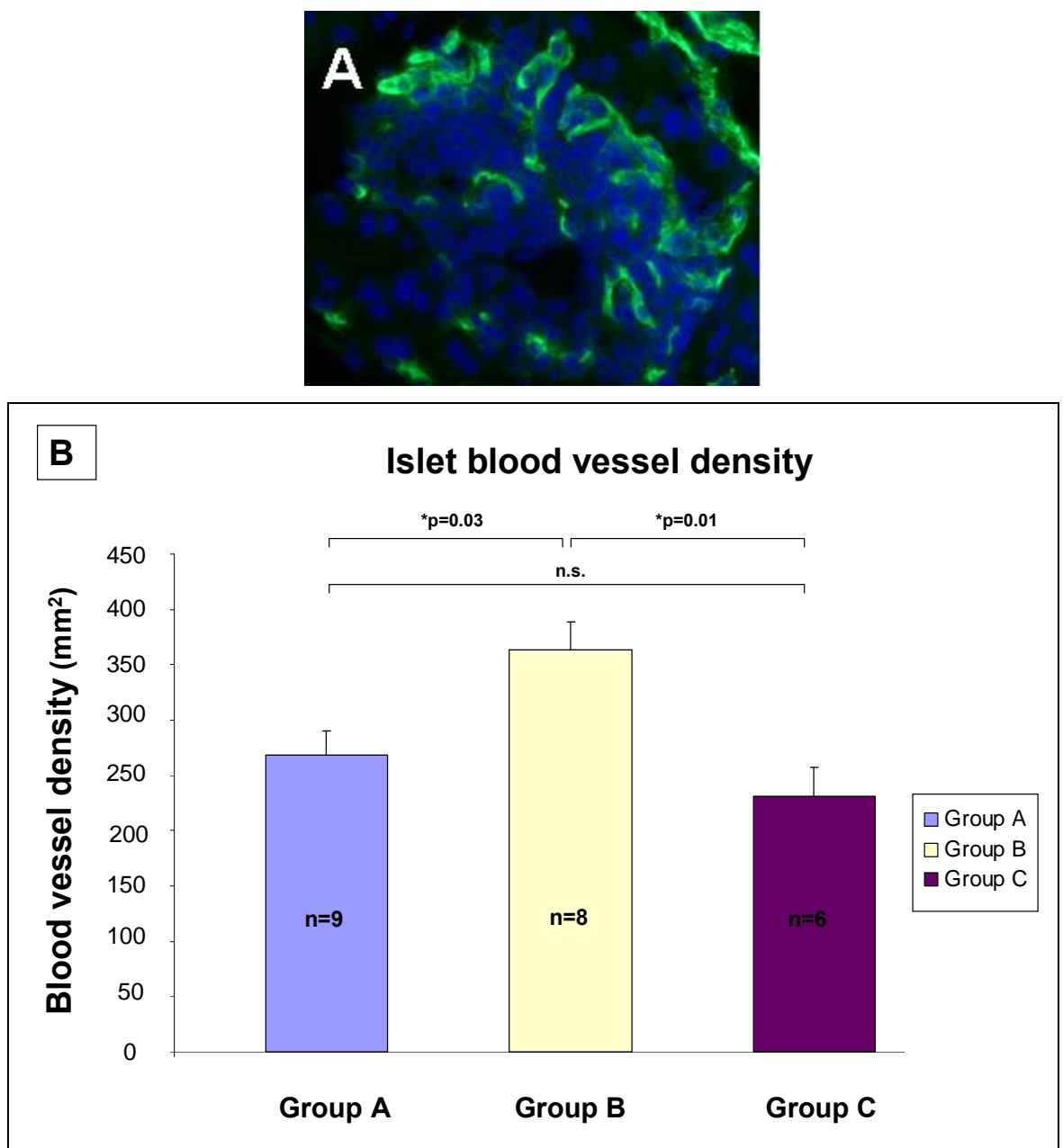


Figure 6.8. Islet blood vessel density. (A) Representative CD31 blood vessel staining in the pancreatic islet (x 200 magnification) (B) Blood vessel density of Group A, Group B and Group C mice

Group B mice have increased blood vessel density compared to control mice (without irradiation & BMT) (Figure 6.8B). In addition, our data demonstrated a significant difference in blood vessel density in Group B mice compared to Group A mice (*P-value: 0.03).

6.4. DISCUSSION

The potential of BM transplantation to treat diabetes has been shown in many animal models. In this study we hoped to demonstrate the potential of wild type and non diabetic NOD BM cells to block the onset of diabetes in non-diabetic NOD mice.

To reconstitute the immune system, recipient mice must receive a lethal dose of irradiation to eliminate mature autoreactive memory B and T-cell lymphocytes. Low dose irradiation (250/450 cGy) of STZ-induced diabetic mice followed by BMT only temporarily delayed the development of hyperglycaemia, as the low dose of irradiation did not kill the majority of T-cell lymphocytes, allowing destructive insulinitis to occur (Urban *et al.*, 2008). High dose irradiation is therefore required to improve the efficiency of BMT to restore normoglycaemia. In our study, the recipient female mice received a lethal dose of irradiation of 1000 cGy before the onset of diabetes, to eliminate mature autoreactive memory B and T-cell lymphocytes in the NOD peripheral lymphoid tissue, allowing new BM cells to reconstitute the NOD immune system and contribute to haematopoiesis. Majority of Group A and B mice that received BM transplantation did not develop diabetes during the time frame, with only 2/18 developing diabetes. The 3-4 week transient delay in the development of diabetes could be attributed to the time taken (approximately 2-3 weeks) for the donor BM to reconstitute the immune system after lethal irradiation. As diabetes occurs spontaneously in the NOD mice, the best available control to measure the effect of donor BM is NOD mice, but without irradiation or BM transplantation. Control mice that did not receive any irradiation and BMT had an increase in blood glucose levels from 18 weeks of age. This is expected as the immune system is still intact and thus will have an earlier onset of diabetes compared to Group A and Group B mice.

It has been documented that BM transplantation can initiate β -cell regeneration in many diabetic animal models. Mechanisms involved in β -cell regeneration by BM cells however are still unclear. BM transplantation into STZ-induced diabetic mice revealed that donor derived cells surrounding islets were CD45⁺ cells (Hasegawa *et al.*, 2007). Lechner *et al.* and Taneera *et al.* similarly found a substantial number of donor-derived haematopoietic (CD45⁺ cells) in the pancreas of irradiated female mice with pancreatic injury transplanted with unfractionated male GFP and eGFP labelled BM cells (Lechner *et al.*, 2004; Taneera *et al.*, 2006). We similarly observed cells surrounding the islets in our BMT mice that were CD45⁺ cells. There is a possibility that haematopoietic cells may have expanded, mobilized into peripheral blood and migrated to the damaged pancreas upon receiving signals from the damaged pancreas to aid in pancreatic repair.

While a few studies have suggested that transplanted BM cells can normalise the blood glucose levels of diabetic animals once diabetes is established (Ianus *et al.*, 2003; Lee *et al.*, 2006; Li *et al.*, 2003), several subsequent studies have not been able to confirm these findings (Akashi *et al.*, 2008; Lechner *et al.*, 2004; Mathews *et al.*, 2004). In our study, we transplanted wild type and non-diabetic NOD BM before the onset of diabetes. The blood glucose range of the non-diabetic recipient mice before transplantation was 5 to 6 mmol/l. All female recipient mice chosen for this study were 10-11 weeks old at the point of transplantation and monitored until 25 weeks of age; the peak diabetes incidence is reportedly between 20-22 weeks of age. Our data showed that only 1/9 (Group A) and 1/8 Group B mice started to develop diabetes between 23-24 weeks of age. Although, insulinitis had already started to develop at this stage, there were still 36-41% of islets free from insulinitis at 14 weeks after transplantation compared to control mice with only 10% Grade 0 islets. Additionally, blood glucose levels of Group A and B mice were generally controlled in the same manner during the whole duration, suggesting that new bone marrow may have a role in inhibiting T-cell-mediated immune responses against newly formed β -cells.

Various models of diabetes have been used to date to investigate the contribution of BM to rescue diabetes. Chemically induced diabetic mice such as the STZ-treated mice are commonly used, but they have their disadvantages. When STZ is given in low doses, it can cause non-specific islet inflammation. Unlike STZ-treated mice, NOD mice are a clinically relevant model of human T1D, as they mimic spontaneous development of diabetes in

humans and exclude an artificial influence for induction of diabetes to recipients. When T-cell depleted allogeneic BM cells were transplanted into NOD mice prior to the onset of diabetes with non lethal and lethal irradiation (700 cGy, 950cGy), reversal of destruction of β -cells was observed (Zorina *et al.*, 2003). Although we did not demonstrate complete reversal of insulinitis in the BMT mice, all mice had controlled blood glucose levels, with a significant number of islets without infiltration and delayed diabetes development.

Induction of haematopoietic chimaerism *via* allogeneic BM transplantation had been shown to arrest progression of autoimmune diabetes in both pre-diabetic and diabetic NOD mice (Kang *et al.*, 2005; Li *et al.*, 1996). Zorina *et al.* demonstrated that chimaerism even at a level of 1%, can reverse insulinitis in pre-diabetic NOD mice (Zorina *et al.*, 2003). This could be attributed to the large number of donor cells of 25×10^6 BM cells as similarly found by Gao *et al.*, where mice transplanted with 6.5×10^6 BM cells have a significant reduction in blood glucose level compared to STZ-induced mice transplanted with 1×10^6 BM cells (Gao *et al.*, 2008). Our mice were transplanted with 2×10^6 BM cells after lethal irradiation of 1000cGy, and we observed a very low engraftment level of BM donor cells (i.e. Y-chromosome positive cells) in the pancreata and spleen of our recipient female NODs. The number of donor BMCs could therefore be an important factor in improving the efficiency of BM engraftment, on the other hand a large number of donor cells could result in the possibility of cells getting caught in the capillary beds of the lung reducing the number of cells actually reaching other organs. Another factor to consider is the nature (intensity) of the stimulus as demonstrated in the second study, where Y-chromosomes were detected in all recipient early diabetic mice.

