EFFECT OF VEGF EXPRESSION IN BIOENGINEERED HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS FOR PROMOTING ANGIOGENESIS IN CHRONIC WOUND HEALING

A THESIS PRESENTED BY
AMITA AJIT

TO

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF
DOCTOR OF PHILOSOPHY

2019

CERTIFICATE

I, AMITA AJIT, hereby certify that I had personally carried out the work depicted in the thesis entitled, “Effect of VEGF Expression in Bioengineered Human Adipose Derived Mesenchymal Stem Cells for Promoting Angiogenesis in Chronic Wound Healing”, except where external help is sought, it is duly acknowledged. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Signature :

Name : AMITA AJIT

Reg. No : 2016/PhD/02

Date: 23.09.2019

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram – 695011, INDIA
(An Institute of National Importance under Govt.of India)

Dr. Lissy K Krishnan
Scientist G, Division of Thrombosis Research
Department of Applied Biology,
BMT Wing, SCTIMST, Trivandrum.

This is to certify that **Ms. Amita Ajit**, in the division of thrombosis research, of this Institute has fulfilled the requirements prescribed for the Ph. D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled, "Effect of VEGF Expression in Bioengineered Human Adipose Derived Mesenchymal Stem Cells for Promoting Angiogenesis in Chronic Wound Healing" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

*Clearance was obtained from the Institutional Ethics Committee for collection of adipose tissue. Approval from Institutional Committee for Stem Cell Research and Therapy was obtained for use of stem cells in this study. Clearance was obtained from Institutional Biosafety Committee for bioengineering stem cells. Institutional Animal Ethics Committee's clearance was also obtained for conduct of *in vivo* experiments using rabbit model.

Dr. Lissy K Krishnan
(Research Guide)

Date:

The thesis entitled

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Submitted by

AMITA AJIT

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR MEDICAL SCIENCES AND TECHNOLOGY,
TRIVANDRUM - 695011

Is evaluated and approved by

Dr. Lissy K Krishnan ……..…….

(Research Guide) (Thesis Examiner)

Dedicated to Dr .R. Prakashkumar, Director KSCSTE- JNTBGRI, who taught me that it is never too late to change careers to pursue your true passion and that context, is everything.

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With great hope on adult stem cells revolutionizing medicine, thank you all.

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ABBREVATIONS

AB/AM	:	Antibiotic/Antimycotic
BM	:	Basal Medium
BMSCs	:	Bone Marrow Stromal Cells
DMEM	:	Dulbecco's Modified Eagle's Medium
EC	:	Endothelial Cell
ECM	:	Extracellular Matrix
ELISA	:	Enzyme-Linked Immunosorbent Assay
FBS	:	Fetal Bovine Serum
Flk-1	:	Fetal Liver Kinase-1
GFs	:	Growth Factors
GFP	:	Green Fluorescent Protein
hADMSCs	:	Human Adipose Derived Mesenchymal Stromal Cells
HBSS	:	Hank's Balanced Salt Solution
Hif-1α	:	Hypoxia-Inducible Factor-1α
Hif-1α-e-hADMSCs	:	hADMSCs engineered with Hif-1α gene

HUVEC	:	Human Umbilical Vein Endothelial Cell
MSCs	:	Mesenchymal Stromal Cells
n-e-hADMSCs	:	non-engineered hADMSCs
CD31	:	Platelet Endothelial Cell Adhesion Molecule
s-e-hADMSCs	:	Secretome from engineered hADMSCs
s-n-hADMSCs	:	Secretome of non engineered ADMSCs
SVF	:	Stromal Vascular Fraction
TCPS	:	Tissue Culture Polystyrene Dish
tPA	:	Tissue Plasminogen Activator
VEGF-A	:	Vascular Endothelial Growth Factor-A
VCAM	:	Vascular Cell Adhesion Protein
Vegf-e-hADMSCs	:	hADMSCs engineered with Vegf-A gene.

SYNOPSIS

Facilitating adequate angiogenesis concurrently with tissue growth is a major challenge in the healing of chronic wounds. Advances in wound research have recognized a variety of angiogenic factors (AFs) for therapy. Of which, vascular endothelial growth factor A's (VEGF-A) autocrine and paracrine potential to activate angiogenesis is highly notable. In wounds, hypoxia persuades the expression of a plethora of genes upon induction of hypoxia-inducible factor-1α (HIF-1α), which mediates adaptive responses that regulate VEGF-A expression for optimal granulation tissue formation and cell proliferation/survival. Although VEGF is a downstream protein regulated by HIF-1α, it is less correlated. Therapeutic angiogenesis hence stresses on combinatorial administration of both HIF-1α and VEGF-A to achieve optimal tissue neovascularization. Despite safety reports, systemic or local delivery of potent AF stand challenging and results in poor outcome pertaining to burst release of GFs, requirement of supra-physiological doses; poor bioavailability, enormous cost and ineffective means of delivery. In the pursuit of delivery vehicles for sustained bioactivity of therapeutic molecules, natural or synthetic materials have been explored. However, their complex design, poor drug release mechanism, biomaterial selection and matrix/carrier fabrication methods, make it hard to mimic natural tissue regeneration. Recent reports favor development of engineered MSCs as suitable vehicles of therapeutic genes. In wound repair, the pivotal role of MSCs implicated by their differentiation, paracrine signaling, and release of extracellular matrix (ECM) proteins including biomolecules is reported. Thus, exploiting MSCs as a delivery vehicle of AF for wound regeneration

seems appealing. Recently, human adipose derived mesenchymal stromal cells (hADMSCs) derived from fat tissue, have gained attention due to its abundance, minimally invasive and standardized tissue collection processes.

Hence in this study it was hypothesized, genetic exploitation of hADMSCs, not only would serve in effective AF delivery, but could boost its limited paracrine activity, in addition to self-differentiation potential. Viral transfection, though efficient, are translation limited due to insertion mutagenesis and immune responses whereas non-viral transfection is less efficient in primary cells, requiring optimization. Neon® Transfection System, an advanced next generation electroporation technique has gained attention in transfecting human primary cells. No reports demonstrate application of Neon® Transfection System on primary hADMSCs indicating delivery of AF (VEGF-A and HIF-1α) and exploration of their combinatorial functional response. The use of Neon® Transfection System to bioengineer hADMSCs for adequate functional delivery of VEGF-A and HIF-1α into the secretome of engineered hADMSCs (s-e-hADMSCs) was explored in this study. To determine the success of this bioengineering approach, the requisites identified were to prove; (1) post transfection, cells maintain stem cell property; (2) sufficient release of target molecules in the secretome of engineered hADMSCs in culture; (3) that the endothelial cells (EC) do respond to the delivered molecules to participate in angiogenesis activity *in vitro* and *in vivo.* The possible endothelial lineage commitment of hADMSCs contributed by the over expressed molecules in HIF-1α engineered hADMSCs secretome (Hif-1α-s-e-

hADMSCs) and VEGF-A engineered hADMSCs secretome (Vegf-s-e-hADMSCs) through autocrine/paracrine mediation also seemed interesting to study.

To establish our hypothesis the following specific objectives were formulated;

1. To establish an efficient non-viral mediated angiogenic factor delivery system in hADMSCs.
2. To analyze the functionality of bioengineered hADMSCs in cellular response.
3. To study the combinatorial effect of over expressed VEGF-A and HIF-1α in angiogenic response *in vitro*.
4. To demonstrate effect of bioengineered hADMSCs and over-expressed factors in vascularization of dermal substitutes *in vitro.*
5. To demonstrate the effectiveness of over-expressed factors within the hADMSCs secretome in *in vivo* angiogenesis and tissue regeneration.

For strong establishment of the effect of over-expressed molecules, comparative analysis of the effect of s-e-hADMSCs with the secretome of non-engineered hADMSCs (s-n-hADMSCs) was the employed strategy, in all the independent experiments that were performed.

The thesis pertaining to this study is presented in 6 Chapters as below;

- **Chapter 1** introduces the area of research, defines the problem, development of the hypothesis and states the objectives. The topics elaborated include; Biology of the human Skin, major skin cells, Homeostasis in skin and cells involved in

skin regeneration, Wounds and their classification, Acute wounds and their healing mechanism, Non healing wounds, Role of Angiogenesis in skin regeneration, Mechanism of angiogenesis and cells involved, Conventional treatments of chronic wounds, Tissue engineered skin substitutes, Growth Factors, Cell Based Therapies, Adipose Derived Stem Cells, Approaches to improve stem cell potential and Stem cells as a suitable delivery vehicle for growth factors.

- **Chapter 2** consists of the literature review of the recent advancements in the field of chronic wound angiogenesis and regeneration based on published literature. Reviewed literature describes various approaches, therapies, advancements and their evolution, highlighting their advantages and major limitations. The topics reviewed include; Mechanism of blood vessel development and molecular mediators, VEGF, Fibroblast Growth Factor, Angiopoietins, Transforming Growth Factor Beta Proteins , HIF-1alpha protein, Cell-matrix interactions in angiogenesis, Matrix metalloproteinases (MMPs) and other proteases, Major extra cellular matrix molecules and markers in wound angiogenesis, Cells in wound angiogenesis, Impaired angiogenesis, Current therapeutic strategies, Growth factor therapy, Cell based strategies, Keratinocyte stem cells, Dermal fibroblasts, Mesenchymal stem cells, Induced pluripotent stem cells, Bioengineering MSCs for improved angiogenic gene delivery, Advantages of combination therapy and influence of cell delivery matrix, Angiogenic strategies in *in-vitro* and i*n-vivo* tissue engineering, Role of 3-D scaffold and cell niche for cell differentiation and influence of supporting cells in the study of angiogenic response, Assays to validate *in-vitro* tissue angiogenesis, *In vivo* models to

study wound angiogenesis, Assays to validate *in vivo* tissue angiogenesis and tissue analysis for determination of angiogenesis and interpretation of the final outcome.

- **Chapter 3** contains details on the materials used and methodologies adapted. Briefly the methods pertain to: Isolation, culture & characterization of hADMSCs, Electroporation and its effect on hADMSCs, Assessment of angiogenic factor release, Effect of released AF on HUVEC: Influence on target cell proliferation, cell migration, tube formation, and receptor (*Flk-1*) up regulation, Participation of hADMSCs in directed endothelial commitment: qRT-PCR and Immunostaining, Assessing effect of transfected hADMSCs on biomimetic matrix coated scaffold for dermal substitutes: Scaffold selection, Fabrication of bio-mimetic matrix and seeding of cells, Proliferation Assay, Analysis for dermal fibroblast and endothelial differentiation markers by RT-PCR Analysis and Immunocytochemistry, Quantification of ECM synthesis, Development of large full thickness excision rabbit wounds, wound healing assessment, Isolation of allogenic rabbit ADMSCs and cell labeling, Transplantation studies, Histological Analysis, Immunohistochemistry and Fluorescent cell monitoring and analysis.

- **Chapter 4** illustrates the results obtained in line with each of the methods described in Chapter 3. The results are illustrated using figures, tables, and graphs. Multiple donor cell experiments established adequate transfection efficiency, acceptable cell viability, and post-transfection phenotype maintenance with safe and transient AF delivery. The delivered AF induced endothelial lineage commitment of engineered/non-engineered heterogeneous hADMSCs in culture. Delivered AF was

established to function in dose dependent manner in terms of effect on HUVEC's response emphasizing requirement for combinatorial AF therapy. Application of this approach in dermal substitutes established angiogenic activity and guided endothelial differentiation, favoring development of vascularized skin substitutes. Application in *in vivo* experiments further proved its efficacy through accelerated angiogenic response and tissue regeneration.

- **Chapter 5** discusses results in light of current concepts and speculations published in literature emphasizing the findings of this study. This study is the first to report Neon® Transfection System for reproducible bioengineering of primary hADMSCs for functional AF (VEGF-A & HIF-1α) delivery, demonstrating endothelial lineage commitment, accelerated angiogenic response, predicting combinatorial effectiveness, and translational safety for regenerative medicine application.
- **Chapter 6** summarizes the study. Effectiveness of Neon® transfection system to safely engage hADMSCs as a transient delivery vehicle for AF is advocated, highlighting retained stemness for guided endothelial differentiation. The limitations of the study are identified and the future prospects are projected.

The present study thus collectively contributes towards (i) a clinically safe and effective non viral method for delivery of two potent AF (VEGF-A and HIF-1α), (ii) directed endothelial lineage commitment mediated by s-e-hADMSCs (iii) additional benefits upon combined AF therapy (Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs) on *in vitro* angiogenic response. This study further establishes the efficacy of this approach by its application in (i) in house generated synthetic dermal substitutes for its

vascularization and (ii) in large full thickness excision wounds for guided tissue regeneration with accelerated angiogenic response *in vivo*.

Future directions: Taken together, our non viral-based approach opens a broad range of experimental opportunities and serves as a proof of principle to improve the success of growth factor delivery and cell-based implants in therapeutic angiogenesis. Separate to this, it will be desirable to know the secreted protein and exosome composition of the secretome, when producing bioengineered MSCs for therapeutic use facilitating a cell free therapy. Development of immortalized and bioengineered cell lines could also be explored for cost reduction in terms of large scale AF production and availability at clinical perspective. Additionally, exploring better vector promoter systems and combinatorial over expression of multiple genes targeting angiogenesis and regeneration might prove difficult in stem cells, but would be a welcome addition to the over expression arsenal, for regenerative medicine.

Other documents included in the thesis are: List of references studied for defining the problem, designing the study and discussing the results, abbreviations, acknowledgements, table of contents, list of figures & tables, list of courses studied, conferences attended, manuscripts published and those under consideration for publishing.

CHAPTER 1: INTRODUCTION

Globally, chronic wounds represent a significant health problem. Of the 150 million diabetic people worldwide, the lifetime incidence of a foot ulcer is ~25%. Nearly 70% of these wounds remain unhealed despite twenty weeks of standard treatment. Considering India alone, it is estimated to have 61.3 million diabetics, which is projected to cross 100 million by the year 2030. Often, reduced ability to re-establish adequate blood supply to the injury site remains the significant cause of wound chronicity in these patients. Various tissue-engineered skins have been developed to replace and restore barrier function to such patients in whom healing has been severely compromised. However, current tissue engineered skin products have short comings such as lack of vascular structures, causing slow vascularization or even transplant failure. Effective solutions to vascularization are of demand and remain challenging. For the past 30 years, the area of wound healing has been an identified key target for research due to variety of health issues that arise from non healing wounds.

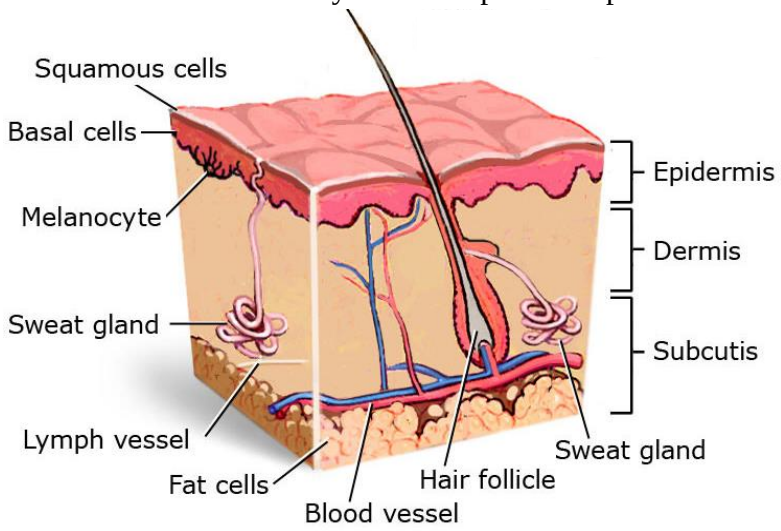
The human skin is the body's largest organ, with a range of functions that support survival. Microscopic observation reveals the skin to be made up of layers, with many smaller elements within these layers that help the skin to perform its protective role. The protective properties imparted by the skin include; protection of the body from water loss and injury due to bumps, chemicals, sunlight or microorganisms ('germs');

control of body temperature; as a sensor to inform the brain of changes in the immediate environment; and synthesis of vitamin D. Addressing the limitations of current skin tissue constructs in replacing and restoring the functionality of skin demands deep understanding of the skin's complexity and structure.

1.1. The biology of human Skin

The skin is composed of two main layers: the superficial layer, the epidermis, which functions as a barrier to the external environment, and the deeper layer, the dermis, made up of connective tissue, which provides the skin with its mechanical properties. Briefly, the epidermis has a mosaic appearance, having cells glued together and its thickness depends on the location of the body. On the palms and soles the epidermis is thick, flexible and resists mechanical injury. On the eyelids it is very thin and allows maximum movement. The epidermis prevents loss of water and body fluids, resists mechanical and chemical injury and protects against bacteria, viruses and parasite infections. The pigment in the epidermis plays an important role in protecting the skin from ultraviolet radiation. The hair follicles, sweat glands, sebaceous (oil) glands and apocrine glands develop from the epidermal cells, but their deeper parts extend into the dermis. The glands open onto the surface of the skin via small ducts.
Hair grows from the hair follicle, which is found in all skin except the palms and soles.
Nails are specialized plates of hard keratin that develop from the epidermis overlying the small bones at the ends of the fingers and toes.

Connecting the epidermis to the dermis is the dermo-epidermal junction which is a complex region where the attached to each other is achieved via specialized cells and molecules which make up the basement membrane. The dermis lies beneath the epidermis and is 20 to 30 times thicker than the epidermis. It is composed of a dense network of specialized proteins (collagen and elastin) organized into fibers of differing sizes and properties. A complex gel of different proteins surrounds these fibers. All together this is known as the extracellular matrix. Within the extracellular matrix are blood and lymphatic vessels, nerves, the bottom part of the hair follicles and sweat glands. Beneath the dermis is the subcutis (the subcutaneous layer), is a specialized area which contains a network of collagen fibres and fat cells (adipocytes). It has protective role in case of external trauma and insulates from cold. It also acts as a main storage site for fat and therefore energy. There are many blood and lymphatic vessels and nerves passing through the subcutis. The thickness of the subcutaneous layer again varies according to the location on the body and from person to person.



(Figure courtesy: healthadvisors.org)

Figure 1: Cross section of the human skin

1.1.1. Major types of cells in skin

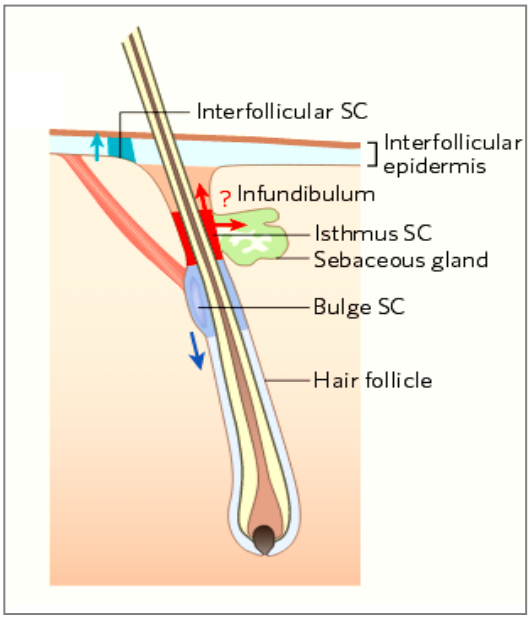
There are 3 main groups of cells in the epidermis: (1) Keratinocytes (skin cells), (2) Melanocytes (pigment cells) and (3) Langerhans cells (immune cells). The main cell in the epidermis is the keratinocyte, which develops from the bottom or basal layer and then migrates upwards over a period of about four weeks to the outer surface (stratum corneum) where it is shed. Langerhans cells are specialized immune cells that are an important part of the body's immune response to foreign materials and infections. The melanocytes are responsible for pigment production. Interestingly, all humans have the same number of melanocytes. The difference in skin colour occurs with the difference in production of the melanin pigment. The melanin pigment protects the cells of the epidermis and the tissues in the dermis from sun damage.

The cells of the dermis are derived from somite mesoderm. Fibroblasts, which secrete the connective tissue matrix, Schwann cells, which will form the perineural structures, and endothelial cells, which form the blood vessels, differentiate very early from the primordial dermal mesenchyme. Adipocytes, which form the fat lobules of the subcutaneous tissue, arise later from the same source. Macrophages, pericytes, and mast cells appear last.

1.1.2. Homeostasis in skin and cells involved in skin regeneration

The physiological process that maintains a constant number of cells in renewing organs is called tissue homeostasis. Stem cells (SCs) that are located in these

organs are responsible for the maintenance of tissue homeostasis and repair following injuries based on their ability to self-renew and give rise to the different cell lineages that form mature adult tissues. The process of skin wound healing is highly organized and coordinated series of events that results in the restoration of tissue integrity and functions. This homeostasis in skin is mainly fuelled by SCs in epithelial tissues. The epidermal region also produces appendages, including sweat glands, and hair follicles and their associated sebaceous glands. The different epidermal compartments undergo constant cellular turnover to replace the dead or damaged cells. This homeostatic process is thought to involve several types of stem cells, each located in a specific epidermal region and contributing to the maintenance of a discrete compartment of the skin (Figure 2).



(*Figure courtesy: cédric Blanpain, 2010)*

Figure 2: Various stem cells ensure skin homeostasis

Arrows indicate the flux of the different stem cell (SC) progeny.

Significant advances have been made in identifying and locating the stem cells that inhabit the skin including epidermal stem cells (interfollicular and bulge stem cells), dermal stem cells, sebaceous stem cells, hair follicle stem cells, sweat gland stem cells, melanocyte stem cells, MSCs, neural stem cells and endothelial stem cells. The majority of stem cells in the skin reside in the ‘‘bulge’’ region of the hair follicle (named bulge stem cells), with only a small fraction of stem cells residing in the basal layer of the interfollicular epidermis (named interfollicular stem cells). These stem cells ensure the maintenance of adult skin homeostasis and hair regeneration, but they also participate in the repair and regeneration of the epidermis after injuries.

In addition to the contribution of stem cells, the skin homeostasis begins through a cascade of events by the actions of numerous cell types which undergo proliferation, differentiation, migration and apoptosis to rebuild the skin. This process involves resurfacing, reconstitution and restoration of the tensile strength of injured skin. Briefly, soon after wounding, the blood vessels close, fibrin aggregates are formed, growth factors (such as PDGF and EGF) are released, and cells associated with inflammation (monocytes, neutrophils) migrate into the wound. During the next 1–3 days, epidermal keratinocytes, almost damaged, migrate to the wound bed reproducing layer of the epidermis. This process, described as reepithelization, is a very crucial step in regeneration of functional epidermis and prevents the development of infections. Also, dermal fibroblasts translocate towards the wound area and start the synthesis of ECM components and take part in ECM remodeling. Fibroblasts in the wound area transform into myofibroblasts whose contraction is responsible for tightening wound

borders. In this stage, myofibroblasts strongly proliferate and synthesize components of ECM while maintaining tissue integrity and promoting its regeneration. Normal cutaneous wound repair is thus broadly characterized by four overlapping classic phases (Haroon *et al* 1999) of healing termed Hemostasis, inflammatory, proliferative, and remodeling phases as depicted in Figure 3.

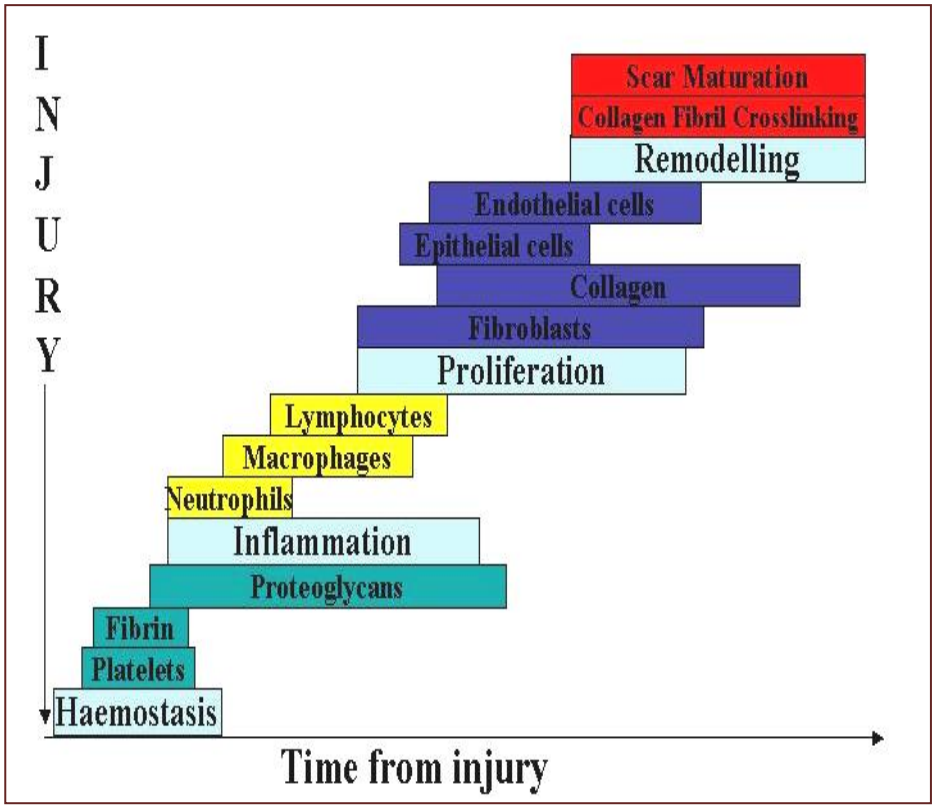


Figure 3: Phases in wound healing.

(a) Hemostasis: Hemostasis is a process which causes bleeding to stop, meaning to keep blood within a damaged blood vessel. It is the first stage of wound healing. This involves coagulation, blood changing from a liquid to a gel. Immediately

after an injury to the skin, small vessels within the wound constrict to provide a measure of hemostasis for 5 to 10 minutes. Platelets aggregate and trigger the clotting cascade by releasing growth factors & cytokines (PFGF & TGF-β) that are important for progression of wound healing.

(b) Inflammation Phase: This is the second phase of wound healing. During this phase blood vessels dilate to allow essential cells; antibodies, white blood cells, growth factors, enzymes and nutrients to reach the wounded area. Signs of inflammation include; Erythema, heat, oedema, pain and functional disturbance. The predominant cells here are the phagocytic cells; 'Neutrophils and Macrophages' mounting a host response and auto-lysing any devitalized 'necrotic / sloughy tissue. This phase lasts for 1-5 days.

(c) Proliferation Phase: During this phase the wound is 'rebuilt' with new granulation tissue along with collagen, into which a new network of blood vessels develops. The production of collagen by fibroblasts is a hallmark of this phase. Fibroblast originating from adjacent uninjured tissue/ MSCs proliferates & accumulates at the wound site. Using fibrin cross-linking fibers they migrate across the wound and adhere to fibronectin, then deposit ground substance and later collagen. The developing collagen matrix stimulates angiogenesis. This process is often termed as Fibroplasia. Meanwhile, epithelial cells migrate across the new tissue to cover the wound. The main cells responsible for this are the basal keratinocytes from the wound edges and dermal appendages such as hair

follicles, sweat glands and sebacious glands. Overall the proliferation phase lasts for 5-21 days.

(d) Maturation/ Remodeling Phase: This is the longest phase which involves cross linking & remodeling of collagen from type III to type I. during this phase, cellular activity reduces and the number of blood vessels in the wounded area regress and decrease. This phase lasts for 3 weeks to 2 years.

1.1.3. Wounds and their classification

There are many different ways in which wounds can be classified. In many cases a wound may consist of a combination of the different classifications. Classifications of the wound can be based as follows;

I. ***Level of contamination****:*

As per the extent of contaminants seen on formation of a wound they are grouped as;

(a) Clean wounds: These kinds of wounds are made under sterile conditions usually during a surgery with no organisms present in the wound and are likely to heal without complications.

(b) Contaminated wound: These wounds are formed by an accidental injury where there are pathogenic organisms and foreign bodies in the wound.

(c) Infected wound: These wounds have pathogenic organisms present and multiplying with clinical signs of infection, look yellow, oozing pus, having pain and redness.

(d) Colonized Wound: Wound is a chronic one and there are a number of organisms present and very difficult to heal.

II. ***Depending on the depth of Injury/wound thickness:***

(a) Superficial wounds: Injury involves only the epidermis and the upper dermis.

(b) Partial thickness wounds: Involves skin loss up to the lower dermis.

(c) Full thickness wounds: Involves skin and subcutaneous tissue.

(d) Deep wounds: involves penetration into natural cavities, an organ or tissue.

III. ***Depending on the wound origin (Open wounds):***

In an open wound, the skin is broken or torn. It can be classified according to the object that caused the wound. There are five types of open wounds:

(a) Incision Wounds: Usually originated as a result of surgical intervention whereby a cut is made through intact tissue for the purpose of exposure or excision caused by a clean, sharp-edged object such as a knife, razor, or glass splinter.

(b) Abrasion wounds: These are superficial wounds in which the epidermis is scraped off. Abrasions are often caused by a sliding fall onto a rough surface.

(c) Laceration wound: Is a deep cut or tear of the skin. Accidents with knives, tools, and machinery are frequent causes of lacerations. The bleeding is rapid and extensive.

(d) Puncture wounds: Is a small hole caused by a long, pointy object. Punctures may not bleed much, but these wounds can be deep enough to damage internal organs.

(e) Avulsion wounds: An avulsion is an injury in which a body structure is forcibly detached from its normal point of insertion by either trauma or surgery.

