COMPARATIVE EVALUATION OF CIRCULATING KERATINOCYTE PROGENITOR CELL PROPERTIES IN DIABETIC VERSUS NON-DIABETIC SUBJECTS

A

DISSERTATION SUBMITTED

BY

INDU A. G

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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DECLARATION

I, **Indu A. G**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Comparative evaluation of circulating keratinocyte progenitor cell properties in diabetic versus non-diabetic subjects"**, under the direct supervision of **Dr. Lissy K. Krishnan, Scientist G, Thrombosis Research Unit,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Comparative evaluation of circulating keratinocyte progenitor cell properties in diabetic versus non-diabetic subjects**" is being submitted by **Indu A. G** in partial fulfilment for the degree of Master of Philosophy Technology in Biomedical Research to be awarded by this Institute. The entire work was done by her under my supervision and guidance at **Thrombosis Research Unit**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, 695012.

Thiruvananthapuram **Name of the guide**

Date Signature

The Dissertation

Entitled

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Submitted
By

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For
Master of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FORMEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM

Evaluated and approved
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Signature **Signature**

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List of Abbreviations

KPC	Keratinocyte Progenitor Cell
PBMNC	Peripheral Blood Mono Nuclear Cell
ECM	Extra Cellular Matrix
rt PCR	Real Time Polymerase Chain Reaction
HbA1c	Glycated Hemoglobin
EGF	Epidermal Growth Factor
VEGF	Vascular Endothelial Growth Factor
CD	Cluster of Differentiation
DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
PCNA	Proliferating Cell Nuclear Antigen
FBS	Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle Medium
DEPC	Diethylpyrocarbonate
cDNA	Complementary Deoxyribo Nucleic Acid
dNTP	Deoxyribonucleotide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TBE	Tris/Borate/EDTA
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
FITC	Fluorescein isothiocyanate
IgG	Immunoglobulin G
BSA	Bovine Serum Albumin
FACS	Fluorescence-activated cell sorting
FSC	Forward Scatter Channel
SSC	Side Scatter Channel

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Synopsis

Wound healing is a natural process of tissue repair. The purpose of wound healing is to restore a skin defect and to regain, at least in part the lost integrity, tensile strength and barrier function of the skin. Wound healing is a well-orchestrated process that involves molecular events of cell migration and proliferation, extracellular matrix deposition and remodeling. This normal wound healing process is disturbed in certain pathologic conditions like diabetes which alter the normal course of events so that healing is impaired, resulting in non-healing chronic wounds. In general, wound healing depends on several factors, including the patient's age and physical condition, the location of the wound and the cause of injury.

Stem cell therapy is being explored as a treatment option to address the pathophysiology of diabetic wounds. Several types of stem cells are being studied to promote healing in diabetic foot ulcers. These can be grossly categorized into allogenic and autologous stem cells, based on their source of procurement. Stem cells mobilize and home to ischemic and wounded tissues where they secrete chemokines and growth factors that promote angiogenesis and extracellular matrix (ECM) remodeling, creating a local environment that is conducive to wound healing. As an easy-to-access source of cells, stem cells are often harvested from peripheral blood but since they circulate in low numbers in the peripheral blood, they have to be culture-expanded before they are used for transplantation. Keratinocyte stem/progenitor cells (KPCs) are also mobilized into the peripheral blood, however, their source is yet to be elucidated. These KPCs are characterized by the presence of p63 marker, which distinguishes the keratinocyte stem cells from the transient amplifying cells. A recent study has shown that $p63^{+}$ KPCs in the peripheral blood could be isolated and differentiated into mature keratinocytes when cultured on a fibrin-based matrix under specific growth conditions (Nair and Krishnan, 2013). So if these KPCs are present in the peripheral blood of diabetic subjects then they may be culture expanded and used for autologous transplantation for treating chronic wounds.

This study hypothesizes that $p63^+$ keratinocyte progenitor cells are present in the peripheral blood of diabetic patients, which could be isolated and culture-expanded for potential application to use as autologous cells in treating chronic wounds. The main objectives include: (i) To detect and compare KPC occurrence in peripheral blood mononuclear cell (PBMNC) fraction of diabetic and healthy subjects, (ii) To isolate and culture the KPCs and differentiate them to keratinocytes using fibrin-based keratinocyte-specific niche, (iii) To track KPC phenotype at the transcriptional and translational level, (iv) To compare the differentiation potential of KPCs from diabetic subjects with those isolated from non-diabetic controls.

Chapter I of the dissertation includes an introduction to the research problem and recent review of literature, which includes topics like the anatomy of skin, stem cells of skin, events in wound healing, diabetes and its associated wound healing complications. Stem cells used in treating chronic wounds, differentiation of peripheral blood mononuclear cells to epithelial cells are also reviewed with appropriate citations. Finally, the chapter describes the gaps identified in the field of use of stem cells in treating chronic wounds, the study hypothesis and specific objectives of the study.

Chapter II includes materials used and methodology adopted for carrying out the study, which includes methods for harvesting and characterization of the stem cells from blood. Isolation of PBMNCs from blood by density gradient centrifugation and characterization of the keratinocyte stem cells by PCR analysis of the marker gene and staining for the marker antigen are described. Culture expansion and differentiation of the KPCs to keratinocytes on a fibrin-based matrix are explained. Details of the cell culture including the morphology analysis on 4^{th} day, 8^{th} day and 12^{th} day by phase contrast microscopy, isolation and quantification of RNA, real time-PCR analysis of differentiation markers are elaborated.

Results and discussion of the study are presented in Chapter III. Data obtained on day 1 of PBMNC isolation confirmed the presence of p63 expressing KPCs in diabetic blood. The frequency of KPC occurrence is comparable to that of non-diabetic individuals. This was analyzed by flow cytometry of PBMNC stained with antibody

against p63. PBMNCs were cultured on fibrin matrix in keratinocyte specific growth medium. The cells showed morphological changes and the cell population became homogenous after day 4. On day 12, the culture was terminated and cells were analyzed for differentiation marker expression. Presence of the differentiation marker CK14 was analyzed by PCR and the amplified products were run on agarose gel, which showed bands for CK14. The differentiation of KPCs to keratinocytes was further confirmed by immunostaining for filaggrin, which is a terminal differentiation marker.

Chapter IV summarizes the study and conclusions were drawn. It has been concluded that p63 expressing keratinocyte progenitors are present in diabetic blood, which can be differentiated to keratinocytes *in vitro* on a suitable fibrin-matrix. More characterization of KPC properties may be required to assess whether the KPCs in diabetics proliferate in culture at the same rate as non-diabetic subjects, so that they may be used for treating chronic wounds.

Chapter I

Introduction

1.1 Background

According to World Health Organization (WHO) facts sheet 2013, 347 million people worldwide have diabetes. In 2012, 4.8 million people died due to diabetes. More than 80% of diabetes deaths occur in low- and middle- income countries. WHO predicts that diabetes will be the 7th leading cause of death in year 2030. India being the diabetic capital of the world has more than 35 million people affected by diabetes currently with future estimation of around 80 million people in 2030 (Wild *et al*., 2004). One of the most significant complications of diabetes is foot ulcers, which often lead to amputations. Foot ulcer in diabetic patient is due to a combination of peripheral vascular disease, peripheral neuropathy, infection and poor foot care. Risk factors such as lower extremity ischemia, neuropathy, elevated HbA1c and retinopathy are indicators for lower extremity amputations (Calhoun *et al*., 2002). Epidemiological data shows that between 40-70% of all limb amputations occur in people with diabetes. Amputations not only affect the physical functional status but also affect their psycho-social status and increase the financial burden by means of hospital stay and treatment and loss of employment. Therefore, an appropriate treatment approach for limb complications has to be implemented.

Standard treatment methods include debridement, infection elimination by antibiotic therapy, use of moisture dressings, and offloading high pressure from the wound bed. In cases where the patients do not respond to standard care, skin replacement therapies by skin transplantation, tissue engineered human skin equivalents and somatic cell transplantation are considered, to prevent amputation. However, cell based therapy yield limited results as they do not address the underlying pathology of wound healing i.e., chronic inflammation and impaired angiogenesis. Somatic cells do not differentiate into other cell types of the epidermis or dermis and they result in wound healing by repair and not regeneration. Stem cell has been found promising in regenerative therapy as they

are multipotent and has good proliferation/regeneration capability. Even though direct transplantation of stem cells is effective for restoration of injured areas, numerous cells and supporting structures will be needed for repairing those tissues that are extensively damaged. As an alternative to direct cell therapy, tissue engineering approach has been introduced, to replace severely damaged structures. Skin substitutes that are available commercially are either acellular or consist of allogenous cells seeded on them. Allogenous cells cause immune reactions that limit their application as skin substitutes. Therefore, there is an urgent need to develop novel therapeutic strategies that could prevent amputation and increase ulcer-healing rate.

Stem and progenitor cell therapy offer the potential for accelerated wound repair. Stem cells such as adult stem and progenitor cells, embryonic stem cells and induced pluripotent stem cells have the ability to self-renew and differentiate into different cell types. In fact, there is a growing body of evidence that the use of stem cells in wound healing promote skin regeneration and scar less wound healing. The primitive stem cells are the embryonic stem cells that are capable of differentiating into three germ layers namely endoderm, mesoderm and ectoderm. With subsequent divisions, they undergo restricted divisions and become multipotent, capable of forming limited cell types. Similarly, adult stem cells are able to self-renew during the lifetime of an organism. These cells continuously produce new cells as part of homeostatic control to replace worn out cells and are seen in metabolically quiescent state in specialized tissues of the body, including the brain, bone marrow, liver, skin, and gastrointestinal tract. But, they are difficult to isolate. Human embryonic stem cells (hESC) have unlimited self-renewal and ideally should be able to give rise to any cell lineage of the body but they could also form teratoma, a tumor tissue of any 3 germ layers. Induced pluripotent stem cells (iPSC) that have been developed by nuclear programming, also give rise to cells of the 3 germ layers, but the tumorigenic potential of these cells could make them form malignant tumors in the host. Adult stem cells like the hematopoietic stem cell (HSC) can be easily harvested from the bone marrow and umbilical cord. They can also be collected from peripheral blood after their mobilization from the bone marrow. These HSC could differentiate into

cells of non-hematopoietic lineages. Mesenchymal stem cells (MSC) are adult stem cells that differentiate to mesodermal cell lineages and are conveniently isolated from bone marrow and used in regenerative medicine applications but, unless there is a proper niche, these cells may differentiate to undesired lineages. Due to the limitations of the hESCs, iPSCs and MSCs which include difficulty in harvesting and the risk of teratoma formation, a source of stem cells that can be easily isolated without causing any morbidity may be preferred. Several multipotent progenitor cells circulate in peripheral blood and harvesting of these progenitors from circulation is relatively easy and causes less morbidity to the patient. Several studies have proven the contribution of circulating progenitors for regeneration of many tissues including the epithelial layer. A study conducted by Medina *et al* (2007), showed that circulating bone marrow derived $CD34^{+}$ cells might have the capacity to trans-differentiate into epithelial-like cells. It has been reported that p63, a transcription factor involved in epithelial development and epidermal differentiation, is present on lineage committed keratinocyte progenitor cells (KPC) and they circulate in low numbers in the peripheral blood. In a particular study, KPCs that express p63 were isolated from peripheral blood and differentiated *in vitro* to mature keratinocyte that expressed epidermal differentiation marker keratin and terminal marker filaggrin (Nair and Krishnan, 2013). KPC requires an appropriate substrate for attachment and multiplication both *in vitro* and *in vivo.* In physiology, expansion of skin stem cells take place on basement membrane that consists of collagen, fibronectin, laminin and growth factors. The fibrin clot that is formed immediately after the injury, acts as a scaffold on which stem cells grow and promote wound healing in almost all regions in the body and distinctively in skin repair. A naturally formed fibrin clot contains adhesive proteins like fibronectin and the growth factors released from platelets and fibroblasts. Studies have shown that exogenous fibrin-based matrix is suitable for differentiation of neural, endothelial and keratinocyte progenitor cell (Nair and Krishnan, 2013). Therefore, the fibrin matrix that was designed for the differentiation of KPC by Nair and Krishnan could be used to aid the differentiation of KPCs from diabetic subjects.

From the background information on therapies developed for treating diabetic non-healing wounds, it is clear that cell-based therapies offer a novel treatment strategy to augment diabetic wound healing, increase ulcer healing rate and prevent amputation. The field of tissue engineering has also developed commercially available skin substitutes for diabetic wound repair. Some of these products are incorporated with cells delivered in bioengineered scaffolds. However, most of them showed only moderate clinical benefit in small clinical trials. Stem and progenitor cell therapy offer great potential for accelerated wound repair. The use of biomaterials helps to control the delivery of cells to the wound. Current delivery options include injection of cells, delivery along with extra-cellular matrix (ECM) or artificial scaffolds and transplantation of tissue engineered construct (Sorrell and Caplan, 2010). The importance of fibrin in wound healing response suggests that it can be exploited to produce matrices with signals incorporated in it. The advantage of fibrin is that it can function as a matrix for presentation of cell-adhesion molecules such as gelatin and fibronectin and growth factors. Therefore, a carefully designed fibrin matrix would support cell growth and enhance wound repair. Such a cell-specific niche was found to support the selective attachment, growth and differentiation of circulating keratinocyte progenitor cells (KPC) to keratinocytes. So, circulating progenitors may be used as autologous source for treating diabetic ulcers. The goal of developing novel wound healing treatments is to reduce the time to complete wound closure and restore the barrier function of the skin. In order to explore the possibility of collecting KPCs from diabetic patients, at the first instance it is required to know if progenitors are available in diabetic subjects. In this study, the major objective is to compare the incidence of circulating KPCs in blood samples collected from diabetic subjects and non-diabetic subjects. It is also important to prove that these circulating progenitors are able to differentiate, if an appropriate niche is provided. Once the keratinocyte progenitor cell differentiation is proved successful, the KPCs may be used for autologous therapy in diabetic subjects to enhance early wound healing.