BM stem cells were initially thought to transdifferentiate into β -cells, causing the reversal of diabetes in STZ-induced mice (Janus *et al.*, 2003). Other studies however demonstrated the incidence of transdifferentiation is very rare and collectively suggested that BM cells can promote regeneration and survival of endogenous β -cells. The identity of new β -cells has been controversial but Dor *et al.* suggested that new β -cells arise primarily from proliferation of existing β -cells (Dor *et al.*, 2004). Further studies concluded that all β -cells have equal potential for growth and maintenance (Brennand *et al.*, 2007). It is also possible that β -cell regeneration could involve the transient dedifferentiation of β -cells followed by proliferation and re-differentiation (Ouziel-Yahalom *et al.*, 2006), although recent lineage

tracing studies of cultured β -cells showed otherwise (Morton *et al.*, 2007; Weinberg *et al.*, 2007). We observed significant β -cell regeneration (Ki-67⁺ Ins⁺ cells) in both groups of BM transplanted mice. Upon receiving signals of β -cells destruction, BM cells may have homed into the islet and contributed to healing by promoting the proliferation of pre-existing β -cells or secreted cytokines and growth factors as a mode of pancreatic repair. Another mechanism that may have delayed diabetes development is pancreatic repair *via* neovascularization as shown by several studies (Hasegawa *et al.*, 2007; Hess *et al.*, 2003; Mathews *et al.*, 2004). These studies suggest that BM, presumably containing EPCs, differentiated into endothelial cells and contributed to the repair of injured islet neovasculature. Although we did not show endothelial cells in the islets are donor-derived cells, we observed an increase of islet blood vessel density in our NOD BM transplanted mice compared to the control mice. Additionally, we cannot exclude the possibility of a small contribution of stem cells or other non β -cells to β -cell regeneration, as we observed some β -cells scattered throughout exocrine tissue suggesting transdifferentiation of exocrine cells, possibly acinar cells into β -cells.

Our data imply that BM cells from either wild type or non diabetic NOD mice administered before the onset of diabetes may have an effect in delaying β -cell destruction evidenced by glycaemic control, reduced inflammation and increased number of proliferating Ki-67⁺ Ins⁺ cells. These data support our assumption that attempts to reverse the disease by promotion of β -cell regeneration need to be carried out before β -cells are completely destroyed. Although we did not reverse the development of diabetes in all our mice, we have collectively shown the potential of BM cells in aiding β -cell regeneration and are an encouraging proof of concept for a regenerative medicine approach to treating diabetes.

CHAPTER 7: ENDOTHELIAL PROGENITOR CELL TRANSPLANTATION INTO NOD MICE

7.1. INTRODUCTION

BM cells have been shown to aid in pancreatic repair in spontaneous or induced diabetic models. The exact mechanisms by which stem cells in the BM initiate repair are still unknown. BM cells consist of three types of stem cells; mesenchymal stem cells (MSCs), EPCs and HSCs. Both MSCs and EPCs were believed to have the paracrine ability to secrete a variety of cytokines and growth factors (Chen *et al.*, 2008; Di Santo *et al.*, 2009). Recently, Di Santo *et al.* demonstrated that EPCs secrete a number of growth factors important in stem cell mobilization and homing such as SDF-1, VEGF and HGF (Di Santo *et al.*, 2009). It has also been reported that EPCs may play a role in the tissue regeneration *via* the formation of new blood vessels. New blood vessels formed *via* neovascularization can deliver essential growth factors, nutrients, inflammatory cells and oxygen to injured sites during the healing process.

Patients having diabetes and vascular disease have reduced numbers of circulating EPCs and impaired function to form tubules, migrate and recruit cells for re-endothelialization after vascular injury (Ii *et al.*, 2006; Loomans *et al.*, 2005). An impairment of EPC function may result in an imbalance between the reparative effort of EPCs and injury by inflammatory leukocytes, thus explaining the reduced ability for neovascularization in diabetes. These alterations decrease vascular regeneration and thus may contribute to the pathogenesis of vascular complications in T1D and T2D. Anti-diabetic strategies such as treatment with thiazolidinedione pioglitazone was shown to increase numbers and functional capacity of EPCs in patients with T2D (Wang *et al.*, 2006). Alternatively, transplantation of healthy EPCs or genetically modified EPCs may be able to improve vascular function *in vivo*.

EPCs generally exist in small numbers in the circulation and therefore culture expansion is necessary prior to transplantation into animal models. Many studies have demonstrated the potential of EPCs to promote neovascularization in myocardial and hindlimb ischaemia

(Kocher *et al.*, 2001; Tamarat *et al.*, 2004), pulmonary arterial hypertension (Takahashi *et al.*, 2004) and liver injury animal models (Taniguchi *et al.*, 2006; Ueno *et al.*, 2006). Recently, Jeong *et al.* demonstrated the intramuscular administration of *ex vivo* expanded BM-EPCs into STZ-induced diabetic mice can reverse diabetic neuropathy manifestations through direct modulation of nerves (Jeong *et al.*, 2009). Paracrine factors such as VEGF-A, FGF-2 and SDF-1 α were highly upregulated in the EPC-transplanted mice. Additionally, blood vessel density and blood flow in nerves were increased suggesting that EPCs had augmented neovascularization in the nerves. Taken together, the dual angiogenic and neurotrophic effects of EPCs may be an attractive therapeutic option in treating other vascular diseases.

Accordingly, we investigated whether transplantation of WT and NOD EPCs could attenuate or reverse T1D in the NOD mouse model by promoting neovascularization and providing paracrine factors.

7.2. METHODS

7.2.1. Mice

Two groups of mice were used as donors in this study, pre-diabetic NOD and wild type (Balb/c) mice. Wild type mice were purchased from Charles River Laboratories and housed in the animal facility at Charterhouse Square. NOD mice were bred and housed in the same facility. NOD mice were initially diagnosed to be diabetic by monitoring the urinary glucose level (Diabur Test 5000) and were subsequently tested for diabetes by measuring blood glucose levels using an Accu-chek AVIVA Blood Monitor. These newly diabetic mice were then transferred within two days to the Biological Resource Unit, Cancer Research UK to receive EPC transplantation.

7.2.2. EPC transplantation experimental plan

EPCs used in this study were c-Kit⁺ lin⁻ cells magnetically isolated from BM and cultured on fibronectin coated plates for 3-4 weeks in endothelial growth media as previously described in Chapter 5 (Figure 7.1.).

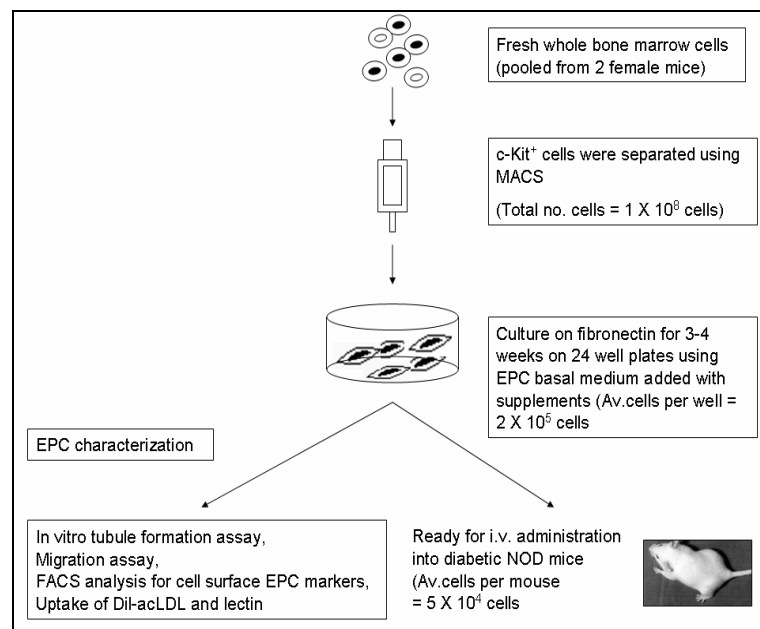


Figure 7.1. General experimental strategy.

c-Kit⁺ cells isolated from BM were grown on fibronectin coated plates for 3-4 weeks and administered into early diabetic and non diabetic NOD mice. (Abbreviations: EPC, endothelial progenitor cells; MACS, magnetic activated cell sorting; i.v., intravenous; FACS; fluorescence activated cell sorting; Dil-acLDL, Dil-labelled acetylated low-density lipoprotein).

NOD mice used in this study (0-3 days diabetic with a blood glucose level in the range of 11.5 mmol/l to 25 mmol/l) were divided into 3 groups (Figure 7.2):

- a) **Group A:** 7 female animals (early diabetic NOD, 15-23 weeks old) have been injected i.v. with 5×10^4 BM-derived EPCs (cultured c-Kit⁺ cells) isolated from wild type mice. Mice were kept for 2-4 weeks unless unwell due to development of diabetes.
- b) **Group B:** 6 female animals (early diabetic NOD, 15-23 weeks old) have been injected i.v. with 5×10^4 BM-derived EPCs (cultured c-Kit⁺ cells) isolated from NOD mice. Mice were kept for 2-4 weeks unless unwell due to development of diabetes.
- c) **Group C:** 7 female animals (early diabetic NOD, 15-23 weeks old) served as a control. These mice did not receive any EPC transplantation and were kept together with Group A and B mice in the animal facility.