IV. *Closed wounds:*

In these kinds of wounds the skin remains intact while the underlying tissue is damaged. Bleeding occurs below the surface. They are just as dangerous as open wounds. The types of closed wounds are:

(a) Hematomas: In these wounds a localized collection of blood outside the blood vessels usually in liquid form within the tissue is seen. It produces elevation and discoloration of the wound edges, discomfort, and swelling.

(b) Crush Injury: This is a kind of injury by an object that causes compression of the body. It is common following a natural disaster or after some form of trauma from a deliberate attack.

(c) Contusions: Usually called bruises, formed by a blow with a blunt instrument, in which the subsurface tissue is injured but the skin is not broken but results in swelling.

1.1.4. Acute wounds and their healing mechanism.

Typically, acute wound healing is a well-organized process leading to predictable tissue repair where platelets, keratinocytes, immune surveillance cells, microvascular cells, and fibroblasts play key roles in the restoration of tissue integrity. The acute wound repair process can be divided into 4 temporarily and spatially overlapping phases: coagulation, inflammation, formation of granulation tissue (proliferative phase), and remodeling or scar formation phase. As discussed earlier, the various phases in acute wound healing strictly follow the cascade of events as predicted in the homeostasis mechanism. Immediately after injury, platelets adhere to damaged blood vessels, initiate a release reaction, and begin a hemostatic reaction, giving rise to a blood-clotting cascade that prevents excessive bleeding and provides provisional protection for the wounded area. Blood platelets release well over a dozen growth factors, cytokines, and other survival or apoptosis-inducing agents. Key components of the platelet release reaction include platelet-derived growth factor (PDGF) and transforming growth factors A1 and 2 (TGF-A1 and TGF-2), which attract inflammatory

cells, such as leukocytes, neutrophils, and macrophages. As leukocytes are phagocytic cells, they release reactive oxygen species (ROS) that are antimicrobial and proteases that clear the wound of foreign bodies and bacteria. Resolution of the inflammatory phase is accompanied by apoptosis of inflammatory cells, which occurs gradually within a few days after wounding.

As the inflammatory phase subsides, the proliferative phase of repair begins. At this stage, growth factors produced by remaining inflammatory cells and migrating epidermal and dermal cells act in autocrine, paracrine, and juxtacrine fashion to induce and maintain cellular proliferation while initiating cellular migration; all these events are required for the formation of granulation tissue while supporting epithelialization. As dermal and epidermal cells migrate and proliferate within the wound bed, the requirement for an adequate blood supply for nutrient delivery, gas, and metabolite exchange leads to wound angiogenesis.

In acute wounds, a robust angiogenic response is thus initiated and sustained. This event fosters the production of proangiogenic factors. Vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and PDGF, initially released by platelets and then by resident cells within the wound bed, are all central mediators of injury-induced angiogenic induction. In response, endothelial cells degrade basement membrane, migrate toward the wound site, proliferate, and form cell-cell contacts and eventually new blood vessels. Reestablishment of a normal blood supply provides a favorable microenvironment for epidermal and dermal cell migration

and proliferation. In turn, this leads to wound re-epithelialization and restoration of epidermal integrity. Fibroblasts proliferate within the wound and synthesize extra-cellular matrix (ECM) forming granulation tissue perfused with newly formed blood vessels. Simultaneously, provisional matrix mainly consisting of collagen III, fibrin, fibronectin, and hyaluronic acid is progressively substituted with ECM mainly containing collagen. Next, wound contraction and matrix remodeling occur.

1.1.5. Non healing wounds

A practical classification of a non healing wound is one that fails to heal spontaneously within 3 months (Saltmarche 2008). Common chronic types of wounds are venous leg ulcers, ischemic wounds, diabetic foot ulcers, and pressure wounds. Unlike the typical case in acute cutaneous wound healing whereby in response to tissue injury, a dynamic process is initiated by multiple signals from both serum and the surrounding extra cellular environment through a consecutive, concerted or synergistic manner towards repair, in non healing wounds this regulation is altered to impair granulation and healing leading to chronic wounds. Local factors like infection, ischemia, foreign bodies' interaction, edema etc and systemic conditions like age, sex hormones, stress, diseases, obesity, medication, alcoholism and smoking, immunocompromised conditions and nutrition contribute to the impairement of wound healing. Whatever be the cause of impairment, chronic wounds are thought to persist in the inflammatory state of wound healing (Drinkwater *et al* 2002). The current

understanding is that locally elevated levels of proteolytic enzymes in the hypoxic microenvironment of the wound bed degrade beneficial growth factors and thereby prevent the wound from progressing into the proliferative phase with laying of granulation tissue and a provisional matrix as a precursor for tissue remodeling and healing. Moreover, key signaling intermediates responsible for coordinating/regulating wound healing angiogenesis and vasculogenesis seem dysfunctional during diabetes. Also, diabetic patients prone to the development of chronic wounds exhibit deficiencies in either endothelial progenitor cells (EPC) bone marrow release or peripheral tissue homing and engraftment. Thus, therapies aimed at correcting EPC - linked deficiencies may prove beneficial for treating diabetes-induced chronic wounds.

1.1.6. Role of Angiogenesis in skin regeneration

Re-establishment of a functional vascular network is one of the most important components of successful wound repair. The process by which new blood vessels sprout from pre-existing vasculature to supply the hypoxic wound bed is highly complex and tightly regulated. Angiogenesis involves endothelial cell (EC) proliferation, differentiation, migration, and organization into a branched tubular network and is controlled by specific interaction of endogenous pro- and antiangiogenic factors with ECs. One of the most potent pro-angiogenic agents is the well-characterized vascular endothelial growth factor (VEGF), a matricellular protein produced by keratinocytes, and macrophages during the early phases of physiological wound repair. VEGF

functions by binding compatible receptors on EC membranes, which initiate and amplify signaling cascades that lead to pro-angiogenic cellular changes. Endogenous inhibitors to this process are produced to aid in the spatio-temporal control of angiogenesis during healing. In diabetic chronic wounds, an imbalance of important angiogenic mediators may be responsible for the observed dysfunctional angiogenic response. Impaired angiogenesis in all chronic wounds lead to further tissue damage resulting from chronic hypoxia and impaired micronutrient delivery.

1.1.7. Mechanism of angiogenesis and cells involved

Although granulation is assigned to the proliferative stage, angiogenesis is initiated immediately after tissue injury and is mediated throughout the wound healing process. Angiogenesis occurs as an orderly cascade of molecular and cellular events in the wound bed: 1. endothelial cell surface has receptors to which angiogenic growth factors bind in preexisting venules (parent vessels); 2.Growth factor receptor binding activates signaling pathways within endothelial cells. Proteolytic enzymes released by activated endothelial cells dissolve the basement membrane of surrounding parent vessels; 4. Endothelial cells proliferate and sprout outward through the basement membrane; 5. Endothelial cells migrate into the wound bed using integrins ($\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha v\beta 1$) which are cell surface adhesion molecules; 6. Matrix metalloproteinases (MMPs) dissolve the surrounding tissue matrix in the path of sprouting vessels; 7. Vascular sprouts form tubular channels that connect to form vascular loops; 8. Vascular

loops differentiate into afferent (arterial) and efferent (venous) limbs; 9. New blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilize the vascular architecture; 10. Blood flow begins in the mature stable vessel. These complex growth factor-receptor, cell-cell and cell-matrix interactions characterize the angiogenesis process, regardless of the stimuli or its location in the body. In the wound bed, the mechanism of angiogenesis can be explained as;

(a) Step 1- Angiogenesis initiation : Basic fibroblast growth factor (bFGF) stored within intact cells and the ECM is released from damaged tissue.Bleeding and hemostasis in a wound also initiate angiogenesis. Cellular receptors for vascular endothelial growth factor are upregulated by thrombin in the wound. Endothelial cells exposed to thrombin also release gelatinase A (MMP-2), which promotes the local dissolution of basement membrane, a necessary early step of angiogenesis. Platelets release multiple growth factors, including platelet-derived growth factor (PDGF), VEGF, transforming growth factor (TGF-α, TGF-β), bFGF, platelet-derived endothelial cell growth factor and angiopoietin-1 (Ang-1). These factors stimulate endothelial proliferation, migration and tube formation.

(b) Step 2-Angiogenesis amplification : Macrophages and monocytes release numerous angiogenic factors, including PDGF, VEGF, Ang-1, TGF-α, bFGF, interleukin-8 (IL-8) and tumor necrosis factor alpha into the wound bed during the inflammatory phase amplifying angiogenesis further. Several growth factors (PDGF, VEGF and bFGF) synergize in their ability to vascularize

tissues.Proteases that break down damaged tissue matrix further release matrix-bound angiogenic stimulators. Enzymatic cleavage of fibrin yields fibrin fragment E, which stimulates angiogenesis directly and also enhances the effects of VEGF and bFGF. Expression of the inducible COX-2 enzyme during the inflammatory stage of healing also leads to VEGF production and other promoters of angiogenesis.

(c) Step 3- Vascular proliferation: Hypoxia is an important driving force for wound angiogenesis. Expression of gene HIF-1α, due to hypoxic gradient between injured and healthy tissue triggers VEGF production. VEGF is present in both wound tissue and exudates. VEGF is also known as vascular permeability factor since it increases permeability of capillaries. Hypoxia also leads to endothelial cell production of nitric oxide (NO). NO promotes vasodilatation and angiogenesis to improve local blood flow.

(d) Step 4 - Vascular stabilization: Vascular stabilization is governed by Ang-1, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2), smooth muscle cells and pericytes. Production of PDGF and recruitment of smooth muscle cells and pericytes to the newly forming vasculature are regulated by binding of Ang-1 to its receptor Tie-2 on activated endothelial cells. A PDGF deficiency leads to poorly-formed immature blood vessels.

(e) Step 5- Angiogenesis suppression: Angiogenesis is suppressed at the terminal stages of healing. As tissue hypoxia is restored, and inflammation subsides, the

level of growth factors decline in the wound. Pericytes which stabilize endothelial cells secrete an inhibitory form of activated TGF-β that impedes vascular proliferation. A cleavage product of collagen XVIII, endostatin, is present surrounding the VBM, and it inhibits wound vascularity.

1.2. Conventional treatments of chronic wounds

When wound healing does not progress normally, a chronic wound may result and this is at significant burden to both the patient and the medical system. Different strategies have been employed for wound treatments, which are discussed below:

1.2.1. Tissue engineered skin substitutes

While skin substitutes have long been used in grafts (mostly from autologous sources) either for significant surgical defects or burns necessitating replacement of a large surface area of tissue, advances in this field have allowed for the development of novel bioprosthetic skin substitutes. These materials generally consist of a biologically derived substance combined with a material to allow for its placement on a wound. Overall, these dressings are quite costly, representing a significant barrier to widespread adoption. Human decellularized dermis products such as AlloDerm and DermaMatrix seem to be the best option to date, with no other decellularized or synthetic scaffold being clinically proven to have better results. Also these quite

advanced bioengineered skin substitutes still have their limitations when compared with human skin, and developments in certain fields are essential to reach full therapeutic potential. The lack of built-in vascular or nervous components makes grafts dependent on host neovascularization and reinnervation. Simultaneous growth of vascular networks within skin substitutes by co seeding grafts with endothelial cells or their progenitors looks promising. Increasing complexity of skin substitutes addressing the deficiencies of skin substitutes over native human skin involves time delays needed for cell culture. It is also important to take the higher costs into account. More research is, however, needed to fully reach the potential of cell seeded scaffolds while the rising production costs can form a serious issue as well.

1.2.2. Growth Factors

Growth factors in wound healing have received significant attention in the field of wound healing in the past few decades. However, the only therapy that has proven to improve healing in a double-blinded randomized controlled trial is platelet-derived growth factor (PDGF). Clinical studies confirm the pivotal role of growth factors in wound healing and their diminished levels in the chronic wound. A variety of angiogenic growth factors have been identified, of which vascular endothelial growth factor A (VEGF-A) is well established as the primary inducer of angiogenesis and lymphangiogenesis. Additionally, VEGF is known to modulate endothelial function via autocrine and paracrine pathways. It is also known that hypoxia in wounds persuades the

expression of a plethora of genes through the induction of hypoxia-inducible factor-1α (Hif-1α), which mediate adaptive responses such as angiogenesis and cell proliferation/survival. The action of Hif-1α as a major regulator of VEGF-A expression and contributor to the maintenance of optimal granulation tissue formation in response to wounding has also been reported. However, results with traditional bolus dosing of a single growth factor have yielded insignificant results, which may be the result of the inherent short half-life of growth factors, hostile microenvironment rich in protease activity, and poor delivery mechanisms. Technologies capable of delivering multiple growth factors in a spatially oriented approach include polymer systems, scaffolds, and hydrogels. With improved delivery systems, treating chronic wounds with growth factors potentially could accelerate healing in a manner not previously achieved with traditional delivery approaches.

1.2.3. Cell Based Therapies

Cell therapy using autologous cells accelerates the wound healing process by reducing the time needed for the host cells to invade the wound tissue and by early synthesis of new skin. Based on previous research, cell-only treatment accelerates the rate of wound healing, but does not show a beneficial effect on wound contraction. Moreover, the procedure of cell therapy is different depending on cells. At present, keratinocytes, fibroblasts, and platelets are actively used in the clinical setting. However, the source of harvest of these cells is a concern. Active transgene expression is limited.

Recombinant keratinocytes generate immune response specific against the transgene product. Most fibroblast therapy products are composed of cryopreserved allogeneic cells. However, the effect has generally shown to be not very dramatic. One of the possible reasons for the limited effect of these products is that it is difficult for grafted frozen cells to recover, colonize, and persist in chronic wound beds which are deficient in oxygen and nutrients. Moreover, cell activities are impaired due to cryopreservation .To establish cell therapy as a standard treatment, more investigation with a larger number of patients is necessary. In addition, further studies are needed to determine the fate of transplanted cells and the number of cells required to show definitive effects. Further studies on the length of time for cells to be maintained after harvesting and still retain viability are also required.

1.3. Adipose Derived Stem Cells

Emerging evidence has shown that adipose tissue is the richest and most accessible source of mesenchymal stem cells. The capacity of adipose-derived stem cells (ASCs) to promote angiogenesis, secrete growth factors, regulate the inflammatory process, and differentiate into multiple cell types makes them a potential ideal therapy for chronic wounds as shown in Figure 4.

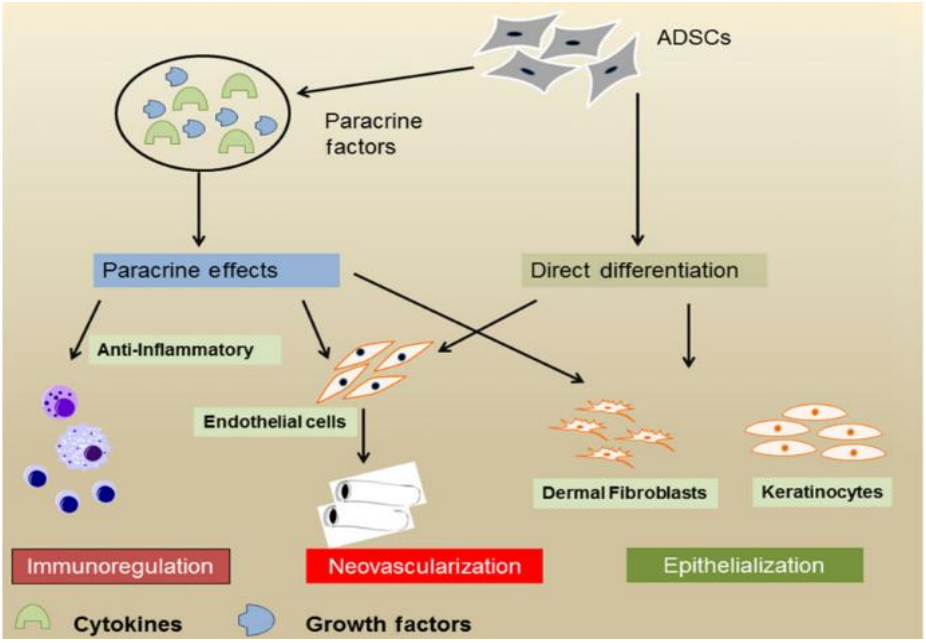


Figure 4: Possible mechanism of skin repair by adipose-derived stem cells.

In response to injury, ASCs may migrate and differentiate into skin cells to repopulate the injured skin or activate the dermal fibroblasts and keratinocytes by secretion of growth factors for accelerated wound healing. Capacity to use allogeneic and autologous cells, multipotency, self renewing capacity, possibility to divide without apoptosis than differentiated cells and ease to isolate with no burden of ethical and immunological concerns as well as the large quantity of cells that can be isolated makes adipose derived stem cells superior in cell based application. ASCs also secrete many different growth factors such as insulin- like growth factor (IGF), hepatocyte growth factor (HGF), transforming growth factor-beta 1 (TGF-β1), and VEGF. In recent studies, these secreted growth factors have shown to be efficacious in preclinical wound healing models of animals.

There is also still significant evidence that these cells have the potential to contribute to keratinocytes in the epidermis, which is why they are exciting targets for improving wound healing. ADMSCs have been shown to promote human dermal fibroblast proliferation in the wound site by secretion of paracrine factors, which ultimately increased the rate of wound healing. Also, ADMSCs under hypoxic conditions due to the inflammation significantly increased the levels of collagen synthesis and helped reduce the wound area.

Further studies have showed that this was achieved by up-regulation of imperative growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Thus, there is conclusive evidence that ADMSCs show immense promise in the future of wound healing treatments. However, extensive studies are required to ensure the development of these new therapeutic strategies. In order to explore the potential behind any new strategy, one must understand the physiology of a wound, the basic mechanism of healing, various growth factors and cells involved in healing and the principle of engineering new strategies.

1.3.1. Approaches to improve stem cell potential.

It is generally observed that *in vivo* MSC survival and engraftment is limited upon transplantation and ADMSCs' paracrine signals are insufficient to produce a significant acceleration in angiogenesis associated healing. Various approaches to augment stem cell's paracrine, autocrine, or endocrine activity, are preconditioning of stem cells, and modification of stem cells via gene expression. Furthermore,

modification of the secretion profile to augment the therapeutic effects of the secretome has been targeted with a better understanding of the effects of stem cell preconditioning or genetic manipulation strategies. Most commonly, bioengineering of cells rely on viral vectors to achieve efficient gene delivery, which raises safety concerns for regenerative application. On the other hand, non-viral methods of gene transfer such as lipofection are associated with their own limitations, such as low efficiency, acute toxicity to exposed cells, and inflammatory responses *in vivo*.

1.3.2. Stem cells as a suitable delivery vehicle for growth factors.

As discussed earlier, the growing demand for improved delivery systems, in treating chronic wounds using growth factors seems important. Addressing this demand for delivery vehicles to sustain the bioactivity of therapeutic molecules in a steady and long-term fashion has lead to the exploration of various natural or synthetic materials. This again has its own limitations such as lack of mass availability, batch to batch quality issues, poor tailoring capacity, and biodegradability with associated toxicity. In addition, limitations due to their simple design, poor drug release mechanism, biomaterial selection and matrix/carrier fabrication methods, make it hard to mimic natural tissue regeneration. On the other hand, advancements in research have thrown light towards the repair and regenerative contributions of mesenchymal stromal cells (MSCs) in injury and disease. Recent studies favor the development of engineered MSCs for use as a suitable vehicle to deliver therapeutic genes in cancer and for islet transplantation. MSCs have hence recently received tremendous attention for the

delivery of therapeutic products to repair neural injury; the amelioration of cardiovascular events; the promotion of bone, cartilage, liver, pancreas, lung, and kidney regeneration. On exploitation, MSCs can serve as programmed molecule transmitters, addressing better bioavailability of the desired molecule. However, the hunt for a safe and effective tool to bioengineer MSCs as suitable delivery vehicle is in need.

1.4. Definition of the problem.

Considering the promises of currently available approaches and their limitations, the following gap areas were identified;

- The pursuit of a safer alternate engineering approach with improved efficacy to over express angiogenic growth factors using non-viral strategies for therapeutic wound angiogenesis stands significant.
- Lack of extensive proof of concept to demonstrate that non viral mediated gene delivery in ADMSCs can promote angiogenesis *in vitro,* can pre-vascularize dermal scaffolds and large area full thickness *in vivo* wounds.
- Lack of studies demonstrating the efficacy of engineered secretome over combined cell and growth factor therapy in large full thickness *in vivo* wounds.

This study thus focuses to address these concerns through a strategy which collectively exploits the potential of growth factors and cell-based therapy by bioengineering of adipose derived stem cells.

1.5. Hypothesis

Genetic exploitation of hADMSCs using non-viral technique, not only would serve in effective & safer AF (VEGF-A & HIF-1α) delivery, but could also boost limited paracrine activity & differentiation potential of stem cells in therapeutic angiogenesis.

1.6. Objectives of the study

To test the hypothesis the following objectives were developed;

1. To establish an efficient non viral mediated angiogenic factor delivery system in hADMSCs.
2. To analyze the functionality of bioengineered hADMSCs in cellular response.
3. To study the combinatorial effect of over expressed AF in angiogenic activity *in vitro*.
4. To demonstrate angiogenic effect of bioengineered hADMSCs in synthetic dermal substitute engineering.

5. To demonstrate the angiogenic effect of bioengineered hADMSCs in full thickness, slow healing large area wounds *in vivo*.

Chapter 1 of the thesis has introduced the area of research, defined the problem, developed the hypothesis and stated the objectives. Chapter 2 consists of the literature review of the specific area of wound angiogenesis in chronic wound healing. Chapter 3 contains details on the materials used and methodologies adapted. The results obtained are illustrated in chapter 4. Chapter 5 discusses the results in light of current concepts and speculations published in literature emphasizing the findings of this study, its limitations and future prospects. Chapter 6 summarizes the study.

CHAPTER 2: LITERATURE REVIEW

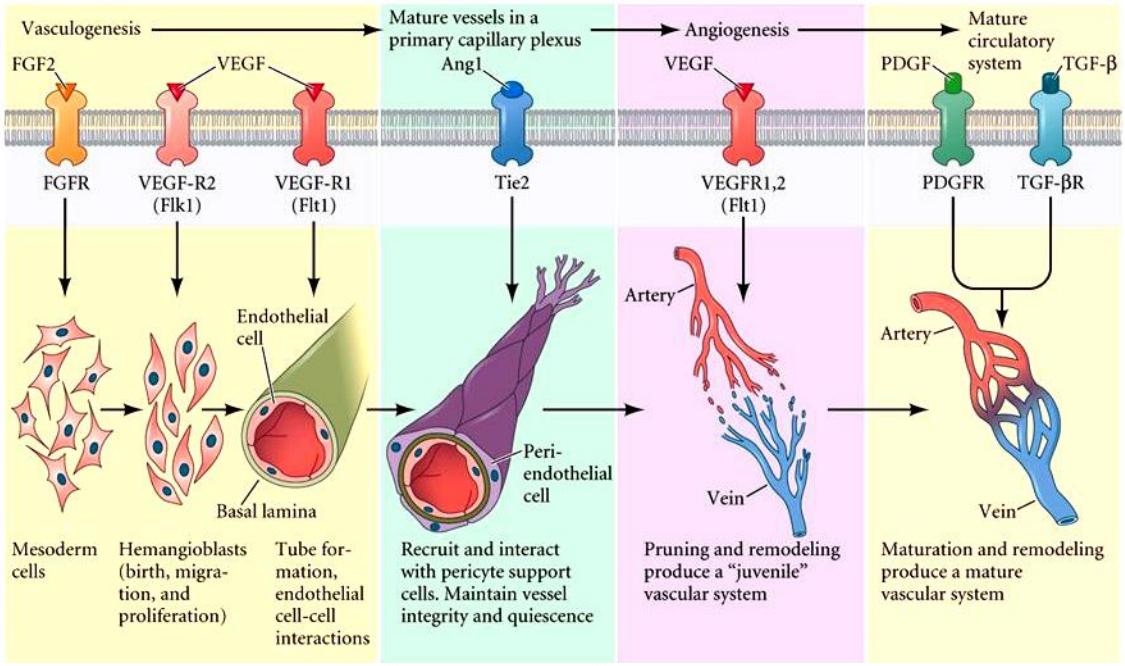
This section summarizes the recent advancements in the field of chronic wound angiogenesis and regeneration based on published literature. Reviewed literature describes various approaches, therapies, advancements and their evolution, highlighting their advantages and major limitations. The embryonic development of blood vessels, the growth factors and cells that play a pivotal role in angiogenesis are also reviewed in detail in this section. The hurdles in generation of a suitable effective therapy for chronic wound angiogenesis are briefly mentioned in this section. The potential of adult stem cells in combination with growth factor delivery for wound angiogenesis and regeneration is also discussed in this section.

2.1 Mechanism of blood vessel development

Blood vessels are made up of several different cell types. The inner layer of blood vessels is composed of endothelial cells (ECs), which are covered on their outer, abluminal surface by perivascular (or mural) cells. Based on marker expression profiles and morphological criteria, these mural cells can be classified as pericytes, which are embedded in the sub-endothelial basement membrane and make direct cell-cell contact with capillary ECs, and vascular smooth muscle cells, which generally cover larger caliber vessels, namely arteries and veins, and lack physical contact with ECs (Armulik et al., 2011). The growth of blood vessels appears to occur via two completely

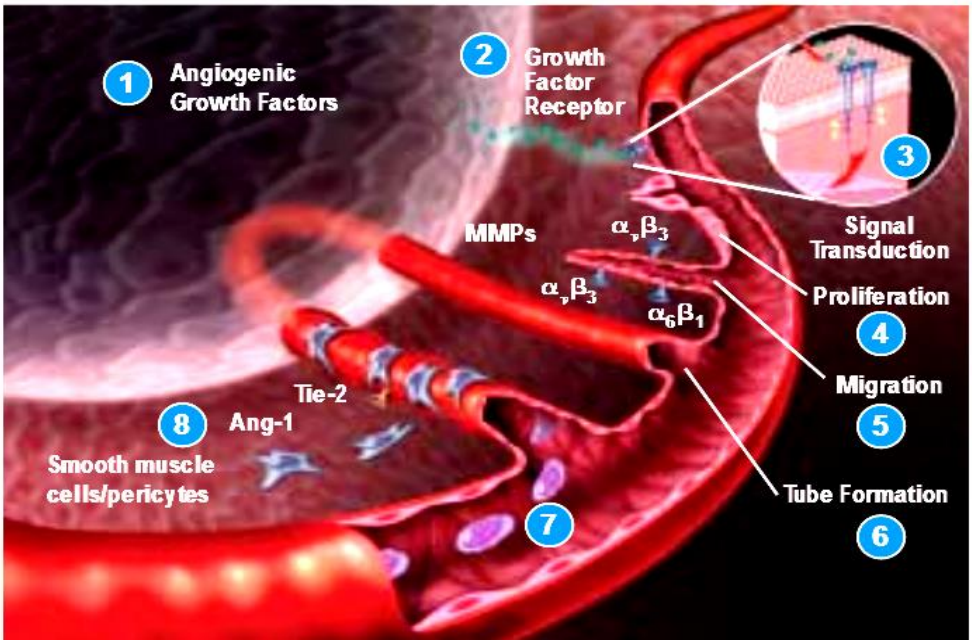
different mechanisms. In early embryogenesis, mesodermal cells differentiate into hemangioblasts (the progenitors of ECs and blood cells), which migrate to specific locations and aggregate to form the first primitive vessels with dispersed endothelial cells (ECs: the cells lining the inner walls of fully-formed blood vessels) organized into a vascular network in a process termed vasculogenesis. Later during development and in adult life, blood vessels are formed by sprouting or splitting of existing blood vessels through a process called angiogenesis(Merks and Glazier, 2006). This involves expansion of existing vascular networks through a series of processes such as EC sprouting, migration, proliferation, vessel anastomosis and pruning.

Angiogenesis requires extensive coordination between the different vascular cell types to ensure that new vessels are fully functional and stable. The expansion of capillary beds, for example, typically involves arteriovenous specification of a subset of ECs, allowing the formation of either arteries or veins. Pericytes and smooth muscle cells are also required, notably for vascular remodeling, stabilization and maturation. There is also increasing evidence indicating that blood vessels form and become specialized in an organ-specific fashion, controlled by local micro environmental signals, leading to specific molecular signatures in ECs (Sivaraj and Adams, 2016). The pictorial representation below explains the above two mechanisms.



Picture courtesy: (Li and Weina, 2011)
Figure 5 – Mechanisms of blood vessel development.

Angiogenesis occurs as an orderly cascade of molecular and cellular events in the wound bed as described in Figure 5 below



Picture courtesy: The Angiogenesis Foundation.
Figure 6 – The angiogenesis cascade of events.

These events include; 1. Angiogenic growth factors bind to their receptors on the surface of endothelial cells in pre-existing venules (parent vessels), 2. Growth factor-receptor binding activates signaling pathways within endothelial cells; 3.Activated endothelial cells release proteolytic enzymes that dissolve the basement membrane surrounding parent vessels, 4. Endothelial cells proliferate and sprout outward through the basement membrane, 5. Endothelial cells migrate into the wound bed using cell surface adhesion molecules known as integrins (αVβ3, αVβ5, and α5β1,), 6. At the advancing front of sprouting vessels, enzymes known as matrix metalloproteinases (MMPs) dissolve the surrounding tissue matrix, 7. Vascular sprouts form tubular channels which connect to form vascular loops, 8. Vascular loops differentiate into afferent (arterial) and efferent (venous) limbs, 9. New blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilize the vascular architecture, 10. Blood flow begins in the mature stable vessel. These complex growth factor-receptor, cell-cell, and cell-matrix interactions characterize the angiogenesis process, regardless of the inciting stimuli or its location in the body(Reinke and Sorg, 2012a).