So to design this study, the literature was reviewed to understand the skin biology, requirements of wound healing and current developments in the field of stem cell biology and their applications in regenerative medicine.

1.2 Review of literature

1.2.1 Skin

The integument or skin is the largest organ of the body, making up 16% of body weight. One of the most important functions is that it forms a physical barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against micro-organisms, ultraviolet radiation, toxic agents and mechanical insults. There are three structural layers to the skin: the epidermis, the dermis and subcutis. Skin is a dynamic organ in a constant state of change, as cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface.

1.2.1.1 Anatomy of skin

Human skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue. (McGrath *et al*., 2008). The superficial epidermis is a stratified epithelium composed of keratinocytes that are formed by division of cells in the basal layer. These cells give rise to several layers as they move outwards and gradually differentiate. The underlying dermis rests on a supporting matrix formed by collagen that forms the major constituent of the dermis. The cellular constituents of the dermis include fibroblasts, mast cells, monocyte and macrophages. The normal epidermis is a terminally differentiated, stratified squamous epithelium. Keratinocytes, which synthesize the protein keratin, is the major cell constituent which moves from the epidermal basement membrane towards the skin surface, forming several well-defined layers during its transit, such as stratum basale or stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum. Protein bridges called desmosomes connect the keratinocytes, which are in a constant state of transition from the deeper layers to the superficial layers. The four separate layers of the epidermis are formed by the differing stages of keratin maturation.

a) Stratum Basale:

The innermost layer of the epidermis, which lies adjacent to the dermis, comprises of dividing and non-dividing keratinocytes, which are attached to the basement membrane by hemidesmosomes. As keratinocytes divide and differentiate, they move from this layer to the surface. The basal cells are small and cuboidal (10–14 nm) and have large, dark-staining nuclei, dense cytoplasm containing many ribosomes and dense tonofilament bundles. The basal layer is composed of cells that are progressing along the differentiation pathway, including interfollicular stem cells, young TA cells, and mature TA cells.

b) Stratum spinosum:

Immediately above the basal cell layer is the spinous/prickle-cell layer. Desmosomes, the intercellular bridges that connect cells appear as prickles at the microscopic level.

c) Stratum granulosum:

The stratum spinosum is succeeded by the *stratum granulosum* or granular layer because of the presence of intracellular granules of keratohyalin. As the cells move up, they flatten, lose their nuclei and cytoplasm appears granular. The cytoplasm of cells of the upper, spinous layer and granular cell layer also contains smaller lamellated granules, which are known as lamellar granules and membrane-coating granules. These are numerous within the uppermost cells of the spinous layer and migrate towards the periphery of the cells as they enter the granular cell layer. They release their lipid components into the intercellular space, which play important roles in barrier function and intercellular cohesion within the stratum corneum.

d) Stratum corneum:

The outermost layer of epidermis is the stratum corneum (corneocytes) which lacks nuclei and cytoplasmic organelles. The cells become flattened and the keratin filaments align into disulphide cross-linked fibers, under the influence of filaggrin (filament aggregating protein), the protein component of the keratohyalin granule, responsible for keratin filament aggregation. The movement of epidermal cells to this layer usually takes about 28days and is known as epidermal transit time. The corneocyte has an insoluble

cornified envelope within the plasma membrane that is formed by cross-linking of the soluble protein precursor, involucrin.

Dermis:-

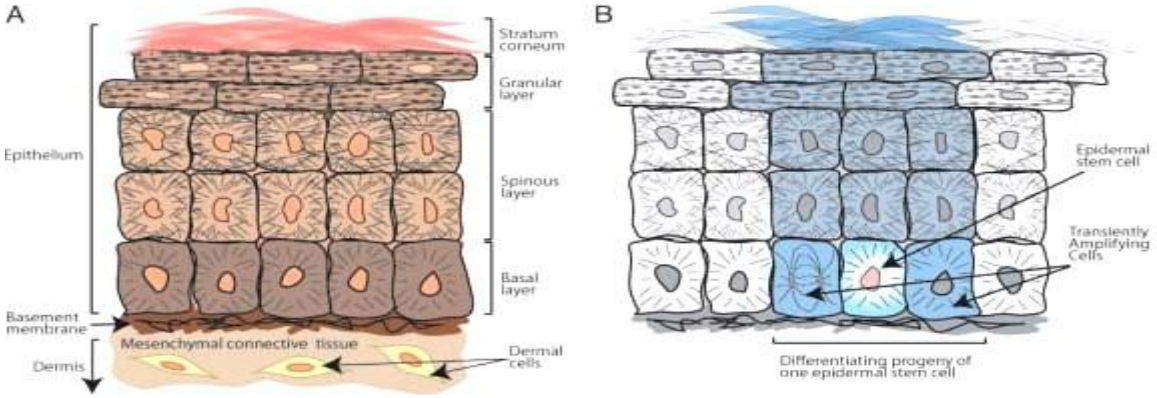
Dermis is found below the epidermis and is composed of a tough supportive cell matrix. The dermis is made up of fibroblasts, which produce collagen, elastin, structural proteoglycans, and mast cells and macrophages. Dermis receives rich blood supply. The basement membrane consists of fibronectins, heparan sulphate proteoglycan, type IV collagen and laminin 5.

In adult life, cell division maintains differentiated tissues and replaces lost cells. In skin tissues, permanently renewing populations are produced by continuous cell turnover from a small population of stem cells into differentiated cells having short lifespan. The epithelial histology is shown in **Fig. 1.2.1.**

1.2.1.2 Stem cells of the skin

The epidermis is a stratified squamous epithelium maintained by cell division within the basal layer. Differentiating cells are displaced outwards through the stratum spinosum to the stratum corneum. Stem cells in the epidermis have large proliferative capacity but divide infrequently. At least three different stem cell populations exist in the skin: inter-follicular stem cells, bulge stem cells, and skin-derived precursors (SKPs). Of these, the inter-follicular stem cells and the bulge stem cells regenerate the epidermis and hair follicle, respectively. Although either of these stem cell types can contribute to the epidermal or hair follicle lineage in response to injury, they only contribute to their own lineage under homeostatic conditions. When inter-follicular stem cells divide, they give rise to daughter cells, termed transit amplifying (TA) cells. After a few rounds of cell division, TA cells permanently withdraw from the cell cycle, and move supra-basally to initiate terminal differentiation. A marker that is expressed in the transient amplifying population is the transcription factor p63. The transcription factor p63 is required for key events in epidermal development and differentiation, including epidermal lineage commitment, keratinocyte adhesion, basement membrane formation, epidermal differentiation, and barrier formation. p63 is required for the induction of epidermal

lineage markers keratin 5 and keratin 14. Stem cells in the bulge region have the capacity to migrate and differentiate into cell lineages like the outer sheath, hair shaft and inter-follicular epidermis. Other markers of the epidermal stem cells include the nuclear-export protein 14-3-3σ (stratiffin), and the cytoskeletal keratins, K19 and K15.



[Courtesy: Laura Alonso and Elaine Fuchs. Stem cells of the skin epithelium, PNAS, 2003.]

Fig. 1.2.1. Skin epithelial histology. A Diagrammatic representation of skin epithelial histology. **B** Diagram of the epidermal proliferative unit.

1.2.2 P63 in keratinocyte differentiation

The transcription factor p63 belongs to the p53 family, with p53 having a well-established role as a tumour suppressor gene (Yang *et al*., 2002). Functional p63 is essential for commitment of the ectoderm to epidermal lineages (Mills *et al*., 1999) and for the maintenance of epidermal stem cell population necessary for epithelial morphogenesis and renewal (Pellegrini *et al*., 2001). p63 is transcribed from two different promoters (TA and ΔN) and generate alternative splice variants (α, β and γ). The TA isoforms (TAp63α, β and γ) contain an N-terminal transactivating domain that is absent in the ΔN isoforms (ΔNp63α, β and γ). p63 is expressed mainly in the basal layer of the epidermis, predominantly as the ΔNp63α isoform, however, other isoforms are expressed at lower levels (Nylander *et al*., 2002). Even though p63 expression is localized to the basal layer of complex epithelia, it is required to maintain the proliferative potential of epidermal stem cells. ΔNp63α expression is high in basal keratinocytes but its expression is reduced to approximately 25% in supra-basal

keratinocytes as the protein is degraded in supra-basal keratinocytes (Barton *et al*., 2010). ΔNp63α induces genes required for terminal differentiation and barrier formation. In addition to regulating epidermal development and differentiation, p63 is also important for cell–cell adhesion within the epidermis by inducing integrins that mediate the adhesion of keratinocytes to the basement membrane. TAp63 isoforms are the first p63 isoforms expressed during embryonic development and that they are expressed prior to the commitment to stratification. ΔNp63 isoforms, on the other hand, are first detected after the surface ectoderm has committed to stratification, but prior to terminal differentiation (Koster and Roop, 2004). When p63 is down regulated, the keratinocytes commit to terminal differentiation.

While TAp63 isoforms are responsible for initial steps toward stratification during development, their activity must be suppressed by ΔNp63 isoforms for proper differentiation to occur. In adult tissues, p63 is expressed in stratified epithelia, whereas its expression is absent from single-layered epithelia (Truong *et al*., 2006). Other genes that contribute to the formation of cutaneous barrier include involucrin, loricrin, filaggrin, keratins 1 and 10, and transglutaminase 1.

1.2.3 Cytokeratins as keratinocyte marker

Keratins (Ks) are the largest subgroup of intermediate filament (IF) proteins that provide mechanical support to tissue architecture and are critical for the maintenance of cell viability and preferentially expressed in epithelial tissues (Coulombe and Omary, 2002). The cytokeratin (CK) family is a highly complex multi-gene family of polypeptides whose molecular weight ranges from 40 to 68 kDa. The classification and numbering (CK1-CK20) is based on the catalogue of Moll *et al*. based on biochemical properties, such as molecular weight and isoelectric point (Moll *et al.*, 1982). Twelve CKs (CK9-CK20) belong to the acidic type A (class I) and eight (CK1-CK8) to the neutral-basic type B (class II) subfamily. Epithelial tissues express different pairs of keratins depending upon the cell type. In stratified epithelia, keratins exhibit a complex expression pattern tightly regulated by the differentiation program of the tissue. The keratins in the basal proliferating layer of these epithelia are K5/K14 (Moll *et al*., 1982).

As these cells move upward and differentiate, K5/K14 levels are gradually reduced and expression of a new pair of keratins is induced, depending upon the tissue type (Fuchs and Green, 1980). Differentiating cells express K1/K10 in skin; K4/13 in internal stratified epithelia, such as esophagus; and K3/K12 in corneal cells. All stratified squamous epithelia express K5/K14 while K8/K18 are seen in all simple epithelia (Omary *et al*., 2009). Keratin 14 (K14) is a prototypic marker of dividing basal keratinocytes and helps in the maintenance of epidermal cell shape; it also provides resistance to mechanical stress. The K5/K14 pair is expressed in the basal layer of the epidermis, which contains epidermal stem cells and transient amplifying (TA) cells, while the K1/K10 pair is synthesized only in postmitotic keratinocytes (Byrne *et al*., 1994). Pan-cytokeratin (PanCK) antibody is a broad-spectrum CK antibody, which can stain all epithelia as well as other cell expressing CKs. CKs belong to the most fundamental markers of epithelial differentiation and their composition reflects both cell type and differentiation status. In most CK-containing cells, the filaments are arranged in a mesh of loose bundles. However, in stratified squamous epithelia, the CK filaments are arranged in dense bundles, tonofibrils, attached to the desmosomes. The basal and myoepithelial cells generally express the stratified-epithelial CK5, CK14 and CK17, whereas the luminal/secretory epithelial cells mainly express simple-epithelial CKs like CK8, CK18, CK19 and CK7 (Byrne *et al*., 1994). The basal layer of the stratified squamous epithelia expresses CK5 and CK14. Staining for these markers helps to distinguish between various kinds of tumors of the epithelium and track the differentiation of epithelial cells.

The normal process of keratinocyte differentiation is to maintain an important barrier between the organism and its environment. This normal self-renewal of keratinocytes is interrupted when an injury occurs which is followed by the wound healing process. Wound healing is a result of a highly coordinated series of events, initiated by formation of a fibrin clot followed by recruitment of inflammatory cells, formation of granulation tissue with anigiogenesis, fibroblast proliferation, and migration of keratinocytes, contraction of the dermis, and scar remodeling. During the process of

tissue repair, keratinocytes are required to activate, migrate, and differentiate for a proper wound healing process.