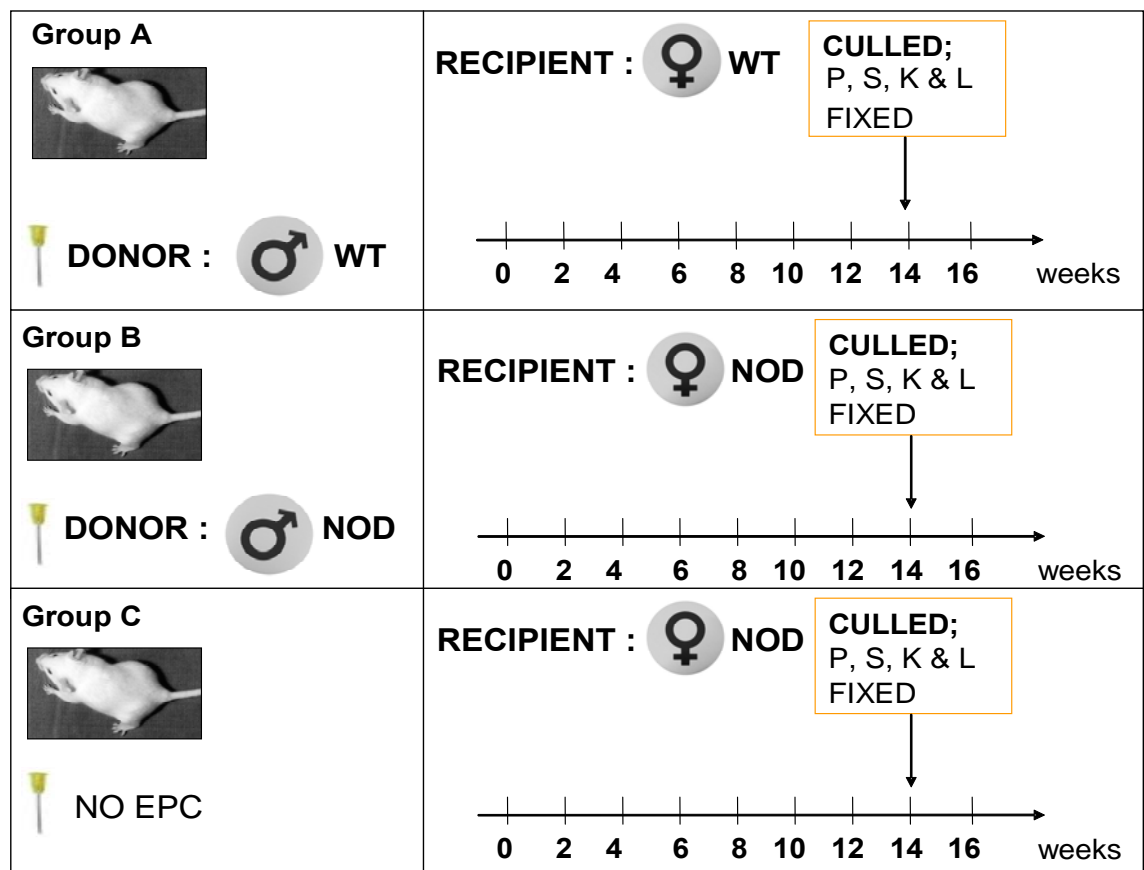


Figure 7.2. Experimental strategy.

Female early diabetic (0-3 days) Group A and B mice were injected i.v. with 5×10^4 cells cultured EPC cells. Group C, control mice did not receive any cultured EPCs (Abbreviations: BMT, bone marrow transplantation; i.v., intravenous; P, pancreas; S, spleen; K, kidney; L, liver)

7.2.3. Gender mis-matched EPC transplantation into NOD mice

Breeding of mice and isolation of BM were carried out under UK Home Office procedural and ethical guidelines (Home Office Project License NO. 70/5962) at the Biological Services Unit, Charterhouse Square. Administration of EPCs and maintenance of chimaeric mice were carried out in the Biological Resource Unit, Cancer Research UK, London Research Institute.

7.2.4. Preparation of EPCs for transplantation

The cells for EPC transplantation were obtained from the BM of wild type (Balb/c) and NOD male mice. c-Kit⁺ derived EPCs were obtained by culture in endothelial growth media, of the enriched c-Kit⁺ fraction from fresh BM of age matched wild type and NOD male mice (approximately 10-12 weeks old) (Figure 7.1.). After 3-4 weeks, adherent cells were washed with PBS before addition of trypsin to remove them from the bottom of the 24-well plate. After 5 mins incubation, cells were washed with EPC growth media at 3 times the volume of trypsin to neutralise the effect of trypsin. Cells were centrifuged at 1500 rpm for 5 mins to separate the supernatant from the cells. The pellet was then resuspended in 200 µl sterile PBS for i.v. tail vein injection into the recipient female mice on the same day. The number of nucleated cells was counted using a haemocytometer by the dye exclusion test using trypan blue (Stem Cell Technologies). Cells were stored on ice for no more than 1 hour before administration. Following administration, the mice were housed in sterile conditions and specific protocols were performed in accordance to each defined study.

7.2.5. Monitoring of diabetes development

In the period after transplantation, blood glucose levels were evaluated once every 7 days using Accu-chek AVIVA Blood Monitor (Roche). At the end point, all mice were killed by cervical 'dislocation' and/or CO₂ inhalation dependent on the type of tissue collected. Pancreas, spleen, kidney and liver were fixed in neutral buffered formalin for 24 hours at room temperature before being embedded in paraffin wax. Mice transplanted with EPCs were monitored for 21-28 days.

7.2.6. Endomucin microvessel staining

In this study, an anti-endomucin antibody was used to stain for endothelial cells lining blood vessels as endomucin is closely related to CD34, a putative mouse EPC marker. Both primary and secondary antibodies were diluted in blocking buffer, made up of goat serum (Dako) diluted in PBS (1:25). Paraffin sections were dewaxed, blocked with 0.3% v/v hydrogen peroxidase in methanol and rehydrated through a descending series of alcohol in PBS (100%, 95% and 70% v/v ethanol), before incubation in 20% v/v acetic acid/methanol for 10 mins. After incubation, slides were rinsed with PBS before addition of goat serum (Dako) for 30 mins. Rat monoclonal anti-mouse Endomucin (V.7C7) (Santa Cruz) antibody was added (1:250) for 40 mins and subsequently washed twice with PBS for 5 mins. Secondary antibody, rabbit biotinylated anti-rat IgG (1:100) (Vector Laboratories) was added to sections and incubated for 35 mins. Sections were washed twice for 5 mins before addition of alkaline phosphatase streptavidin (1:50) (Vector Laboratories) for 25 mins. After washing sections twice for 5 mins, sections were developed according to the manufacturer's instructions using alkaline phosphatase substrate kit 1 (Vector Laboratories). Sections were washed in PBS twice for 5 mins. Next sections were stained with haematoxylin for 2 mins and washed with excess in tap water for 1 min. Sections were then dipped in acid water before counterstaining with eosin. After counterstaining, sections were dehydrated for 2 mins in 70%, 90% and 100% v/v ethanol for 5 mins followed by clearing in xylene twice for 1 min before mounting with DEPEX (BDH Laboratory Supplies Inc.) and coverslipping.

For scoring of blood vessel density, see section 2.6.2.

7.3. RESULTS

7.3.1. Mice that received an EPC transplant prior to onset of hyperglycaemia

In order to study the possible contribution of EPCs in delaying hyperglycaemia, sex-mismatched EPCs from wild type (Group A) and non-diabetic NOD mice (Group B) were transplanted into female early diabetic mice. At approximately 14 days after EPC transplantation, Group A mice and control mice had an average survival rate of 60% compared to 100% survival rate for Group B mice (Figure 7.3). One mouse from Group B survived past 21 days until 28 days, which was the end point of the study.

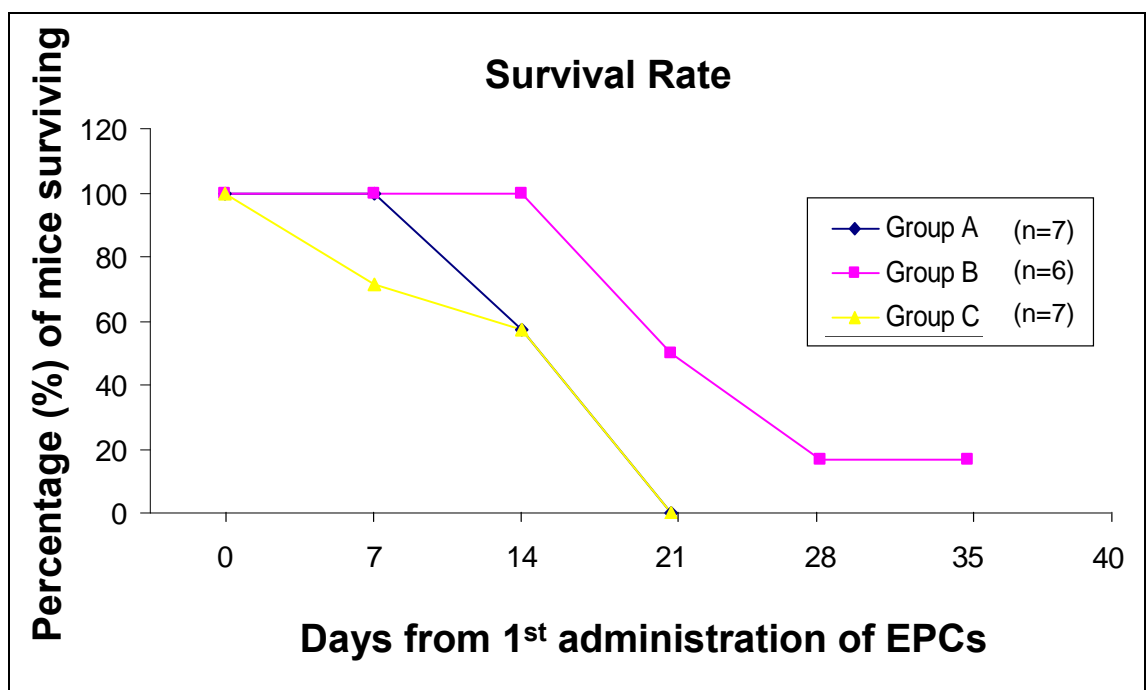


Figure 7.3. Survival rate of mice (%) after administration of EPCs.

Group A (blue), mice administered with wild type EPCs; Group B (pink), mice administered with NOD EPCs; Group C (yellow), control mice without administration of EPCs.

7.3.2. EPC contribution in the pancreas

To characterise EPC engraftment, Y-Fluorescent In Situ Hybridization (Y-FISH) analysis was performed on paraffin embedded sections containing pancreata, kidney, liver and spleen. All staining on test sections was carried out in parallel with male (as previously done, Figure 6.2A) and female control sections (Figure 7.4A&B). Optimisation of parameters such as duration and concentration of pepsin digestion were carried out in all sections for all tissue samples, as certain tissue sections were more resistant to the effects of pepsin digestion than others.

Y-chromosome-positive cells were detected in the pancreas and spleen, but not kidney or liver of all female recipient mice that received an EPC transplant. The majority of the Y-chromosomes were detected inside the islets (Figure 7.4C). These observations indicate that injected EPCs have mobilized and engrafted in the damaged pancreas. To differentiate between the host- and donor-derived endothelial cells, in situ hybridisation for the Y-chromosome was combined with immunohistochemical characterisation of endomucin, an endothelial marker. After repeated experiments, our results demonstrated that donor-derived cells were very rarely of the endothelial lineage due to the absence of Y-chromosome detection in majority of endomucin⁺ cells (endothelial cells) (Figure 7.4F).