2.2 Molecular mediators in angiogenesis

Extensive research into the molecular mechanisms involved in vessel formation has identified proangiogenic factors such as vascular endothelial growth factor (VEGF) and the angiopoietins, together with antiangiogenic factors such as the thrombospondins and transcription factors leading to their expression like Id1 (Bikfalvi and Bicknell, 2002). Some of these major molecules in angiogenesis are viewed below;

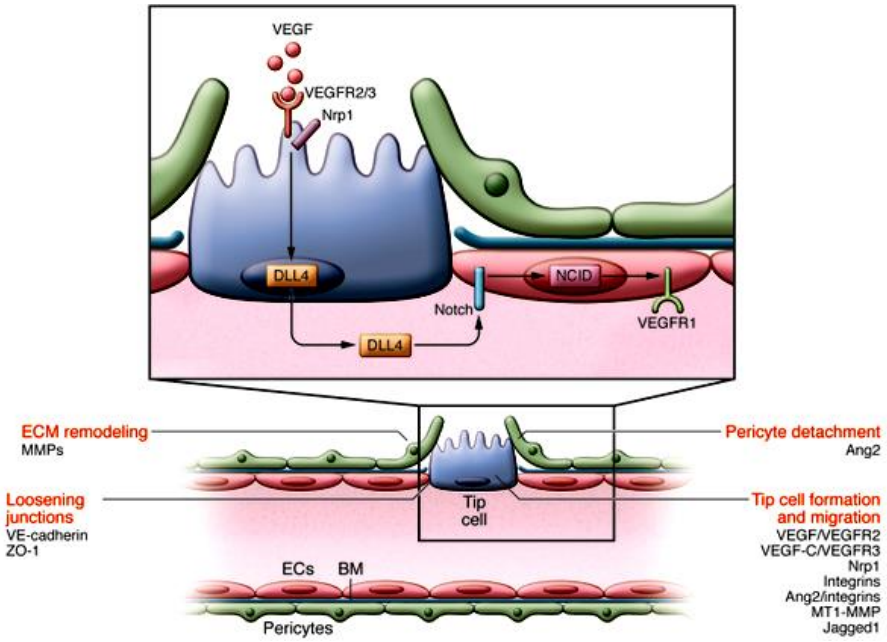
2.2.1 Vascular endothelial growth factor

VEGF is one of the most well studied growth factors involved in EC migration, mitogenesis, sprouting, and tube formation. The VEGF is a homodimeric glycoprotein that consists of six family members: VEGF-A, B, C, D, E and placental growth factor (PLGF).The human VEGF gene produces eight isoforms generated through alternative splicing. The isoforms VEGF121, VEGF145, VEGF165, VEGF 189, and VEGF206 are referred to as VEGF-A, B, C, D, and E respectively (Leung et al., 1989). Forms of VEGF bearing the heparin sulfate-binding domain are sequestered on cell surfaces or embedded in the extracellular matrix. VEGF-A on the other hand is freely diffusible and tends to be in greatest abundance at sites of injury. The various iso-forms suggest that VEGF may have role not only in initiating angiogenesis in adult tissues but also in maintaining long-term vascular stability and homeostasis (Ng et al., 2001). Up regulated VEGF and VEGF-receptor (VEGF-R) mRNA has been detected in the tips of invasive angiogenic sprouts, and antibody blockade of VEGF significantly decreases microvessel outgrowth (Gerhardt et al., 2003). This heparan sulfate binding molecule exists in several isoforms resulting from alternative splice variants from a single gene product. The soluble or membrane bound form binds most commonly to 2 transmembrane receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1).A third transmembrane receptor neuropilin-1 (Nrp-1) binds the VEGF-A splice variant of VEGF, is involved in capillary morphogenesis, and appears to be required for EC filopodial tip directionality during angiogenesis. VEGF also promotes von Willebrand factor release, integrin expression, interstitial collagenase expression, plasminogen

activator (PA) and plasminogen activator receptor (PA-R) expression, and increases both vascular permeability and fenestration, which accounts for its original name vascular permeability factor (Ucuzian et al., 2010).

In the context of vessel sprouting, it requires coordination between migrating tip cells and proliferative stalk cells. ECs at the leading edge extend filopodia and migrate toward angiogenic signals. At the forefront, where VEGF levels are highest, VEGF activates VEGF receptor 2 (VEGFR2) to stimulate tip cell migration. VEGFR2 internalization and activation of ERK1/2 signaling are important for sprouting, likely because rapid receptor turnover and signaling is essential for ECs at the vascular front to respond strongly and quickly to angiogenic signals. Signaling through VEGFR2 is enhanced by the coreceptor neuropilin-1 (Nrp1), which promotes tip cell function. Although the Nrp1 cytoplasmic domain (and signaling) is dispensable for angiogenesis, it is essential for separation of arteries and veins (Welti et al., 2013). The specification between tip cells and stalk cells is regulated by Notch (Geudens and Gerhardt, 2011). ECs with activated VEGFR2 compete for the tip position by increasing expression of the Notch ligand Delta-like 4 (DLL4), which binds to Notch receptors on neighboring ECs and releases the Notch intracellular domain (NICD). NICD acts as a transcriptional regulator, decreasing Vegfr2 and Nrp1 expression while increasing the levels of VEGFR1, which traps VEGF (Krueger et al., 2011) and renders stalk cells less responsive to VEGF. Hence, Notch blockade induces vessel hyper branching. The tip cell position is fluid: EC interchange occurs at the leading edge, with ECs with the highest VEGFR2 and lowest VEGFR1 levels migrating to the tip position (Krueger et

al., 2011). Competition and position exchange couple VEGFR levels to leadership, ensuring that the tip cell is optimally equipped to sense the VEGF gradient.



Picture courtesy:(Welti et al., 2013)
Figure 7 –VEGF mediated tip cell selection.

In the context of wound healing, the function of VEGF in wound repair has been extensively studied. One of the most potent inducers of VEGF during wound healing is hypoxia. As metabolic demand increases in injured tissues and oxygen tension is lowered VEGF production is stimulated in a variety of cells at the wound site ((Detmar et al., 1997)). Additionally, hypoxia preferentially stimulates expression of the VEGF receptor Flt-1 (VEGFR1) which influences the function of a number of inflammatory cells. The role of VEGF and VEGFR1 in endothelial cell survival is in fact more important. This is of critical importance during wound neovascularization where growing blood vessels are subjected to intermittent stress and a host of potentially lethal

stimuli at the wound site. Following injury, platelet release VEGF where it promotes endothelial proliferation and migration. Macrophages attracted to the wound site produce TNF-a, which in turn induces VEGF expression in a host of resident cell populations including keratinocytes and fibroblasts. As pro-inflammatory, immunomodulatory and angiogenic mediators accumulate at the wound site and as the hypoxic gradient is established, VEGF expression is further enhanced. The resulting neovascular network restores tissue perfusion and helps drive tissue regeneration. VEGF thus stimulates angiogenesis and also influences wound closure and epidermal repair, granulation tissue formation, and the quality of repair—both in terms of the strength of the healed wound and the amount of scar tissue that is deposited.

2.2.2 Fibroblast Growth Factor

Fibroblast growth factors (FGFs) comprise a large family of polypeptide growth factors that are involved in a variety of cellular processes including cell migration, chemotaxis, proliferation, angiogenesis, differentiation, cell survival and apoptosis. (Wagner and Siddiqui, 2007). FGF, which is both stored in the vascular basement membrane to serve as a reservoir supply, and is upregulated during active angiogenesis. The two most commonly studied forms are FGF-2 or basic FGF (bFGF) and FGF-1 or acidic FGF (aFGF) which bind most commonly to the receptor tyrosine kinases FGFR-1 or FGFR-2. FGF1 and FGF2 are synthesized by a number of major cell types involved in angiogenesis and wound- healing processes, including inflammatory cells, vascular endothelial cells and dermal fibroblasts and can be released from mechanically wounded

endothelial cells (Li et al., 2003). *In vitro*, FGF binding of FGFR-1 increases EC migration and promotes capillary morphogenesis when cultured on collagen gels, and activates signaling pathways mediated in part by protein kinase-C (PKC), phospholipase A and numerous others. The observation that FGF-2 enhances endogenous VEGF production, and that VEGF is required for the FGF-2-induced expression of placental growth factor demonstrates the existence of cross talk and synergism between FGF and other growth factor pathways. During granulation tissue formation, FGF2 also promotes endothelial cell migration by induction of endothelial cell-surface αV3 integrin, which mediates the binding of endothelial cells to ECM. In addition, αV3 integrin expression promotes endothelial cell migration via localization of matrix metalloproteinase MMP-2 onto endothelial cell surface, which in turn facilitates the collagen degradation and endothelial cell migration (Li et al., 2003).The FGFs, like VEGF, also stimulate EC synthesis of proteases including plasminogen activator and metalloproteinases, important for extracellular matrix digestion in the process of angiogenesis. Although FGFs are potent EC mitogens, they are not EC specific, and also serve as ligands for other cell types including vascular smooth muscle cells and fibroblasts. Unlike VEGF, however, the common forms of FGF (FGF-1 and 2) lack a secretory signal sequence; hence in a clinical trial perspective, FGF gene transfer have consequently required either modification of the FGF gene or use of another of the FGF gene family with a signal sequence(Vale et al., 2001). Also, recent literature reports that bFGF treatment specifically induces an angiogenic phenotype of CD34+ /procollagen fibrocytes in

granulation tissues, which could be a main contributor to bFGF-induced angiogenesis in wounds(Nakamichi et al., 2016)

2.2.3 Angiopoietins

Angiopoietins (Ang-1 through Ang-4) are known to be endothelial cell specific growth factors and they seem to be important partners of VEGF. All four angiopoietins bind to the receptor Tie-2 tyrosine kinase with immunoglobin and epidermal growth factor homology domain or Tek, which is expressed specifically on endothelial cells (Fagiani and Christofori, 2013). Ang-1 and Ang-2 are the two well-studied angiopoietins. Ang-1 and Ang-2 both bind Tie-2, but only Ang-1 can phosphorylate the receptor(Tsigkos et al., 2003). Ang-1 and VEGF are both endothelial cell-specific growth factors. They play separate but essential roles in and have additive effects on angiogenesis. Ang-1 protects endothelial cells from apoptosis through PI3 kinase/Akt-dependent pathway and facilitates endothelial cell survival. In vitro studies have demonstrated that Ang-1-induced endothelial cell migration and sprouting leads to formation of tubule-like vascular structures(Gamble et al., 2000). During wound repair, the expression of Tie-2 was found to be upregulated in the granulation tissue as new vessels develop. While Ang-1 is found constitutively expressed in adult, Ang-2 is highly induced at sites of active angiogenesis.

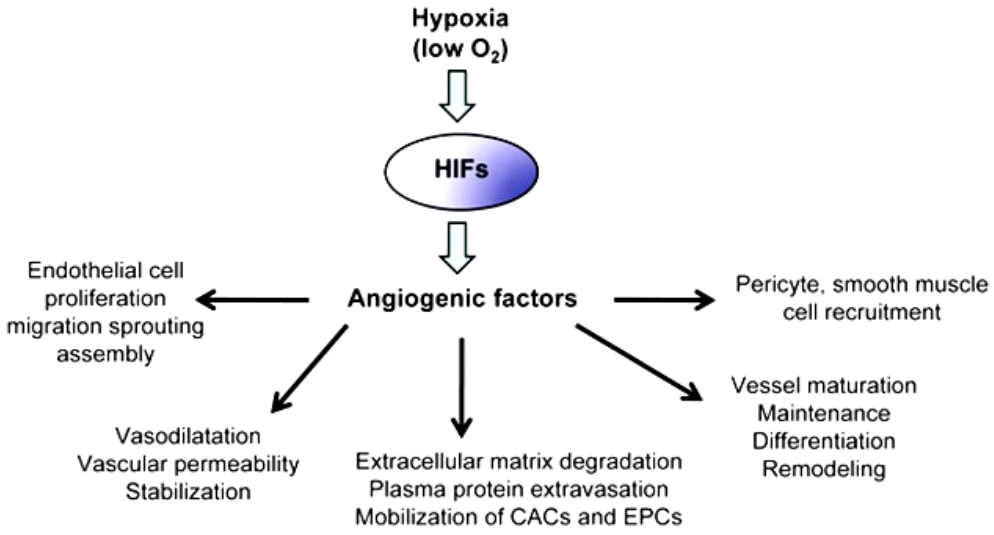
2.2.4. Transforming Growth Factor Beta Proteins (TGF-β)

TGF-β has been found to promote angiogenesis through stimulating endothelial cell migration, differentiation, and tubule formation as well as ECM deposition and upregulation of their integrin receptors. The stimulatory effect of TGF-β on angiogenesis in vivo may be, in part, an indirect by recruitment of inflammatory cells that secondarily release angiogenic factors like VEGF, FGF, and PDGF. TGF-β was also found to increase the secretion of VEGF from dermal fibroblasts and in concert with hypoxia induces the promoter activity of human VEGF(Falcone et al., 1993) .

2.2.5. HIF-1alpha protein

Hypoxia-inducible factor 1alpha (HIF-1alpha) is the inducible subunit of the HIF-1 transcription factor that regulates genes involved in the response to hypoxia, some of which promote neovascularity. The HIF pathway regulates a host of pro-angiogenic genes, including vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, Tie2, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and monocyte chemoattractant protein–1 (MCP-1)(Krock et al., 2011). HIF-regulated pro-angiogenic factors execute the HIF-specific angiogenic program by increasing vascular permeability, endothelial cell proliferation, sprouting, migration, adhesion, and tube formation (Figure 6). The breadth of pro-angiogenic HIF target genes

and the comprehensive list of angiogenic processes regulated by them truly make HIF a "master regulator" of angiogenesis. Interestingly, hypoxia also regulates vessel branching through modulation of Notch signalling. HIF-1α directly binds to the Notch intracellular domain (NICD) and augments its transcriptional activity. Additionally, the Notch ligand Dll4 is a transcriptional target of both HIF-1α and HIF-2α in the endothelium (Skuli et al., 2009). ECs trailing the leading edge begin to form tubes that extend the existing vascular network. Hypoxia enhances formation of endothelial tubes in vitro, and this pro-angiogenic effect is dependent on EC expression of HIF-1α. Once established, the immature vasculature recruits vascular support cells, including pericytes and smooth muscle cells, and forms a basement membrane(Patel et al., 2005). This process, known as vessel normalization, involves HIF-2α, which directly regulates expression of the basement membrane component fibronectin. Various studies thus demonstrate that HIF regulates nearly every aspect of angiogenesis, which has made it an attractive therapeutic target in many human diseases.



Picture courtesy:(Krock et al., 2011)

Figure 8: Regulation of angiogenesis through the hypoxia-inducible factor (HIF) – induced angiogenic factors.

2.3 Cell-matrix interactions in angiogenesis

The proteolysis and remodelling of the extra cellular matrix (ECM) is a critical aspect in all phases of angiogenesis, affecting EC migration, invasion into the perivascular tissue, and the morphologic formation of luminal structures (Ucuzian et al., 2010). The major players in cell matrix interactions are discussed below;

2.3.1 Matrix metalloproteinases (MMPs) and other proteases

The matrix metalloproteinases (MMPs) are a family of extracellular endopeptidases that selectively degrade components of the extracellular matrix. The MMPs are clearly implicated in angiogenesis. The most direct and compelling evidence for this conclusion is that MMP inhibitors, both synthetic and endogenous, inhibit angiogenic responses both *in vitro* and *in vivo*(Hiraoka et al., 1998). The MMPs are a family of secreted and membrane-associated neutral endopeptidases with a diverse spectrum of substrates. These enzymes are produced by a variety of cell types, including epithelial cells, fibroblasts, and inflammatory cells. MMPs reportedly produced by endothelial cells are MMP-1, MMP-2, MMP-9, and MT-1-MMP. Of these, MMP-2 and MT-1-MMP are the most studied for their role in angiogenesis. However, from in vitro analysis of protease activity, it is evident that collectively the MMP family can degrade all known extracellular matrix components. The role of proteases in angiogenesis includes growth of endothelial cells or vascular explants in amnionic membranes, fibrin clots, type I

collagen, or basement membrane matrices(Stetler-Stevenson, 1999). In brief, the mechanism by which MMP may promote angiogenesis is basement membrane and ECM components degradation. The disruption of the basement membrane allows the migration of endothelial cells from existing vessels to the newly created. Moreover, MMP9 plays a key role, in releasing ECM bound factors and increasing their bioavailability which, are important mechanism in angiogenesis. The main factor that stimulates angiogenesis and release by MMP is VEGF (vascular endothelial growth factor). MMP also trigger the integrin intracellular signalling. On the other hand, MMPs can also inhibit the process of angiogenesis by releasing angiostatin caused by plasminogen cleavage and by the influence on endostatin production caused by collagen XVIII cleaving (Jabłońska-Trypuć et al., 2016). The major role in angiogenesis is played by MMP-2, MMP-9, and MT1-MMP. Other MMPs may play a supporting role by complementing the main activity of MMPs.

2.3.2 Major extra cellular matrix molecules in angiogenesis.

Type IV collagen, the main protein component of all basement membranes (BMs), has a crucial role in endothelial cell proliferation and cell behaviour. Various studies report the dependence of angiogenesis on secretion and subsequent extracellular deposition of collagen type IV(Neve et al., 2014). Laminin of BM appears to be involved prevalently in the regulation of the late stages of angiogenesis: it is responsible for cessation of endothelial cells proliferation and pericytes recruitment and vessels stabilization through Notch signalling activation (Bahramsoltani et al., 2014).

Fibronectin is a widely distributed glycoprotein and is a component of plasma in a soluble dimeric form and of cell surface and ECM in a dimeric and multimeric form and is localized in ECM underlying endothelial cells. The arginine-glycine-aspartic acid (RGD) motif was the first sequence of fibronectin found to possess cell-adhesive properties (Sarin et al., 2005) .The binding of RGD sequence to the integrins α5β1 and αvβ3 in endothelial cells initiates the polymerization of fibronectin locally synthesized (Brennan and Hocking, 2016), regulates cytoskeletal organization , and stabilizes cell-matrix adhesion . Additionally, fibronectin controls endothelial cell survival during angiogenesis *in vivo*(Chavakis, 2002).

2.4 Major molecular markers in wound angiogenesis

Angiogenesis plays a crucial role in wound healing by invading the wound clot and organizing into a microvascular network throughout the granulation tissue. This dynamic process is highly regulated by signals from both serum and the surrounding extracellular matrix (ECM) environment (Honnegowda et al., 2015). VEGF and fibroblast growth factor-2 (FGF-2), play key roles in wound angiogenesis. These growth factors are often sequestered in the extracellular matrix, and are liberated by extracellular proteolytic activity in the microenvironment. Again, hypoxia is a potent stimulator of VEGF transcription mediated by HIF-1α(Pathak et al., 2008). VEGF exerts biologic functions through 2 related receptor tyrosine kinases: vascular endothelial growth factor receptors-1 and -2 (VEGFR1 or FLT1, and VEGFR2 or Flk1/KDR). In addition, the VEGF signalling pathway may also involve neuropilin-1 and neuropilin-2, which are used as co

receptors by VEGFR2 and VEGFR1. The interaction of VEGF with its receptors is a critical part of angiogenesis. VEGFR2 mediates most of the mitogenic, survival, and vascular permeability effects of VEGF. The receptors for VEGF—VEGFR1 and VEGFR2—are induced in dermal capillaries during wound healing and during delayed hypersensitivity reactions. Given that, VEGFR 2 plays such an important role in many aspects of blood vessel growth, it would be advantageous to monitor VEGFR2 gene expression in various conditions involving angiogenesis (Zhang, 2004). It is also observed that the expression of VEGF correlated with expression of mRNAs encoding other angiogenic cytokines (angiopoietin-1 and angiopoietin-2), endothelial cell receptor tyrosine kinases (Flt-1, KDR, Tie-1), and endothelial cell adhesion molecules (VE-cadherin, PECAM-1)(Shih et al., 2002).

2.5 Cells in wound angiogenesis

Neovascularization represents an essential component in uncompromised wound healing due to its fundamental impact from the very beginning after skin injury until the end of the wound remodeling. The (micro) vasculature contributes to the initial hemostasis, reduces blood loss and establishes a provisional wound matrix. Blood clot-derived cytokines and growth factors drive the recruitment of pivotal cells that are crucial for the healing process. This provisional wound microenvironment depicts the starting point for new vessel formation and regeneration thereby ensuring the nutritive perfusion of the wound and the delivery of immune cells that remove the cell debris. This complex yet well orchestrated transition phase from the inflammatory to the proliferative stage of

wound repair is a topic of intensive current research(Sorg et al., 2017). In this context, the role of cells involved specifically in wound angiogenesis is reviewed briefly. On injury, neutrophils are recruited to the site of the skin injury and are present for 2–5 days. They release mediators such as TNF-α, IL-1β and IL-6, which amplify the inflammatory response and stimulate VEGF and IL-8 for an adequate repair response. Approximately 3 days after injury macrophages enter the zone of injury and support the ongoing process by performing phagocytosis of pathogens and cell debris as well as by the secretion of growth factors, chemokines and cytokines which influence cell proliferation and protein synthesis, as well as by the production of proteases and their inhibitors, influence ECM content and remodelling. Within the healing wound, macrophages are rarely the sole source of any of these described factors, and many other cell types within the wound, including other immune cells, keratinocytes, fibroblasts, endothelial cells and adipocytes also produce the same factors(Koh and DiPietro, 2011). The blood clot contains fibrin molecules, fibronectin, vitronectin and thrombospondins, forming the provisional matrix as a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells and as a reservoir of growth factors. In addition, platelets influence the infiltration of leukocytes by the release of chemotactic factors. Both platelets and leukocytes release cytokines and growth factors to activate the inflammatory process (IL-1α, IL-1β, IL-6 and TNF-α), stimulate the collagen synthesis (FGF-2, IGF-1, TGF-β), activate the transformation of fibroblasts to myofibroblasts (TGF-β), start the angiogenesis (FGF-2, VEGF-A, HIF-1α, TGF-β) and already support the reepithelialization process (EGF, FGF-2, IGF-1, TGF-α) (Reinke

and Sorg, 2012). The first step in new vessel formation is the binding of growth factors to their receptors on the endothelial cells of existing vessels, thereby activating intracellular signaling cascades. The activated endothelial cells secrete proteolytic enzymes which dissolve the basal lamina. Thus, the endothelial cells are now able to proliferate and migrate into the wound, a process also known as 'sprouting'. The endothelial cells orientate themselves at superficial adhesion molecules, e.g. integrins (αvβ3, αvβ5, α5β1). Furthermore, they release matrix metalloproteinases at the front of proliferation, lysing the surrounding tissue for the ongoing endothelial proliferation. The newly built sprouts form small tubular canals which interconnect to others forming a vessel loop. Thereafter, the new vessels differentiate into arteries and venules and mature by a further stabilization of their vessel wall via the recruitment of pericytes and smooth muscle cells. Finally, the initial blood flow completes the angiogenic process. High density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles are seen in this phase. This high amount of cellular compounds appears to form the so called granulation tissue. The dominating cell in this granulation phase is the fibroblast, which fulfils different functions such as the production of collagen and ECM substances (i.e. fibronectin, glycosaminoglycans, proteoglycans and hyaluronic acid)(Turksen, 2017).

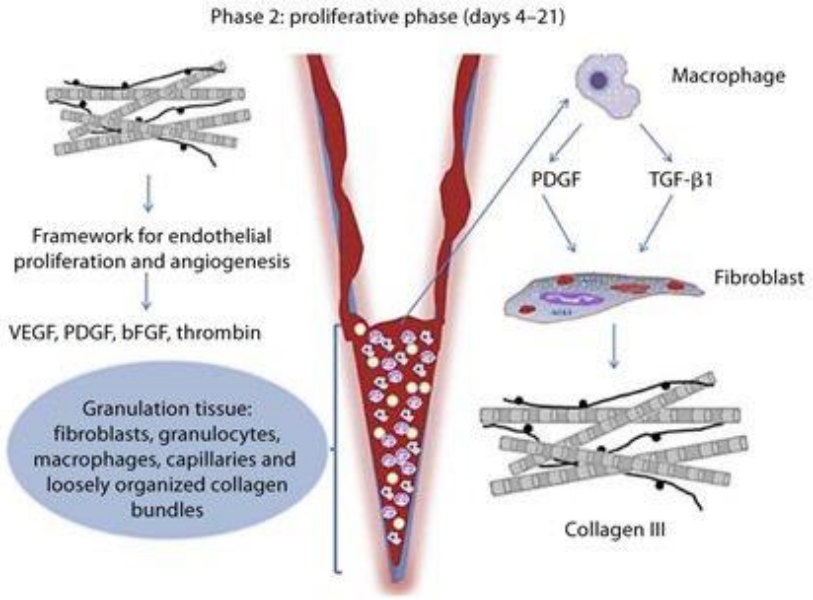


Figure 9: cells involved in wound angiogenesis at the proliferative phase.

2.6 Impaired angiogenesis in non healing wounds.

The hallmark of the proliferative phase of wound healing is robust angiogenesis. Following the oxygen gradient that was established by injury, numerous proangiogenic factors are produced in wounds. These factors, the most notable of which is VEGF, stimulate capillaries to form nascent immature loops and branches. VEGF has been shown to be one of the most important angiogenic factors in wounds, and its production lies downstream of hypoxia. Hypoxia following injury activates hypoxia-inducible factor-1 (HIF-1), a transcriptional activator that promotes angiogenesis by upregulating target genes such VEGF-A. VEGF-A, the main isoform in the wound, binds to its receptors on endothelial cells, directing vessel growth (Okonkwo and DiPietro, 2017). VEGF and other pro-angiogenic factors guide vascular growth to areas of low oxygen starting from the wound periphery into the wound bed. In healing wounds, angiogenesis

supports and intersects with the other ongoing proliferative activities and with the remodelling phase of repair(Reinke and Sorg, 2012). Moreover angiogenesis in normal wound healing relies on a delicate balance between the promotion of vessel growth and proliferation and the promotion of vessel maturation and quiescence. On the other hand, in non healing wounds, insufficient angiogenesis plays a significant role showing decreased vascularity and capillary density. Macrophages, an important cell type of the innate immune system that are required for wound repair, have been shown to have altered functions in diabetic wounds. In normal wounds, macrophages switch from a proinflammatory to pro-reparative phenotype, with the latter supporting tissue regrowth. In diabetic wounds, macrophage deficits include altered phenotypes that fail to stimulate tissue repair(MacLeod and Mansbridge, 2016). Since macrophages are an important source of VEGF and other pro-angiogenic mediators in wounds, the macrophage deficit may be linked to the documented decrease in wound angiogenesis that is seen in diabetic wounds. Impairment in the vasculature integrity is also a prime characteristic of diabetes mellitus. Endothelial cells (ECs) counteract with sustained and prolonged hyperglycemia leading to integrity and functional loss that in turn increases susceptibility for apoptosis along with many other impairments (Raghav, 2018). Research studies found that patients with diabetic foot ulcers have decreased VEGF and VEGFR-2 expression in tissues. VEGF mRNA levels are declined in wounds of diabetic mice as compared with normal mice(Zhou et al., 2015).

2.7 Current therapeutic strategies

2.7.1. Growth factor therapy

A range of GFs including platelet derived GF (PDGF), VEGF, EGF, FGF, TGF, keratinocyte GF (KGF), insulin like GF and HIF have been well documented in reviews for their potential in accelerating the wound healing process. While the clinical results of using growth factors and cytokines are encouraging, many studies involved a small sample size and are disparate in measured endpoints. Therefore, further research is required to provide definitive evidence of efficacy(Barrientos et al., 2014). In the context of therapeutic wound angiogenesis, VEGF has been the most potent proangiogenic growth factor, which is extensively studied. Though, administration of growth factors (GFs) are thought to offer an off the shelf treatment; the dose and time dependent efficacy of the GFs together with the hostile environment of diabetic wound beds impose a major hindrance in the selection of an ideal route for GF delivery. As an alternative, the delivery of therapeutic genes using viral and nonviral vectors, capable of transiently expressing the genes until the recovery of the wounded tissue offers promise(Laiva et al., 2018). The development of implantable biomaterial dressings capable of modulating the release of either single or combinatorial GFs/genes may also offer solutions to this overgrowing problem. Most importantly, achieving favourable benefit–risk balance remains the key to clinical translation. The need to overcome the limitations described above has led to exploring into novel GF delivery systems/techniques that serve to protect the GFs from degradation but at the same time allow controllable release and

reduce the frequency of administration. Alternatively, gene delivery approaches that use deoxyribonucleic acid (DNA) encoding for therapeutic genes could potentially provide a more stable and effective approach to allow sustained and controlled release of therapeutic factors(Gainza et al., 2015). Having recognized the difficulty imposed upon by wound environment on the bioactivity of localized therapeutics, adoption of an interdisciplinary approach for improving the therapeutics delivery is highly anticipated as a promising solution. Gene delivery has the advantage that it can sustain the production of protein of interest *in situ*; however, the lack of clinically approved chemical vectors and the concerns associated with potentially immunogenic viruses still maintains as major limitations. In particular relevance to diabetic wound healing, *in vitro* studies relating to gene transfer or angiogenic effect of a particular GF should be performed with cells harvested from diabetic patients, and the succeeding result could be applied for development of more advanced tissue engineered wound dressings(Laiva et al., 2018).

2.7.2 Cell based strategies

Over past decades, advances in stem cells and regenerative medicine have offered exciting opportunities of developing cell-based alternatives and demonstrated the potential and feasibility of various stem cells for wound healing(Li and Maitz, 2018).