1.2.4 Wound healing

Wounds in the skin are very common discomfort and can range from acute to chronic wounds. Wounds cause leakage of blood from damaged blood vessels. Clot formation occurs immediately after a cut, which then serves as a temporary shield protecting the damaged wound tissues, and provides a temporary matrix over which cells can migrate and initiate the repair process. Inflammatory cells and then fibroblasts and capillaries invade the clot to form a granulation tissue that draws the wound margins together (Hunt *et al*., 1999). The clot consists of platelets embedded in a mesh of cross-linked fibrin fibers that are derived from the cleavage of fibrinogen by thrombin. The clot also serves as a reservoir of cytokines and growth factors that are released as activated platelets begins to degranulate. This early cocktail of growth factors initiates the wound closure process by providing chemotactic signal that recruits inflammatory cells from the circulation to the wound site (Nwomeh *et al*., 1998). This in turn initiates the reepithelialization of the tissues, connective tissue contraction, and stimulates the characteristic wound angiogenic response.

Different phases of wound healing include:

i. Inflammatory phase

Neutrophils and monocytes are attracted to wound sites by a huge variety of chemotactic signals during this phase (Hunt *et al*., 1999). The signals include growth factors released by degranulating platelets, peptides cleaved from bacterial proteins and the by-products of proteolysis of fibrin and other matrix components. Both neutrophils and monocytes are recruited from the circulating blood to the wound site. Neutrophils that arrive at the wound site within minutes of injury ward off contaminating bacteria and release pro-inflammatory cytokines that serve as a signal to activate local fibroblasts and keratinocytes (Nwomeh *et al*., 1998). After the neutrophil infiltration ceases they are phagocytosed by macrophages. Macrophages continue to accumulate at the wound site by recruitment of monocytes and if macrophage infiltration is prevented, then healing is

severely impaired. Macrophages phagocytose any remaining pathogenic organisms and other cell and matrix debris. Once activated, macrophages also release several growth factors and cytokines at the wound site amplifying the earlier wound signals released by degranulating platelets and neutrophils. (Martin P, 1997).

ii. Reepithelialization

In normal skin, the basal keratinocyte layer is attached to a specialized matrix, the basal lamina. The keratinocytes' primary anchoring contacts are hemidesmosomes, which is bound to laminin in the basal lamina by integrins. The keratinocytes express new integrins as the hemidesmosome starts to dissolve and relocalize collagen receptors, in order to crawl over the temporary wound matrix and underlying wound dermis. After the onset of migration, epidermal cells just back from the wound margin proliferate and provide a pool of extra cells to replace those lost during the injury (Hunt *et al*., 1999). Migrating keratinocytes express high amount of tissue-type plasminogen activator and urokinase-type plasminogen activator. They activate plasmin, which dissolves the fibrin clot thereby helping keratinocytes cut through the clot. Members of the matrix metalloproteinase (MMP) family, which cleave matrix proteins like collagen type IV, collagen type VII, that helps in keratinocyte crawling, are also up-regulated by keratinocytes (Falanga, 2005). Once the wound surface is covered by a monolayer of keratinocytes, epidermal migration ceases and a new stratified epidermis with underlying basal lamina is reestablished from the margins of the wound. Epidermal growth factor (EGF), transforming growth factor–α (TGF-α), and heparin binding epidermal growth factor (HB-EGF), which acts as mitogens driving wound closure, are the key regulators of keratinocyte proliferation at the wound edge (Mast and Schultz, 1996). After the reepithelialization, the underlying contractile connective tissue shrinks in size to bring the wound margins toward one another. The resident fibroblasts near the wound margin begin to proliferate and after 3 to 4 days, they migrate towards the wound clot and lay down collagen-rich matrix. Growth factors present at a wound site like the PDGF and TGF-β act as chemotactic factors for wound fibroblasts proliferation. Other growth factors such as the FGF and VEGF released at the wound site by damaged endothelial

cells and macrophages promote angiogenesis, which enhances wound healing (Gillitzer and Goebeler, 2001).

Cutaneous wounds are the result of disrupted skin integrity. The healing process depends on local wound factors, systemic mediators, the underlying disease, and the type of injury. These factors determine whether normal healing or abnormal healing process, also called chronic wound healing occurs. Chronic wounds are those wounds that do not follow the well-defined process of physiologic healing. One of the most common pathologic factors that contribute to delayed wound healing is diabetes.

1.2.5 Diabetes mellitus

Diabetes affects approximately 374 million people worldwide and by 2030 these numbers are projected to double. Diabetes mellitus is a group of metabolic diseases in which a person has high blood glucose either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced by the pancreas. The high blood sugar causes polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Kolluru *et al*., 2012). Type I diabetes results from the failure to produce insulin due to autoimmune destruction of cells, and may require the injection of insulin to control symptoms. In type I diabetes, the pancreas cannot synthesize enough insulin to maintain euglycemia (McCance *et al*., 1997). This type of diabetes is more common among children and young adults and insulin injections are used for treatment, thus type I diabetes is also referred to as insulin dependent diabetes mellitus (IDDM) or Juvenile Diabetes. In type II diabetes there is normal production of insulin hormone but the body cells become resistant to insulin and cells fail to use insulin properly. Cells and tissues are not responsive to insulin, so glucose remains elevated in the bloodstream. Type II diabetes is manifested by middle-to-late-aged adults and is commonly called noninsulin dependent diabetes mellitus (NIDDM) or Adult Onset Diabetes (MacKinnon, 1999). Due to improper production or resistance to insulin, diabetic patients cannot metabolize carbohydrates, proteins or fats. Diabetic patients' cells do not make use of glucose from the blood due to abnormal insulin metabolism, resulting in hyperglycemia. Over time, this high glucose levels in the bloodstream leads

to complications such as vision loss, cardiovascular diseases, kidney disorder, and nerve damage.

1.2.6 Diabetic wound healing

The diabetic foot ulcer (DFU) is one of the leading causes of hospital admissions for people with diabetes and is a major morbidity associated with diabetes, often leading to pain, suffering, and poor quality of life for patients. Diabetic foot ulcers (DFUs) are estimated to occur in 15% of all patients with diabetes and lead to diabetes-related lower limb amputations (Reiber *et al*., 1999). Prolonged diabetes leads to defective angiogenesis. Diabetic individuals are unable to combat infection due to defective immune responses. Nerve damage occurs in diabetic patients and as a result, there is lack of peripheral sensory function. Diabetic individuals have diffuse atherosclerotic vessel disease that diminishes blood perfusion leading to a disruption in wound oxygenation and healing (Carmeliet, 2000). Diabetic Foot Ulcer (DFU) may also become a portal for systemic infection leading to bacteremia, septicemia, and may ultimately result in limb amputation. Importantly, delayed healing of these diabetic wounds is also characterized by impaired angiogenesis, decreased cell and growth factor response, diminished peripheral blood flow and decreased endothelial cell proliferation. Keratinocyte cell migration is inhibited due to reduced number of EGF receptors and activation of glucocorticoid pathway. At the non-healing edge of DFUs, keratinocytes and fibroblasts show absence of migration and hyperproliferation. However, cells in the adjacent non-ulcerated area display normal phenotype yet impaired physiology and respond to cellular therapy and administration of growth factors. Excessive ROS production in diabetic patients is one of the primary factors contributing to wound healing deficiencies (Efron *et al*., 2000). Nitric Oxide that is produced during the healing process regulates and augments wound repair. During wound-induced hypoxia, VEGF released by macrophages, fibroblasts, and epithelial cells induces the phosphorylation and activation of eNOS in the bone marrow, resulting in an increase in NO levels, which triggers the mobilization of bone marrow EPCs to the circulation. The chemokine SDF-1α promotes the homing of these EPCs to the site of injury, where they participate in

neovasculogenesis (Gallagher *et al*., 2007). SDF-1α expression is decreased in epithelial cells in the diabetic wound, which prevents EPC homing to wounds and therefore limits wound healing. In contrast to normal wound healing, the inflammatory response in poorly healing diabetic wounds is prolonged, which generates an equally intensified protease response, in particular MMPs and neutrophil elastase (Falanga, 2005). These inflammatory reactions may be the result of bacterial contamination and recurrent painless tissue trauma. Bacterial endotoxins, fragments of extracellular matrix, and cell debris maintain this inflammation and large number of neutrophil granulocytes accumulate near the wound. The granulocytes also secrete pro-inflammatory cytokines, particularly TNF-α and interleukin (IL)-1β. Both of these cytokines are capable of directly stimulating the synthesis of MMPs (Chen *et al*., 1999). MMPs are expressed by inflammatory cells, fibroblasts, keratinocytes, and help in the migration of keratinocytes and reconstruction of new basement membrane. Imbalance in the MMP levels contributes to pathogenesis of non-healing chronic wounds (Falanga, 2005). In chronic wounds, the high level of proteases in the wound site leads to a disrupted and uncoordinated wound healing process by degrading the matrix proteins and growth factors that are essential for healing.

1.2.7 Role of stem cells in wound healing

Patients with critical limb ischemia have a prognosis for both survival and limb salvage, despite treatment with conventional approaches like pharmacotherapy or revascularization, because most patients with this condition have generalized atherosclerosis. In 2002, Tateishi *et al*. reported the first clinical evidence that injection of bone marrow mononuclear cells (BMMNC) into patients with critical limb ischemia yielded improvements with respect to rest pain and pain-free walking distance (Tateishi *et al*., 2002). Since then, many clinical studies of autologous cell therapies, including BMMNC and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (MPBMNC), have been conducted. After 2006, MPBMNC and BMMNC were implanted into more than 100 patients with peripheral arterial disease worldwide, and both therapies proved beneficial for patients with critical lower limb

ischemia. For BMMNC implantation, long-term clinical studies have shown comparable safety and efficacy relative to conventional revascularization therapies.

1.2.7.1 Bone marrow stem cells in wound healing applications

Bone-marrow derived cells have been a promising option in regenerative therapy. Several evidences suggest that bone marrow contains stem cells that have the potential for differentiation into a variety of tissues (Badiavas and Falanga, 2003). Diabetic patients have impaired mobilization of EPCs from the bone marrow and decreased accumulation of these cells in the wounds. Therefore, bone-marrow derived cells could be an unlimited source of progenitor cells. In patients who did not respond to standard therapies like the bioengineered skin and grafting, local application of bone marrow derived autologous cells resulted in wound closure. Hyperbaric oxygen therapy and co-administration of stromal cell-derived factor-1-alpha (SDF-1α) in murine model resulted in recruitment and homing of EPCs to the wound site (Gallagher *et al*., 2007). Another novel approach is enabling the lineage commitment of stem cells to keratinocyte lineage by exposing the stem cells to various cytokines, growth factors, and extracellular matrix components.

1.2.8 Mobilization of stem cells onto peripheral blood

Studies have shown that hematopoietic progenitor cells (HSPCs), as well as pluripotent very small embryonic-like stem cells (VSELs), are mobilized into peripheral blood (PB) in patients and experimental animals in response to tissue injury (Drukala *et al*., 2012). This phenomenon indicates that stem/progenitor cells circulate in peripheral blood to improve tissue damage. In steady-state conditions, a majority of these circulating cells are hematopoietic stem progenitor cells (HSPCs). However, other cell types are also seen in peripheral blood like the very small embryonic-like stem cells (VSELs). A chemokine SDF-1α is involved in trafficking of stem cells from the bone marrow. The number of circulating HSPCs increases in response to i) systemic or local inflammation, ii) strenuous exercise iii) tissue injury iv) pharmacological agents (Massberg *et al*., 2007). The existence of this intrinsic mechanism suggests that this phenomenon could be

exploited therapeutically by, increasing the number of circulating stem cells by administration of pharmacological mobilizing agents.

1.2.9 Differentiation of embryonic stem cells to keratinocytes

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of early mouse embryos. They can be propagated stably in an undifferentiated state *in vitro* in the presence of the cytokine leukemia inhibitory factor (LIF) (Aberdam D, 2004). When the LIF is withdrawn, ES cells aggregate to form embryoid bodies (EB) from which the cells differentiate spontaneously into all cell types, representative of the three germ layers. ES cells can be manipulated and enriched for a particular lineage like the keratinocytes, neurons, chodrocytes (Hegert *et al*., 2002) or adipocytes (Dani *et al*., 1997), by providing the necessary chemicals, cytokines and medium conditions. The *in vitro* differentiation of ES cells may provide an excellent model for studying the cellular and molecular mechanisms of early epidermal development and eventually the generation of donor cells for transplantation therapies. A study reported that EB differentiation recapitulates embryonic epidermal differentiation with the sequential appearance of epidermal markers starting with K8/K18 pair, the first keratins to be expressed in simple epithelia, followed by k14 keratinocyte cells (Bagutti *et al*., 1996). Appearance of involucrin demonstrated that EB could produce terminally differentiated keratinocytes. Although EB cells could produce keratinocytes *in vitro*, the nature of the stimuli responsible for the commitment was unknown. While EB formed keratinocytes after 21 days of culture ES cells formed k14 positive keratinocytes on the 8^{th} day when cultured on matrix derived from human normal fibroblasts (HNF) and NIH-3T3 cells. Therefore, ES cell culture would be a better option for *in vitro* differentiation of keratinocytes (Coraux *et al*., 2003).