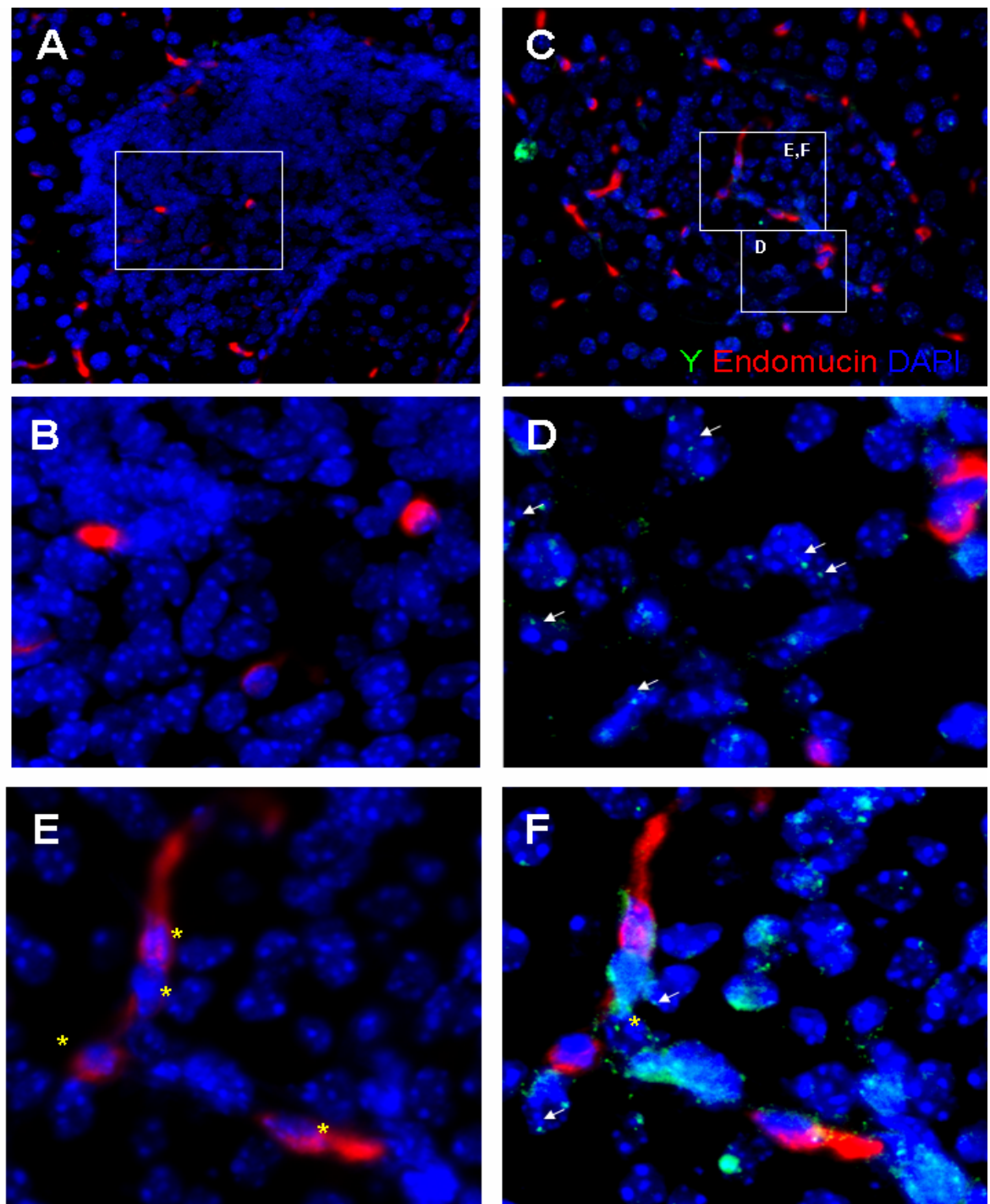


Figure 7.4. Y-chromosomes detected in female recipient transplanted with NOD EPCs (A) Female control mouse pancreas demonstrating the FITC labelled Y-chromosome probe (fluorescent green) does not label female (XX) (B) Close up image of female cells (C) Endothelial cells labelled with endomucin (red), nuclei (blue), Y-chromosomes (green). Dashed lines circle an islet (x 200 magnification) (D) Y-chromosomes are present in most of the cells in the islets. (E) Endothelial cells stained with endomucin antibody (asterisk) (F) Donor-derived cells present close to blood vessels (white arrows) and a single donor-derived endothelial cell (asterisk)(x 400 magnification).

7.3.3. Larger number of Grade 0 and 1 islets in EPC transplanted animals

Insulinitis was gauged using H&E stained sections and carried out by an observer blinded to the treatment group.

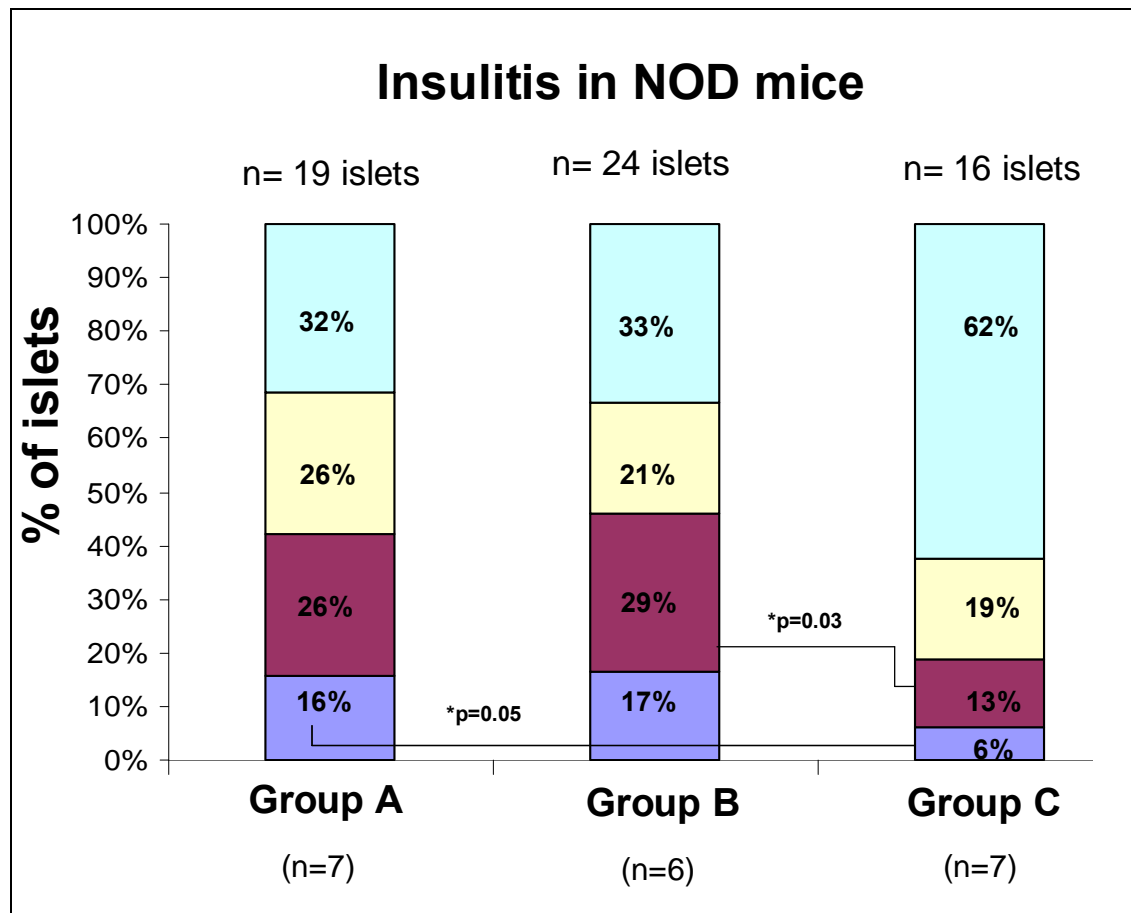


Figure 7.5. Grades of insulinitis observed in Group A, B and C mice. Grade 0, normal islet ; Grade 1, peri-insulinitis ; Grade 2, moderate insulinitis; Grade 3, severe insulinitis. The data are the sum of islets from 6-7 mice for each group (Abbreviation: n= number of islets scored)

To study the effect of EPC transplantation on lymphocyte homing into islets, insulinitis was scored in pancreatic sections from 6-7 mice each in both group A and B mice (using grading scheme shown on Figure 4.6). Insulinitis was quantified in all islets present in two H&E-stained sections from each animal, each section being separated from the next by at least 100 μm . After 21 days of receiving wild type EPCs, 16% of islets from Group A mice showed little infiltration and destruction of pancreatic β -cells (grade 0 insulinitis) whereas only 3% of islets from control mice were grade 0 (*P-value: 0.05). However 29% of islets

in group B mice had peri-insulinitis (grade 1) compared to 13% islets in the control mice (*P-value: 0.03) (Figure 7.5). The percentage of islets with Grade 0, 1, 2 and 3 insulinitis were almost the same in Group A & B mice, thus indicating that the EPC effects on inflammation are the same regardless of the strain of the donor mouse.

7.3.4. Little change in average blood glucose levels before and after EPC transplantation

Blood glucose levels of Group A and Group B mice were in the range of 15-25 mmol/l before EPC transplantation. Average blood glucose level of Group A, B and control mice at 21-28 days after EPC transplantation did not change significantly (Figure 7.6).