2.7.2.1. Keratinocyte stem cells

The epidermis is mainly comprised of keratinocytes which are renewed and sustained by keratinocyte stem cell (KSC) populations anchored at the membrane in the epidermal-

dermal junction, the hair follicle bulge and the sebaceous gland. KSCs, expressing K5, K14 and p63, are well known for regulating epithelial stratification, hair folliculogenesis and wound repair. The keratinocytes can be easily isolated and expanded in numbers under in vitro cell culture conditions. Keratinocytes produce various bio-factors and cytokines including interleukin (IL)-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, and IL-20 and tumour necrosis factor alpha (TNF-α), which are important for regulating skin regeneration and wound healing. All these properties make them the most important and widely used cells for therapeutic application in burn wound care(Mcheik et al., 2016).

2.7.2.2. Dermal fibroblasts

Dermal fibroblast cells play an important role in normal skin and skin wound healing. They produce the key ECM proteins in the dermis including laminins, fibronectins, collagens, elastic fibres, non-collagen molecules and bio-factors to regulate cell function, migration and the cell-matrix and cell-cell interactions in normal skin homeostasis and wound healing. Dermal fibroblast cells produce many growth factors and cytokines including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF)-AA, transforming growth factor-beta1 (TGF-β1), keratinocyte growth factor (KGF), IL-6 and IL-8 and tissue inhibitors of metalloproteinases.

2.7.2. 3. Mesenchymal stem cells

Numerous reports demonstrate that both autologous and allogenic MSCs, administered both systemically or and locally, exhibit therapeutic potentials promoting cutaneous wound healing and tissue regeneration via the paracrine bio-factors and cytokines and the multi-potency in tissue regeneration. Under specific niche conditions and molecular stimulations, MSCs could be induced to differentiate into multiple tissue-specific cell lineages including osteoblasts, adipocytes, chondrocytes, tenocytes, myocytes, endothelial cells, vascular smooth muscle cells, keratinocytes and sweat gland-like structures. MSCs could produce anti-fibrotic factors and modulate development of hypertrophic scarring(Foubert et al., 2017). Adipose-derived stem cells (ADSCs) and ADSC-conditioned medium appear highly effective in promoting hair growth, and development of functional sweat gland-like structures was observed from transplanting differentiated bone marrow mesenchymal stem cells (BM-MSCs) (Duscher et al., 2016). MSCs promote angiogenesis and vascular stability that are critical for delivering the essential nutrient supply for tissue regeneration and wound healing(Jackson et al., 2007).

2.7.2.4. Induced pluripotent stem cells

iPSC-derived cells could secrete proteins including VEGF, FGF-2, TGF-β, S100A4, GRO, GM-CSF, MCP-1, IL-6 and IL-8 that promote increased proliferation, contraction and migration(Greene et al., 2016). iPSCs as patient specific or allogenic devices demonstrated potential values for clinical applications, research and drug discoveries in

laboratory research. Their safety and efficacy are still to be assessed mainly due to the use of retroviral vectors, associated risk, genetic instability and potential immunogenicity (Revilla et al., 2016). To avoid the risks of mutagenesis and oncogenic transformation associated with retroviral vector, iPSCs are also generated with non-integrative reprogramming strategies using plasmid vectors, episomal plasmid vectors, modified/microRNA or even direct delivery of reprogramming protein factors(Okita et al., 2013).

It is important to point out that cell therapy devices and associated procedures are now highly regulated by global authorities to ensure their bio-safety and efficacy. While animal sourced reagents could cause concerns, unresolved issues also exist in the potential applications using MSCs and iPSCs. It is also important to understand that MSCs from different sources could have different immunomodulation capabilities and varied capacities to proliferate and differentiate to various cells. If allogeneic MSCs are used, their immunogenicity could have impact on their in vivo durability. More studies are needed to define those issues as they could potentially affect the therapeutic outcomes (Hu et al., 2018). In addition, other factors including wound depth, wound bed preparation, and wound infection further complicate the application of cell therapy and lead to variable outcomes. Although progresses have been made to demonstrate the potential and feasibility of various stem cells for chronic wound healing, there are still scientific and technical issues that should be resolved to facilitate the full potential of the cellular devices. More evidence is needed from research and large, randomly controlled trials to define the clinical efficacy and safety of cell therapy(Li and Maitz, 2018).

2.8 Bioengineering MSCs for improved angiogenic gene delivery

Advances in wound research have recognized a variety of angiogenic factors (AFs) for therapy. Of which, as mentioned earlier, vascular endothelial growth factor A (VEGF-A) is a notable inducer of angiogenesis. In addition to modulating endothelial function through autocrine effects, VEGF-A's paracrine potential to activate angiogenesis has been well interrogated(Honnegowda et al., 2015). In wounds, it is understood that hypoxia persuades the expression of a plethora of genes upon induction of hypoxia-inducible factor-1α (Hif-1α). This in return mediates adaptive responses that regulate VEGF-A expression for optimal granulation tissue formation and cell proliferation/survival(Ciarlillo et al., 2017).Systemic or local administration of these potent angiogenic factors are reported to be safe as per accumulated trials, and offers off-the-shelf availability. However, many hurdles lie ahead before this approach could serve as a true human therapy for promoting angiogenesis. At clinical translational level, the burst release of growth factors, proteolytic degradation; requirement of supra-physiological doses; poor bioavailability, enormous cost and ineffective means of growth factor delivery stand challenging and results in poor outcome(Chu and Wang, 2012).The pursuit for delivery vehicles to sustain the bioactivity of therapeutic molecules in a steady and long-term fashion has lead to the exploration of various natural or synthetic materials. This again has its own limitations such as lack of mass availability, batch to batch quality issues, poor tailoring capacity, and biodegradability with associated toxicity. In addition, limitations due to their simple design, poor drug release mechanism, biomaterial selection and matrix/carrier fabrication methods, make it

hard to mimic natural tissue regeneration(Wang et al., 2017).On the other hand, advancements in research have thrown light towards the repair andregenerative contributions of mesenchymal stromal cells (MSCs) in injury and disease. Recent reports favor the development of engineered MSCs for use as a suitable vehicle to deliver therapeutic genes in cancer and for islet transplantation.(Hu et al., 2010),(Chulpanova et al., 2018),(Saulite et al., 2017),(Wu et al., 2011),(Ramírez et al., 2015). In wound repair, the pivotal role of MSCs implicated by their mechanism of differentiation and paracrine signaling,leading to the release of extracellular matrix (ECM) proteins and biomolecules such as GFs, cytokines and chemokines is reported (Lee et al., 2016).Thus, the suitability of exploiting MSCs as a delivery vehicle of angiogenic growth factors for wound regeneration seemed promising. MSCs have recently received tremendous attention for the delivery of therapeutic products to repair neural injury; the amelioration of cardiovascular events; the promotion of bone, cartilage, liver, pancreas, lung, and kidney regeneration (D'souza et al., 2015a) .On exploitation, MSCs can serve as programmed molecule transmitters, addressing better bioavailability of the desired molecule (D'souza et al., 2015a).At clinical aspect, in addition to multilineage differentiation capacity, MSCs regulate immune responses, inflammation and scarring and also possess powerful tissue protective and reparative mechanisms, making these cells attractive for wound healing therapy(Julianto and Rindastuti, 2016).

In the context of an ideal MSC source, human Adipose derived mesenchymal stromal cells (hADMSCs) derived from fat tissue, has gained much attention. Its abundance,

minimally invasive and standardized tissue collection processes and similarity to bone marrow stromal cells (BMSCs) in terms of differentiation potential stands advantageous(Tremolada et al., 2016a). On genetic exploitation of hADMSCs, not only would it serve as an effective delivery vehicle of angiogenic growth factors, but its insufficiency to independently drive adequate angiogenesis in healing could also be addressed(Nauta et al., 2013) through boosted paracrine activity, in addition to its retainedself-differentiation potential. Of the various tools used in engineering MSCs for controlled growth factor delivery, the use of viral vectors such as adeno-associated virus, lenti-virus and retrovirus was efficient but limited for application at translational level. These limitations included the integration of the gene into the host chromosome resulting in undesirable, overproduction of angiogenic factors that inhibit blood vessel formation and the trigger of immune responses against viral components. Other gene transfer methods using non viral vectors, such as synthetic polymers, ultrasound and conventional electric field, are reported less efficient with the requirement for further optimization(Chu and Wang, 2012).

2.9 Advantages of combination therapy using stem cells and bioengineered cells and influence of cell delivery matrix.

Controlled vascular growth is an important component of successful tissue regeneration, as well as for treating various ischemic diseases such as stroke, limb ischemia, and myocardial infarction. Several strategies have been developed to promote vascular growth, including growth factor delivery and cell-based therapy. Despite the efficacy

observed in the animal disease models, these methods still face limitations such as the need for supraphysiological doses for growth factor delivery, or insufficient paracrine release by cells alone(Keeney et al., 2013). One potential strategy to overcome the above limitations is to combine stem cell therapy and gene therapy, whereby stem cells are genetically programmed *ex vivo* prior to transplantation to overexpress desirable therapeutic factors. This approach has been demonstrated in various disease models including hindlimb ischemia, heart disease, bone healing and neural injury, *etc*. However, most gene therapy techniques rely on viral vectors, which are associated with safety concerns such as potential immunogenicity and insertional mutagenesis. Combining stem cells, growth factors through efficient non viral methods could provide a promising strategy for engineering tissues and successful cellular delivery. Virally modified, VEGF-overexpressing mesenchymal stem cells (MSCs) were reported to enhance angiogenesis (Jabbarzadeh et al., 2008) *in vivo* and improve myocardial function. Genetic modification of MSCs with Akt or Bcl-2 gene also improved the therapeutic efficacy of cell transplantation in treating myocardium. Nonviral delivery systems, such as polyethylenimine and Lipofectamine, offer an alternative (Yang et al., 2010) but are often associated with toxicity and typically provide significantly lower transfection efficiency than a viral-based approach. Previous work has shown that untransfected stem cells themselves may secret a broad spectrum of cytokines (Yang et al., 2010) that can mediate ischemic tissue survival and repair. Together with the up-regulated production of VEGF by transfection, these paracrine factors secreted by the stem cells may lead to enhanced angiogenesis, decreased cell apoptosis, and better tissue

survival, relative to VEGF protein alone. Also, recent studies have demonstrated that delivering multiple pro-angiogenic GFs either in combination or sequentially can help to stimulate rapid neovascularization during wound healing(Park and Gerecht, 2014).

In the context of cell delivery matrices, Matrices used for cell delivery are especially important, as they provide support to transplanted cells that is essential for promoting cell survival, retention, and desirable phenotypes. Current materials used as delivery vehicles include natural materials, synthetic polymers, and ceramics. Natural materials such as purified collagen, fibrin, hyaluronic acid, alginate, and chitosan have been extensively used in regenerative medicine and tissue engineering. Materials derived from natural sources can be biologically active, promote cell adhesion and growth, and enzymatically or hydrolytically degradable. However, natural materials display lot-to-lot variability, risk of immunogenicity and pathogen transmission, and structural complexity that renders modifications difficult. Synthetic polymers provide an alternative to natural materials as cell-delivery vehicles. Because of their defined chemical composition, synthetic materials are often reproducible and can be modified to control material properties such as degradation rate, mechanical properties, and shape. As most synthetic polymers lack cell adhesion sites, polymers usually need to be chemically modified to support cell adhesion and other bio-functionalities, and this yields the opportunity of engineering specificity into the material. Tuning of the degradation rate is a strong advantage over ceramics and natural polymers, as synthetic polymers can be used to deliver therapeutic molecules at controlled and defined rates(Cheng and García, 2013). Although material stiffness may play a role in cell differentiation in well-controlled

cultures *in vitro*, stiffness is generally coupled with degradability properties which, for *in vivo* cell delivery applications, modulate engraftment, proliferation, cell survival, and vascularization. The matrix environment should provide directional cues to the transplanted cells in terms of adhesive ligands, integrin specificity, and a carefully engineered growth factor microenvironment. However, current materials are associated with one or more challenges in biocompatibility, shear-induced cell death, lack of control over cellular phenotype, lack of macroporosity and remodeling, and relatively weak mechanical strength. Future cell-based strategies must hence consider the temporal and spatial complexities of the *in vivo* environment and the effect or role of cellular retention and engraftment for regenerative medicine. Future considerations for engineering a cell delivery vehicle include dynamic or triggered changes in bioactivity , as well as multiple growth factor delivery for combinational effects in directing cell fate(Salimath et al., 2012). Understanding, harnessing, and engineering these aspects into a cell-delivery vehicle may finally enable us to use cells to their full potential as a tissue repair therapy strategy.

2.10. Angiogenic strategies in *in-vitro* and *in-vivo* tissue engineering

In tissue engineering, the generation and functional maintenance of dense voluminous tissues is mainly restricted due to insufficient nutrient supply. Larger three-dimensional constructs, which exceed the nutrient diffusion limit, become necrotic and/or apoptotic in long-term culture if not provided with an appropriate vascularisation(Kress et al., 2018). In tissue engineering research, angiogenesis is hence essential to promote micro-

vascular network inside engineered tissue constructs, mimicking a functional blood vessel *in vivo*. Micro-vascular network can be used to maintain adequate tissue oxygenation, nutrient transfer and waste removal. One of the problems faced by angiogenesis researchers is to find suitable *in vitro* assays and methods for assessing the effect of regulators on angiogenesis and micro-vessel formation(Ishak et al., 2014). In tissue engineering, bio-printing of vascular structures poses a highly promising attempt in copying the native environment for cellular nutrient supply and waste removal. However, the technology is not yet able to generate fully functional capillary structures and clinically applicable vascular vessel systems(Kress et al., 2018; Yu et al., 2013). Other approaches in regenerative medicine try to induce pre-vascularized structures *in vivo* to improve tissue integration and functional restoration. Pre-vascularization is induced either by *in vivo* implantation of a proangiogenic scaffold prior cell transfer or by endothelial cell vascular self-assembly before implantation, which led to promising results of graft integration into host tissues. However, these strategies mostly need two surgical interventions and cells are seeded rather loosely onto a pre-vascularized scaffold. It also takes several weeks until the pre-formed vessel-like system is fully connected to the host's blood system(Takebe et al., 2014). Therefore, both approaches limit clinical applicability since larger tissues ideally require an immediate adequate perfusion with nutrients and oxygen. Common *in vitro* strategies aim to promote the vascularization of engineered tissues by either using growth factors–releasing scaffolds, or by co-culturing mature endothelial cells (EC), or bone marrow-/adipose tissue stromal cell-derived endothelial progenitors cells (EPC) with mesenchymal stem/stromal cells

(MSC) or perivascular cells or by using pre-formed micro-fabricated engineered vasculature. Despite being valid approaches, these strategies present limitations. These limitations include difficulty in matching growth factor type and time-releasing profile, identifying the proper cell types and their ratio, and selecting suitable fluid shear stresses (SS) within the micro-scaffold. Moreover, an *in vitro* 3D model able to summarize the key components of the angiogenic process, like the dynamic interplay between EC and other vascular/mural cells (e.g. smooth muscle cells, pericytes and MSC), the supporting extracellular matrix (ECM) and/or the basement membrane deposition, and the exposure to the blood hydrodynamic-based shears does not yet exist(Cerino et al., 2017).

2.11 Role of 3-D scaffold and cell niche for cell differentiation and influence of supporting cells in the study of angiogenic response.

Traditional two-dimensional (2D) cultures require stem cell growth to occur in monolayers atop stromal layers that support stem cell proliferation or atop membranes with or without growth factors. Furthermore, 2D culture systems face difficulties in meeting the requirements of many downstream applications due to the inherent heterogeneity, limited scalability or reproducibility and incompatibility with the development of *in vitro* models that accurately simulate the native stem cell niche. The precise control of cell behaviour is a crucial aspect that must be taken into account when using in vitro stem cell models. 3D culture on other hand can significantly improve stem cell viability and function offering a higher degree of efficiency, consistency, and predictability to the resulting stem cell manufacturing platform, which makes it a more

promising tool for preclinical research(Meng et al., 2014). Although significant advances have been made recently in the development of artificial kidneys, pancreata, livers, cardiac muscle, skeletal muscle, and blood vessels a better understanding of the cellular mechanisms that guide stem cell behavior in native and engineered 3D microenvironments would facilitate even greater progress. Current efforts aim to provide proper 3D structural, biochemical, mechanical, and stimulatory environments for stem cells(Bardelli and Moccetti, 2017). Mature cells cultured in 3D matrices exhibit altered phenotypes that inhibit their proliferative nature and enhance their ability to form higher order structures. Furthermore, mature cell growth in a 3D matrix enhances stem cell potential by providing the dynamic interface that naturally occurs between the stem cells and the matrix(Cerino et al., 2017). For example, in an angiogenesis study by Benelli and Albini, the authors found that vascular endothelial cells could form capillary-like structures with a lumen when cultured on basement membrane gels. Further reports showed that cell density and time affect the morphology/differentiation of vascular endothelial cells grown on 3D basement membranes. Additional evidence has suggested that differentiated cells that were cultured in 3D matrices can be more readily transplanted into animals for further investigation(Yamamoto et al., 2014).

Moreover, with the growing hope of cell-based regenerative medicine, successful implementation of such approaches hinges on the ability to predictably and reliably control cell fate decisions. In the body, such decisions are governed not only via genetic and epigenetic means (i.e., cell intrinsic), but also by many different microenvironmental features (i.e., cell extrinsic), including soluble and insoluble morphogens, cues from

other cells, and the extracellular matrix (ECM). Collectively, these factors constitute the stem cell niche, which helps maintain stem cell quiescence by regulating self-renewal and promotes their ability to support tissue homeostasis by controlling differentiation. However, the complexity and integration of these various elements remains poorly understood. Artificial stem cell niches may augment efforts to identify the specific cues that define stem cell niches, and thereby pave the way for the successful use of stem cells in regenerative medicine(Putnam, 2014). While complete recapitulation of stem cell microenvironments *ex vivo* has yet to be achieved, an improved understanding of *in vivo* niches will greatly facilitate this goal.

In the context of angiogenesis, the vascular niche is described as below; the walls of large and medium-sized vessels (arteries and veins) are composed of three concentrically arranged layers. These are the tunica intima (lumen/blood facing region), the tunica media and the outer tunica adventitia, with endothelial cells, smooth muscle cells and fibroblasts or adventitial stromal cells being the predominant cell types associated with these three respective regions. The adventitia also contains nerves, which control vessel wall contractility, and microvessels penetrating the media and intima are formed in the adventitia(Watt et al., 2010). Endothelial cells within the intima of large vessels, such as the aorta, were reported to be mostly quiescent in the adult under normal homeostatic conditions. Over the past years, the view of the vascular system as a sole provider for oxygen and nutrients has dramatically changed. First, endothelial cells, which form the inner layer of the blood vessel wall, are in direct contact at the luminal face with blood-derived elements and on the outer face with other

various parenchymal cell types facilitating the communication between all these components, creating a functional vascular niche. Besides endothelial cells, pericytes have recently moved into the focus of attention as an essential player for different roles of the vascular niche. Classically, pericytes contribute to vascular homeostasis such as vessel stabilization, blood flow regulation, and the formation of the blood brain barrier. However, a number of studies have shown that pericytes display unexpected functions, beyond vascular homeostasis, as this cell type supports tissue repair and regeneration. Indeed, pericytes are multipotent being able to give rise to cells that form the fibrotic scar after acute injuries. In addition, pericytes may act as stromal cells and modulate the function of neighbouring local stem and progenitor cells in their regenerative activities(Rivera et al., 2017).

Ultimately there are a number of significant challenges researchers must overcome before a stem cell and biomaterial-based option for wound healing therapy can be used in the clinical setting. In addition to overcoming the difficulty of obtaining a renewable source of stem cells in large quantities, major advances in both the understanding of the local cues necessary for stem cell survival and function and development of biomaterials necessary to promote these functions is important. These polymer-based biomaterials should be tested in a variety of in vivo and in vitro studies prior to their application with the stem cells on a clinical level. The future lies in utilizing high throughput arrays to test the functionality of new biomaterials for their usage in stem cell delivery in short periods of time while wasting few materials, ultimately allowing for a more rapidly developed end product. Further progress in this field involves utilizing a hybrid

approach to produce personalized tissue engineered constructs by using patient specific cells, biomimetic matrices (e.g., collagen, gelatin, chitosan, Fibrin etc.) and bioactive stimuli (e.g., Fibrin, laminin, silk protein, GAG etc.) to promote a regenerative healing. Furthermore, multiple cell types may also be employed with spatial control to generate skin tissues ex vivo, along with the requisite vascular supply(Dash et al., 2018).

2.12. Assays to validate effect of bioengineering in in-vitro tissue angiogenesis.

Investigations of the molecular mechanisms of angiogenesis require assays that resolve individual aspects of the angiogenic process with precision, accuracy, and reproducibility. *In vitro* angiogenesis assays (i.e., tubulogenesis, proliferation) only recapitulate a few steps of the angiogenic process and, though very reproducible, are not necessarily accurate reflections of blood vessel formation in contrast to *in vivo* models. In general, *in vitro* assays offer superior precision and control of components of the angiogenic process because they are isolated from confounding variables resident in the whole organism(Irvin et al., 2014). Fundamentally, these *in vitro* studies are based on purified endothelial cell cultures or carefully controlled co-culture with another cell type (i.e., fibroblasts, immune cells, pericytes, tumor cells). These assays allow researchers to study particular mechanisms or drug intervention in defined elements of angiogenesis while controlling nearly all other influencing variables. Such studies have been helpful in the identification of selective target molecules and/or key pathways controlling endothelial cell functions. *In vitro* endothelial cell assays typically assess proliferation, migration, and tube formation(Staton et al., 2009). The quantitative

capacity of these *in vitro* studies is particularly important, as it provides a confidence that is not readily acquired with more complicated *in vivo* experiments. The major and commonly used *in vitro* angiogenesis assays are described below(Cimpean et al., 2011);

(1) Proliferation: Assays that monitor endothelial cell proliferation in culture have the benefit of being rapid, reproducible, precise, and quantifiable. These assays can be used to analyze and compare the basal growth of endothelial cells isolated from a variety of sources, including primary human cell cultures (from aorta, dermal vasculature, or adipose tissue), cells obtained from syngeneic or transgenic mice, such as the immortomice(Shao and Guo, 2004), and established cell cultures. The rate of growth determines the ability of endothelial cells to respond to external stimuli (i.e., matrices, forces, growth factors). Measures of endothelial cell proliferation include traditional proliferation assays that can be achieved with manual count or automated cell counters, MTT assays that measure metabolic reduction in cells of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] from a yellow tetrazole to a purple formazan, and tritiated-thymidine or BrdU (Bromo-deoxyuridine) incorporation into duplicating DNA.

(2) Migration: Migration assays are commonly used to determine the ability of an intrinsic molecular mechanism or an externally provided regulator, including pro- and/or anti-angiogenic factors, matrices (natural or bioengineered materials), and/or cell types (i.e., fibroblasts and/or immune cells) to promote or

diminish endothelial cell migration. Boyden chambers position endothelial cells on one side of a filter (with pores of different-sized cut-offs) and facilitate migration of the cells toward a chemoattractant on the other side of the filter, or toward an extracellular matrix coated on the other side of the filter. Quantitation of endothelial cell migration is accomplished by analyzing the number of cells that completely traverse the filter in a finite time. The possibility to "create" chemotactic gradients provides the advantage of allowing detailed mechanistic analyses of endothelial cell migration. Although frequently used, drawbacks of the Boyden chamber migration assays include the difficult and time-consuming nature of quantitation, loss of the chemotactic gradient over time, and the inability to incorporate mechanical stimuli. In addition to the Boyden chamber, the "scratch assay" can be used as a two-dimensional model of endothelial cell migration. In a scratch assay, an area of an endothelial cell monolayer is denuded via scratching or other means, and the ability of endothelial cells to migrate into the denudation is measured over time. These scratch assays have the advantages of being fast and allowing continuous monitoring of angiogenesis

(3) Tubulogenesis: Tube formation assays are conducted by placing endothelial cells on or within an extracellular matrix (fibrin, collagen, or Matrigel) and monitoring tube formation over time. Quantization is accomplished by counting the lengths and/or number of the formed tubes and/or the number of branch points. Tube formation assays can be two-dimensional (plating on top of a thin layer of extracellular matrix) or three-dimensional (placing cells within an

extracellular matrix). These assays are rapid, reliable, and sensitive to composition and mechanical properties of the extracellular matrix(Staton et al., 2009)

2.13 *In vivo* models to study the effect of bioengineering on wound angiogenesis

Due to the complex cellular and molecular activities of angiogenic reactions, *in vivo* studies are more informative than *in vitro* studies providing that the biology of the assay and the experimental design are relevant; *in vitro* studies of ECs are, however, in many instances a necessary complement to *in vivo* experiments. *In vivo* assays typically investigate drug effects on angiogenesis and validate observations about the molecular mechanisms of angiogenesis from *in vitro* studies. As a result, these assays have proven indispensable to the understanding of angiogenesis and the development of angiogenic therapies. Since newly formed microvessels are delicate, histological microscopy provides the most detailed information on *in vivo* angiogenesis. Moreover, mammalian systems are considered to be more representative of human pathophysiology than, for example, the embryonic avian chorioallantoic membrane (CAM) or embryonic zebrafish assay(Norrby, 2006).

In the context of studying angiogenesis in wound regeneration, the *in vivo* wound healing assay model seems to be a useful model to evaluate angiogenesis and vascular maturation/remodelling. Wound healing models are usually performed on the skin. Usually, two circular, trans-dermal wounds are created on the back of anesthetized animals, allowing one wound to serve as control while topical treatment can be

administered on the other. Wound size, scar formation and re-epithelization of the wounds should be recorded daily by photography and by measuring the wound area with calipers, In this model, treatment is given either systemically by oral administration or injection, or preferably topically on just one of the two wounds, can consist of pro- or anti-angiogenic compounds, and their effects on both the regenerative angiogenesis as well as on vessel morphology and function is determined post mortem after the regenerated tissue has been excised, fixed and stained.

Currently, genetically manipulated mice have been generated for most of the genes important for angiogenesis and vascular biology. Such mice include conditional or global knock-outs or mice over-expressing angiogenic factors in defined tissues. As the murine and human vasculatures are highly similar, these tools have been very valuable in defining the role of angiogenesis and distinct angiogenic factors in the onset and progression of various human diseases(Eklund et al., 2013). Moreover, small animals like mice and rabbits are inexpensive, easily obtainable, and require less space, food, and water. Additionally, they often have multiple offspring, which develop quickly allowing experiments to proceed through multiple generations. Small animals usually have accelerated modes of healing compared to humans, thus experiment duration lasts for days, as opposed to weeks or months in human experiments. Again, small mammals can easily be altered genetically and provide a wound model capable of approximating defective human conditions such as diabetes, immunological deficiencies, and obesity(Perez and Davis, 2008). Despite the convenience and frequent use of small mammals in wound healing research, there are some limitations. Small mammal models

also have several anatomical and physiological differences. Small mammals have a follicular pattern and hair growth cycle, which is different in comparison to humans and tend to be covered in dense fur(Plikus and Chuong, 2008). They have relatively thin epidermal and dermal layers and are considered "loose-skinned." Although some hairless and tight-skinned mouse models exist, skin thickness and healing mechanisms differ from those in humans. The epidermis is extremely thin and consists of only a few cell layers where the hair follicles are empty, and the subepithelial layer is rich with bundles of skeletal muscle fibers. Particular problems arise in animals with a subcutaneous panniculus carnosus muscle, which aids wound repair by contraction. Although rat, mouse, rabbit, and guinea pig wound models exist, swine skin is the most similar to humans and has been shown to be an excellent tool to evaluate wound healing therapies(Sharpe and Martin, 2013). Porcine skin is structurally similar to human skin with similar epidermal thickness and dermal-epidermal thickness ratios(Summerfield et al., 2015).Pig skin and human skin share similar patterns of hair follicles and blood vessels. Biochemically, pigs contain dermal collagen and a dermal elastic content that is more similar to humans than other commonly used mammals (Marquet et al., 2014). Additionally, pigs and humans have similar physical and molecular responses to various growth factors (Marquet et al., 2014).Unfortunately, pigs have a significant cost disadvantage to smaller animals and because the amount of wound contraction varies depending on where the wound is made, strict standardized procedures must be used(Perez and Davis, 2008). Pigs also grow quickly and can become difficult to handle should the study continue past a few months.

2.14. Assays to validate the effect of bioengineering in guided *in vivo* tissue angiogenesis

Considering the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions, a single assay that is optimal for all situations has not yet been described, although ingenious ways have been developed for measuring angiogenic processes. However, the most consistent limitation in all these approaches has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenic response. (Jain et al., 1997) summaries, "An ideal assay for quantitative angiogenesis studies must satisfy the following requirements: (1) the release rate [R] and the spatial and temporal concentration distribution [C] of angiogenic factor(s)/inhibitor(s) should be known for generating the dose response curves; (2) if neoplastic cells are used as a source of angiogenic factors, they should be genetically well defined in terms of oncogene expression and production of growth factors (stimulators and inhibitors); (3) the assay should provide a quantitative measure of the structure of the new vasculature (e.g., vascular length [L], surface area [A], volume [V], number of vessels in the network [N], fractal dimensions of the network [Df], and extent of basement membrane [BM]; (4) it should provide a quantitative measure of the functional characteristics of the new vasculature (e.g., EC migration [MR], proliferation rate [PR], canalization rate [CR], blood flow rate [F], and vascular permeability [P]; (5) there should be a clear distribution between newly formed and pre-existing host vessels; (6) tissue damage should be avoided, since it may lead to formation of new vessels; (7) any response seen in vitro should be confirmed in vivo; (8) such an assay should permit long-term and, if

possible, non-invasive monitoring; and (9) it should be cost-effective, rapid, easy to use (routine), reproducible and reliable". Unfortunately, no single assay fulfils anything like all of these criteria. Current *in vivo* models (i.e., sponge assays, chorioallantoic membrane assays, cornea angiogenesis assays) evaluate an entire process that is biologically accurate, but they possess little access to and limited control of individual aspects, thereby reducing their reproducibility(Irvin et al., 2014). There is no "gold standard" assay, and therefore angiogenesis studies rely heavily on the appropriate selection of multiple assays.