1.2.10 Differentiation of bone-marrow derived cells to keratinocytes

Studies have indicated that stem/progenitor cells derived from BM could home to injured tissues and participate in regeneration (Wu *et al*., 2007). Culture expanded bone marrow-derived mesenchymal stem cells (BM-MSCs) promote healing of diabetic wounds (Rafii *et al*., 2003). BM-MSCs have the ability to traffic, survive in blood, and

migrate to injured tissue. Systemic infusion of BM-MSCs resulted in engraftment of cells in various organs like skin, intestine, lungs etc. A subpopulation of CD45–/CD34– cells exist in the peripheral blood of mice and humans, which resemble BMMSCs and FACS analysis showed that they constitute about 2-8% of total nucleated cells in peripheral blood. Circulating MSCs were also found in the peripheral blood of patients receiving granulocyte colony-stimulating factor (G-CSF) treatment (Richman *et al*., 1976). It has been reported that hypoxia causes mobilization of BM-MSCs to the peripheral blood thus increasing the levels of circulating MSCs (Chen *et al*., 2001). After wounding of the skin, putative BMMSCs in the wound also increased. However, BM-MSCs are known to quickly age during culture expansion, and may undergo autonomous differentiation toward osteoblasts. With improved culture conditions, MSCs can retain their primitive properties better. BM-MSCs significantly enhanced wound healing in diabetic mice and normal mice. BMMSC-treated wounds exhibited significantly faster wound closure, with increased re-epithelialization, cellularity, and angiogenesis (Borue *et al*., 2004). Culture expanded autologous BM-MSCs were topically applied to the wounds in human subjects in a matrix of fibrin. The tissue biopsy analysis showed signs of the survival of implanted BM-MSCs and generation of new elastic fibers in the wounds. The reports suggest that BMMSCs could be exploited for regenerative therapies.

1.2.11 Trans-differentiation of BM-derived MSCs

Bone marrow contains a subset of hematopoietic stem cells as well as mesenchymal stem cells (MSC) accounting for roughly 0.01–0.001% of the bone marrow derived cell population (Sasaki *et al*., 2008). MSCs have the capacity to proliferate and differentiate into tissue-specific cells in response to cues provided by different organs. Induction of mechanical stress in skin results in the release of various cytokines, especially chemokines which recruit blood-circulating MSCs to the injured area. The chemokines help in stem cell mobilization into the peripheral blood as well as to the sites of wound healing. The MSCs that accumulate at the wound site transdifferentiate into skin cell types and aid in wound repair. These MSCs when injected contribute to wound repair via accumulation in wound site and a specific chemokine

(SLC/CCL21)/chemokine receptor (CCR7) interaction recruits circulating MSCs into the wound site, resulting in the stimulation of wound repair via the promotion of angiogenesis (Sasaki *et al*., 2008). Wounding stimulated engraftment of bone marrow cells to the skin and induced these cells to differentiate into non-hematopoietic skin structures. A specific chemokine, CTACK, is one of the major regulators involved in the migration of keratinocyte precursor cells from bone marrow into skin. However, there is no reported evidence for direct tissue-specific regeneration from the bone marrow derived cells.

Another study reported that systemically infused BM-MSCs showed long-term engraftment in multiple organs like skin, kidney, liver, intestine, in baboons after lethal body irradiation. This indicates that BM-MSCs have the ability to traffic in blood and migrate to injured tissues (Filshie R., 2002). Circulating MSCs were also found in the peripheral blood of patients receiving granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment (Richman *et al*., 1976). Studies also show that cells derived from the BM not only contribute to fibroblast-shaped cells in the dermis but also to cells in the epidermis such as a keratinocyte phenotype. BM-MSCs and keratinocyte crosstalk appears to be necessary for structural organization of the epidermis. The biological activities and therapeutic potential of BM-MSCs are impaired in elderly individuals and patients with chronic diseases such as diabetes. In human trials, autologous cells were culture expanded and topically applied to the wounds in fibrin matrix. Tissue biopsy analysis showed survival of these implanted BM-MSCs and generation of new fibers in wounds. Development of single specific marker to recognize MSCs would allow a better understanding of their role in physiology.

1.2.12 Trans-differentiation of Bone-Marrow derived cells to epithelial-like cells

Transdifferentiation is a process in which the original commitment of a cell is changed to give rise to unexpected peripheral mature cells, especially after inductive signals such as tissue damage or even transplantation (Medina *et al*., 2009). This phenomenon, also called cell plasticity, constitutes an important characteristic of bone

marrow-derived cells to repopulate somatic tissues. Cell plasticity is also exhibited by human circulating $CD14^+$ monocytes, which show the ability to trans-differentiate into various cell types. Medina and workers postulated that monocytes can migrate into injured areas where they are exposed to either fibrogenic or antifibrogenic stimuli from the local repertoire of cytokines and growth factors and also, releasable factors from these PBMNC/monocytes-derived trans-differentiated cells may act over fibroblasts to induce either collagen or MMP over expression.

Bone marrow-derived stem cells have the potential to trans-differentiate into unexpected peripheral cells (Medina *et al*., 2007). The circulating bone marrow-derived stem cells might have the capacity to trans-differentiate into epithelial-like cells and release matrix metalloproteinase-1-modulating factors such as 14-3-3σ for dermal fibroblasts. The protein 14-3-3σ is considered a specific marker for epithelial cells. Medina and workers described a releasable form of keratinocyte-derived 14-3-3σ that induces matrix metalloproteinase (MMP)-1 expression in dermal fibroblasts. Because of its diverse expression pattern and antifibrotic effects on dermal fibroblasts, 14-3-3σ serves as a promising protein to elucidate further the functional commitment of PBMNCs into epithelial-like cells. Isolated PBMNCs cultured in defined keratinocyte medium induced trans-differentiation into epithelial cells. A releasable form of 14-3-3σ from PBMNC-derived epithelial-like cells regulates MMP-1 expression in dermal fibroblasts. Findings from this study provide new insights into the potential role of PBMNCs and circulating precursor cells to generate epithelial-like cells. The understanding of this particular cell trans-differentiation would facilitate the treatment of chronic non-healing wound.

1.2.13 Identification of p63 as marker for keratinocyte stem cells

The renewal of stratified epithelia in mammals is governed by keratinocyte stem cells. They generate rapidly dividing transient amplifying cells, which undergo several divisions and become terminally differentiated. P63 distinguishes stem cells from their TA progeny in squamous epithelia (Pellegrini *et al*., 2001). The number of keratinocyte stem cells declines with age. Once the stem cells mature to TA cells they show reduced

expression of p63 but high PCNA. In human hair follicles, p63 is expressed by keratinocytes forming the outer root sheath as well as by bulb keratinocytes surrounding the surface of follicular papilla. In human epidermis, and stratified epidermal cultures, p63 is expressed in the nuclei of cells that are either proliferating or possess the ability to multiply (Parsa *et al*., 1999). p63 is expressed by keratinocytes that possess the ability to proliferate and not simply by keratinocytes that are duplicating their DNA. Several clonogenic data shows that p63 is restricted to keratinocyte stem cells. It has also been observed that p63 cells do not necessarily express PCNA *in vivo* but all cultured p63 clones possess PCNA. This indicates that epithelial stem cells are slow cycling *in vivo*, but actively proliferating in culture. Epidermal SCs, TA cells and early-differentiated cells have the ability to form fully differentiated epidermis both *in vivo* and *in vitro* (Li *et al*., 2004). Once keratinocytes process profilaggrin into filaggrin they undergo specific changes that mark late (terminal) differentiation (Presland *et al*., 2001).This keratinocyte stem cell marker will be of crucial importance for proper clinical application of epithelial cultures in cell therapy.

1.2.14 Fibrin as a scaffold for growth and differentiation of cells

Certain somatic stem cells mobilize to remote damaged tissue sites and get differentiated into cell lineages and participate in organ repair and regeneration (Asahara 1997; Orlic *et al*., 2001). Recent research has demonstrated that bone marrow derived endothelial progenitor cells (EPC) circulate in peripheral blood and play an important role in neoangiogenesis of ischemic tissue and endothelial cell (EC) repair following damage (Asahara *et al*., 1999). A local injury or damage may release homing signals that guide the progenitor cells to the target tissue. (Werner *et al*. 2003). Fibrin network forms at the injury site and provides a support matrix for the cells to proliferate and aid in healing and neovascularization. Fibrin can function as a matrix for presentation of other cell-adhesion molecules such as gelatin and fibronectin (FN) and growth factors, while fibrin may be readily resorbed to promote remodeling through normal fibrinolysis. Fibrin has been shown to be an effective substrate for EC adhesion and proliferation on biomaterial surfaces (Pankajakshan and Krishnan, 2009). In physiological conditions,

multiple-growth factors are actively involved at the site of a wound to promote angiogenesis and tissue regeneration. Presence of VEGF in the coated matrix supports EC migration and survival. The scaffold must be similar to the natural ECM and it should assist cell attachment and deposition of ECM. Addition of suitable growth factors to the scaffold may further support growth and differentiation of cells and the composed fibrin matrix could be used for the growth and differentiation of other type of cells like the neurons and keratinocytes.

1.2.15 Presence of $p63^+$ keratinocyte progenitors in blood

A study conducted by Nair and Krishnan showed that $p63^+$ keratinocyte progenitor cells circulate in peripheral blood (Nair and Krishnan, 2013). These cells were isolated from the PBMNC fraction and cultured on a specific fibrin-matrix. The fibrin matrix was specifically designed to support the proliferation and differentiation of keratinocyte progenitor cells. The fibrin matrix was composed of hyaluronic acid, bovine hypothalamus extract, VEGF and EGF. The differentiation medium containing DMEM/F12 supplemented with FBS, ascorbic acid, insulin, hydrocortisone and growth factors were found to be ideal for the growth of KPCs. In the study it was observed that by 12^{th} day the cell population became homogenous and majority of the cells were $p63^+$ and $CK14^+$ indicating that the cells differentiated to keratinocyte lineage. The study proved that KPCs are capable of differentiating on a matrix and therefore the cells could be used for transplantation on a fibrin sheet.

1.3 Gaps identified

Although literature supports the fact that circulating PBMNCs can be differentiated into keratinocytes, if they are present in similar frequency in diabetic subjects is not understood. In addition their potential to proliferate and differentiate is similar to the KPC present in healthy subjects is also not clear. Occurrence of MSC is known to be reduced as one gets older. Mostly, the diabetic wounds are prevalent in older people; therefore, age-related changes in the availability of KPC also need to be evaluated.

1.4 Research hypothesis

This study hypothesizes that $p63^+$ keratinocyte progenitor cells may be present in the peripheral blood of diabetic patients, which could be isolated and culture-expanded for potential application to use as autologous cells in treating chronic wounds. In circulation, the monocyte fraction is heterogenous with several multi potent adult progenitor cells (MAPC) and therefore, transplantation of crude preparation may cause differentiation into undesirable lineages. Therefore, homogenous cell that differentiate into a specific lineage is an essential requirement for achieving successful regeneration after cell transplantation.

To obtain pure KPC, cell-specific niche consisting of fibrin, other adhesive proteins, growth factors and hyaluronic acid may be adopted. If the presence of keratinocyte progenitor cells in circulation is proven in diabetic subjects then the autologous cells may be used in bio artificial skin substitute construction for regeneration of chronic wounds.

1.5 Research objectives

i. To detect and compare KPC occurrence in peripheral blood mononuclear cell (PBMNC) fraction of diabetic and non-diabetic healthy subjects.
ii. To isolate and culture the KPCs and differentiate them to keratinocytes using fibrin-based keratinocyte-specific niche.
iii. To track KPC phenotype at the transcriptional and translational level.
iv. To compare the differentiation potential of KPCs from diabetic subjects with those isolated from non-diabetic controls.

In order to achieve the objectives, a study plan was prepared and the proposal was submitted to the Technical Advisory Committee (clinical) and Institutional Ethics Committee (IEC) of SCTIMST. The committee approved collection of 10 ml blood from diabetic patients who took treatment at SCTIMST for cardiovascular problems. Ten patients in the age group of 50y to 60y were selected and blood samples were collected from SCTIMST clinics, for the study. The details of materials and methods used for the study are described in chapter II of this dissertation.

Chapter II

Materials and methods

2.1 PBMNC isolation

Materials:

Centrifuge (Biofuge stratus, Hereaus, UK), Histopaque 1077 (Sigma-Aldrich, USA), HBSS with Antibiotic-Antimycotic cocktail (Gibco®, USA), Nunc tubes and culture dishes (Thermo Scientific, MA).

Method:

Blood was collected from selected patients who underwent cardiovascular surgery at SCTIMST hospital after obtaining their informed consent. PBMNC isolation was done according to an established protocol (Asahara *et al*, 1997 and Sreerekha and Krishnan, 2006). The blood was transferred into a fresh Nunc tube and centrifuged at 1200*g* for 10minutes at 24ºC. After the centrifugation, white PBMNC band was collected into a fresh tube, diluted with HBSS and carefully layered over Histopaque 1077 taken in a fresh centrifuge tube. The tube was centrifuged at 400*g* for 30minutes at 24ºC. After the centrifugation, the white band of PBMNC settled above the Histopaque was carefully aspirated out. The collected PBMNC white fraction was diluted with HBSS to more than double the volume of PBMNC obtained, and the cells were centrifuged down at 250*g* for 10minutes at 24ºC. The cell pellet obtained was re suspended using KPC medium and added into a bare tissue culture polystyrene (TCPS) and incubated at 37ºC. After the incubation, medium was gently aspirated out, fresh medium was added and plates were incubated for 48 hours at 37ºC.

2.2 Culture of PBMNC on KPC-specific matrix

Materials:

Nunc cell-culture plates, 5µg ml^{-1} of insulin (Sigma-Aldrich, USA), 0.3mM ascorbic acid (Sigma-Aldrich, USA), 0.4µg ml^{-1} hydrocortisone (Sigma-Aldrich, USA), 50µg ml^{-1} VEGF (Sigma-Aldrich, USA), 10ng ml^{-1} EGF (Sigma-Aldrich, USA), 5% FBS (Gibco®, USA), antibiotic-antimycotic cocktail (Gibco®, USA), DMEM/F12 medium (Gibco®, USA).