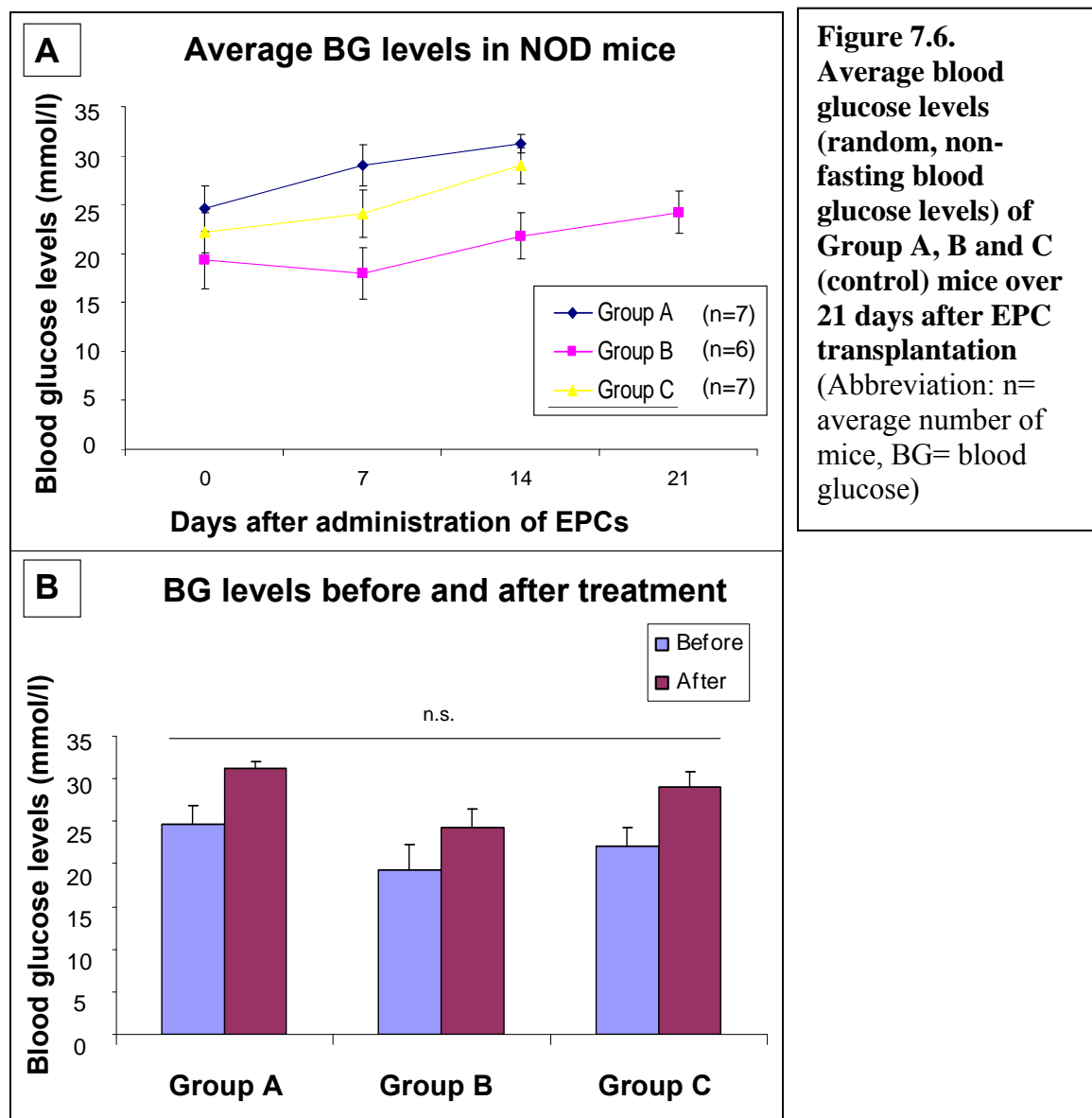


Figure 7.6. Average blood glucose levels (random, non-fasting blood glucose levels) of Group A, B and C (control) mice over 21 days after EPC transplantation (Abbreviation: n= average number of mice, BG= blood glucose)

7.3.5. Evidence of islet regeneration by EPC transplantation once disease is established

To quantify β -cell proliferation and regeneration in the pancreata after EPC transplantation, pancreatic sections were stained with Ki-67 and insulin antibodies. All Ki-67 and insulin antibody staining on test sections was carried out in parallel with positive wild type mouse gut and pancreas sections respectively. Majority of islets in control mice were Grade 3 islets, thus have none or very few Insulin^+ cells (Figure 7.7C,D). There was both Ki-67^+ Insulin^+ cells (Figure 7.7A,B) and Ki-67^+ Insulin^- cells present in the islets of mice that received WT or NOD EPCs.

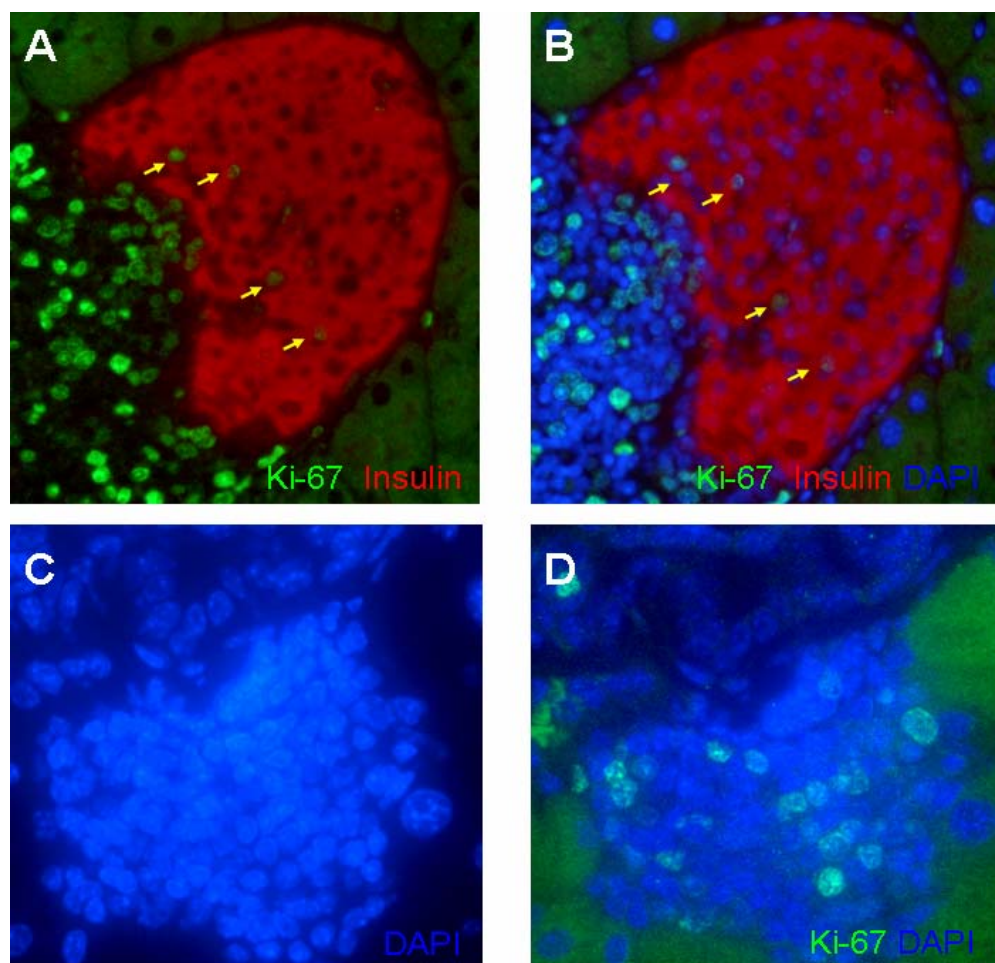


Figure 7.7. Insulin and Ki-67 immunohistochemistry.

(A) Insulin^+ cells which are also Ki-67^+ (arrows) in grade 2 islet from female recipient mouse that received NOD EPCs (B) Merged image of Ki-67^+ Insulin^+ cells stained with nuclear stain, DAPI (x 400 magnification) (C) Nuclear stain in a grade 3 islet of a female control mice (Group C control mice). No Insulin^+ cells were detected (D) Merged image of Ki-67^+ DAPI^+ cells (x 200 magnification)

In the absence of insulin staining, it was obvious that a number of the Ki-67⁺ cells present would most likely be proliferating inflammatory cells (Figure 7.7B).

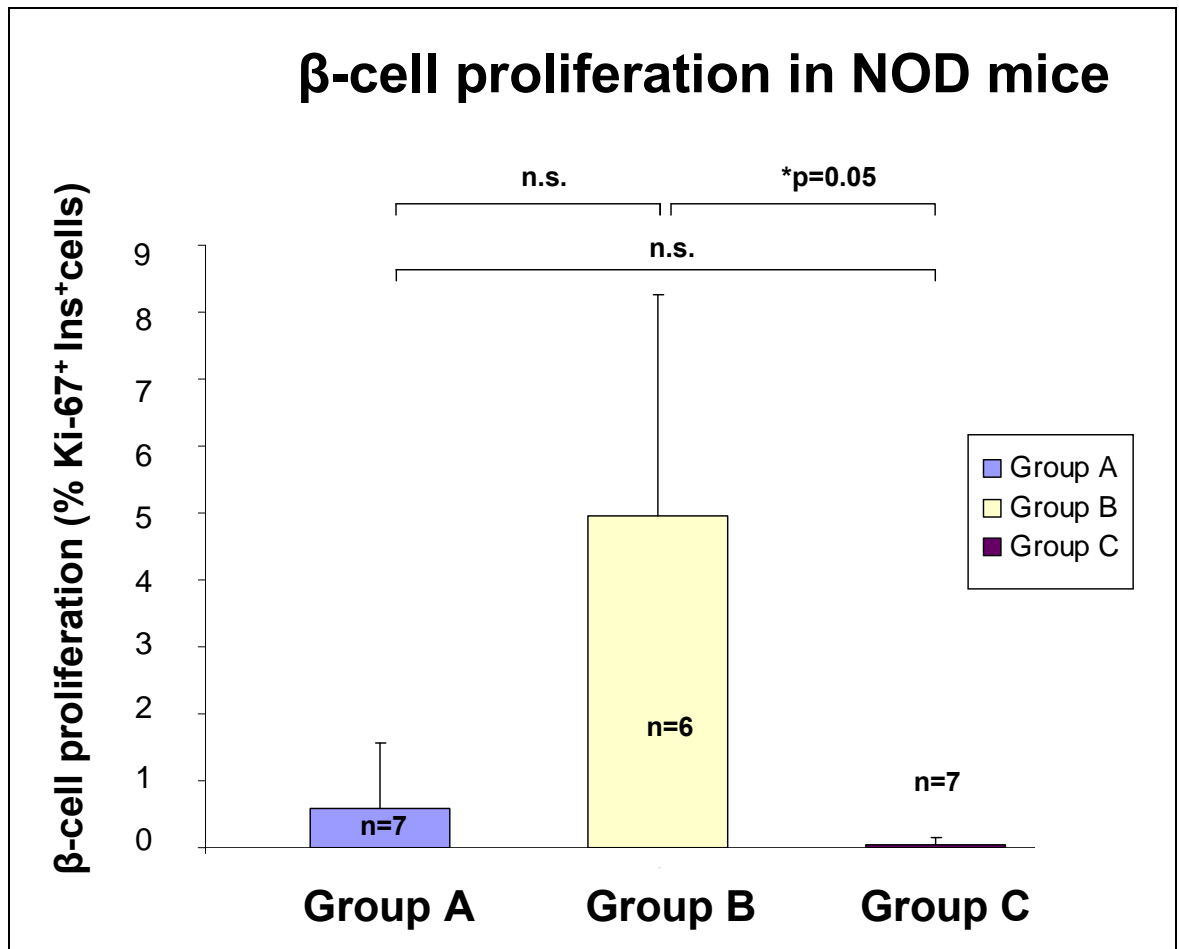


Figure 7.8. Ki-67 labelling index of Ins-positive cells of Group A, B and C mice. Statistical significance was tested by Student's t-test (Abbreviations: n= average number of mice; Ins= insulin)

Staining for Ki-67 and insulin revealed a higher level of proliferation in the islets of NOD EPC transplanted mice (Group B mice) compared to control mice and WT EPC transplanted mice (Group A mice) (Figure 7.8).