2.14.1. Tissue Analysis for determination of angiogenesis and for interpreting the final outcome

Some of the most commonly used assays in determination of angiogenesis quality through post in-*vivo* model study are,

(1) **Hematoxylin/Eosin staining:** Following deparaffinization with xylene and rehydration using 99.7 %, 95 % and 70 % solutions of ethanol, the tissue slides are stained for 3-5 min. with hematoxylin. This results in a clear blue/purple staining of the nuclei of the cells. Eosin is then used to stain the cytoplasmic contents of the cells pink/red. Depending on the different compartments within the cell, different shades of blue to pink can be observed. With this method, the tissue can be studied with regard to the size and distribution of cells. Blood vessels however, cannot be visualized using this method.

(2) **Whole mount immunohistochemistry:** The use of whole mount immunohistochemistry allows investigations of the vasculature in tissues, especially in regard to its structure and functionality. Different primary antibodies can be used to visualize endothelial cells. These antibodies target for example CD31, CD34 or isolectin all of which are expressed on endothelial cells. This provides a general overview of the vasculature in the tissue and its structure, i.e. whether the vessels are organized or disorganized, if their diameter is normal or if they are dilated and also gives information on the presence of microvessels and capillaries that might have been newly formed. The tissue vascularity can be assessed by calculating the area of stained vessel signals per field.

(3) **Immunohistochemistry on paraffin sections:** In this approach, in contrast to whole mount immunohistochemistry where the thickness of the tissue varies as it is cut by hand, rather uniform tissue sections are prepared using a microtome. Immunohistochemistry on paraffin sections is based on the same principle as whole mount immunohistochemistry: the tissue is incubated with a primary antibody against a protein of interest. A fluorochrome or horse radish peroxidase (HRP)-labeled secondary antibody is then used to visualize the protein of interest. It is helpful to counterstain the tissue with DAPI, propidium iodide or Hoechst (for fluorescent stainings) or hematoxylin (for chromogenic HRP stainings) to visualize the nuclei of cells and thereby provide additional information on the structure of the tissue. To study active angiogenesis, proliferating endothelial cells can be visualized by double staining with

antibodies against an endothelial cell marker, such as CD31, and a proliferation marker, such as PCNA or Ki67.

(4) Hypoxia staining: Since hypoxia is one of the driving forces of angiogenesis, detection of the presence of hypoxia in tissues could also be performed. Hypoxyprobe-1 (pimonidazole hydrochloride) staining can be used to detect cell and tissue hypoxia. Pimonidazole hydrochloride has a molecular weight of 290.8 kD, ultraviolet absorbance at 324 nm and a plasma half-life of approximately 25 minutes in mice. The detection of hypoxia in tissues can be performed by intravenous or intraperitoneal injection or oral ingestion of pimonidazole hydrochloride at a dosage of 60 mg/kg, 15-90 minutes before sacrificing mice. Dissected tissues should be fixed in 4% PFA followed by paraffin embedding. Paraffin-embedded tissue sections of 3-5 μm is further stained using a peroxidase conjugated anti-pimonidazole antibodies and counterstained with nuclei staining.

2.15. Summary

From the literature reviewed in this chapter, the current strategies used in addressing wound angiogenesis have been explored. The mechanism of normal wound angiogenesis, molecular deviations in non healing wounds, current cell and growth factor therapy strategies, their limitations and the current interest of effectively tuning ADMSCs to deliver potent angiogenic factors has been reviewed. The lack of a suitable strategy for effective tuning of ADMSCs has been identified. No studies have been

reported so far exploring the use of neon transfection system in exploiting ADMSCs to deliver HIF-1 α and VEGF-A independently. More over the need for more studies in demonstrating the success of combination therapy with special emphasis to requirement of assessing these factors *in vitro* and *in vivo* has been projected. In view to the importance of cell matrix delivery and the atmost need for establishment of angiogenesis in tissue engineered constructs, it is hence clear, that the problem identified for the current study is significant.

CHAPTER 3: MATERIALS AND METHODS

This chapter describes materials and methods used for genetic engineering of human ADMSCs and their application demonstrated in tissue constructs and in acute wound healing animal models for the prospective application in therapeutic angiogenesis. Major focus was to standardize conditions for efficient transfection and delivery of two major pro-angiogenic molecules (VEGF-A and Hif-1α) in hADMSCs. The participation of hADMSCs via autocrine or paracrine differentiation towards endothelial lineage and angiogenesis is also evaluated and described. Biodegradable, electrospun ter-polymeric scaffolds combined with biomimetic matrix was developed to make 3-D structure, for differentiation of transfected hADMSCs towards dermal lineage cells i.e endothelial and fibroblasts, as part of generating an in house vascularised tissue construct. Differentiation of cells to both dermal fibroblasts and endothelial cells were tracked using state-of-art techniques. Application of transfected hADMSCs in diabetic rabbit and non diabetic rabbit acute wounds is also described. The details of materials used and methodologies adopted are described in this chapter.

3.1. Isolation, culture & characterization of hADMSCs

Adipose tissue was collected from cardiac surgery department, SCTIMST with Institutional Ethics Committee's (IEC-SCTIMST) and Institutional Committee for Stem Cell Research (IC-SCR-SCTIMST) approvals. The hADMSCs were isolated as per the modified protocol published by (Zhu et al., 2008) . Briefly, adipose tissue was

washed 3-4 times with Hank's Balanced Salt Solution (HBSS) and enzymatically digested using 0.05% collagenase NB4 Standard Grade (SERVA Electrophoresis) in a shaking incubator at 120 rpm, 37° C for 30 minutes. Enzymatic digestion was neutralized with 10% FBS, followed by centrifugation at 400g for 6 minutes. The pellet was re-suspended with HBSS, filtered through a 70µm cell strainer (BD Falcon) and centrifuged at 400g to remove floating adipocytes. Basal medium (BM) constituting low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco), and antibiotic/antimycotic (AB/AM) solution (Invitrogen) was used to gently re-suspend and seed the stromal vascular fraction (SVF) onto a tissue culture polystyrene dish (TCPS). Cells were incubated at 37° C with 5% atmospheric CO2. Plastic adherent, spindle-shaped cells were grown to ~80% confluence, trypsinized and propagated till passage 3 (P3). P3 cells were characterized as stem cells in accordance to the standards laid by the International Society for Cellular Therapy (ISCT); expression of CD90, CD105, and CD73 as positive cell surface markers, CD34, CD14 as negative cell surface markers [Table 1], was quantified by flow cytometry using FlowJo software (Tree star inc., USA) .

SL. NO	Name	Isotype	Source	Catalog No.
1	CD 105 PE	Mouse IgG	Santa Cruz	sc-18838
2	CD 73 PE	Mouse IgG	Biolegend	344003
3	CD 90	Mouse IgG	Thermo Scientific	MA1-35307
4	CD 14 FITC	Mouse IgG	Millipore	CBL453F
5	CD 34 AF 647	Mouse IgG	Santa Cruz	sc-7324

Table 1: List of antibodies used for characterization of hADMSCs
FITC, fluorescein isothiocyanate; IgG, immunoglobin G; PE, phycoerythrin; AF, Alexa Fluor

The plasticity of hADMSCs was also verified using commercially available Adipogenic-, Chondrogenic- and Osteogenic differentiation kits (StemPro Invitrogen), as per manufacturer's instruction. The acquisition of the adipogenic, chondrogenic and osteogenic lineage was further confirmed by staining the cell cultures with 2% Oil Red-O solution, Toluidine blue and Alizarin Red, respectively.

3.2. Transfection of hADMSCs

Passage 3 (P3) hADMSCs from at least 6 independent donors, at 5 x 10^6 cells/ml were transfected with Institute Biosafety Committee's approval, using commercial ready to use mammalian plasmid encoding human VEGF-A transcript variant 4 gene with C terminal green fluorescent protein (GFP) Spark tag (Cat No: HG11066-ACG, Sino

Biological Inc, China) [Figure 10(A)]and human HIF-1α gene with C terminal green fluorescent protein (GFP) Spark tag (Cat No: HG11977-ACG, Sino Biological Inc, China) [Figure 10(B)].The ORF, preceded with a cytomegalovirus (CMV) promoter, is reported to confer transient expression (Moore et al., 2010). Neon® Transfection System (Cat No: MPK1096, Invitrogen) was optimized at parameters: pipette tip type: 10µl, pulse voltage: 1500v, pulse width: 20ms and pulse number 3 (Halim et al., 2014). Transfected cells were maintained in basal medium (BM) and culture supernatant was harvested every 48h.

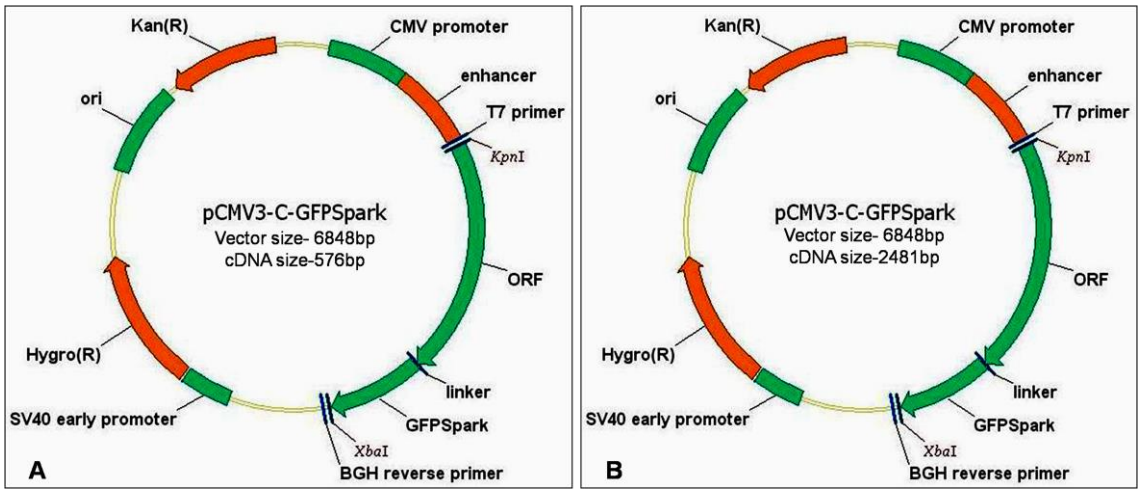


Figure 10: Vector Map: *(A) pCMV3-C-GFPSpark plasmid (Kan: Kanamycin resistant gene) with 575bp cDNA region containing human VEGF-A gene sequence, with the cytomegalovirus (CMV) promoter. (B) pCMV3-C-GFPSpark plasmid (Kan: Kanamycin resistant gene) with 2481bp cDNA region containing the human Hif-1α gene sequence, with the cytomegalovirus (CMV) promoter. Color images availed from the online source at www.sinobiologicals.com.*

3.3. Effect of electroporation on hADMSCs

3.3.1. Cell viability assay

Cell viability was determined 48h post-electroporation of three independent donors, by MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] assay as described (Zeng et al., 2007) in duplicates for each sample. Briefly, Briefly, transfected hADMSCs were seeded in 96-well, flat bottom culture plates at a density of 5000cells/well in basal growth medium and incubated for 48h at 37°C in 5% CO2 atmosphere. The wells were washed with PBS and 100µL of the working MTT dye in DMEM media was added and further incubated for 2h, 200µL of DMSO was added and incubated in dark for 5 min. Non electroporated hADMSCs were used as the control in this assay. The relative viability of electroporated hADMSCs was examined by measuring the amount of purple formazan formed by the MTT reaction using a microplate reader (iMark Microplate Reader, BIO-RAD).The experiment was done in triplicate and absorbance was read at 570 nm. Percentage cell viability was calculated as T/C × 100 (where T is Test absorbance and C is Control absorbance).

3.3.2. Transfection efficiency

24h post-transfection, cells in culture were analyzed for GFP expression (Leica Microsystems, DMIRB, Germany) to confirm gene transfer. Transfection efficiency was

determined as percentage of GFP expressing cells in 20 randomly chosen fields using imageJ software, as described(Zhao et al., 2016) , in 3 separate donor cell experiments.

3.3.3. Stem-ness of transfected Cells

The influence of Neon® transfection system on cell's morphology from three independent experiments was assessed using phase contrast microscopic. Bioengineered hADMSCs was subjected to tri-lineage differentiation (adipogenic, osteogenic and chondrogenic) using commercial induction medium (Invitrogen, USA), to confirm maintenance of stem cell property. Briefly, 10,000 cells/cm^2 was cultured in BM for 24 hours. The medium was then replaced with adipogenesis/osteogenesis/chondrogenic differentiation medium respectively and the cells were incubated at 37°C with 5% CO2 . The differentiation medium was replenished every fourth day for an induction period of 21 days. After induction, the cells were fixed with 3.7% formaldehyde, rinsed with PBS and the differentiation was confirmed by Oil Red O / Alizarin Red/ Toluidine blue staining, respectively.

3.3.4. Transfection outcome on angiogenic factor release

VEGF-A and HIF-1α being released systemically/into cell culture supernatants (Heikal et al., 2018; Choudhry and Harris, 2018), were quantified using enzyme-linked immunosorbent assay (ELISA) kits (cat No.ELH-VEGF-1; Ray Biotech and Cat.No:ab171577, Abcam) as per manufacturer's instruction, respectively, using a

micro-plate reader (iMark Microplate Reader, BIO-RAD). Angiogenic factor (AFs) release in the secretome of VEGF-A over-expressed hADMSCs (Vegf-s-e-hADMSCs) and in the secretome of HIF-1α over-expressed hADMSCs (Hif-1α-s-e-hADMSCs) from 3 independent donor cell transfection experiments were quantified on 2d, 4d, 6d, 8d, 10d, 12d & 14d. Secretome from non electroporated hADMSCs (s-n-hADMSCs) on respective time periods served as comparative control. Since VEGF-A is a downstream target of HIF-1α, its concentration in Hif-1α-s-e-hADMSCs was also examined.

3.5. Effect of transfection on endothelial lineage commitment

3.5.1. Transcriptional level Analysis

Effect of hypoxic exposure on transfected hADMSCs in directing endothelial trans-differentiation was evaluated using selected endothelial specific markers by reverse transcriptase-quantitative real-time polymerase chain reaction (qRT-PCR). VEGF-A electroporated hADMSCs (Vegf-e-hADMSCs), HIF-1α electroporated hADMSCs (Hif-1α-e-hADMSCs) and non-engineered hADMSCs (n-hADMSCs) were subjected to hypoxia (5% O2, 95% N2 at 25psi) for 6h in a hypoxia chamber set up (Stem Cell Technologies) and subsequently cultured for 3d. Total RNA was extracted, using Trizol (Invitrogen, USA) according to manufacturer's protocol. 1 µg of RNA was converted to cDNA using superscript III reverse transcriptase enzyme (Invitrogen). 250ng cDNA was mixed with respective forward and reverse primers (Table 2) and 12.5µl of OrionX 2X Real Time PCR master mix was added (Origin, India).40 cycles of reaction was

performed using Chromo 4 system (Bio-Rad, USA) at annealing temperatures ; 54℃, 55℃ 48°C and 57℃ respectively. For each gene, assessment of quality and specificity was performed by examining PCR melt curves. Cycle threshold (Ct) value of target gene was analyzed after normalization to Ct values of GAPDH. Fold change ($2^{-\Delta\Delta Ct}$) was calculated by comparing mRNA expression levels extracted from n-hADMSCs. Reference gene was GAPDH. The experiment was performed in triplicates from three independent donor cells.

Name	**Forward**	**Reverse**
CD31	5'CAGTCATTACGGTCACAAT3'	5'CTGAGGACACTTGAACTTC3'
tPA	5'ATGGGAAGACATGAATGCAC3'	5'GAAAGGGGAAGGAGACTTGA3'
VCAM	5'CCTCCTTAATAATACCTGCCATTG-3'	5'TCTGTGCTTCTACAAGACTATATGA3'
Flk-1	5'CCTCTACTCCAGTAAACCTGATTGGG3'	5'TGTTCCCAGCATTTCACACTATGG3'
GAPDH	5'CGCTCTCTGCTCCTCCTGTT'9	5'CCATGGTGTCTGAGCGATGT'9

Table 2. List of primers used for mRNA quantification for assessment of endothelial lineage commitment

3.5.2. Translational level Analysis

Transfected hADMSCs on exposure to hypoxia were assessed for endothelial specific markers at protein level. Cells were fixed with 4% paraformaldehyde for 10min, washed in PBS, blocked with 3% BSA for 30 min, incubated with 100µl anti-human CD31 (Cat No.303110, Biolegend) at 1:500 dilution and 100µl anti-human Flk-1 (Cat No.sc-6251, Santa Cruz Biotechnology) antibody at 1:500 dilution for 1h, followed by corresponding secondary antibody (Cat No. ab6787,Abcam) at 1:1000 dilution. Cells were stained with diamidino-2-phenylindole (DAPI) for 10 min and examined by fluorescence microscopy (Leica Microsystems,,DMIRB, Germany).n-hADMSCs served as comparative control.

3.6. Effect of released angiogenic factor on HUVEC

Endothelial cell culture assays involving well-controlled conditions, for assessment of angiogenic effects, as per consensus guidelines on angiogenesis bioassays(Nowak-Sliwinska et al., 2018)have been adopted in this study.

3.6.1. Isolation and characterization of HUVECs

With informed consent, human umbilical cord was collected from a local maternity & child birth hospital at Trivandrum, and human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously reported(Prasad Chennazhy and Krishnan, 2005). The isolated HUVECs were characterized by immunostaining of

endothelial cell specific markers (CD31 and vWF) and Dil-Ac-LDL-uptake assay kit (Life technologies Ltd; UK) as per manufacturer's instruction.

3.6.2. Effect on HUVEC Proliferation

P3 HUVECs at 5000cells/cm^2 was seeded into fibrin coated plates (23) with serum-free BM, at 37°C with 5% CO2. After 1h, s-e-hADMSCs, generated by collecting transfected cell culture supernatants, pooled at 2d, 4d, 6d, 8d, 10d, 12d & 14d, centrifuged at 400g, for 6 minutes and quantitatively assessed for over expressed AFs by ELISA, as described earlier were added to HUVEC culture medium at random concentrations of over expressed AF; 500pg/ml, 1500pg/ml and 3500pg/ml, in the respective seeded cell plates. To analyze combinatorial effect of s-e-hADMSCs, VEGF-A and HIF-1α at 1:1, with total AF concentration of 500 pg/ml, 1500pg/ml and 3500pg/ml was added to seeded cells. HUVEC treated with s-n-hADMSCs (dose normalized with total protein in s-e-hADMSCs) and BM served for comparative analysis. At 48h, trypsinized cells were loaded onto a hemocytometer. Counts were performed manually in triplicate by one analyst under a 10x objective (Leica Microsystems, Germany) as per standard methodology(Cadena-Herrera et al., 2015).

3.6.3. Effect on HUVEC migration

Influence of s-e-hADMSCs on HUVEC migration was assessed by scratch wound assay. HUVECs seeded into 10cm^2 culture wells were grown to confluency. Using a 200µl

micropipette tip, monolayer was scratched forming ~1mm width wound. Detached cells were washed off and replenished with serum free BM. Cytosine β-d-arabinofuranoside hydrochloride (Sigma-Aldrich; 10 μM), a proliferation inhibitor was used 1h before addition of s-e-hADMSCs at predetermined dose of 500pg/ml of AF (test sample) and s-n-hADMSCs (control normalized to total protein in test). S-e-hADMSCs containing VEGF-A and HIF-1α at 1:1, with total AF at 500 pg /ml was tested for combinatorial effect. Wound edge cell migration was examined (Leica Microsystems,DMIRB, Germany) at different time intervals. Better visualization, was attained by actin immunostaining at 48h, and multiple images were captured. Observations from triplicate experiments were quantified using imageJ analysis software.

3.6.4. Effect on HUVEC tube formation

To assess angiogenic response by s-e-hADMSCs, P3 HUVECs at 5,000cells/well from three independent donors were seeded in triplicates on GF reduced Matrigel (Sigma). A predefined AF concentration of 500pg/ml in s-e-hADMSCs was added to cultures (tests) and compared with s-n-hADMSCs treatment (normalized to total protein in test). S-e-hADMSCs containing VEGF-A and HIF-1α at 1:1, with total AF concentration at 500 pg /ml was tested for combinatorial effect. Number of nodes/high-powered field was quantified using phase contrast microscope (Leica Microsystems, Germany). Multiple images were captured at 2h, 16h, 24h and 48h and quantified using ImageJ software.

3.6.5. Effect on VEGF Receptor *(Flk-1)* up regulation

Secretome treated cells in tube assay as described earlier were fixed with 4% formaldehyde, blocked with 3% BSA and stained with anti human Flk-1 primary antibody (1:500, Cat No. Cat No.sc-6251, Santa Cruz Biotechnology) for 1h at room temperature, incubated with corresponding secondary antibody (1:2000, Cat.No. ab150113, Abcam) for 1h at room temperature and analyzed using fluorescence microscope (Leica Microsystems,DMIRB, Germany).

3.7 Effect of biomimetic matrix coated Tissue Engineered Skin Grafts (TESG) on transfected hADMSCs

3.7.1. TESG fabrication

In house fabricated TESG were gifted by Ms. Rashmi R, Division of Thrombosis Research, SCTIMST. As per the protocol reported earlier, (Nair et al., 2014a) this in-house developed and validated terpolymer PLGC was synthesized and electrospun. The electrospun fiber matrices were dried under vacuum at room temperature for 24 hours, cut into patches of the required size for each experiment, and sterilized using ethylene oxide. A biomimetic fibrin composite matrix favouring fibroblast differentiation was deposited on the polymer as described and reported earlier.(Pankajakshan et al., 2008) Briefly, the biomimetic matrix comprised cryoprecipitated human fibrinogen concentrate (2mg/ml), thrombin (5IU/ml), PDGF(50µg/ml), FGF (10ng/ml), gelatin (2%) and HA (50µg/ml) prepared in-house as described previously.(Anilkumar et al., 2011)

Approximately, 100 µl/cm2 of the biomimetic matrix was layered on the sterile scaffold, incubated for 30 min at 37˚C, lyophilized (Edwards, Modulyo 4K), and stored at 4˚C–6˚C until use.

3.7.2. *In vitro* cell growth on TESG

Two approaches with varying cell seeding density was performed

Approach 1 (Low seeding density): Transfected hADMSCs (Vegf-e-hADMSCs and Hif-1α-e-hADMSCs) were independently and combinatorially seeded on the lyophilized biomimetic matrix coated circular scaffolds of 1mm diameter. Non-electroporated hADMSCs were used as the Control cells. Test and control scaffolds were seeded at a density of 5000 cells/cm^2. The BM containing Vitamin C at 50µg/ml of medium was replenished every third day and the culture was terminated after 20d for each experimental analysis.

Approach 2 (High seeding density): This approach was targeted to increase cell seeding density, meaning larger number of cells available to participate towards comparatively better attainment of pre-vascularisation. Hence, engineered hADMSCs (Vegf-e-hADMSCs and Hif-1α-e-hADMSCs) were independently seeded on the lyophilized biomimetic matrix coated circular scaffolds of 1mm diameter at 10000 cells/cm^2. Non-electroporated hADMSCs were used as the Control cells. Test and control scaffolds were seeded at a density of 10000 cells/cm^2. The BM containing Vitamin C at 50µg/ml

of medium was replenished every third day and the culture was terminated after 20d for each experimental analysis. Based on the unfavourable ECM quantification results obtained on combinatorial seeding of (Vegf-e-hADMSCs and Hif-1α-e-hADMSCs) on TESG at low seeding density, this group was eliminated from further investigation in the second approach.

3.7.3. DNA quantification

DNA quantification was performed on extracted DNA at the start and end test periods of the bioengineered hADMSCs culture on TESG, using Trizol (Invitrogen, USA) according to manufacturer's protocol. The quantity of extracted DNA was assessed by measuring the absorbance at 260nm (Nanodrop, Thermoscientific) and the 260/280nm absorbance ratio for all measured samples was between 1.8 and 2.(Desjardins and Conklin, 2010)

3.7.4. Assessment of angiogenic factor release

The release of secretive VEGF-A protein (Beckermann et al., 2008) and Hif-1α (Heikal et al., 2018; Choudhry and Harris, 2018) in bioengineered hADMSCs seeded TESG culture supernatants (Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs respectively), obtained from three independent donor experiments for three independent time periods, were quantified using enzyme-linked immunosorbent assay (ELISA) kits (cat No.ELH-VEGF-1; Ray Biotech and Cat.No:ab171577, Abcam) as per manufacturer's instruction,

respectively, using a microplate reader (iMark Microplate Reader, BIO-RAD). Cell culture supernatants from scaffolds seeded with non-engineered hADMSCs (s-n-hADMSCs) served as comparative control.

3.7.5. RT-PCR Analysis

Effect of transfected hADMSCs delivering angiogenic factors in directing paracrine/autocrine mediated endothelial and fibroblast differentiation in TESG was evaluated using selected endothelial, fibroblast and vascular smooth muscle cell specific markers by reverse transcriptase-quantitative real-time polymerase chain reaction (qRT-PCR). VEGF-A electroporated hADMSCs (Vegf-e-hADMSCs), Hif-1α electroporated hADMSCs (Hif-1α-e-hADMSCs) and non engineered hADMSCs (n-e-hADMSCs) were seeded onto TESG and cultured as per approach 1 and 2 mentioned above. Total RNA was extracted, using Trizol (Invitrogen, USA) according to manufacturer's protocol. 1 µg of RNA was converted to cDNA using superscript III reverse transcriptase enzyme (Invitrogen). 250ng cDNA was mixed with respective forward and reverse primers (Table 3) and 12.5ul of OrionX 2X Real Time PCR master mix was added (Origin, India). 40 cycles of reaction was performed using Chromo 4 system (Bio-Rad, USA) at annealing temperatures ; 55℃ for CD31 gene, 56°C for GAPDH, 58°C for eNOS, 59℃ for FSP-1, 59℃ for Fibrillin-1 and 50℃ for α-SMA (ACTA2) gene respectively. For each gene, assessment of quality and specificity was performed by examining PCR melt curves. The cycle threshold (Ct) value of target gene was analyzed after normalization to

the Ct value of the reference gene. Fold change (2–ΔΔCt) was calculated by comparing expression levels of mRNA extracted from n-e-hADMSCs. The reference gene was GAPDH. The experiment was performed in triplicates from three independent donor cells.

Name	**Forward**	**Reverse**
CD31	5'CAGTCATTACGGTCACAAT3'	5'CTGAGGACACTTGAACTTC3'
eNOS	5'CGGCATCACCAGGAAGAAGA3'	5'CATGAGCGAGGCGGAGAT3'
Fibrillin-1	5'TGATGGCTCCTACAGATGTGAATGC-3'	5'GACACGGCTGGCAAGGTTCC3'
FSP-1	5'AGCTTCTTGGGGAAAAGGAC3'	5'CCCCAACCACATCAGAGG3'
α-SMA (ACTA2)	5'GAGTTACGAGTTTGCCTGAT3'	5'AGACTCCATCCCGATGAA3'
GAPDH	5'CGCTCTCTGCTCCTCCTGTT'9	5'CCATGGTGTCTGAGCGATGT'9

Table 3. List of primers used for mRNA quantification for assessment of dermal lineage commitment in TESG

3.7.6. Immunocytochemistry

Transfected hADMSCs on application in the fabricated TESG were assessed for endothelial and fibroblast specific markers at translational level. Cells were fixed with 4% paraformaldehyde for 10min, washed in PBS, blocked with 3% BSA for 30 min, incubated with anti-human CD31 (Biolegend) and anti-human Fibrillin-1 (Abcam) antibody for 1h, followed by corresponding secondary antibody (Abcam). Cells were stained with diamidino-2-phenylindole (DAPI) for 10 min and examined by

fluorescence microscopy (Leica Microsystems,,DMIRB, Germany). n-e-hADMSCs served as comparative control.

3.7.7. Quantification of ECM synthesis

The assay to determine the collagen content in the decellularized TESG was carried out using sirius red according to published protocols.(Choi et al., 2012) The absorbance of the extracted collagen–sirius red complex was measured in a 96-well plate at 540 nm using a microplate reader (iMark Microplate Reader, BIO-RAD). The concentration of collagen deposited on the TESG was estimated using calibration standards made from type 2 collagen (bovine achilles tendon, Sigma). The soluble elastin content on the decellularized TESG was quantified using a Fastin elastin assay kit (Biocolor) according to the manufacturer's instructions. The elastin deposited was estimated using calibration standards supplied with the kit.