2.2.1. Preparation of KPC-specific matrix-coated culture plate

Cryoprecipitate prepared in-house from screened and pooled human plasma for clinical purpose was used. About 100 mg of fibrinogen powder was reconstituted. When 2 ml sterile deionized water was added to the lyophilized powder in the vial, a stock solution of 50 mg ml^{-1} was obtained. Thrombin obtained has been purified in-house using cryo-poor plasma by diethylaminoethyl (DEAE) cellulose ion exchange chromatography for clinical use. The lyophilized powder with ~250 IU $vial^{-1}$was reconstituted with sterile water to get the working solution of thrombin.

Bovine hypothalamus extract (BHE) was prepared in-house as per the protocol described by Maciag *et al* (1979) which contain BMP4, FGF and VEGF. Hyaluronic acid was prepared from human umbilical cord according to the published protocol (Kaye *et al*, 1951, Anilkumar *et al*, 2011) was obtained from thrombosis research unit as lyophilized powder. The lyophilized powder was reconstituted in water to get a stock solution of 1mg ml^{-1}.

Culture dishes were coated with fibrin based matrix according to established procedure of Chennazhy & Krishnan (2005) with modified fibrinogen composition. The modified fibrinogen composite (FC) consisted of fibrinogen (2 mg ml^{-1}), fibronectin (200 µg ml^{-1}), gelatine (0.2%), BHE (25 µg ml^{-1}), EGF (20 ng ml^{-1}) and HA (0.1 mg ml^{-1}). To obtain composite coated surface, culture dishes were incubated with thrombin (5 IU ml^{-1}) for 30 min at 37^{o} C. After incubation, excess thrombin solution was removed and

fibrinogen composite was spread on the surface (8µl cm^{-2}). The fibrin was allowed to polymerize at 37^{o} C for 30 min and were then frozen at -70^{o} C for at least 2h, lyophilized (Edwards, Modulyo 4K, UK) and stored at 4°C till use.

2.2.2 Preparation of KPC-specific medium

The complete keratinocyte culture medium contained 10% new born calf serum, 5 µgml^{-1} insulin, 0.3 mM ml^{-1} ascorbic acid, 25 µg ml^{-1} BHE, 0.5 µg ml^{-1} hydrocortisone and 20 ng ml^{-1}EGF and 10 µl ml^{-1} antibiotic-antimycotic.

2.2.3 Culture of PBMNC

After the 48 hour incubation of PBMNC (Section 2.1) at 37ºC, the cells were flushed out with fresh KPC medium. Cells were counted using hematology analyzer and the collected cells were seeded onto the coated wells at a seeding density corresponding to ~10^{6} cells per cm^{2} in each well. The plates were incubated at 37ºC and the medium was changed every 24 hours for three consecutive days and afterwards on alternative days. Cell attachment, morphological changes and survival was monitored on the 4th day, 8th day and 12th day under a phase contrast microscope. Culture was terminated after a defined length (12 days).

2.3 Analysis of marker expression

2.3.1 Isolation of RNA from cultured cells

Materials:

DEPC-treated eppendorfs and tips, TRIzol (Invitrogen, USA), Chloroform (LiChrosolv®, Merck, Germany), 100% Isopropanol (EMSURE®, Merck, Germany), 70% Ethanol (Changshu Yangyuan Chemicals, China), Centrifuge, DEPC (Sigma-Aldrich) water.

Method:

PBMNC was isolated from blood using Histopaque 1077 gradient centrifugation. After the final wash with HBSS, the supernatant was discarded and the pellet obtained was resuspended with 1ml TRIzol and transferred to DEPC treated 1.5ml eppendorf. After a short incubation at room temperature for 5minutes, 200µl chloroform/1ml TRIzol originally used, was added to the eppendorf. It was vortexed vigorously for a few seconds until a light pink colour was developed and was incubated at room temperature for 2-3 minutes. The content was centrifuged at 12,000*g* for 15 minutes at 4ºC. After the centrifugation, the upper aqueous phase was transferred to a fresh tube, 500 µl of isopropanol was added to it and the contents were gently mixed and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 12,000*g* for 10 minutes at 4ºC. The pellet obtained was washed with 70% ethanol at 7500*g* for 5 minutes at 4ºC. After the centrifugation, the supernatant was discarded and the pellet was left to dry for about 10-15 minutes. The dry pellet was dissolved in 50 µl of DEPC water.

2.3.2 RNA quantification using Qubit

Materials:

Qubit® RNA Assay Kit (Molecular Probes®, Invitrogen, USA), Qubit® Fluorometer, PCR tubes (Axygen®, USA).

Method:

Working solution was prepared by diluting the Qubit RNA reagent in Qubit RNA buffer at 1:200 dilution. RNA standards were prepared by diluting 10µl of each standard in 190µl of Qubit working solution. The working solution was used to dilute the sample and the standards. 1µl of the sample was diluted with 199µl of the working solution. The tubes were incubated at room temperature for 2 minutes. The Qubit 2.0 Fluorometer was calibrated using both the RNA standards. The samples were then inserted into the fluorometer and the readings were recorded.

2.3.3 Estimation of the purity of RNA

Materials:

HP 8543 UV-Vis Spectrophotometer (Hewlett-Packard, USA), Quartz Cuvettes, DEPC water.

Method:

RNA samples were diluted at 1: 1000 dilution in DEPC water. The spectrophotometer was set on 260/280 ratio mode. Blank was set with DEPC water. The RNA sample was inserted into the spectrophotometer and absorbance was measured.

2.3.4 cDNA synthesis

Materials:

Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen, USA), 10mM dNTP mix (Invitrogen, USA), Recombinant RNase OUT (Invitrogen, USA), DEPC water, 5x First strand buffer (Invitrogen, USA), 0.1M DTT (Invitrogen, USA), SuperScript III Reverse Transcriptase (Invitrogen, USA), Master cycler from Eppendorf (Hamburg, Germany).

Method:

To prepare cDNA, 1µg of RNA sample was mixed with 1µl each of oligo (dT) primer and 10mM dNTP mix and the volume was made upto 18 µl with DEPC water. After adding all the components, the mixture was pre-heated for 5 minutes at 65ºC in the PCR machine. The reaction mix was chilled on ice and cDNA synthesis was undertaken by the addition of 5x first strand buffer, 0.1M DTT, RNase OUT and Superscript III reverse transcriptase. The reaction volume was adjusted to 25µl with DEPC water and incubated at 50ºC for 60 minutes, then reaction was inactivated at 70ºC for 10 minutes.

2.3.5 Real Time-PCR

Materials:

cDNA samples, TAp63 primers (IDT, USA), ΔNp63 primers (IDT, USA), GAPDH primers (IDT, USA), real time-PCR MasterMix for SYBR® assay No ROX (Eurogentec, Belgium), DEPC water, white PCR tubes (BIO-RAD, Hercules, CA), PCR flat cap strips (BIO-RAD, Hercules, CA), Real Time-PCR Chromo4 system (MJ Research, now part of Bio-Rad, Hercules, USA).

Method:

The PCR reaction mixture contained 4µl of template cDNA, 12.5µl of real time-PCR mastermix, 1µl each of forward and reverse primers and the total volume was made upto 25µl with DEPC water. GAPDH was used as internal control. The primer sequence used for each molecule is given in table 1. Samples were assayed under the following conditions 95ºC for 15minutes followed by 40 cycles of 95ºC for 15seconds, 54ºC for 30seconds and 72ºC for 30seconds. The PCR products were then subjected to melting curve analysis from 60ºC to 90ºC to make sure that a single PCR product is evolved.

Table 2.3.5. Primers used for PCR reactions.

Gene	Forward primer	Reverse primer	Amplicon size
GAPDH	5′-gcttgtcatcaatggaaatccc-3′	5′-tccacacccatgacgaacatg-3′	210bp
TAp63	5′-aagatggtgcgacaaacaag-3′	5′-agagagcatcgaaggtggag-3′	234bp
ΔNp63	5′-ggaaaacaatgcccagactc-3′	5′-gtggaatacgtccaggtggc-3′	294bp
CK5	5′-cttgtggagtgggtggctat-3′	5′-ccacttggtgtccagaacct-3′	439bp
CK14	5′-gaccattgaggacctgagga-3′	5′-attgatgtcggcttccacac-3′	157bp

2.3.6 Qualitative analysis of PCR products

Materials:

Agarose (Sigma-Aldrich, USA), Ethidium Bromide (Biogene, USA), UltraPure™ TAE Buffer (Invitrogen, USA), bromophenol blue, 100bp DNA ladder (Invitrogen, USA), Electrophoresis power supply EPS 301 (Amersham Pharmacia-Biotech, UK), UV transilluminator (Spectroline, USA), Gel documentation unit (AlphaImager®, USA).

Method:

PCR products were analysed on 1% Agarose gel. Agarose (Sigma-Aldrich, USA) was weighed and melted by adding 1X TBE buffer and allowed to cool. After cooling ethidium bromide (Biogene, USA) was added and mixed well. Comb was placed in the gel casting tray and molten agarose was poured into it. After the gel was solidified, comb was removed and the gel was placed in electrophoresis tank filled with 1X TBE buffer. The samples were loaded by mixing with gel loading dye bromophenol blue. 100bp DNA ladder (Invitrogen, USA) was loaded as marker. Electrophoresis was carried out at 100V in an electrophoresis power pack. The bands were visualized under UV transilluminator. Gel was imaged in gel documentation unit.

2.3.7 Immunofluorescence

Materials:

37% Formaldehyde (SDFCL, Mumbai), Triton® X-100 (Sigma-Aldrich, USA), BSA 98% (Sigma-Aldrich, USA), PBS (pH 7.4), Leica IM-DRB Fluorescent microscope (Leica, Germany), FITC conjugated cytokeratin-14 (Chemicon International, CA), PE conjugated Cytokeratin-5 (Santa Cruz Biotechnology, Texas), Mouse monoclonal IgG Filaggrin (Santa Cruz Biotechnology, Texas), Actin Texas Red-x Phalloidin (Invitrogen, USA), Mouse monoclonal antibody to p63 (Abcam, Cambridge, USA), Goat Anti-mouse monoclonal IgG-PE (Santa Cruz Biotechnology, Texas), Goat anti-mouse monoclonal IgG-FITC (Santa Cruz Biotechnology, Texas).

Method:

After the medium was discarded from the culture plates, all the wells were washed with PBS twice and the cells were fixed using 3.7% formaldehyde for 20 minutes at room temperature. After the fixation, cells were permeabilized using 0.2% Triton X-100 for 5 minutes at room temperature. After the permeabilization, 0.5% BSA in PBS was added to all the wells for blocking non specific binding sites and the plates were incubated at room temperature for 30 minutes. Cells were washed three times with PBS after each step. Primary antibody was added at 1:25 dilution and the plates were incubated at 4ºC overnight. After the incubation, the wells were washed with PBS and the cells were incubated with conjugated secondary antibody for 1h in dark. The plates were then observed under fluorescence microscope.

2.3.8 Flow Cytometry

Materials:

37% Formaldehyde (SDFCL, Mumbai), Triton® X-100 (Sigma-Aldrich, USA), BSA 98% (Sigma-Aldrich, USA), PBS (pH 7.4), FACS ARIA (BD Biosciences, CA, USA), Mouse monoclonal antibody to p63 (Abcam, Cambridge, USA), Goat anti-mouse monoclonal IgG-FITC (Santa Cruz Biotechnology, Texas).

Method:

Cells in culture for 1 hour were harvested by flushing out and washed with PBS. The cells were fixed with 3.7% formaldehyde in PBS for 20 minutes, washed with PBS, and permeated with Triton X-100 (0.1%) in PBS for 5 minutes. The cells were treated with 1% bovine serum albumin in PBS for 30 minutes and incubated with non-conjugated p63 (1:25) for 2 hours. The cells incubated with primary p63 antibodies were washed and treated with FITC-conjugated secondary antibody (1:200) for 1 hour. After the staining procedure, the cells were washed and analyzed by using a flow cytometer. The percentage of marker-expressed cells was calculated by using BD FACS Diva software.

Chapter III

Results and discussion

The keratinocyte stem cells that are present in defined locations direct the renewal of stratified epithelia in mammals. These stem cells generate transient amplifying cells and then get terminally differentiated into mature keratinocytes. These stem and TA cells give rise to clones that have great proliferative potential *in vivo* and are able to achieve epithelial regeneration when transplanted as grafts of autologous keratinocytes in burn victims. These evidences show that their stemness can be preserved in culture. Keratinocyte stem/progenitor cells are also present in small quantities in circulating blood and they are identified by the presence of p63 marker. This stem/progenitor cells can be isolated and cultured for its use in regenerative applications. The objective of this study was to understand whether the $p63^{+}$ keratinocyte progenitor cells were present in comparable amounts in peripheral blood of diabetic subjects and non-diabetic subjects and if they proliferate and differentiate at the same rate in culture. Therefore, the first step in this study was to isolate KPCs from peripheral blood of diabetic subjects. The percentage of $p63^{+}$ keratinocyte stem cells were analyzed by flow cytometry. After their presence was further confirmed by real time-PCR analysis of the p63 marker, these cells were cultured in specific fibrin-coated plates to track their differentiation potential. The morphological changes were analyzed in phase contrast microscope and analysis of differentiation marker was done using PCR and immunofluorescence.