7.3.6. Increased islet neovascularization in both groups of mice

To evaluate the contribution of BM-derived EPCs to pancreatic repair by neovascularization, blood vessel density of pancreatic sections was assessed using endomucin as a marker to stain for endothelial cells lining blood vessels. Endomucin (clone v.7c7) was chosen in this study instead of CD31 as endomucin is closely related to CD34, a putative mouse EPC marker.

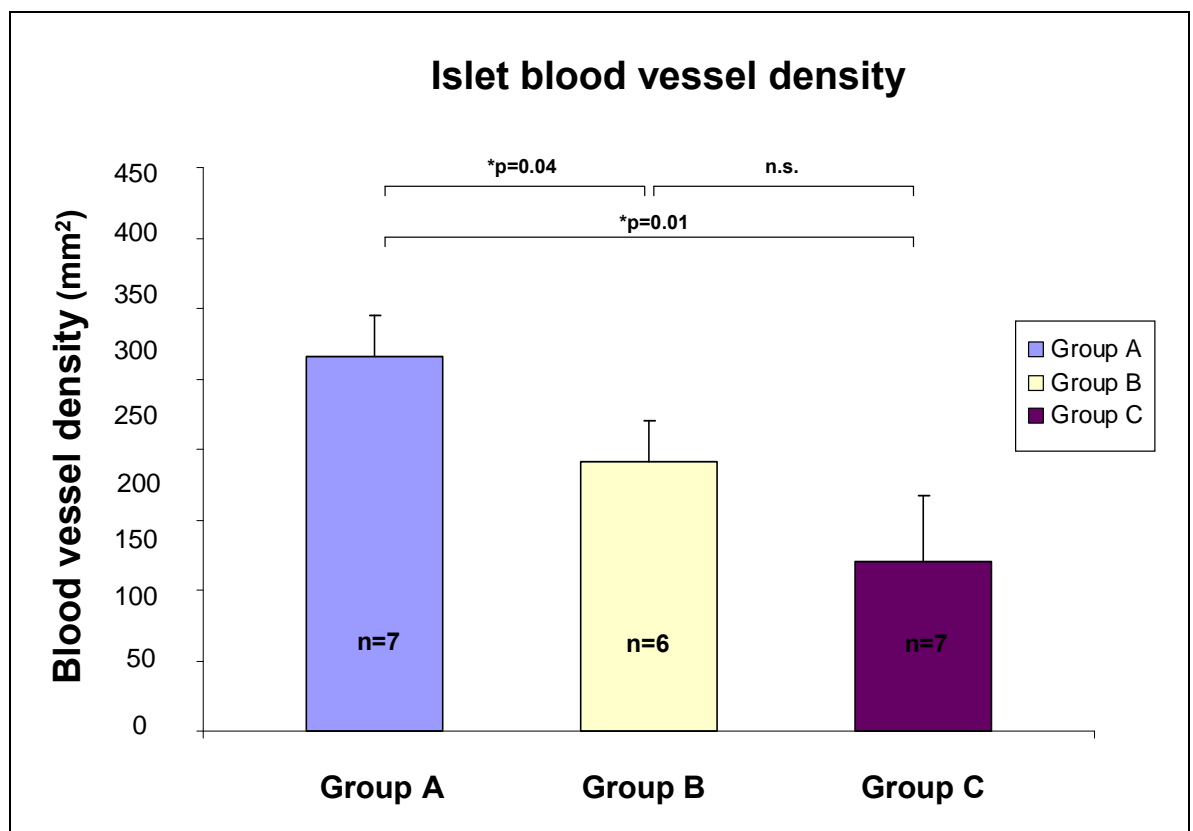


Figure 7.9. Islet blood vessel density of EPC transplanted mice (Group A, B, C mice)

Group A mice (WT EPC transplanted mice) had increased blood vessel density compared to Group B (NOD EPC transplanted mice) and control mice (Figure 7.9). There were no significant differences in blood vessel density between Group B and control mice.

7.4. DISCUSSION

EPCs isolated from BM, umbilical CB and PB have been shown to improve angiogenesis/vasculogenesis in disease models through two mechanisms, vasculogenesis, the recruitment of BM-derived EPCs that migrate and differentiate in response to signals to create new blood vessels, and angiogenesis, the endothelial sprouting of pre-existing vessels. Some new vessels can be formed by a combination of angiogenesis and vasculogenesis. In our study, we observed very few donor-derived endothelial cells as witnessed by co-localization of the Y-chromosome with the endothelial cell marker, endomucin in the islets of Langerhans. This suggests that donor EPCs had differentiated into endothelial cells, although very infrequently. We found in many occasions, the presence of a cluster of donor-derived cells near the blood vessels which may possibly be undifferentiated EPCs. Additionally, we observed an increased islet blood vessel density in WT EPC transplanted mice compared to control mice. These observations thus led us to hypothesise that this process is due to a greater contribution by angiogenesis than vasculogenesis as similarly observed by Jeong *et al.* using a diabetic neuropathy animal model (Jeong *et al.*, 2009).

EPCs are mobilized by various stimuli such as tissue injury, ischaemia and hypoxia. In this study, the NOD mouse is used as a model of T1D where β -cells in the islets are destroyed by inflammatory cells resulting in hyperglycaemia. Using Y-FISH analysis, we detected Y-chromosomes in the pancreas and spleen but neither in the kidneys nor liver of recipient mice. This suggests that EPCs injected intravenously may have been stimulated to enter the systemic circulation and migrate to the damaged islets in response to signals from stressed β -cells. Our observations support previous findings from Hess *et al.* and Mathews *et al.* that BM-derived stem cells preferentially engraft damaged pancreatic tissue, further supporting the notion that tissue damage is necessary for the recruitment of BM-derived cells (Hess *et al.*, 2003; Mathews *et al.*, 2004).

In addition to differentiating into endothelial cells to contribute to wound healing, various studies demonstrated that EPCs are able to produce paracrine factors including cytokines and growth factors. These factors released from transplanted EPCs may have important roles in the regulation of neovascularization, fibrosis, inflammation and endogenous repair

(Burchfield and Dimmeler, 2008). Our results demonstrated that NOD EPC transplantation can initiate and augment proliferation of the pancreatic β -cells (Ki-67⁺ Ins⁺ cells), compared to the lower level of proliferation detected in early diabetic mice that did not receive EPCs. We postulate that these EPCs release a mixture of physiologically relevant cytokines and growth factors, inducing a permissive environment for differentiated cells as well as progenitor cells of the recipient to mediate pancreatic repair. Additionally, we observed a higher number of Grade 0 and Grade 1 islets in WT and NOD EPC transplanted mice compared to control mice, indicating the potential effect of EPCs in controlling inflammation in diabetic mice. Ohnishi and colleagues have shown that BM-derived MSCs may possess anti-inflammatory properties as they reported reduced inflammation in the heart, in particular histiocytic infiltration (marked by CD68⁺ cells) in a rat model of acute myocarditis (Ohnishi *et al.*, 2007). The authors concluded that the anti-inflammatory effects in the transplanted rat were due to paracrine factors released from the MSCs. There is a possibility therefore that the delayed inflammation in our BM-derived EPC transplanted mice could be due to the immunosuppressive factors released by these cells.

The blood glucose range used in this study was 15 mmol/l to 25 mmol/l. As NOD mice spontaneously develop diabetes, it is difficult to enrol mice with the same diabetes severity, however we tried to minimise the differences by enrolling only mice that were 0-3 days diabetic. One mouse from our control mice (Group C) without insulin or EPC therapy became severely diabetic after 7 days and this pattern was subsequently followed by other mice in the group, until the maximum time period of 21 days. The survival rate of Group B mice was slightly better where mice started to get severe diabetic symptoms only from day 14 onwards. In contrast, one mouse from the NOD EPC transplanted group (Group B) managed to survive past 21 days and was culled at 28 days only due to timeframe set for the experiment. The blood glucose level of that particular mouse before EPC transplant was 17.28 mmol/l and decreased to a normoglycaemia level of 9.89 mmol/l after 28 days. This particular mouse did not exhibit any physical signs of diabetes compared to the other diabetic mice. There is a possibility therefore that the mouse treated with NOD EPCs was protected from hyperglycaemia, thus extending its survival.

Early outgrowth cells or early EPCs have a limited ability to form vascular tubes *in vivo* and *in vitro*, but augment neovascularization by secretion of growth factors such as VEGF, which plays a role in revascularization of injured tissues, HGF, G-CSF and IL-8 (Ziegelhoeffer *et al.*, 2004). Recently Di Santo *et al.* discovered that culture expanded EPCs in hypoxic conditions release high concentrations of growth factors with angiogenic properties, namely bFGF and TNF α , IL-8, SDF-1, HGF, angiogenin, PDGF-BB, VEGF-A (Di Santo *et al.*, 2009). When conditioned media is injected intramuscularly into rat hindlimb ischaemic mice, neovascularization was augmented in terms of increased blood flow and capillary density. Although we did not measure the levels of growth factors in the EPC transplanted mice, we postulate that islet blood vessel density was increased due to both paracrine release of pro-angiogenic factors as well as by modest differentiation into endothelial cells.

It has been demonstrated that T1D and T2D patients with cardiovascular problems have reduced numbers of EPCs and are dysfunctional in ways of homing to the site of injury and formation of tubules, demonstrated *in vitro* (Loomans *et al.*, 2005; Tepper *et al.*, 2002). When autologous (dysfunctional) progenitor cells from CAD patients was transplanted into patients with myocardial infarction, EPCs prove to be ineffective in improving neovascularization and heart function (Heeschen *et al.*, 2004). In line with previous observations, our results demonstrated that NOD EPCs did not increase the blood vessel density of diabetic recipient mice (Group A mice). On the other hand, recipients of healthy EPCs (i.e. WT EPCs) had a significant increase in blood vessel density.

Our data imply that EPC cells from either WT or non-diabetic NOD mice transplanted during the early stages of diabetes may have an effect in delaying β -cell destruction, evidenced by reduced inflammation. Although there were no significant changes in glycaemic control in the majority of the EPC transplanted mice, hyperglycaemia was successfully reversed in one recipient mouse transplanted with NOD EPCs. In summary, our study showed that exogenous EPCs transplanted into early diabetic mice can promote β -cell regeneration, and control hyperglycaemia possibly *via* neovascularization and/or release of paracrine factors. BM-derived EPC transplantation therefore may be a feasible approach in the future to improve the regeneration of other organs and tissues.