3.8. Full thickness large area excision rabbit wound healing assay

3.8.1. Creation of large excision acute wounds

Two acute, square 4x4 cm^2 full thickness excision skin wounds were created per animal for understanding wound healing in large wounds (Salgado et al., 2013). The wounds were symmetrically distributed on the back of the diabetic animal. The created wounds were grouped into five for transplantation of the secretome of engineered hADMSCs with over expressed VEGF, engineered hADMSCs with over expressed HIF-1α, non

transfected allogenic rabbit ADMSCs, allogenic rabbit ADMSCs with secretome of over expressed VEGF, allogenic rabbit ADMSCs with secretome of hADMSCs with over expressed HIF-1α and untreated wounds.

3.8.2 Isolation of allogenic rabbit ADMSCs and cell labelling

Adipose tissue from rabbits (~10 g) was surgically collected and ADMSCs were isolated and expanded in culture by standard protocol, as described in section 3.1.Passage 3 rabbit ADMSCs were then labelled with the fluorescent vital dye, PKH26 (General PKH26-GL cell linker kit, Sigma ®), as per the manufacturer's instruction.

3.8.3. Transplantation studies

Among the created full thickness wounds, control sites were treated with standard betadiene ointment. Treatment sites constituted of allogenic cells with or without the transfected hADMSC secretome or with the transfected hADMSCs secretome as described in section 3.9.2. The test samples were injected sub-dermally along the margin of the dorsal wound at four injection sites (Pelizzo et al., 2015). Each wound group received either 1.0×10^{6} cells or transfected hADMSCs secretome containing ~1400pg of VEGF or Hif-1α, independently or in combination (1:1) as tabulated below.

Group I	Sham wounds (un treated control)
Group II	Treated with VEGF-s-e-hADMSCs
Group III	Treated with HIF-1α –s-e-hADMSCs
Group IV	Treated with s-n-hADMSCs
Group V	Treated with rADMSCs and VEGF-s-e-hADMSCs
Group VI	Treated with rADMSCs and HIF-1α –s-e-hADMSCs
Group VII	Treated with rADMSCs

Table 4: List of wound healing groups for transplantation study

After transplantation, a transparent bio-occlusive adhesive tape like Comfeel Plus Transparent Dressing was placed over the wounds. The adhesive tape on the skin of rabbits was tested prior to this experiment for any skin irritation or allergic reaction. The transparent dressing was changed every alternate day to maintain wet wound conditions. Rabbits did not receive any immune suppression. General conditions of the animals and wound healing were monitored daily. Wounds were photographed using a digital camera. Animals were sacrificed after 28 days, wound sites were harvested and tissue was processed for evaluation of wound healing.

3.8.4 Bio-distribution of rADMSCs following sub-dermal administration

Explanted wound skin samples from groups (test and control) that received labelled rADMSCs were assessed for retainment of transplanted cells within the site of

transplantation using IVIS Spectrum in vivo Imaging System (PerkinElmer, USA). Tissues were fluorescently imaged (745/800 em/ex filters) using epi-fluorescence on an IVIS spectrum. Images were then analysed using Living Image 4.3.1 software, regions of interest (ROI) were drawn and average radiant efficiency was calculated after subtracting the background signal (Luli et al., 2016). A color bar was used to represent the cell density in the tissue. From top to bottom of the color bar, red indicated a relative high cell density and blue represented a relative low cell density.

3.9 Histological Analysis

Skin samples were taken from the wounds with the adjoining normal skin and fixed in 10% neutral-buffered formalin. After fixation, the tissues were embedded in paraffin, and 5 μm thickness sections were stained using hematoxylin and eosin (H&E), Direct Red 80 for collagen fibers, and CD31 for detection of neo-vascularization.

3.9.1. H & E Staining

To compare the re-epithelization rate and the amount of inflammatory infiltration, tissue sections were stained with haematoxylin and eosin (H&E). Tissue specimens were fixed in a 10% neutral buffer formalin solution, processed routinely in an automatic tissue processor (ASP300, Leica, Germany) and then embedded in paraffin. 5 micron thin sections were cut using a rotary microtome (RM2550, Leica, Germany) and stained with

hematoxylin and eosin stain. Histopathologic examinations were assessed by light microscope (Nikon E 600, Nikon, Japan) and photomicrographs were captured using the camera (Nikon DsR1, Nikon, Japan) attached to the microscope. A single pathologist at the Institute, blinded to all information regarding the groups performed the gross and histopathologic examination of the tissues.

3.9.2. Immunohistochemistry

Effective wound healing requires vascularization of the newly formed tissue. As granulation tissue forms, the number of blood vessels in the dermis increases. A significant increase in the density of blood vessels compared to uninjured skin is commonly observed during the proliferative phase of healing as a result of angiogenesis (Johnson and Wilgus, 2014). To determine effect of transplantation in accelerated neo-vascularization, an endothelial specific marker CD31, was assessed in each group by immunohistochemical analysis**.** As per the protocol described by (Pelizzo et al., 2015) tissue sections were deparaffinized, hydrated and pretreated for antigen retrieval using a pressure cooker in 10 mM citrate buffer. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 15 min. Nonspecific antibody binding was blocked by incubation with a protein blocker (Fetal Bovine Serum). Endothelial cells were immunohistochemically identified with anti-human CD31/PECAM1 antibody (Biolegend), which cross-reacts with rabbit endothelial cells. The sections were hence incubated with 1:500 anti-human CD31 antibody or bovine serum albumin as a negative

control for overnight at 4℃, then incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin (Abcam) for 2h at RT, followed by a 5 min incubation with 3,3′-diaminobenzidine (DAB; Sigma). Finally, the sections were mounted and examined under a light microscope. Image analysis was conducted with ImageJ. Neoplastic granulation area was selected with the freehand selection tool. DAB-stained areas were distinguished with the Colour Deconvolution plugin. A threshold was set to analyze the brown images using bins. The percentage of the positive area was measured with the Analyze Particles function (Ruifrok and Johnston, 2001).

3.9.3. Direct Red 80 Staining

To assess the degree of collagen synthesis and arrangement, Direct Red-80 (Sigma) (also called pico-sirus red stain) was used (Fujita et al., 2017). Briefly, de-waxed and hydrated paraffin sections were nuclei stained with Weigert's haematoxylin for 8 minutes and washed for 10 minutes in running tap water. The picro-sirius red stain was then added and incubated for 1h which gives near-equilibrium staining. The sections were then washed with two changes of acidified water, dehydrated in three changes of 100% ethanol, cleared in xylene and mounted in a resinous medium. Bright-field images were captured. The fiber density of neoplastic granulation under the wounds was analyzed using 20x images of random areas. The percentages of colored area in the images were quantitatively assessed with ImageJ.

3.9.4. Semi-quantitative analysis of histological parameters

The re-epithelization rate defined as the presence of differentiated multi-layered epithelium, the amount of inflammatory infiltration defined as the presence of inflammatory cells, and the degree of collagen defined as collagen content in granulation tissue of dermis were observed by microscopically as explained above and assessed using a semi-quantitative scale, with scores ranging from 1 to 4 as reported in Table 5. Healing status was graded as good (19-24), fair (12-18) and poor (8-11) as adopted (Gupta and Kumar, 2015) and modified.

Score	**4**	**3**	**2**	**1**
Amount of granulation tissue	Profound	Moderate	Scanty	Absent
Inflammatory infiltrate	Negligible	A Few	Moderate	Plenty
Collagen fiber orientation	Vertical	Mixed	Horizontal	-
Pattern of collagen	Reticular	Mixed	Fascicle	-
Amount of early collagen	Profound	Moderate	Minimal	Absent
Amount of mature collagen	Profound	Moderate	Minimal	-

Table 5: Parameters assessed to calculate healing score

3.10 Statistical Analysis

All experiments were performed at least independent times with three different donor cells, in triplicates. Statistical comparisons were performed by using one-way analysis of variance (ANOVA) or student's t test, as appropriate. Post-hoc Bonferroni correction for multiple comparisons was used. Mean values and standard deviations (SD) were calculated for all parameters and represented in graphical form. Significance is labeled in graphs with *** ($P<0.0001$); ** ($P<0.01$); and * ($P<0.05$). Number of replicate experiments carried out is indicated in legends.

CHAPTER 4: RESULTS

The results of this study are illustrated in this chapter; findings are subdivided into four main parts. First part illustrates non-viral mediated bioengineering of hADMSCs for delivery of angiogenic factors; second part demonstrates functionality of the delivered angiogenic factors *in vitro*; the third part, establishes angiogenesis in in house generated TESG on application of bioengineered hADMSCs and in the fourth part functionality of the delivered angiogenic factors *in vivo* is described.

4.1. Characteristics of hADMSCs

Isolated hADMSCs from multiple donor tissues showed typical plastic adherent property with spindle shaped morphology as shown in Figure 11 (A). Passage 3 hADMSCs on subjection to differentiation, showed adipogenic, osteogenic and chondrogenic lineage differentiation as shown in Figure 11 (B-D), respectively. Flow cytometry analysis showed high expression for positive cell surface markers; CD90 (>90% positivity), CD105 (>95% positivity), CD73 (>95% positivity) and minimal expression for negative cell surface markers; CD45 (<9% positivity), CD14 (<10% positivity) in accordance to the laid standards as shown in Figure 11 (E-I).

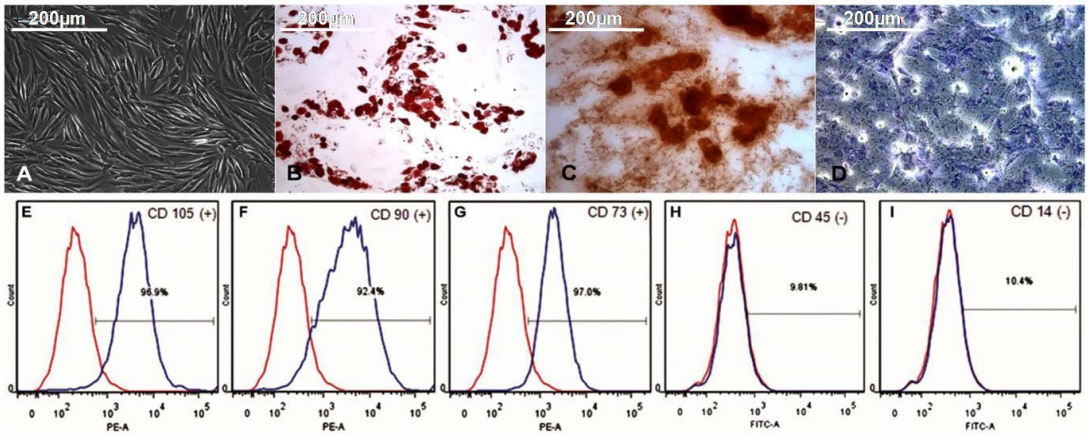


Figure 11: Representative data on hADMSCs characteristics *(A) Micrograph of isolated and cultured hADMSCs showing typical spindle shape morphology, (B) Oil red-O staining confirms the presence of lipoid substances in adipogeneic induced differentiation of hADMSCs (C) Alizarin Red staining confirms the presence of calcium in osteogenic induced differentiation of hADMSCs (D) Toluidine blue staining confirms the presence of acidic proteoglycan present in cartilage tissues for chondrogenic induced differentiation (scale bar = 200µm) (E-I) Flow cytometric analysis of Stem cell marker expression in hADMSCs showing positive for CD105, CD90 & CD73 and negative for CD45 & CD14.*

4.2. Characteristics of HUVEC

Endothelial cell culture assays using HUVECs involving well-controlled conditions, for assessment of angiogenic effects, as per consensus guidelines on angiogenesis bioassays(Nowak-Sliwinska et al., 2018) have been adopted in this study. HUVECs used for *in vitro* studies showed typical cobblestone morphology as shown in Figure 12 (A). Further, uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) [Figure 12 (B)],

detection of CD31 and Von Willebrand factor (vWF)[Figure 12 (C-D)] upon immunocytochemistry confirmed endothelial cell phenotype.

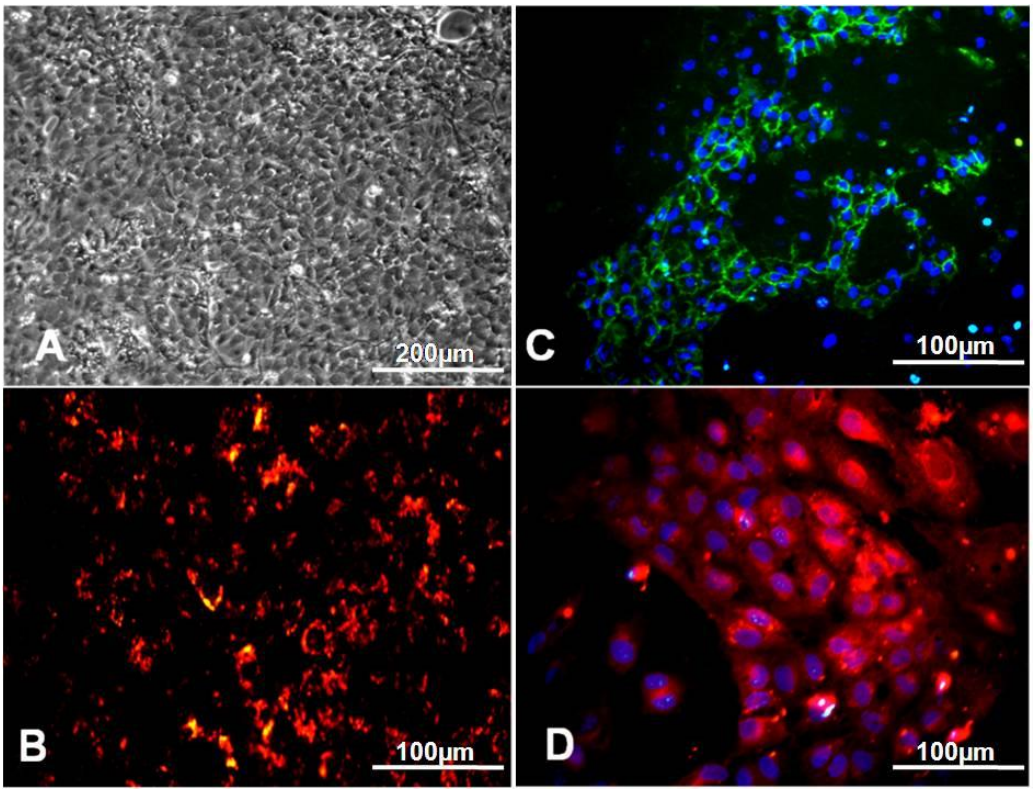


Figure 12: Demonstration of HUVEC characteristics. *(A) Micrograph of Human Umbilical Cord Vein Endothelial Cells (10x) (B) Uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) (20x), (C) CD31 staining (20x) and (D) vWF staining (40x).*

4.3. Characteristics of transfected cells

Microscopic examination of engineered hADMSCs verified no morphological change after plasmid electro-transfer as seen in [Figure 13(A)]. The feasibility of Neon® transfection system was confirmed by GFP expression for VEGF-A and Hif-1α groups at 24h, respectively [Figure 13(B-C)]. Transfection efficiency accounted to ~50% for

VEGF-A and ~47 % for Hif-1α groups, 24h post transfection, inline to the efficiency reported in effective primary cell transfection (Halim et al., 2014; May et al., 2017) [Figure 13 (D)]. Transfection efficiency in terms of protein delivery is described later. Retention of adipogenic, osteogenic and chondrogenic lineage was observed post transfection [Figure 13 (E-G)] similar to control n-e-hADMSCs. MTT assay post transfection showed reduction of cell viability to 70±0.05 percentage, starting from 98±0.03 percentage viable hADMSCs [Figure 13(H)].

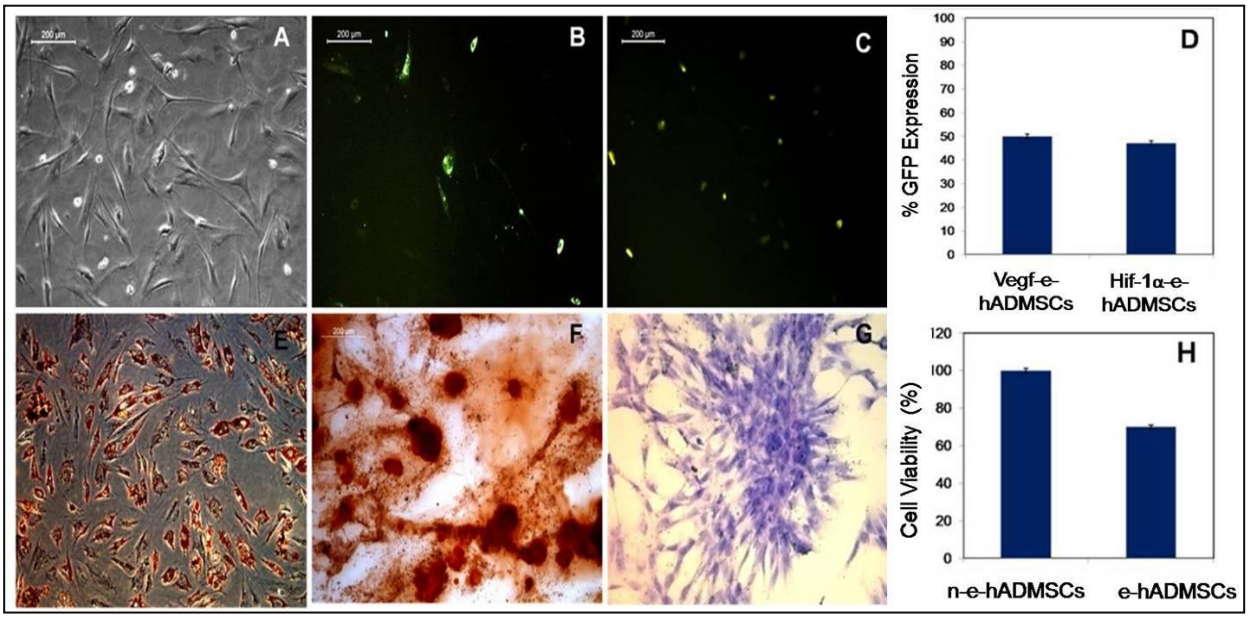


Figure 13: Representative images demonstrating stemness of transfected hADMSCs. *(A) Micrograph showing electroporated hADMSCs confirming no significant change in its morphology after electroporation. (B-C) Micrograph of VEGF-e-hADMSCs and Hif-1α-e-hADMSCs expressing GFP respectively, at 24h post transfection. (D) Transient transfection efficiency using imageJ software analysis from NIH, in 20 randomly chosen fields was carried out 24h post transfection to monitor the percentage of GFP+ cells. (E-G) Micrograph depicting hADMSCs maintain multipotency (adipogenic, osteogenic & chondrogenic, respectively) after electroporation (Scale bar = 200µm, n=3) (G) Effect of electroporation on cell viability at 48h post transfection (n=3).*

Periodic microscopic observation of the transfected cells determined GFP expression to be transient with complete decline by 21d [Figure 14], correlating to earlier reports (Halim et al., 2014).

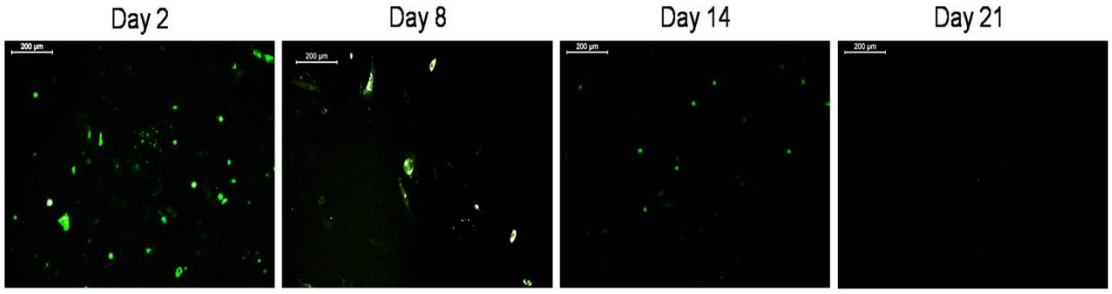


Figure 14: Representative images demonstrating transience of transfection. *Micrograph of electroporated hADMSCs at varying time periods, showing pattern of GFP expression. Decline in number of GFP+ cells determined 21d established transiency.(Scale bar = 200µm, n=3)*

4.4. Release profile of over expressed proteins

4.4.1. Quantity of VEGF-A

Neon® transfection system mediated delivery of VEGF-A in Vegf-s-e-hADMSCs was detected by ELISA as shown in Figure 15 (A). The release pattern of VEGF-A was observed to be elevated and significant, in comparison to its base level expression in s-n-ADMSC(D'souza et al., 2015b). Hif-1α, being an upstream inducer of VEGF, the concentration of VEGF-A in the Hif-1α-s-e-hADMSCs was assessed and found to be elevated in comparison to its expression in s-n-hADMSCs as shown in Figure 15(B).

4.4.2. Quantity of HIF-1α

Hif-1α estimated in Hif-1α-s-e-hADMSCs was significantly elevated compared to s-n-hADMSCs [Figure 15(B)]. Detected basal level expression of Hif-1α in s-n-hADMSCs connects with earlier reports on the presence of normoxic Hif-1α expression by hMSCs for regulation of metabolic fate and maintenance of multipotency (Palomäki et al., 2013).

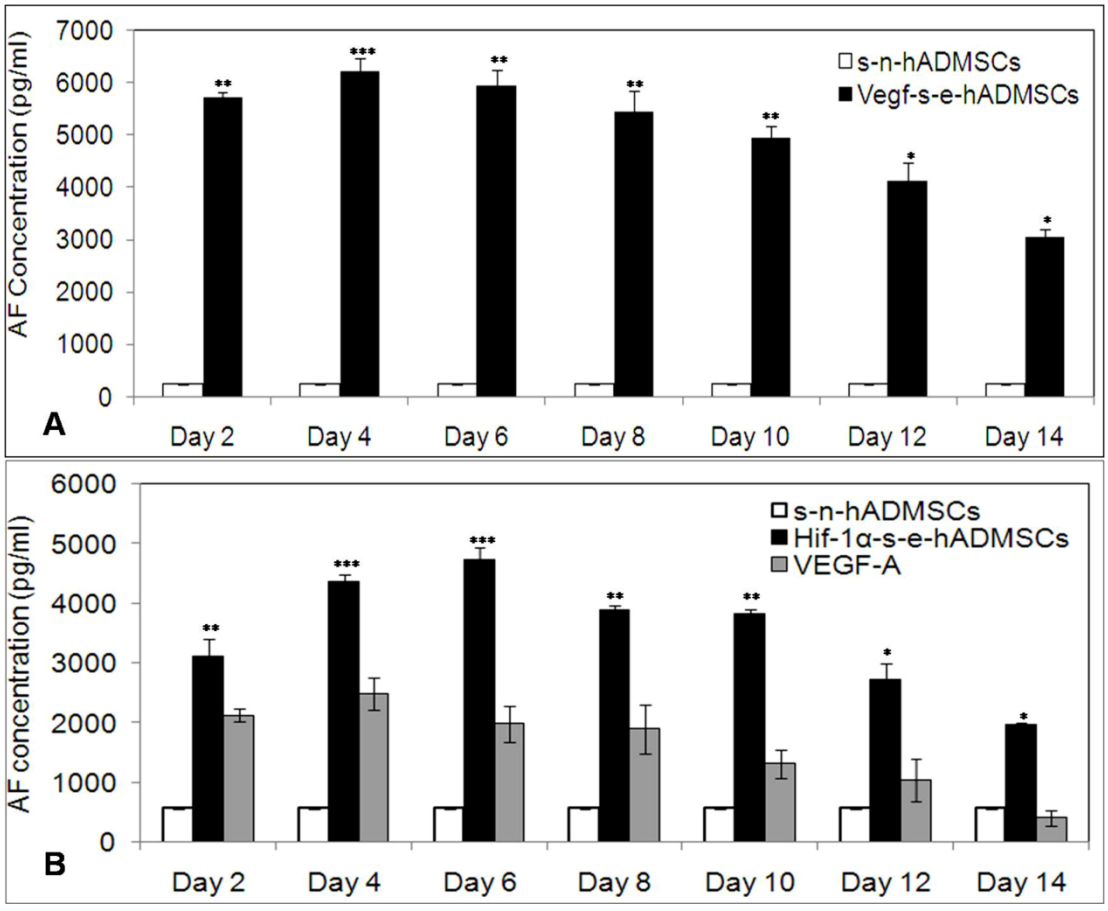


Figure 15: Graphical representation of release profile demonstrating transience of expression *(A) Measurement of VEGF-A in VEGF-s-e-hADMSCs on comparison with s-n-hADMSCs of the same donor at the respective time periods by ELISA. (B) Measurement of VEGF-A in Hif-1α-s-e-hADMSCs and Hif-1α in Hif-1α-s-e-hADMSCs*

*on comparison with s-n-hADMSCs of the same donor at the respective time periods by ELISA. *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.5. Lineage commitment to EC

4.5.1. Quantitative Gene expression

The expression of tissue plasminogen activator (tPA), platelet endothelial cell adhesion molecule (CD31), vascular cell adhesion protein (VCAM) and fetal liver kinase 1 (*Flk-1)* was significantly higher in the electroporated population than in n-e-hADMSCs [Figure 16]. Hif-1α-e-hADMSCs showed up-regulation of endothelial genes; tPA (~16 fold), CD31 (~107 fold) and VCAM (~43 fold). Vegf-e-hADMSCs showed up-regulation of endothelial genes; CD31 (~85 fold), VCAM (~104 fold). Interestingly, *Flk-1* expression was ~16 fold up-regulated in Vegf-e-hADMSCs with no up-regulation in Hif-1α-e-hADMSCs.

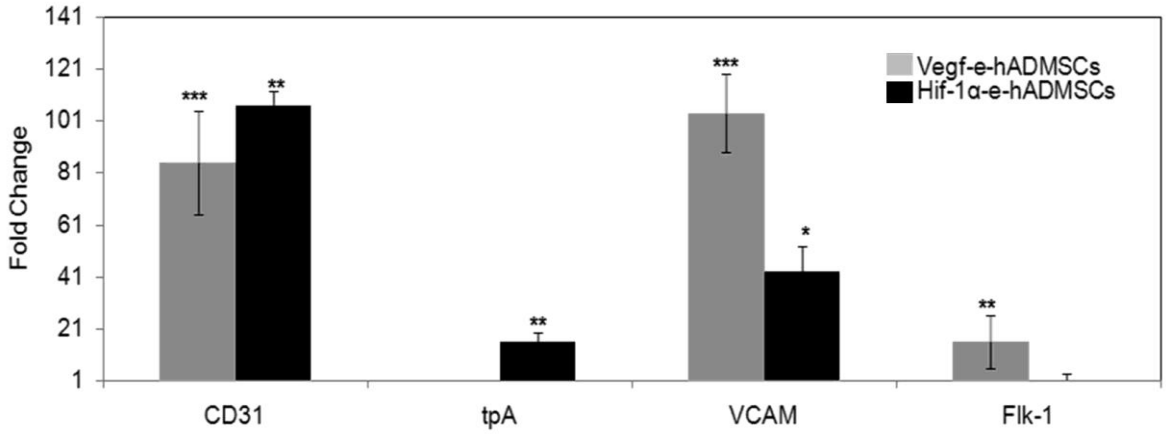


Figure 16: Graphical representation of EC marker up regulation in transfected cell upon culture. *Gene up-regulation profiles of endothelial markers relative to non-transfected hADMSC: CD31, tPA, VCAM and Flk-1 was determined and seen to be significant. No bar plot is shown for tpA in Vegf-e-hADMSCs group as no transcripts were detected for tpA. Similarly, Flk-1 was not up regulated in Hif-1 transfected cells. Fold change is indicated relative to GAPDH expression on each day of analysis using the 2-ΔΔC_t method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** ($P<0.001$); ** ($P<0.01$); and * ($P<0.05$) vs non electroporated hADMSCs. (Number of samples, n=3).*

4.5.2. Immunocytochemistry of CD31 and Flk-1

The exposure of engineered hADMSCs to hypoxia in directing efficient endothelial induction was positively determined by Immunostaining of endothelial functional markers; CD31 and *Flk-1* [Figure17]. The detection of these markers in engineered hADMSCs compared to their absence in n-e-hADMSCs suggests that delivered AF, through autocrine/paracrine mediation directs endothelial determination.