3.1 Isolation of PBMNCs

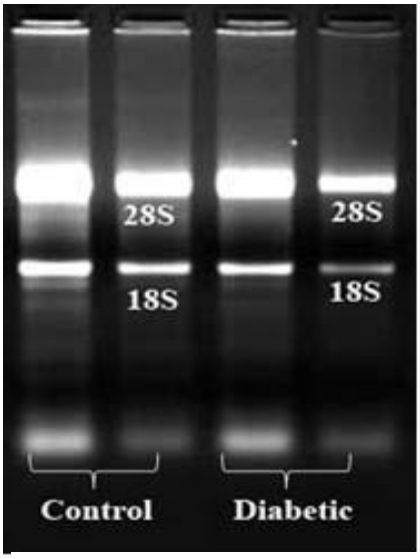
PBMNCs were isolated from selected age-matched and sex-matched diabetic patients after obtaining informed consent. Male patients in the age group of 50 to 60 were chosen and their diabetes was confirmed by verifying Random Blood Sugar (RBS) and HbA1c levels. Diabetic patients having RBS more than 126mg/dl and HbA1c above 7% were chosen for the study. Ten ml blood was collected in heparinized tubes and PBMNCs were isolated using the standardized ficoll gradient centrifugation protocol. Samples

were obtained from 10 diabetic patients and 10 age-matched and sex-matched non-diabetic individuals were selected as control. The isolation protocol resulted in concentrated PBMNC band at the interphase of platelet poor plasma and ficoll. On reconstitution, between 1.5 to 3 million leukocytes were obtained and the yield was comparable in both diabetic and non-diabetic samples. One hour incubation in bare tissue culture wells resulted in elimination of platelets and lymphocytes in suspension, which was carefully aspirated and discarded. The heavier cells which were settled also contained few RBCs. Such unattached but settled PBMNC fraction was flushed out and upon counting using hematology analyzer the yield of cells was ~1 million. There was donor to donor variation, but there was no distinction between the two subject groups. This fraction was used for (1) for analyzing p63 expression using real time-PCR, and by flow cytometry and (2) for differentiation experiment by seeding on keratinocyte specific matrix. The protocol adopted for PBMNC isolation resulted in consistent yield irrespective of the subject group.

3.2 Identification of KPC using p63 as marker

3.2.1 RNA isolation and cDNA synthesis

Total RNA obtained from PBMNC as described in section 3.1 was between 2.5-4µg. All isolations were consistent and relatively pure with A26/A280 ratio ranging from 1.6- 1.9. The integrity of the RNA obtained was checked in Agarose Gel Electrophoresis (AGE) using 1.5% agarose gel. The AGE image of the RNA obtained in shown in **Fig.3.2.1**.



Lane 1 Lane 2 Lane 3 Lane 4

Fig.3.2.1. Agarose gel image of RNA. Lane 1 and 2 contains RNA from control (non-diabetic) sample and lane 3 and 4 contains RNA from diabetic sample.

The AGE image of the RNA obtained shows that there was no degradation of RNA. Immediately cDNA was synthesized from 1µg of RNA to maintain the integrity of the molecule of interest.

3.2.2 Real Time-PCR analysis

The expression of p63 was analyzed by amplification of the cDNA by real time-PCR. The protocol used was taken from published literature. The primer used resulted in the product as per the published literature as well. TAp63 expression was obtained after 40 cycles of amplification. The amplification protocol used resulted in products with similar/comparable Ct values in both diabetic and non-diabetic samples. **Table 3.2.2.** shows Ct values obtained for 4 subjects in each group. For GAPDH, which was used as housekeeping gene, also the results were consistent and comparable.

Table 3.2.2. Ct values obtained from real time-PCR analysis.

Group	Ct target gene	Ct gapdh	ΔCt non-diabetic
Non-diabetic 1	24.2	13.3	10.9
Non-diabetic 2	23.72	13.95	9.77
Non-diabetic 3	24.11	14.2	9.91
Non-diabetic 4	26.38	13.06	13.32
Group	**Ct target gene**	**Ct gapdh**	**ΔCt diabetic**
Diabetic 1	25.47	13.35	12.12
Diabetic 2	24.7	13.48	11.22
Diabetic 3	24.4	14.2	10.2
Diabetic 4	25.43	13.94	11.49

The amplification of the genes was determined from the amplification plot and melt curve analysis confirmed specificity of the product. The gene expression was calculated by ddCt (delta delta Ct) method. In real time-PCR analysis Ct number is defined as the threshold level of fluorescence which is the observed value in most of the real time-PCR experiments. Delta Ct (dCt) for each gene (target or reference) is calculated by subtracting the Ct value of target sample from that of reference sample.

$$\mathbf{dCt = Ct_{sample\ gene} - Ct_{gapdh}}$$

ddCt is calculated by subtracting dCt value of control sample from dCt value of treatment.

$$\mathbf{ddCt = dCt_{diabetic} - dCt_{non\text{-}diabetic}}$$

The ratio of target gene expression in diabetic versus non-diabetic is calculated by the formula $\mathbf{2^{-\,ddCt}}$. From cells in culture for 1 hour, only one isoform of p63 (TAp63) was expressed while the other isoform (ΔNp63) was not expressed. This clearly shows that p63 marker is expressed in keratinocyte progenitor cells or transient amplifying cells that is found in the peripheral blood.

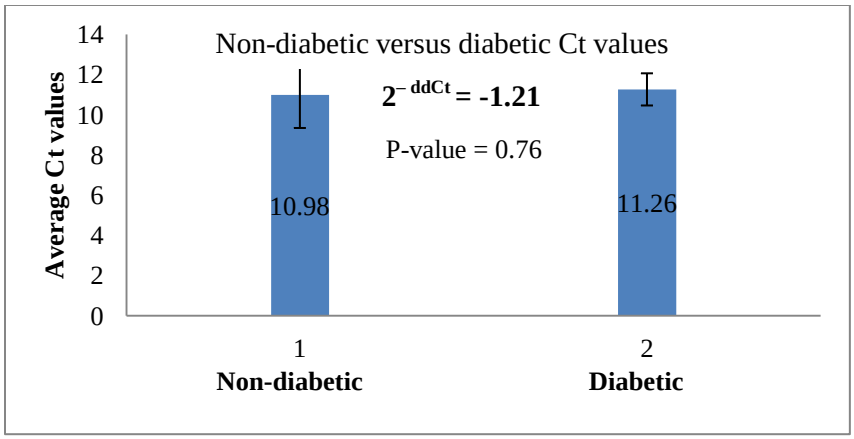


Fig. 3.2.2 Histogram representing the average Ct value obtained for real time-PCR analysis of p63 gene. The standard deviation is shown as error bars.

From the expression analysis of p63 gene, diabetic samples showed 1.2-fold decrease when compared to non-diabetic controls, which is not a significant decrease.

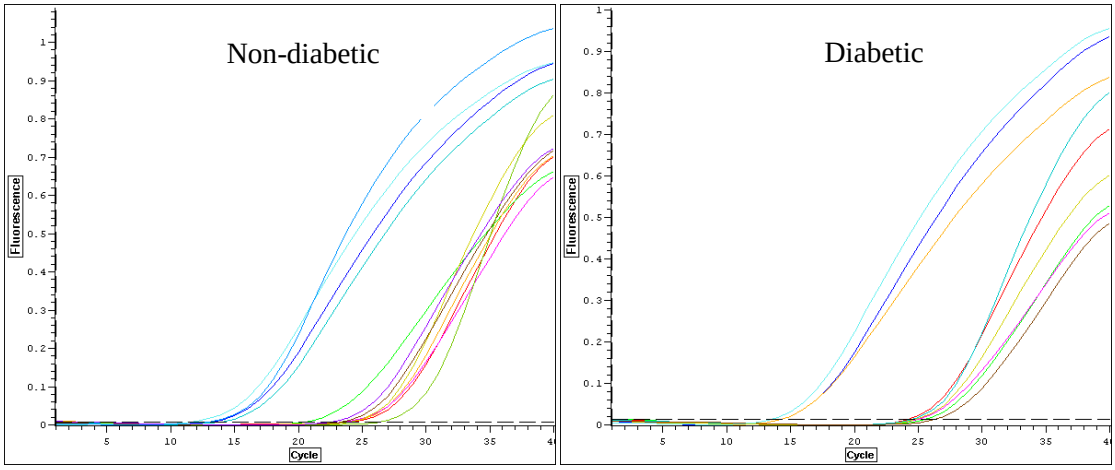


Fig.3.2.3. Amplification plot of real time PCR. Representative amplification plot for TAp63, ΔNp63 and GAPDH in non-diabetic and diabetic subjects.

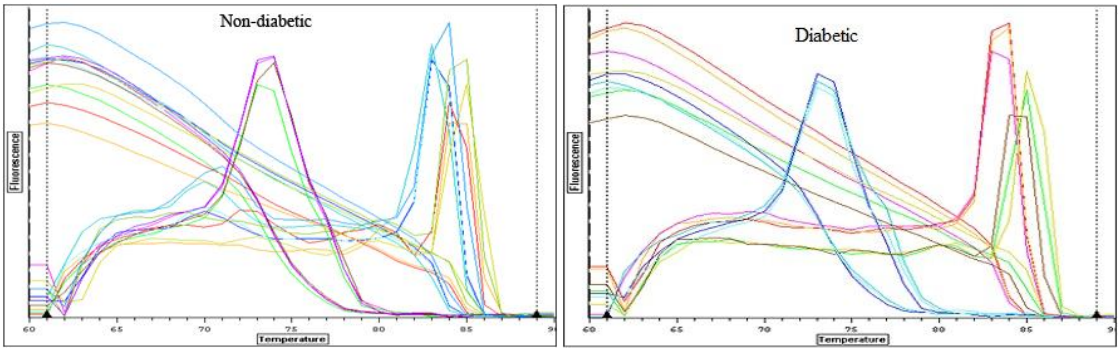


Fig.3.2.4. Melt curve of real time PCR. Representative melt curve for TAp63, ΔNp63 and GAPDH in non-diabetic and diabetic subjects.

The melt curve plots show that TAp63 and GAPDH produced a single peak indicating the specificity of the product. The peak obtained for ΔNp63 could probably be a dimer of low Tm.

The PCR amplified products were run on 1% agarose gel (**Fig.3.2.5**). TAp63 gene, after the amplification showed band corresponding to 234bp; both in samples obtained from diabetic and non-diabetic subjects.

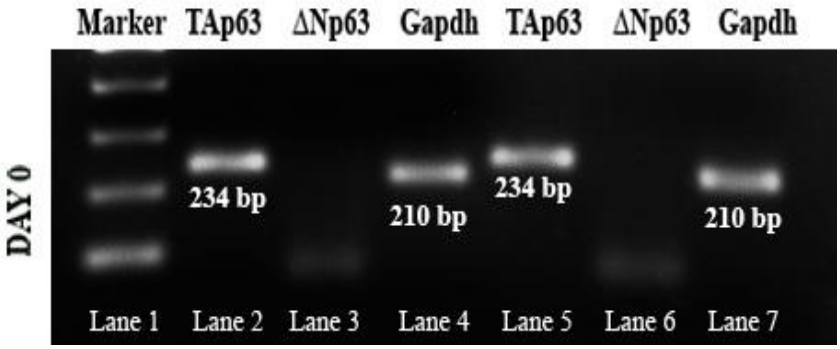


Fig.3.2.5 Agarose gel image of RT-PCR amplified products of diabetic and non-diabetic samples. Lane 1 represents 100bp marker, lane 2, 3 and 4 represent amplified products of diabetic sample and lane 5, 6 and 7 represent amplified products of non-diabetic control sample.

The observation suggests the presence of p63 expressing keratinocyte progenitor cells in PBMNC isolated from the samples.

3.3 Estimation of $p63^+$ KPC in PBMNC

Flow cytometry analysis was done to compare the percentage of circulating $p63^+$ KPCs in diabetic and non-diabetic subjects. In order to avoid false positive results by adding excess antibody during flow cytometry analysis, optimum concentration of the antibody was determined by staining PBMNCs using two different concentrations (1:25 and 1:50) of antibody.

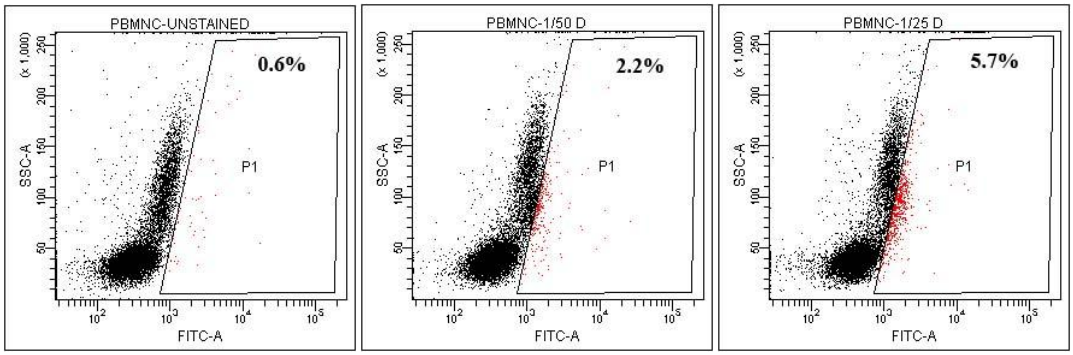


Fig.3.3.1. P63 antibody staining of PBMNC. 1st dot plot represents the unstained control, 2nd represents cells stained with 1:50 dilution and 3rd represents cells stained with 1:25 dilution of antibody. The percentage of $p63^+$ cells obtained in shown in the dot plots.