CHAPTER 8: GENERAL SUMMARY AND FUTURE DIRECTIONS

8.1. GENERAL SUMMARY

Pancreas and islet transplantations have so far proved to be a successful treatment for T1D, however due to insufficient supply of donor pancreata and islets and reported immune system rejection, alternative strategies such as stem cell therapy have been considered. In recent years, adult stem cells have emerged to have the ability to cross lineage boundaries and form lineages within other tissues. Studies have so far tried to replace damaged β -cells with newly derived insulin-secreting cells from BM and cord blood, however efforts have proved futile as these cells did not demonstrate appropriate characteristics of functional β -cells and were thus unsuitable for transplantation therapy. Besides the ability to transdifferentiate into other cell lineages, extrapancreatic adult stem cells were believed to have the ability to release paracrine factors which can promote a regenerative environment *in vivo*.

Through this thesis, I have investigated two strategies for enhancing pancreatic regeneration and produced data demonstrating their potential application in T1D therapy. The first strategy involves the study of the contribution of BM cells and BM-derived EPCs to promote β -cell regeneration in the damaged pancreas of NOD mice.

Based on various reports demonstrating the ability of EPCs to promote neovascularization in animal models of disease, I decided to first isolate and measure the number of circulating EPCs using typical murine EPC markers that exist in both non-diabetic and diabetic NOD mice using FACS analysis (Chapter 3). Following are a number of observations:

- 1) EPCs exist in very low numbers in mouse PB and BM.
- 2) EPC number is markedly reduced in the BM of diabetic mice, but is conversely increased in the bloodstream, indicating the possibility of recruitment of BM-derived EPCs to mobilize into the blood circulation upon receiving signals from the damaged pancreas.

To fully understand the EPC recruitment process and contribution to the damaged pancreas, BM-derived EPCs were first isolated using magnetic isolation (Chapter 5) and next transplanted into early diabetic NOD mice (Chapter 7). Two different methods were used to expand BM-derived EPCs and cells were assessed based on phenotypic and functional endothelial properties. I could draw the following conclusions:

- 1) BM-derived EPCs isolated using magnetic isolation of c-Kit⁺ cells express markers of murine EPCs and appears to be late EPCs.
- 2) BM-derived EPCs isolated using selective media did not express the panel of EPC markers and have limited proliferative activity. Magnetic isolation therefore is the method of choice for the isolation of BM-derived EPCs.
- 3) In the early diabetic NOD mouse, transplanted WT and NOD EPCs homed to the damaged pancreas and engrafted in the islets and spleen.
- 4) Transdifferentiation of EPCs into endothelial cells occurs very infrequently.
- 5) EPCs may have a role in β -cell regeneration but neovascularization is not the major mechanism.

Parallel to the EPC transplantation study, fresh whole BM was transplanted into pre-diabetic mice and monitored for 14 weeks (Chapter 6). I could draw the following conclusions:

- 1) The majority of enrolled mice had delayed β -cell destruction evidenced by better glycaemic control, reduced inflammation and increased number of proliferating β -cells.
- 2) Diabetes intervention is best performed before the complete destruction of β -cells.

In addition to promoting endogenous β -cell regeneration using EPCs, I investigated an alternative strategy to block β -cell destruction *via* apoptosis by injecting intraperitoneally, a protease peptide inhibitor for JNK domain (XG-102) into early diabetic NOD mice (Chapter 4). I could draw the following conclusions:

- 1) Blocking the interaction between JNK1 and c-Jun substrate using XG-102 had very little effect on blocking β -cell apoptosis in early diabetic NOD mice.

- 2) XG-102 may have a role in reducing lymphocyte homing into the pancreas.
- 3) JNK1 may not have an important role in β -cell apoptosis.

8.2. GENERAL DISCUSSION

Our data demonstrated the potential of both BM stem cells and protease peptide inhibitor in delaying diabetes and promoting pancreatic repair. Following are a number of issues and limitations which need to be studied in more detail before stem cell or drug therapy for diabetes can be applied to the clinical setting:

8.2.1. Issues to be addressed before EPC transplantation therapy can become a reality

Research has demonstrated the potential of EPCs for future therapeutic applications in improving angiogenesis/vasculogenesis. Successful neovascularization has been demonstrated in various disease models using EPCs, however to translate the knowledge obtained from these experiments to clinical therapy, several issues need to be considered and addressed:

- i) *EPC source* – EPCs from patients with endothelial dysfunction have limited proliferative potential and are reduced in numbers. Therefore, EPCs from a healthy donor is required for successful cell transplantation.
- ii) *Recipient* – Patients need to be assessed of their suitability, such as their age and other cardiovascular risk factors which may reduce the availability and function of EPCs. Additionally, unfavourable microenvironment present in the ischaemic tissue might impair the effectiveness of cell transplantation.
- iii) *Ex vivo* expansion of EPCs - A standard source and use of gold-standard markers need to be established to avoid any variation in therapeutic efficacy.
- iv) *Transplantation* – Dosage of cells for transplantation needs to be optimised to overcome problem of loss of EPCs during early stage of transplantation. Choice of EPC delivery also needs to be considered for successful initiation of vascularization and tissue regeneration *in vivo*.

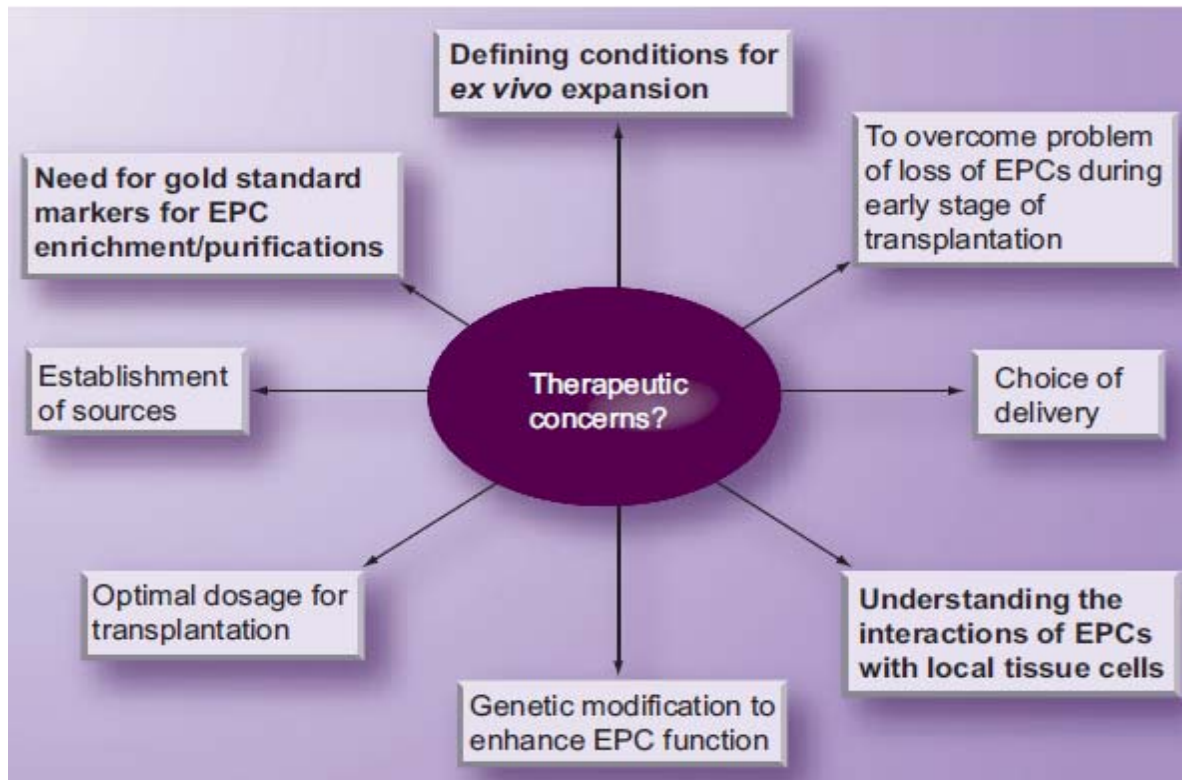


Figure 8.1 Issues needing to be addressed before vascular therapy becomes an everyday occurrence. Newly derived EPCs need to be isolated and characterized using a standard procedure to avoid any variations. Additionally, it is important to optimize transplantation procedures such as the technique of delivery and number of cells to initiate vascularization *in vivo*. (Abbreviation: EPC, Endothelial progenitor cell).

8.2.2. Cultured EPCs versus fresh EPCs

Assessments of EPC contribution in this study were performed on cultured EPCs because they are low in numbers when freshly isolated, but this is how they are currently defined and isolated. The culture process can result in maturation of EPCs to more mature endothelial cells therefore limiting the efficacy of the EPC contribution. This therefore poses a risk when transplanted into patients. Additionally, Li *et al.* reported low senescence in *ex vivo* expansion of c-Kit⁺ cells which may thus affect the low potential for inducing therapeutic angiogenesis in their animal model (Li *et al.*, 2004). In our study, the majority of cells were viable as ascertained using the trypan blue exclusion test when counting cells for culture or transplantation. This technique however is not very sensitive and just gives an overview of the viability of cells. This problem can be addressed by first isolating viable

cells using magnetic cell sorting or flow cytometry for cell viability markers such as propidium iodide (PI) and 7-actinomycin D (7-AAD), before enriching with a lineage positive antibody (Lu *et al.*, 2008). Caution therefore is warranted as cell loss can occur after several separation steps by magnetic beads or flow cytometry.