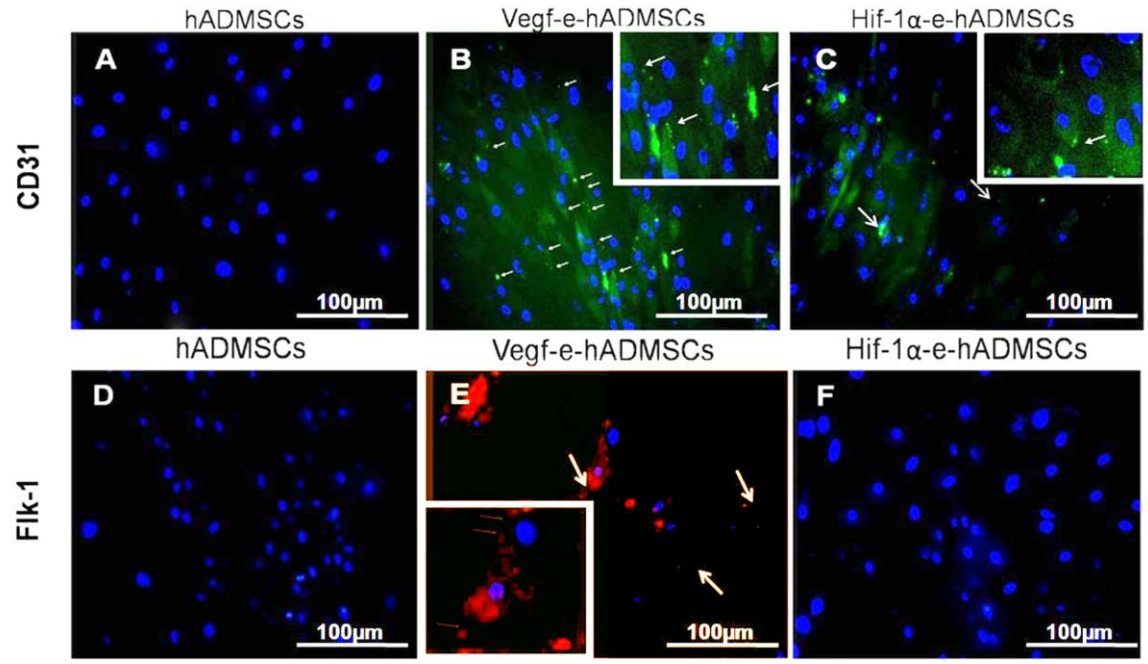


Figure 17: Representative images of EC proteins upon culture of transfected hADMSCs*: In the Vegf-e-hADMSCs (B) and Hif-1α-e-hADMSCs (C) group, many CD31-positive cells (green) are seen in the images (n=3, magnified region of interest at 40x) but absent in the non engineered hADMSCs group (A). Few Flk-1-positive cells are observed (red) in the images of the Vegf-e-hADMSCs (E) but absent in the non engineered hADMSCs (D) and in the Hif-1α-e-hADMSCs group (F). (n=3, magnified region of interest at 40x).*

4.6. Response of HUVEC cells

4.6.1. Effect on Proliferation

Effective concentration ranges of VEGF-A (500pg/ml, 1500pg/ml and 3500pg/ml) significantly increasing EC proliferation compared to s-n-hADMSCs treatment is demonstrated [Figure 18 (A)]. In terms of dose-dependent action, significant proliferation was achieved at 500pg/ml. However, increasing concentration of Hif-1α (500pg/ml, 1500pg/ml and 3500pg/ml) showed dose-dependent increase in proliferation

[Figure 18 (B)]. Combinatorial AFs (VEGF-A + Hif-1α) in s-e-hADMSCs adding up to (500pg/ml, 1500pg/ml and 3500pg/ml), showed dose-dependent increase in cell proliferation [Figure 18 (C)]. Neither VEGF-A nor Hif-1α at 250pg/ml, independently increased EC proliferation significantly. However, on combination at 1:1, the proliferative effect at all concentrations was enhanced compared to their independent action.

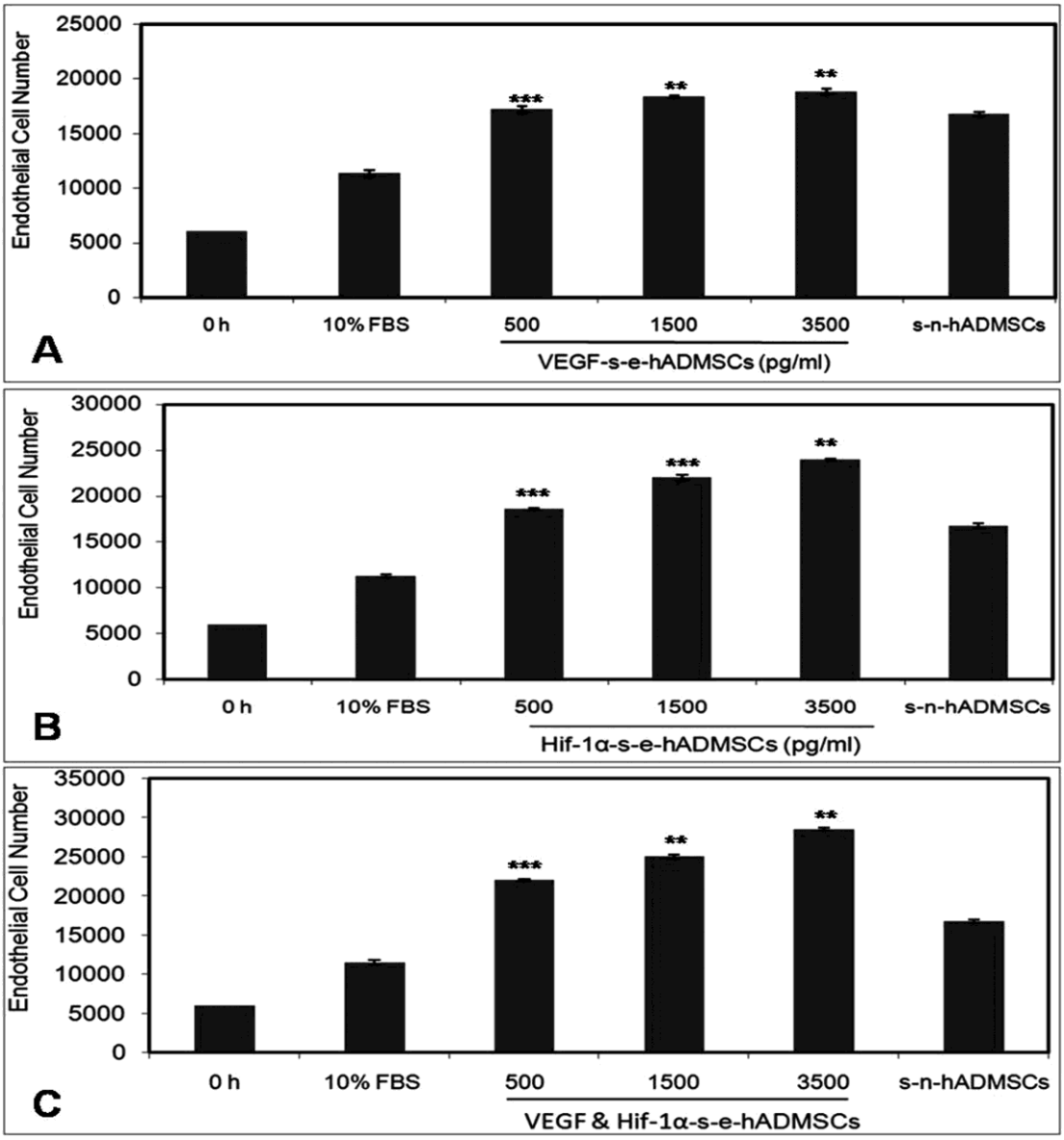


Figure 18: Graphical representation of EC proliferation upon adding secretome in culture: *(A) Dose dependent action of VEGF-s-e-hADMSCs showing significant increase in proliferation of HUVEC cells at 48h. (F) Dose dependent action of Hif-1a-s-e-hADMSCs showing significant increase in proliferation of HUVEC cells at 48h (C) Dose dependent action of VEGF+Hif-1α-s-e-hADMSCs showing significant increase in proliferation of HUVEC cells at 48h , *** (P<0.001); ** (P<0.01); and * (P<0.05) vs. control (non treated cells), (n=3).*

4.6.2. Effect on Migration

In comparison to scratch wounds treated with s-n-hADMSCs the effect of Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs, showed significant wound closure in 48h, with no commendable effect in 24h [Figure 19&20]. However, the combinatorial effect of Vegf-s-e-hADMSCs & Hif-1α-s-e-hADMSCs was much prominent by 24h [Figure 19& 20]. The combinatorial effect at 24h with consistent progress by 48h may have clinical relevance.

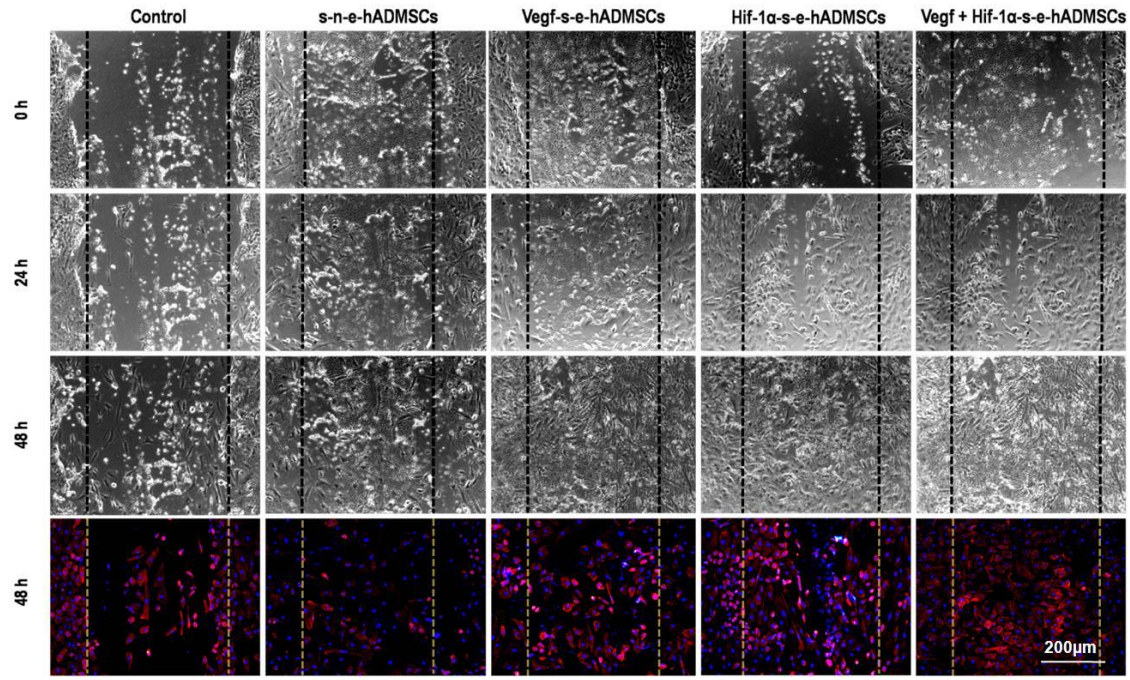


Figure 19: Representative images demonstrating effect of secretome on EC migration. *Cell migration was detected by scratch wound assay. Representative wound closure images from six different conditions of treatment at different time points are shown. Actin staining of the cell cytoskeleton (red) and its respective DAPI staining of the nuclei (blue) is shown for better visualization of the migration effect at 48h. All images shown were acquired using a 10x objective. The white scale bar in the lower right picture represents 200µm (n=3).*

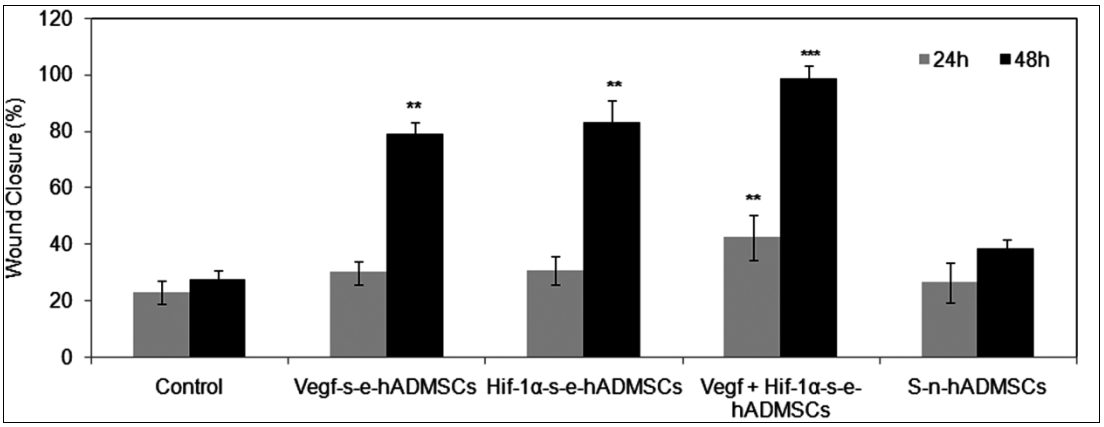


Figure 20: Graphical representation computed EC migration and wound closure. *Cell migration was calculated and expressed as the percentage of cell coverage to the initial cell-free zone. Values are presented as means ± SD of three independent experiments, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non treated cells, n=3.*

4.6.3. Effect on endothelial tube formation

Tube formation and elongation in ECs exposed to Vegf-s-e-hADMSCs was remarkably high [Figure 21 (B)]. In cells treated with Hif-1α-s-e-hADMSCs, establishment of distinct elongated tube-like structures was not prominent [Figure 21(C)]. On combinatorial treatment (Vegf-s-e-hADMSCs and Hif-1α-e-hADMSCs), angiogenic response was remarkable with higher number of loops [Figure 21 (D)] compared to independent and s-n-hADMSCs effects [Figure 21 (A)]. Quantitative angiogenic response is significantly higher in combinatorial treatment [Figure 21 (E)].

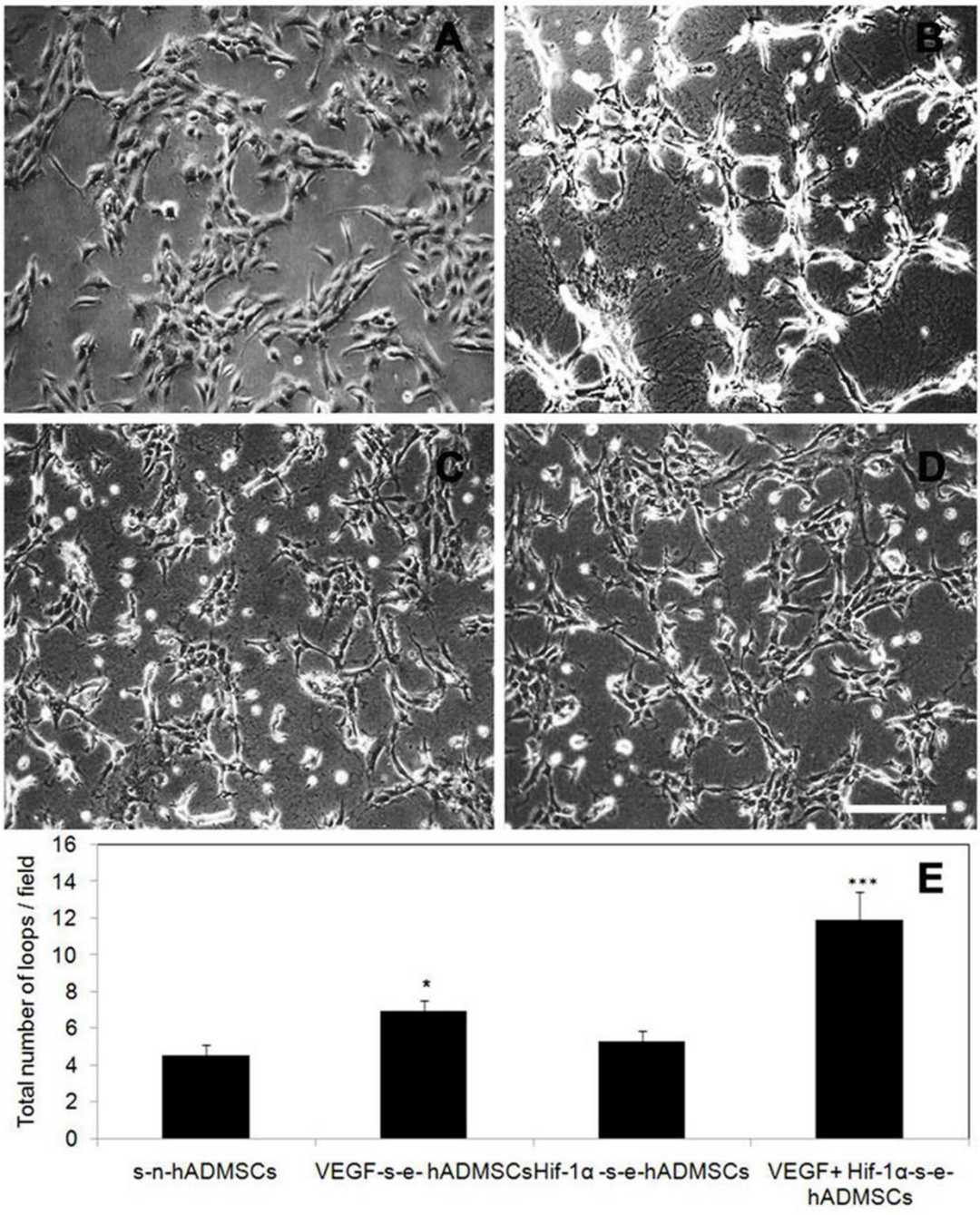


Figure 21: Demonstration of the effect of secretome on endothelial tube formation in culture. *Representative tube formation images from four different conditions of treatment are shown (A) HUVEC cultured in s-n-hADMSCs as control, (B) HUVEC cultured in VEGF-s-e-hADMSCs (C) HUVEC cultured in Hif-1a-s-e-hADMSCs (D)*

*HUVEC cultured in both VEGF+ Hif-1α-s-e-hADMSCs (E) Total number of loops per field was calculated and values are presented as means ± SD of three independent experiments, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs s-n-hADMSCs treated cells and within groups. All images shown were acquired using a 10x objective. The white scale bar in the lower right picture represents 200μm, n=3.*

4.6.4. Effect on VEGF-R expression

The detection of upregulated *Flk-1* in HUVECs treated with s-n-hADMSCs and s-e-hADMSCs is demonstrated by immunostaining [Figure 22 (A-D)]. Striking difference in *Flk-1* expression in Vegf-s-e-hADMSCs treated cell is noted [Figure 22 (B)]. Hif-1α-s-e-hADMSCs treated [Figure 22 (C)] and s-n-hADMSCs treated cells [Figure 22 (A)], showed no significant *Flk-1* expression. On combinatorial treatment (Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs), *Flk-1* expression was evident [Figure 22 (D)]. High density of *Flk-1* detected in correlation to nodes was observed, indicating higher expression upon loop formation. Demonstrated results thus emphasize EC activation through receptor coupling. Poor *Flk-1* expression on independent treatment with Hif-1α-s-e-hADMSCs advocates additional benefit of combination therapy.

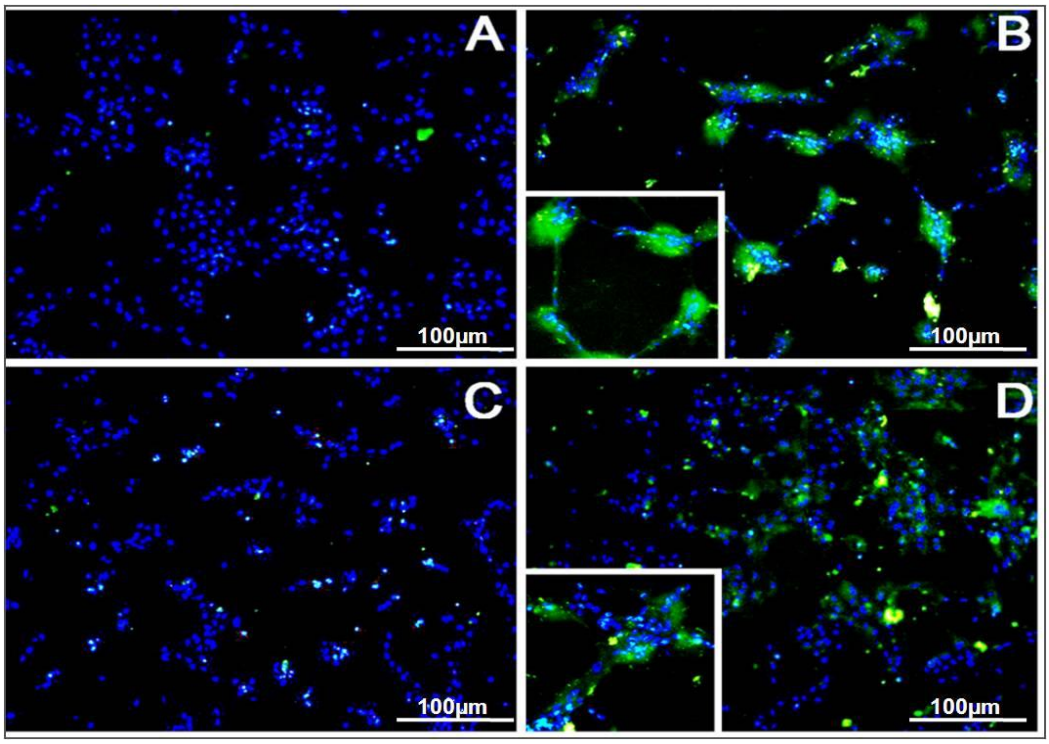


Figure 22: Representative micrographs depicting up regulation of Flk-1in HUVEC. *(A) s-n-hADMSCs as control (10x) (B) VEGF-s-e-hADMSCs (10x), inset (40x) showing strong Flk-1 detected in correlation to nodes upon loop formation (C) Hif-1a-s-e-hADMSCs (10x) (D) VEGF+Hif-1α-s-e-hADMSCs (10x), inset (40x) showing nodes expressing Flk-1, confirmed the onset of angiogenesis in vitro in groups treated with Vegf-s-e-hADMSCs both independently and in combination. Flk-1 expression was not observed in other groups, (n=3).*

4.7. Role of transfected hADMSCs on in vitro tissue engineering

Various materials with natural and synthetic structures are used to promote angiogenesis in the context of skin tissue engineering. Considering reproducibility and accessibility, synthetic materials are likely for medical translation services (HUSSAIN et al., 2017). Hence, in the current study an in house fabricated TESG using electro-spun polymer

PLGC was syringe coated with a fibroblast specific matrix as described earlier(Nair et al., 2014b)and lyophilized for subsequent use in this study is shown in Figure 23.

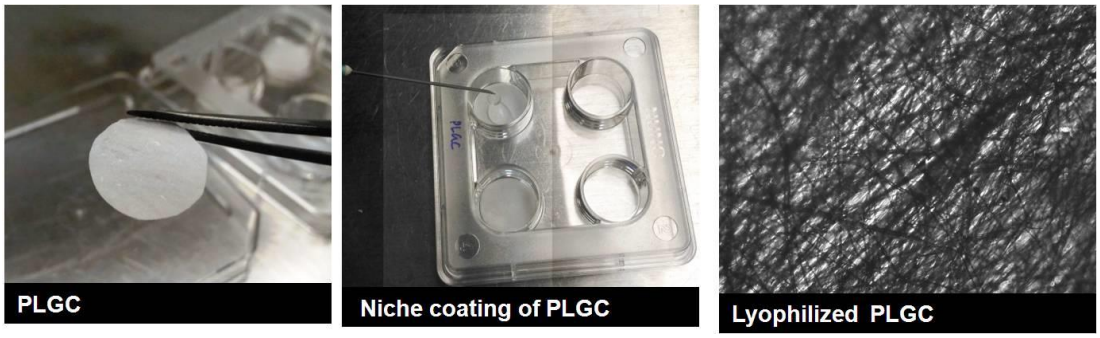


Figure 23: Representative images of scaffolds before cell seeding. *The first figure in the panel depicts gross appearance of the electrospun PLGC scaffold with a soft and smooth texture favourable for skin application. The second figure in the panel depicts syringe based niche coating of the electro-spun PLGC. The last figure in the panel shows phase contrast micrograph of lyophilized PLGC- fibroblast niche coated scaffold.*

4.7.1. Effect on Cell Proliferation

Cell doubling was evident in all groupswith initial seeding density of 5000cells/cm^{2}and 10000cells/cm^2 respectively [Figure 24 (A-B)]. Significant proliferation rate was evident at 20d upon culture of the e-hADMSCs to that of the non engineered hADMSCs. Increase in cell number was comparable among the Vegf-e-hADMSCs groups and the non engineered hADMSCs (n-e-hADMSCs) at seeding density of 5000cells/cm^2 [Figure 24 (A)] but significantly higher at seeding density of 10000cells/cm^2 [Figure 24 (B)].

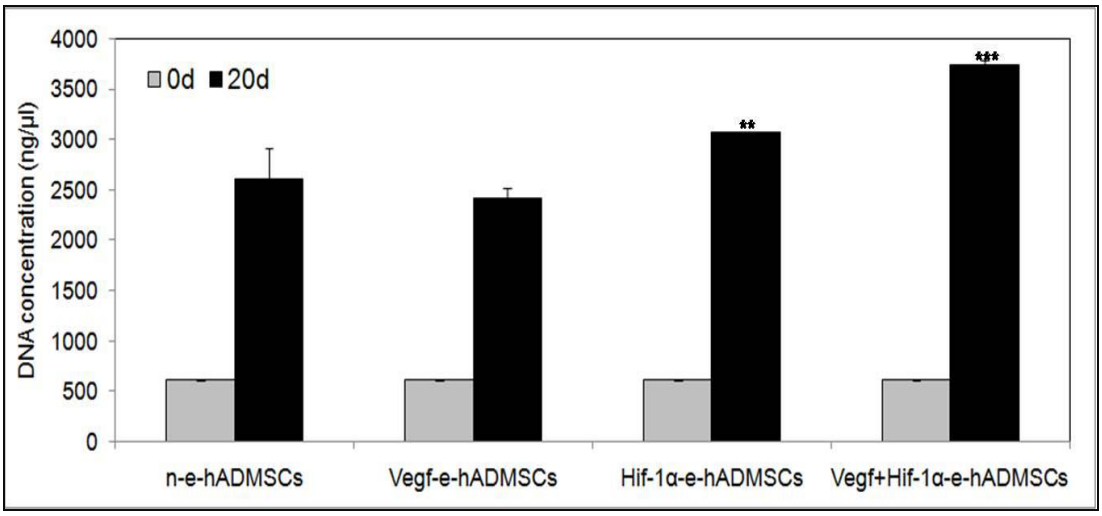


Figure 24 (A): Graphical representation of hADMSCs proliferation upon seeding 5000cells/cm^2: *Hif-1a-e-hADMSCs independently, and in combination showing significant increase in proliferation at 20d. *** (P<0.001); ** (P<0.01); and * (P<0.05) vs. control (n-e-hADMSCs), (n=3).*

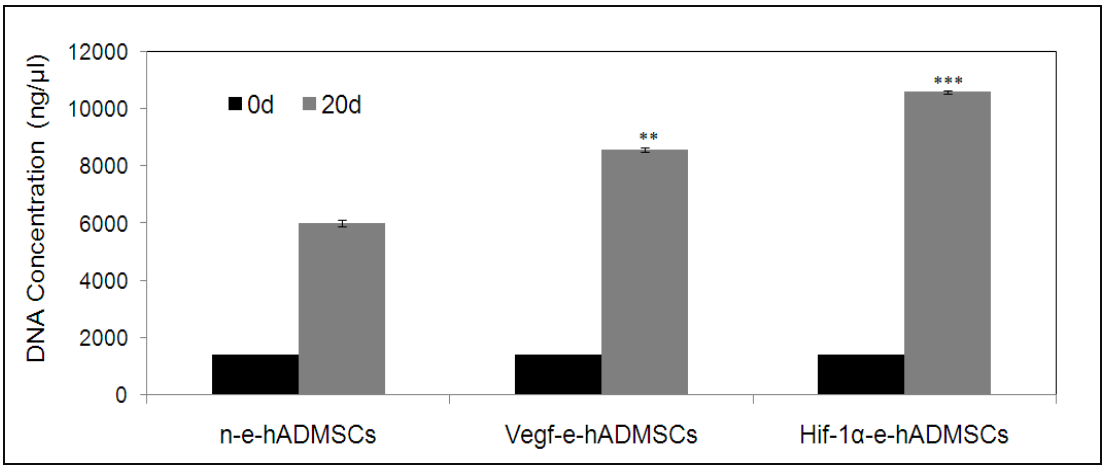


Figure 24 (B): Graphical representation of hADMSCs proliferation upon seeding 10000cells/cm^2: *Vegf-e-hADMSCs and Hif-1a--e-hADMSCs independently, showing significant increase in proliferation at 20d, upon high seeding density. *** (P<0.001); ** (P<0.01); and * (P<0.05) vs. control (n-e-hADMSCs), (n=3).*

4.7.2. AF release during TESG culture

Release of VEGF-A from tissue engineered skin grafts (TESG) seeded with Vegf-e-hADMSCs was detected by ELISA as shown in Figure 25. The release pattern of VEGF-A was observed to be elevated and significant, in comparison to n-e-hADMSCs. Hif-1α release estimated in TESG seeded with Hif-1α-e-hADMSCs was also significantly elevated compared to n-e-hADMSCs [Figure 26]. Moreover, in comparison to the release pattern of angiogenic factors independent of the effect of scaffolds as described in the earlier section 4.4., their detection at longer durations may be assumed to be a result of their binding to the scaffolds surface with sustained release to the surrounding tissue, supporting angiogenic outcome upon *in vivo* transplantation(Saberianpour et al., 2018).