The results showed that 2.2% cells were $p63^+$ at 1:50 dilution while 5.7% of cells were $p63^+$ at 1:25 dilution of the antibody (**Fig. 3.3.1**). So 1:25 dilution was used for further analysis to ensure that sufficient quantity of antibody was added to stain all $p63^+$ cells in the PBMNC population. Secondary antibody used for both dilutions were the same and therefore, chances of false positive cells is eliminated. PBMNCs isolated from diabetic and non-diabetic subjects were analyzed for estimating the percentage of $p63^+$ cells. All cells from the FSC-versus-SSC plot were gated and the gate having <0.6% was selected for detection of the positively stained population. Among the cells obtained after 1 hour incubation in bare culture dish, few were $p63^+$ in the gated region. The $p63^+$ cells were prominent in the monocyte region of the FSC-versus-SSC plot. The percentage of $p63^+$ KPC obtained is shown in **Fig.3.3.2.**

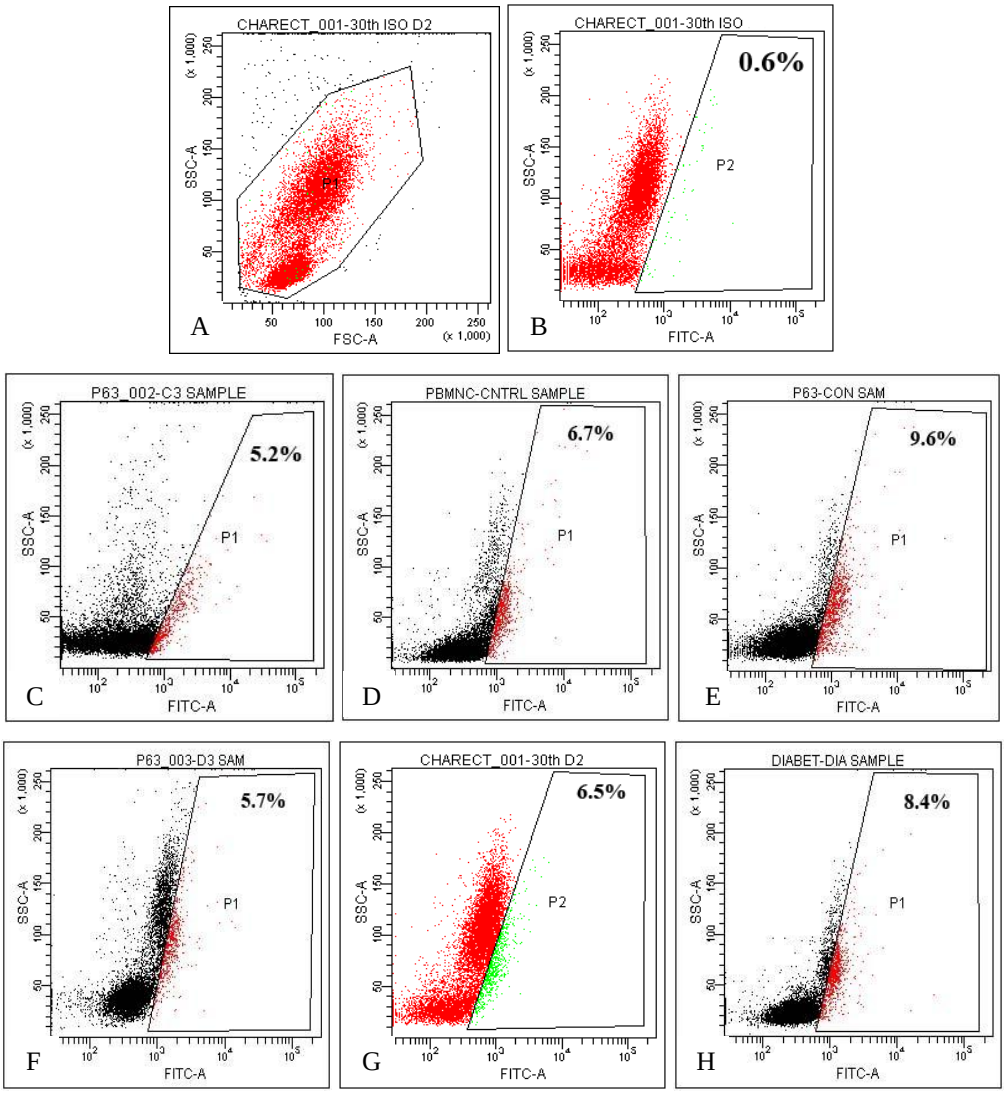


Fig.3.3.2. Percentage of p63^{+} cells in diabetic and non-diabetic subjects.

A and **B** represent the FFC-versus-SSC plot and gated population, **C**, **D** and **E** represent percentage of p63^{+} cells in non-diabetic controls and **F, G** and **H** represent percentage of p63^{+} cells in diabetic samples.

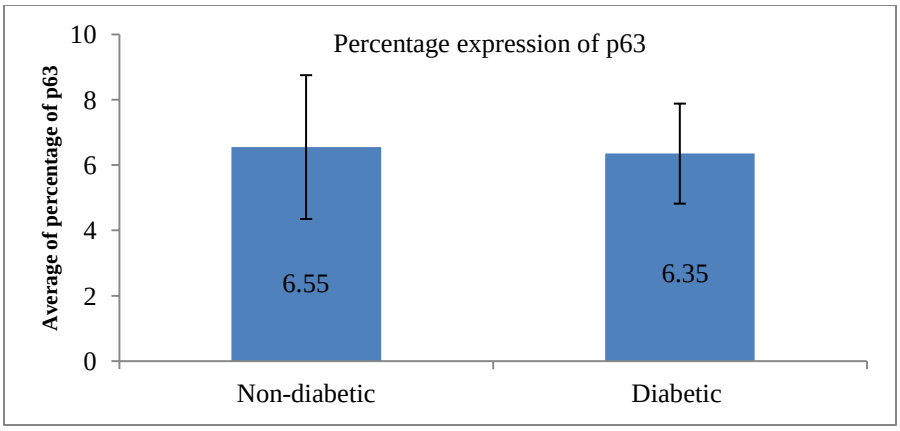


Fig. 3.3.3. Histogram representing the average percentage obtained for p63 gene expression. The standard deviation is shown as error bars.

Comparable results were obtained for the p63 expression in both diabetic and non-diabetic subjects. P-value obtained is 0.8. This shows that there is no significant difference in the percentage of p63 expression in both the groups. Thus, the Real Time-PCR and flowcytometry data together confirm the presence of $p63^+$ KPCs in peripheral blood of diabetic subjects and their expression is comparable with that of the non-diabetic control subjects.

3.4 Differentiation experiments

Objective of the second phase of the study was to identify whether the $p63^+$ KPCs from diabetic subjects could differentiate into mature keratinocytes when cultured on keratinocyte-specific fibrin matrix. Both 4-well plates with surface area 1.75 cm^2 and 96-well plate with surface area 0.3 cm^2 were prepared and used. The decision to use the particular culture area was based on the end use of the cultured cells. For isolation of RNA 4 well plates were used whereas for immunostaining 96-well plates were used, so that economic use of antibody was possible. Since it has been reported earlier that KPCs require cell-cell contact for proliferation and differentiation, the seeding density was adjusted to $1x10^6$cells/cm^2. Cultures were grown in DMEM/F12 media, kept under 5% CO_2 incubator at 37ºC. Cells were visualized under phase contrast microscope. Morphological changes observed in cultured cells are shown in **Fig.3.4.** All images

shown are cultures of PBMNC isolated from diabetic subjects. Similar results were found in the case of non-diabetic subjects as well. Previous study from our lab has also reported that after 12 days of culture on keratinocyte specific matrix, the KPCs may progress into terminal differentiation (Nair and Krishnan, 2013); therefore, the culture was terminated on day 12 for analyzing differentiation marker cytokeratins and cells in culture on day 15 was analyzed for terminal differentiation marker expression.

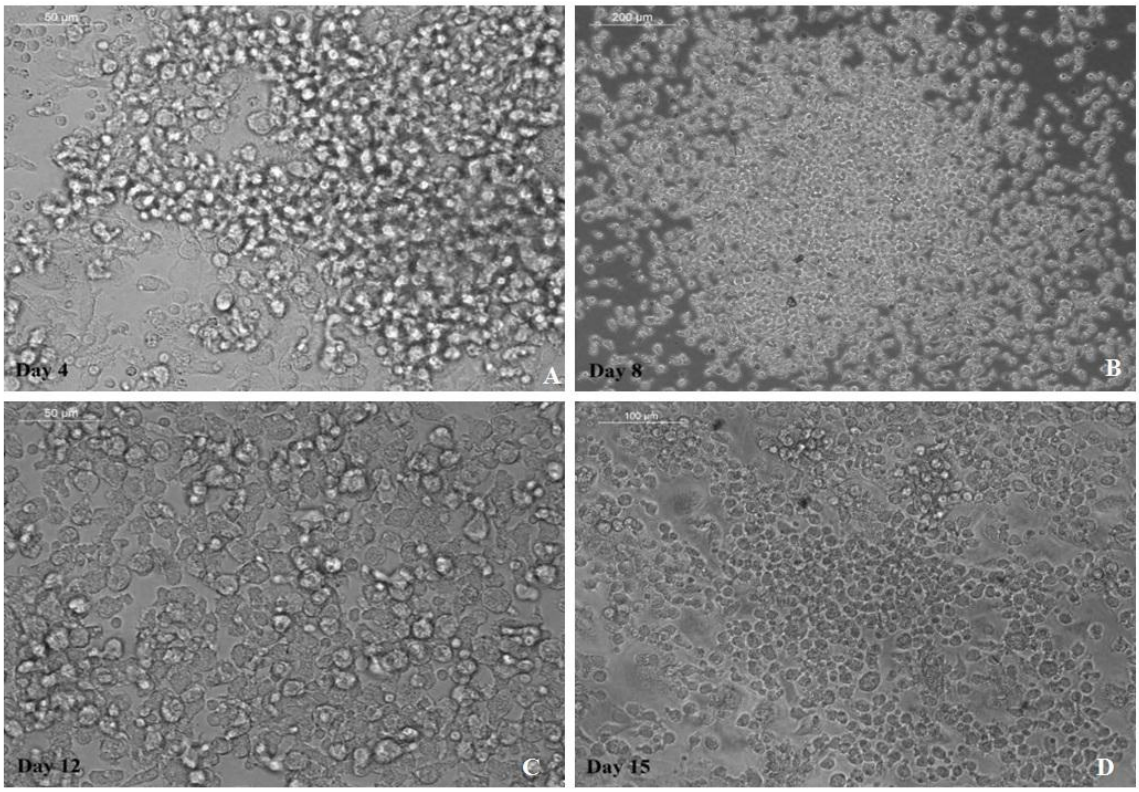


Fig.3.4. Phase contrast images of KPC culture on different days. A represents cells on day 4, **B** represents cells on day8, **C** represents cells on day12 and **D** represents cells on day15. **B** and **D** are at low magnification (scale bar 200µm and 100µm respectively); **A** and **C** are at high magnification (scale bar 50µm).

3.5 Expression of differentiation marker after culture

3.5.1 PCR analysis

Cells in culture on day 12 were flushed out using TRIzol and total RNA was isolated by TRIzol/chloroform method of RNA isolation. The RNA yield and purity in 25µl of the RNA suspension are given in **Table 3.5.1.** cDNA was synthesized using Superscript III reverse transcriptase and Oligo dT primers. PCR reaction was carried out using 4µl of cDNA for 40 cycles. The amplified products were visualized on 1% Agarose gel. TAp63, GAPDH and differentiation marker CK14 showed up specific bands on the gel, however, for CK5 and ΔNp63, the bands seen in the figure are non-specific based on the reported molecular weight. The gel images of the amplified products are shown in **Fig.3.5.1.**

Table 3.5.1. Yield and purity of the RNA from culture.

Sample	Yield (ng)	Purity (260/280)
Non-diabetic 1	81.5	1.66
Non-diabetic 2	105.3	1.61
Non-diabetic 3	885	1.58
Diabetic 1	110	1.55
Diabetic 2	62.5	1.82
Diabetic 3	72.3	1.77

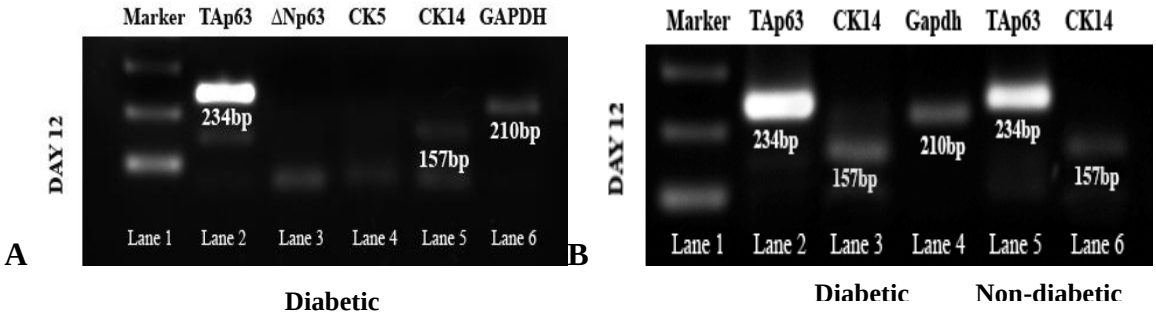


Fig.3.5.1. Agarose gel image of PCR amplified products. A Representative gel image showing PCR amplified products of KPCs in culture of diabetic sample. **B** Representative gel image showing PCR amplified products of KPCs in culture of diabetic and non-diabetic samples of genes that showed specific products, where, Lane 2 and 3 represent amplified products of diabetic and lane 5 and 6 show amplified products of non-diabetic samples. GAPDH was used as the internal control.