8.2.3. Practical uses and limitations of cell-permeable peptides

Drug delivery is an important aspect in achieving a therapeutic effect in human or animal models. Previous therapeutic approaches such as protein and gene therapy have limited success due to poor permeability of peptides and oligonucleotides through the cell membrane. Other delivery methods include invasive methods such as microinjection and application of membrane disrupting agents which can result in high toxicity, immunogenicity and low delivery yield to the desired site (Morris *et al.*, 2008). Substantial progress has been made with the development of cell-penetrating peptide (CPP), a delivery system that overcomes previous limitations of entry restriction into the cell. A commonly used CPP is TAT discovered in 1988, a transcription activator of the human immunodeficiency virus type 1 (HIV-1) viral genome (Frankel *et al.*, 1988). Target molecules can be linked to the TAT, be delivered to the desired site in a highly efficient manner. In our study, we used XG-102 or D-JNK1 inhibitory peptide which was created by linking the 20-amino acid JNK-binding motif of JIP-1 to 10-amino acid HIV TAT transported sequence (Borsello *et al.*, 2003). This peptide was designed to competitively block interaction between JNK1 to c-Jun and other substrates, thereby disrupting the MAPK/JNK cell death signal cascade. In addition to our study using XG-102, previous studies have shown the ability of this peptide to protect neurons against cell death in both rat model of middle cerebral artery occlusion and cerebral ischaemia (Borsello *et al.*, 2003; Hirt *et al.*, 2004). Although XG-102 has produced therapeutic results in animal models, its translation to use in human therapy is hampered by the cost of the molecule. For example the XG-102 dose used by Borsello and colleagues was 11mg/kg which corresponds to approximately 1g for the successful treatment in humans (Borsello *et al.*, 2003). Additionally, as XG-102 has a long-lived biological activity inside cells and the TAT-linked design prevents leakage from cells, it could potentially be toxic and thus further investigations are warranted prior to its introduction for clinical use, especially in small organs like the pancreas (Barr *et al.*, 2002).

8.2.4. *NOD mouse versus human T1D*

The NOD mouse is the most commonly used animal model of human T1D to date due to the ability to spontaneously develop diabetes, with shared immunological features such as defective central and peripheral tolerance. Therefore, the NOD mouse is commonly used to improve our understanding of diabetes pathogenesis and extensively used as a preclinical tool for the development of new therapeutic strategies. Agents such as insulin, anti-CD3 and TNF- α have all been shown at various stages of diabetes in the NOD mouse to protect β -cells from damage (Harrison *et al.*, 1996; Hayward and Shreiber, 1989; Seino *et al.*, 1991). Such strategies are highly motivating, but caution is often warranted as therapies that worked in NOD mice may not be suitable for humans. For example oral injections of insulin protected pre-diabetic mice from developing diabetes whereas this strategy did not delay or prevent T1D in non-diabetic relatives at risk of developing T1D (Diabetes Prevention Trial of oral and subcutaneous insulin)(Skyler *et al.*, 2005). This discrepancy could be due to the fact that the NOD mouse only represents one pathogenic mechanism of diabetes which may be relevant only to a small number of human patients (Raz *et al.*, 2005). The time course of diabetes development between the NOD mouse and humans are also quite different, where humans develop diabetes at or before puberty whereas NOD mice develop the disease much later. Additionally, the incidence of diabetes is equal in human females and males; however this is not the case in NOD mice where female NOD mice predominantly develop diabetes. Future preclinical therapeutic strategies need to take the differences between the NOD mouse and human T1D into consideration, to improve future translational efforts.

8.2.5. *Variation in clinical measurements*

Many studies have successfully demonstrated the delay or prevention of disease using therapeutic agents and biomolecules. Recently Shoda *et al.* analyzed past studies and discovered there is a wide variability of the definition of diabetes and treatment efficacy for the treatment of diabetes using animal models (Shoda *et al.*, 2005). For example Akashi *et al.* defined Akita mice to be diabetic based on fed glucose levels of above 250 mg/dl. Treatment efficacy however was only based on two factors, reduction in blood glucose

level and donor chimaerism (GFP⁺) cells in the peripheral blood and pancreas (Akashi *et al.*, 2008). In all my experiments, diabetic status of the mice was diagnosed by monitoring urinary glucose levels for glycosuria and then confirming by measuring blood glucose levels exceeding 11.5 mmol/l (i.e. 207 mg/dl) with multiple hyperglycaemic measurements since first diagnosis. Treatment efficacy was determined based on blood glucose levels, survival rate, chimaerism using Y-FISH technique, insulinitis, proliferating β -cells and insulin staining. Our clinical measurements are similar to the study by Zorina and colleagues, where they defined diabetes using glycosuria and blood glucose measurements and efficacy based on blood glucose levels, chimaerism in blood using FACS, evaluation of islet destruction, proliferating β -cells and insulin staining (Zorina *et al.*, 2003). The variability that exists among current studies in terms of clinical measurements and outcomes may complicate the translation of results from mice to humans. For example the definition of diabetes in animals may reflect different states of diabetes progression, thus effecting therapeutic intervention. A standard definition for diabetes and treatment efficacy thus needs to be established for utility for future human clinical trials.

8.2.6. Paracrine growth factors for augmentation of vasculogenesis

In our study, EPCs were isolated from BM and cultured for 3-4 weeks in standard endothelial growth media. Some studies have demonstrated that EPCs transfected with VEGF or preconditioning of cells with G-CSF growth factors can augment neovascularization in rat myocardial ischaemia and mouse hindlimb ischaemia models (Iwaguro *et al.*, 2002; Kocher *et al.*, 2001). Following these studies, Chen *et al.*, implanted a layered scaffold in a mouse model of hindlimb ischaemia. The implant delivered spatially controlled gradients of VEGF in the mouse, resulting in increased blood vessel density and rapid restoration of hindlimb blood flow (Chen *et al.*, 2007). Interestingly, Di Santo and colleagues recently demonstrated that EPCs are not necessary to augment neovascularization, instead conditioned media from EPCs grown in hypoxia for 72 hours can achieve the same effect in rat hindlimb ischaemia (Di Santo *et al.*, 2009). This cell-free based therapy offers great potential for therapeutic applications.

8.2.7. Limitations in established techniques for the assessment of chimaerism

Analysis of chimaerism after allogeneic BM cell transplantation is important for assessing engraftment and the early detection of graft failure. In sex mis-matched transplanted mice, where male cells were transplanted into female mice, the obvious method in distinguishing donor cells from host cells is the detection of the Y-chromosome in the recipient mice. In our BM and EPC transplanted studies, Y-chromosome fluorescent in situ hybridization (Y-FISH) technique was used to identify Y-chromosomes in the female mice. In the first batch of BM transplanted mice, very few Y-chromosomes were detected in the spleen and pancreas. Similarly, de Weger and colleagues identified very low numbers of Y-chromosomes in the host tissue, which did not correlate with the numbers they had transplanted (de Weger *et al.*, 2008). Although the degree of injury/stimulus may possibly be the reason for low BM engraftment, the authors suggested that this discrepancy could be due to technical factors, where the apparent lack of a demonstrable Y-chromosome in half of the sections could be due to the location of the Y-chromosome outside the plane of the section. Analysis of polymorphic DNA sequences, i.e. short tandem repeats (STR) or variable number of tandem repeats (VNTR), is another widely used procedure used in the assessment of chimaerism. Although STR-PCR and Y-FISH method allow rapid and quantitative evaluation of engraftment, their use is limited in the monitoring of minimal residual disease due to its sensitivity of 0.1-5% (Elmaagacli *et al.*, 2001). In recent years, other sensitive methods such as real time PCR on the analysis of Y-chromosome (Y-PCR) and single nucleotide polymorphism PCR (SNP-PCR) have been developed. Based on Koldehoff *et al.* study, they demonstrated the SNP-PCR method using 10 different SNP loci and were able to detect patients with a high risk of relapse after transplant significantly earlier than the STR-PCR method (Koldehoff *et al.*, 2006). Additionally, the authors suggested that Y-PCR is a more sensitive method than interphase XY-FISH method where Y-PCR could detect 3 times more relapses by mixed chimaerism compared to XY-FISH.

8.2.8. Effect of endothelial dysfunction

EPC number is reduced and functionally impaired in patients with diabetes, rheumatoid arthritis and cardiovascular disease. When BM-derived progenitor cells (Sca-1⁺ cells) from

type 2 diabetic Lepr^{db} mice were injected into skin wounds of diabetic mice, these cells failed to enhance wound vascularization and were unable to form tubules and incorporate efficiently in wound structures (Stepanovic *et al.*, 2003). Non-diabetic cells from Lepr^{db} mice however were able to promote neovascularization compared to diabetic cells, suggesting that although there is a numerical dysfunction of EPCs in Lepr^{db} mice, the remaining EPCs may still have some angiogenic function. In our study, we observed that transplanted wild type EPCs can promote revascularization in early diabetic NOD mice based on increased islet blood vessel density compared to when non-diabetic NOD EPCs were transplanted. When these observations are gathered together, the wild type or healthy EPCs have the best efficacy in promoting neovascularization, followed by non-diabetic and diabetic cells. This warrants the need to investigate whether endothelial dysfunction exists from birth or onset of the disease which may affect future intervention strategies.

8.3. FUTURE DIRECTIONS

8.3.1. Investigate the mechanisms involved in β -cell regeneration

It has not been shown before that EPCs may have a role in β -cell regeneration. In light of the observations made in this thesis, the mechanism by which EPCs promote β -cell regeneration needs to be identified and studied more extensively. For example, EPCs can release paracrine factors that could recruit and mobilize stem cells and simulate tissue repair. Different EPC subtypes should be studied and growth factors released need to be identified to allow direct use in future therapy. As EPCs are mainly derived from the BM, they may also have immunosuppressive activity as shown for BM-derived MSCs.

8.3.2. Strategies to promote mobilization and homing

Stimuli such as tissue injury, ischaemia and hypoxia can initiate mobilization of stem cells and EPCs from the BM. EPC mobilization and homing properties are impaired in diabetic mice. The introduction of growth factors such as GM-CSF & SDF-1 and IL-1 β has been shown to promote mobilization and homing of endogenous EPCs. Therefore the combination of EPC transplantation and growth factors would be able to further augment neovascularization in the mice. Additional procedures to improve the mobilization and

homing of EPCs to the damaged site could also include inducing ischaemia prior to transplantation.

8.2.3. Strategies to improve EPC function

Hypoxia is a well known stimulatory factor to mobilize EPCs into the circulation (Shintani *et al.*, 2001; Takahashi *et al.*, 1999). Recent studies have shown that EPCs grown in hypoxic condition of 1% oxygen concentration have an effect on EPC proliferation, differentiation, tubule formation and paracrine release. The culture of EPCs in reduced oxygen concentrations before transplantation into animal models would allow the evaluation of whether hypoxic preconditioning can enhance therapeutic vasculogenesis.

8.2.4. Optimising treatment efficacy

XG-102 and BM-derived EPCs were injected into newly diabetic NOD mice with the aim of delaying or preventing diabetes in the NOD mice, but very few residual β -cells were left. Data collected in these studies, demonstrate that treatment timing is therefore important. As NOD mice develop diabetes spontaneously, future intervention therefore should be carried out not only at one time point but several other time points in the development of diabetes of the NOD mouse. Additionally, other factors such as the dose and length of post-treatment also need to be considered and optimized.

CHAPTER 9: REFERENCES

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