The results indicate that after culture the cells expressed only CK14, other than the TAp63 which was already present in KPC at the time of PBMNC isolation. The major problem faced in most of the cultures was low number of KPC, which limited the quantity of RNA obtained from cells after culture. Since the experiment was started using 10 ml of blood from patients, the experiments could not be repeated to improve the RNA yield.

3.5.2 Characterization by Immunofluorescence

To confirm keratinocyte lineage commitment and differentiation, cells in culture on day 12 were fixed and stained for panel of 5 markers; p63 as progenitor marker, CK5, CK14 and panCK as differentiating cell markers and filaggrin as terminal differentiation marker. Fluorescence images are shown in **Fig.3.5.2.** Immunofluorescence images showed that p63 is localized in the nuclear region whereas CKs are present in the cytoplasm. Filaggrin appeared as a string of granules in the cytoplasm.

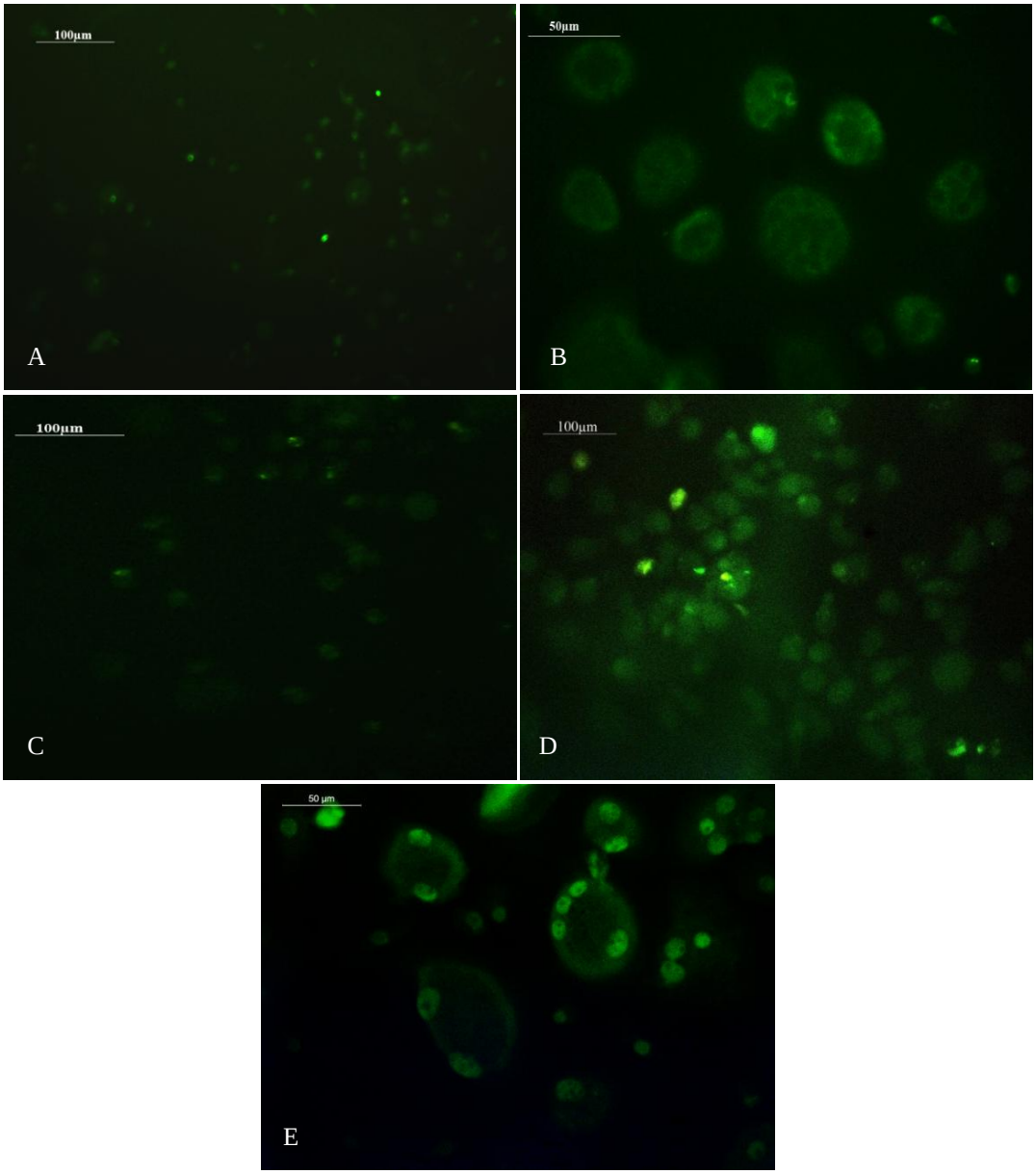


Fig.3.5.2. Immunofluorescence images of differentiation markers. A Represents cells on day 12, stained for p63 antibody, **B** Represents cells on day12, stained for CK14, **C** represents cells on day 12 stained for CK5 **D** represents cells on day 12 stained for panCK and **E** represents cells on day 15 stained for filaggrin antibody. **B** and **E** are shown at high magnification (scale bar 50µm), **A**, **C** and **D** are at low magnification (scale bar 100µm).

The immunofluorescence analysis demonstrate that cells expressed p63 as well as CK14 on day 12. The observation suggests that $p63^{+}$ cells may have survived and it appeared

that the cells that survived have differentiated into keratinocyte lineage with CK expression on day 12. KPCs from diabetic subjects adhered on the fibrin matrix and cells appeared to have proliferated at comparable rates with that of non-diabetic subjects. The differentiation experiment was carried out using PBMNCs from 5 patients. It was also observed that the survival and expression of markers depended on the initial yield of PBMNCs. When the yield and seeding density of cells were poor, the proliferation and marker expression was also poor. Seeding density of PBMNCs and cell-cell contact is crucial for KPC differentiation. Cells proliferated well when the seeding density of the PBMNCs was maintained at $1x10^6$ cells/cm^2 or more on the keratinocyte-specific matrix.

The molecules present in the fibrin matrix and the growth medium together would have influenced the growth of KPCs and keratinocyte lineage commitment. Studies have shown that insulin improves wound healing by stimulating keratinocyte migration and differentiation at the wound margins which is dependent on activation of the PI3K-Akt pathway (Liu *et al*., 2009). Epidermal growth factor has been found to induce differentiation of cells by activating MAPK signaling pathway that ultimately culminates in cell division and differentiation. VEGF facilitates tissue repair by increasing vascular permeability, allowing the efflux of inflammatory cells into the site of injury, and increasing the migration and proliferation of pre-existing endothelial cells (Galiano *et al*., 2004). A study reported that incorporation of hyaluronic acid (HA) with fibrin can create an effective wound care matrix which promotes water retention and wound healing potential (Anilkumar *et al*., 2011). Diabetes impairs numerous components of wound healing, including hemostasis and inflammation, matrix deposition, and angiogenesis. Cutaneous wounds in diabetics have altered blood flow, impaired neutrophil activity, and a dysfunctional inflammatory state associated with abnormal chemokine expression. So, inclusion of specific growth factors and adhesive proteins in the biomimetic matrix and growth medium was essential to enhance survival and differentiation of KPCs.

Stem cells have been used widely in skin tissue engineering applications and an important step in use of stem cells is that they should retain multiplication potential when transplanted. Circulating keratinocyte progenitors is an easy-to-access source of stem

cells but the complex pool of multipotent stem cells in circulation makes it difficult to isolate a pure population of keratinocyte stem cells. Therefore, a defined protocol has to be designed for enabling the expansion and differentiation of KPCs to keratinocytes. Studies have reported that bone marrow-derived endothelial progenitor cells (BM-EPCs) are released into the peripheral blood stream in response to tissue damage and ischemia. These EPCs then home to areas of tissue damage and facilitate endothelial wound healing and angiogenesis (Asahara *et al*., 1997). In diabetics, however, BM-EPCs show impaired mobilization and homing, resulting in a decreased number of circulating EPCs (cEPCs), subsequently delaying wound healing. EPCs in patients with diabetes also have decreased proliferative potential and increased rates of apoptosis. So, it is possible that diabetic subjects may show a decreased number of circulating KPCs as well, which have decreased proliferation potential. So, this study was initiated to find out if KPCs are present in comparable levels in the peripheral blood of diabetic subjects as that of non-diabetic subjects and whether it could be isolated and expanded in culture.

The presence of KPCs in diabetic circulation has been proven and their differentiation into mature keratinocytes on a specific matrix has also been demonstrated. So, the hypothesis was proven true that p63 positive keratinocytes may be present in diabetic subjects as well. It was observed that KPCs are in comparable levels in peripheral blood of diabetic and non-diabetic subjects and they proliferated and differentiated on fibrin matrix and expressed markers of terminal differentiation. Even though these cells may be promising for use in transplantation, the proteolytic hostile environment in chronic wounds may limit its application and therefore the stem cells need to be delivered on a protective vehicle such as fibrin sealants. This is a preliminary finding of presence of circulating KPCs in diabetic individuals. More detailed studies are required to prove that KPC differentiation takes place in similar manner in diabetic and non-diabetic subjects.

Chapter IV

Summary and Conclusion

4.1 Summary

Several multi potent stem cells are present in the peripheral blood of individuals that could be isolated and expanded in culture for use in regenerative applications. These multi potent stem cells are present in the mononuclear fraction of the peripheral blood. Several studies have proven the contribution of circulating progenitors for regeneration of many tissues including skin. It has been reported that p63, a transcription factor involved in epithelial development is present on lineage committed keratinocyte progenitor cells (KPC) and they circulate in low numbers in the peripheral blood. These cells when cultured on a specific fibrin matrix supplemented with growth factors and adhesive proteins differentiate into mature keratinocytes that expresses terminal differentiation markers. The goal of the study was to identify if such circulating KPCs that express p63 are present in diabetic individuals and whether these cells could be differentiated to keratinocytes *in vitro* for autologous application in chronic diabetic wounds.

Blood was collected from selected diabetic patients after obtaining IEC approval and informed consent from each patient; PBMNC was then isolated using ficoll gradient centrifugation. The presence of KPCs in PBMNC was analyzed by real time-PCR analysis of p63 gene expression and the number of KPCs in total PBMNC population was analyzed by flow cytometry, which showed that KPCs are present in comparable levels in diabetic and non-diabetic individuals. The Ct values obtained shows that p63 expression is 1.2-fold decreased in diabetic subjects than non-diabetic subjects, which is not a significant decrease. So, the KPCs were cultured on fibrin matrix to determine their differentiation potential. Culture of KPCs was done on specific fibrin-coated plates. The fibrinogen composite comprised of growth factors, hyaluronic acid and gelatin was layered on culture plates treated with 5IU ml^{-1} thrombin and this fibrin matrix was used to support survival and differentiation of KPCs. Cells were cultured in DMEM/F12

media supplemented with 5% FBS, growth factors, ascorbic acid and insulin to enable keratinocyte differentiation. Cells were observed under phase contrast microscope for morphological changes. KPCs from diabetic blood proliferated and formed colonies similar to that of non-diabetic subjects. The culture was terminated on 12^{th} and 15^{th} day for analysing differentiation marker expression.

To know whether the cultured KPCs differentiated on fibrin matrix, expression of differentiation markers were analyzed. Total RNA was isolated from cultured cells by TRIzol/chloroform method and PCR was carried out to know the marker expression. Differentiation was tracked by analyzing the presence of differentiation marker CK14. The PCR amplified products were run on agarose gel and distinct bands were seen for p63, CK14 and GAPDH. To confirm keratinocyte lineage commitment and differentiation, the cells were stained with 4 different markers including p63, CK14, panCK and filaggrin. On the 12^{th} day the cells were fixed and stained for p63, CK14 and panCK and cells in culture on day 15 was stained for terminal differentiation marker filaggrin. The antigens CK14, panCK and filaggrin stained the cytoplasmic region while p63 expression was restricted to the nucleus. Thus, the differentiation of circulating KPCs to keratinocytes was achieved when cells were cultured on fibrin matrix.

4.2 Conclusions

i. KPCs that express p63 are present in peripheral blood of diabetic individuals and their levels are comparable with those present in non-diabetic individuals.

ii. The presence of KPCs in diabetic individuals is 1.2 lower than non-diabetic individuals but there is no significant change between the groups.

iii. KPCs isolated from diabetic individuals could differentiate into keratinocytes *in vitro*.

iv. Fibrin matrix carefully designed for KPC differentiation enabled differentiation of KPCs from diabetic subjects to CK14 expressing keratinocytes.

v. This study suggests that in the long run, circulating KPCs from diabetic individuals may be suitable for autologous transplantation on a fibrin sheet for healing of chronic wounds.

4.3 Future prospects

i. To compare the expression levels of differentiation markers of cultured KPCs in diabetic and non-diabetic subjects.

ii. Study the detailed mechanism by which KPCs differentiate into keratinocytes.

iii. To study the differentiation and proliferation potential of KPCs collected from patients with ischemic limb.

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