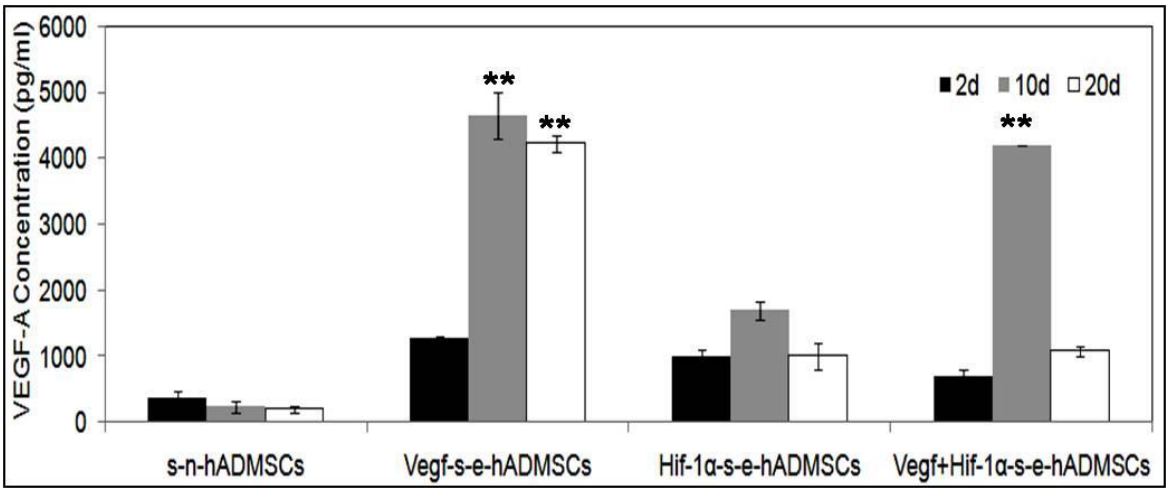


Figure 25: Release profile of VEGF-A in TESG seeded with e-hADMSCs upon culture*: Measurement of VEGF-A in VEGF-s-e-hADMSCs on comparison with s-n-hADMSCs of the same donor at the respective time periods by ELISA. *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

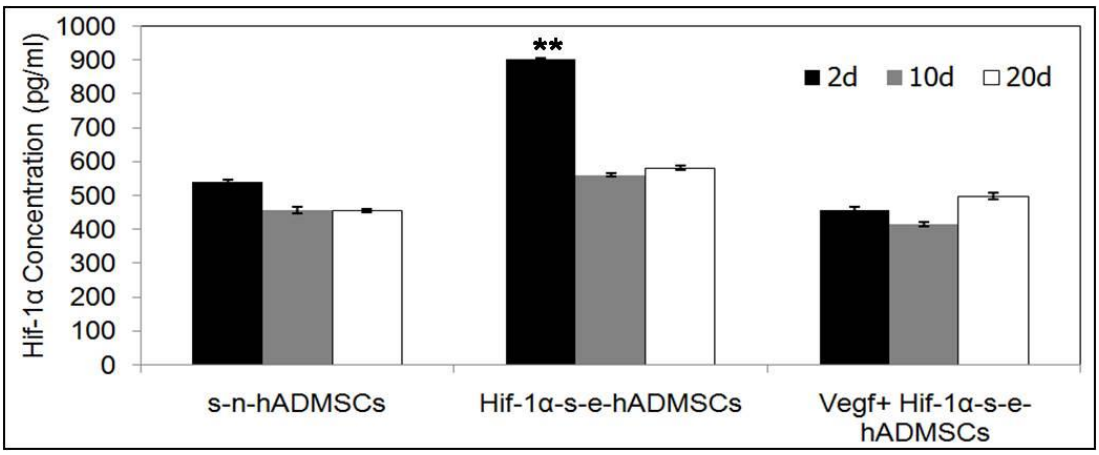


Figure 26: Release profile of Hif-1α in TESG seeded with e-hADMSCs upon culture. *Measurement of Hif-1α in Hif-1α-s-e-hADMSCs on comparison with s-n-hADMSCs of the same donor at the respective time periods by ELISA. *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.7.3. Genotype characteristics of cells on TESG

4.7.3.1. Endothelial lineage commitment

The expression of platelet endothelial cell adhesion molecule (CD31) and endothelial NO synthase vascular cell adhesion protein (eNOS) was significantly higher in TESG seeded with the electroporated population than with n-e-hADMSCs at both seeding densities as shown [Figure 27(A-B)]. At seeding density of 5000cells/cm^2, Hif-1α-e-hADMSCs showed up-regulation of endothelial genes; CD31 (4 fold) and eNOs (5 fold). Vegf-e-hADMSCs showed up-regulation of endothelial genes; CD31 (2.5 fold) and eNOs (8 fold). eNOs expression was 2 fold up-regulated in combination group (Vegf-e-hADMSCs +Hif-1α-e-hADMSCs). It may be noted that Flk-1 expression was not detected in any of the groups [Figure 27(A)]. However at seeding density of

10,000cells/cm^2, Hif-1α-e-hADMSCs showed up-regulation of endothelial genes; CD31 (4.5 fold) and eNOs (3 fold). Vegf-e-hADMSCs showed up-regulation of endothelial genes; CD31 (5 fold)and eNOs (4.5 fold). At higher seeding density, Flk-1 expression was 1.1 fold up-regulated in Vegf-e-hADMSCs but remained un-detected in Hif-1α-e-hADMSCs.

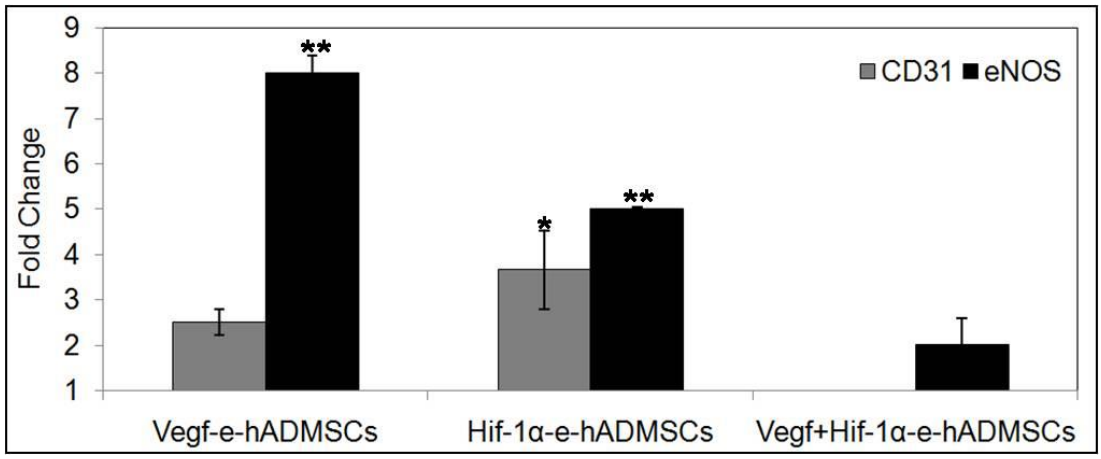


Figure 27(A): Graphical representation of EC marker expression in low density seeded TESG culture. *Gene up-regulation profiles of endothelial markers; CD31 and* eNOs *was determined and seen to be significant. No bar plot is shown for CD31 in Vegf-e-hADMSCs+* Hif-1α-e-hADMSCs *group as no transcripts were detected for CD31, suggesting transcripts at negligible background levels.Fold change is indicated relative to GAPDH expression on each day of analysis using the 2-ΔΔCt method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** (P<0.001*); *** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

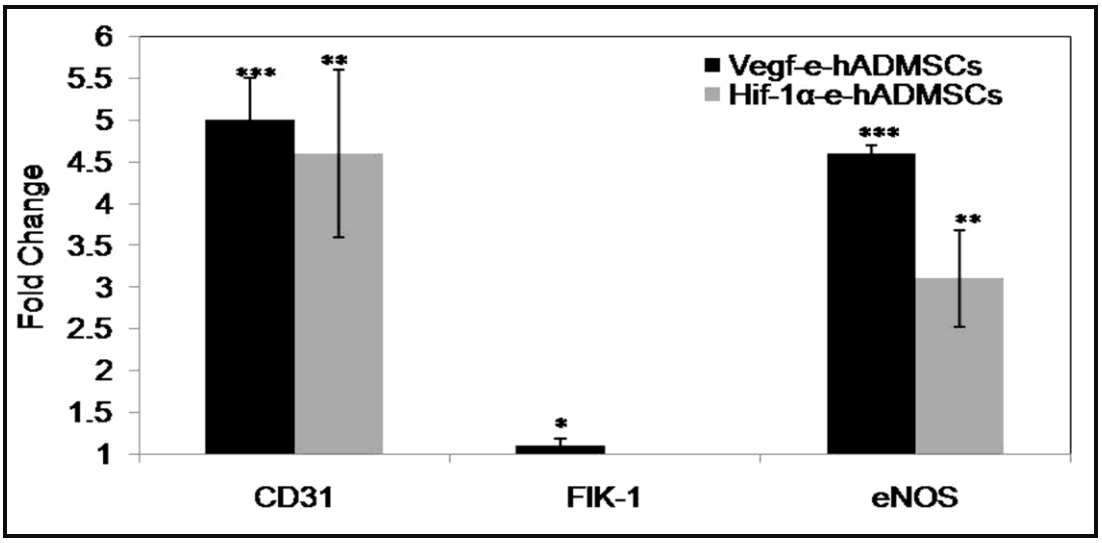


Figure 27(B): Graphical representation of EC marker expression in high density seeded TESG culture. *Gene up-regulation profiles of endothelial markers; CD31,* eNOS and *Flk-1 was determined and seen to be significant. No bar plot is shown for Flk-1in Hif-1α-e-hADMSCs group as no transcripts were detected suggesting transcripts at negligible background levels. Fold change is indicated relative to GAPDH expression on each day of analysis using the 2-ΔΔC_t method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** ($P<0.001$); ** ($P<0.01$); and * ($P<0.05$) vs non electroporated hADMSCs, n=3.*

4.7.3.2. Fibroblast lineage commitment

Fibroblast-specific protein 1 (FSP-1), a member of the S100 super family of cytoplasmic calcium-binding proteins, is predominately expressed in fibroblasts(Sun et al., 2015). Fibrillin-1(Fbln) is also a connective tissue protein produced by fibroblasts(Kissin et al.,

2002).Hence, the expression of FSP-1 and Fbln was investigated to determine co-existence of fibroblasts along with endothelial cells within the dermal graft at both seeding density of 5000cells/cm^{2}and 10000cells/cm^{2}respectively [Figure 27(C-D)]. At seeding density of 5000cells/cm^2, Vegf-e-hADMSCs showed up-regulation of fibroblast genes; FSP-1 (2 fold) and Fbln (45 fold). Hif-1α-e-hADMSCs showed negligible expression of fibroblast genes. Fbln expression was 5 fold up-regulated in combination group (Vegf-e-hADMSCs +Hif-1α-e-hADMSCs) [Figure 27(C)]. However at seeding density of 10,000cells/cm^2, Hif-1α-e-hADMSCs showed up-regulation of fibroblast genes; FSP-1 (3.5 fold) and Fbln (4.2 fold). Vegf-e-hADMSCs showed up-regulation of fibroblast genes; FSP-1 (2.5 fold) and Fbln (4 fold) [Figure 27 (D)].

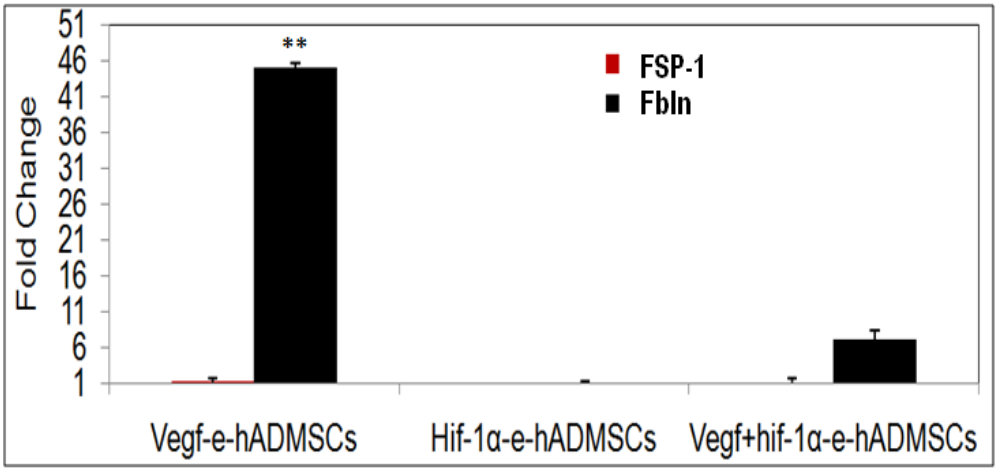


Figure 27(C): Graphical representation of fibroblast marker expression upon low density TESG culture. *Gene up-regulation profiles of fibroblast markers; Fbln was determined and seen to be significant in Vegf-e-hADMSCs group. Fold change is indicated relative to GAPDH expression on each day of analysis using the 2-ΔΔCt method, upon normalization with results obtained from the non electroporated*

*hADMSCs. Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

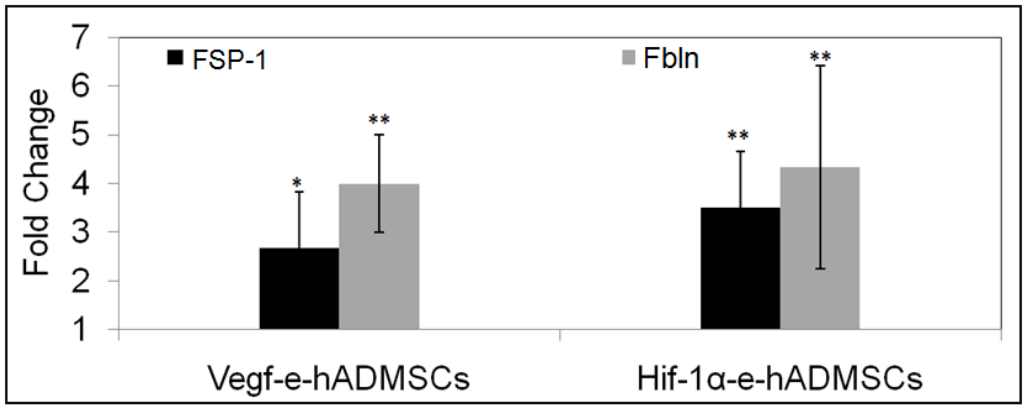


Figure 27(D): Graphical representation of Fibroblast marker expression upon high density TESG culture. *Gene up-regulation profiles of fibroblast markers; Fibroblast specific protein-1 and Fbln was determined and seen to be significant. Fold change is indicated relative to GAPDH expression on each day of analysis using the 2-ΔΔCt method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.7.3.3. Smooth muscle cell lineage commitment

Accumulating studies have revealed that both vasculogenesis and angiogenesis require vascular progenitor cell homing or recruitment, proliferation, and differentiation into endothelial cells (ECs) and smooth muscle cells (SMCs), among other cell types(Zhang et al., 2018). Tissue-engineered human blood vessels have been constructed in vitro using mixtures of vascular smooth muscle cells, dermal fibro-blasts, and human umbilical vein endothelial cells (HUVEC) in a collagen matrix or by using cells alone grown along a tubular support for lumen formation(SUPP et al., 2002). In this context of

attaining neo-vascularisation within the TESG, α-smooth muscle actin (α-SMA), an early-phase SMC differentiation marker was analysed for the detection of smooth muscle lineage commitment cells. Significant up-regulation at the mRNA level was detected in the TESG seeded with e-hADMSCs on comparison to n-e-hADMSCs, on culture for 20d, at low seeding density [Figure 27 (E)] and high seeding density [Figure 27 (F)].

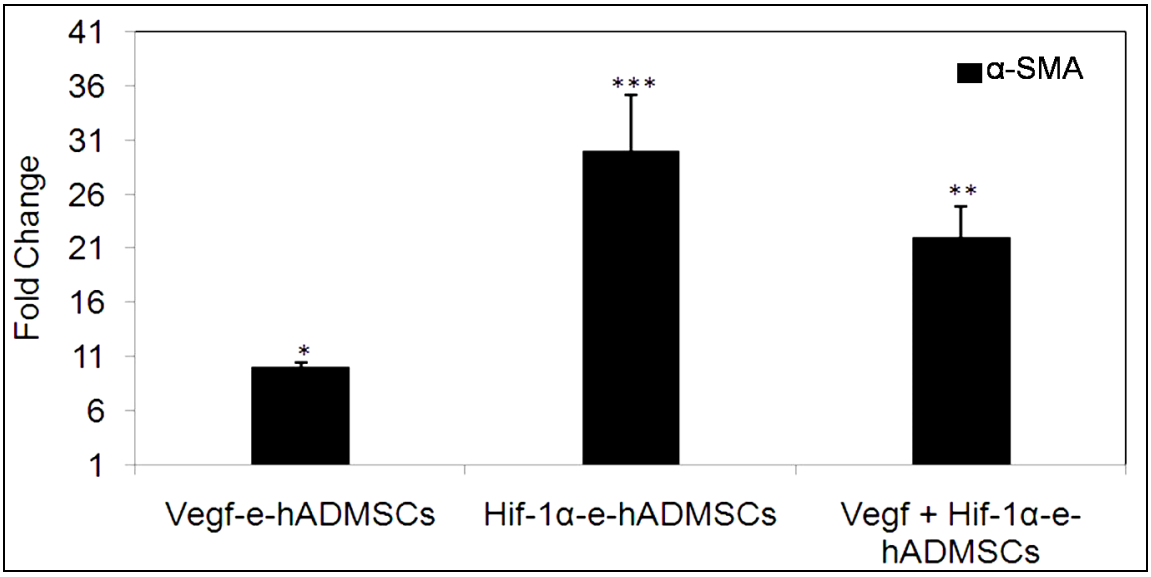


Figure 27(E): Graphical representation of vascular smooth muscle marker expression upon low seeding density TESG culture. *Gene up-regulation profiles of* α-smooth muscle actin (α-SMA) *was determined and seen to be significant in e-hADMSCs groups. Fold change is indicated relative to GAPDH expression on each day of analysis using the* $2^{-\Delta\Delta Ct}$ *method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** (P<0.001*); *** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

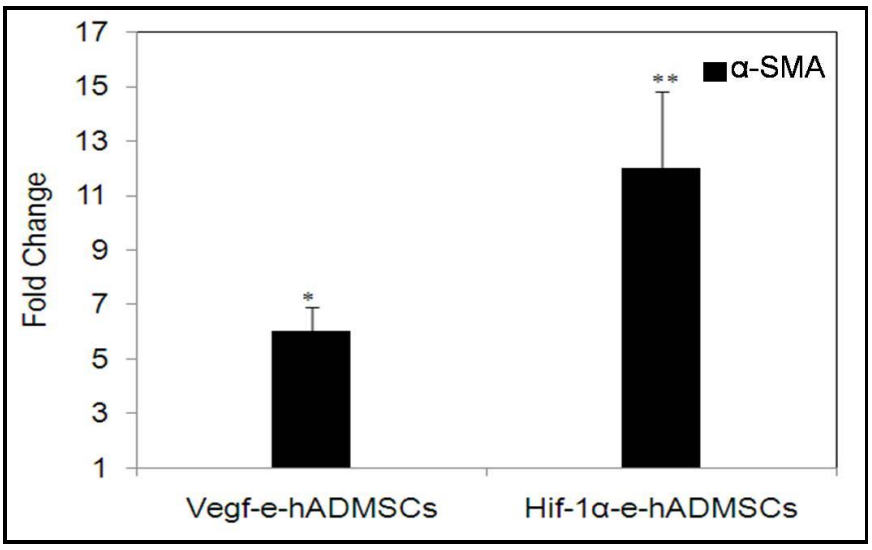


Figure 27(F): Graphical representation of vascular smooth muscle marker expression upon high seeding density TESG culture. *Gene up-regulation profiles of α-smooth muscle actin (α-SMA) was determined and seen to be significant in e-hADMSCs groups. Fold change is indicated relative to GAPDH expression on each day of analysis using the* $2^{-\Delta\Delta Ct}$ *method, upon normalization with results obtained from the non electroporated hADMSCs. Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.7.3.4. Immunochemical characteristics of cells on TESG

The exposure of engineered hADMSCs to fibroblast matrix specific TESG, in directing efficient vascularisation through endothelial lineage commitment was positively determined by immunostaining of endothelial functional marker CD31 in dermal grafts seeded at both 5000cells/cm^2[Figure 28 (A]and at 10000cells/cm^2[Figure 28 (B)]. The detection pattern of CD31 in TESG with higher seeding density depicted elevated levels of its expression similar to earlier reports (Rogozhnikov et al., 2016), upon 3D culture .The detection of CD31 in engineered hADMSCs compared to their absence in n-e-

hADMSCs suggests that delivered AF, through autocrine/paracrine mediation directs endothelial determination.

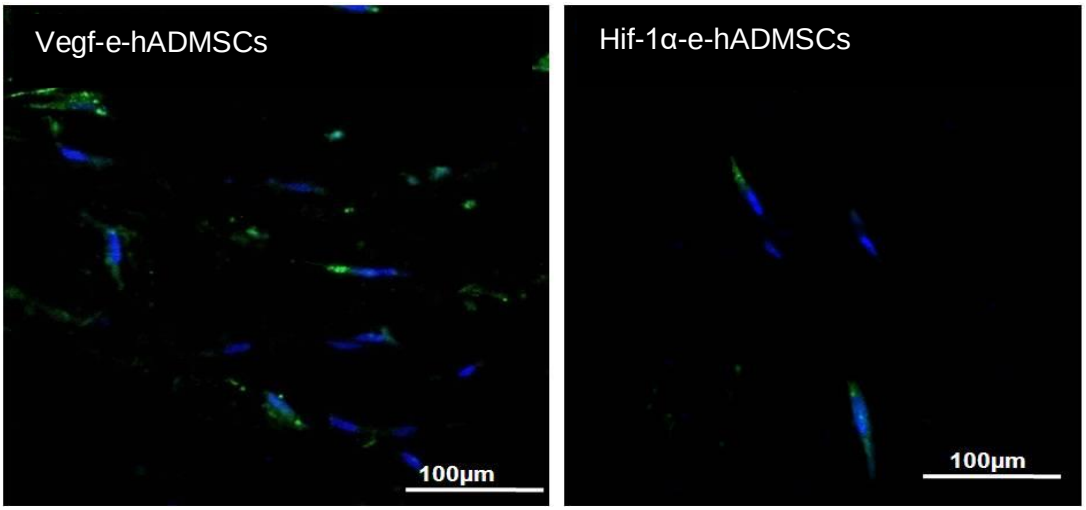


Figure 28(A): Representative images demonstrating EC marker on low density TESG culture: *In the Vegf-e-hADMSCs and Hif-1α-e-hADMSCs group, many CD31-positive cells (green) are seen in the images (20x) (n=3).*

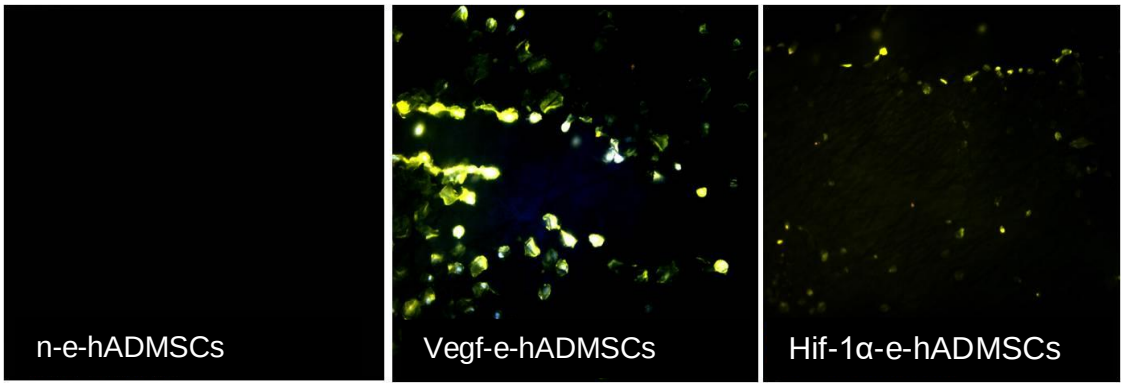


Figure 28(B): Representative images demonstrating EC marker on high density TESG culture. *In the Vegf-e-hADMSCs and Hif-1α-e-hADMSCs group, many CD31-positive cells (green) are seen in the images and absent in the n-e-hADMSCs group (20x) (n=3).*

Despite exposure to fibroblast specific differentiation niche, the presence of fibroblast marker Fibrillin at translational level of analysis was faint as shown in [Figure 29] at both seeding densities.

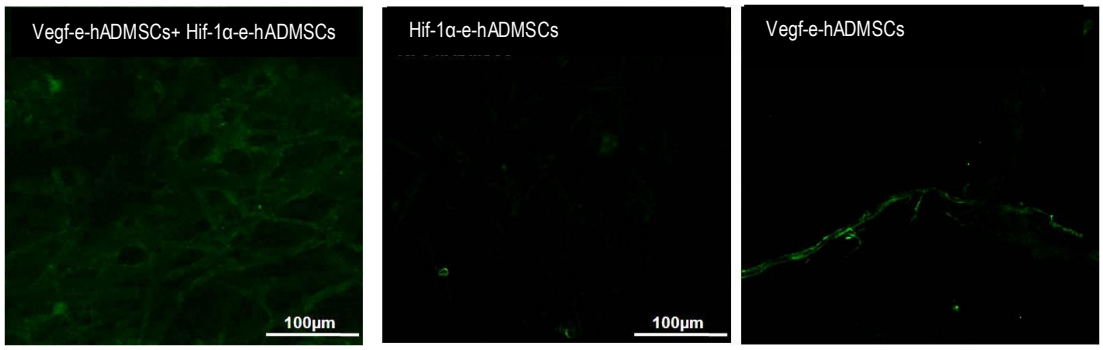


Figure 29: Representative images demonstrating fibroblast marker TESG culture: *In the Vegf-e-hADMSCs and combination group (Vegf-e-hADMSCs+ Hif-1α-e-hADMSCs) group, weak Fbln positivity (green) is seen in the images (20x) (n=3), but were absent in the Hif-1α-e-hADMSCs group.*

4.7.3.5. Quantification of ECM synthesis

The de-cellularized TESG seeded with e-hADMSCs, with n-e-hADMSCs as control at 5000cells/cm^2 were comparable after 20d of cell culture showing uniform deposition of collagen and elastin on the hybrid scaffold [Figure 30 (A)], indicating that the angiogenic growth factor release did not alter collagen and elastin content significantly. Whereas at 10000cells/cm^2, elastin deposition was observed to be elevated than collagen, favouring stable TESG [Figure 30 (B)]. Hence, e-hADMSCs at higher seeding density favoured structural support and elasticity within the scaffold as demanded in the dermis(Strong et al., 2017).

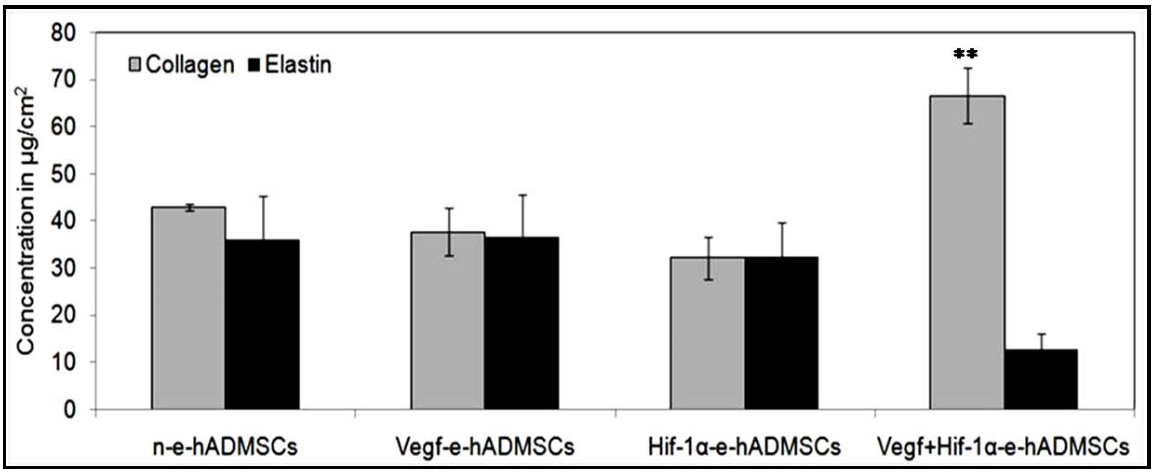


Figure 30(A): Graphical representation of ECM deposited on TESG at low seeding density. *De-cellularized grafts were subjected to digestion and quantitative analysis of collagen and elastin. Angiogenic growth factor release from e-hADMSCs did not alter collagen and elastin content significantly. Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

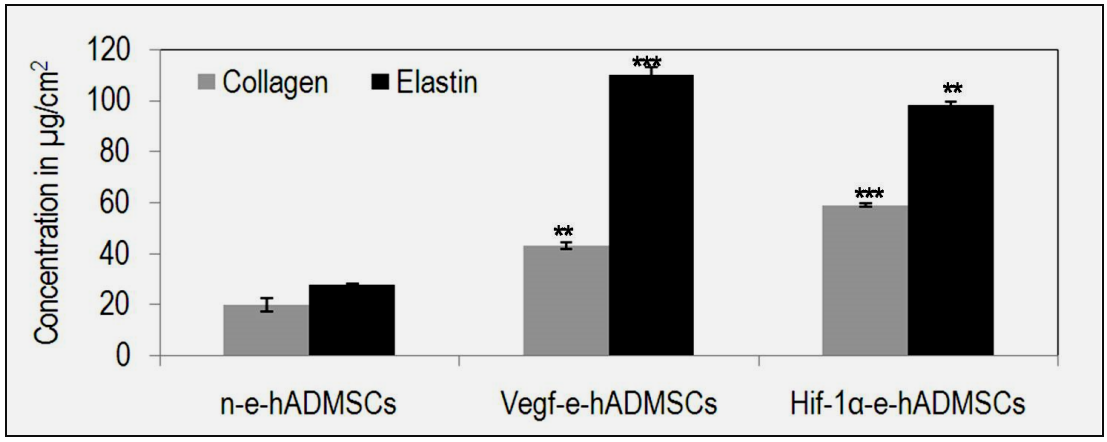


Figure 30(B): Graphical representation of ECM deposited on TESG at high seeding density. *De-cellularized grafts we subjected to digestion and analysis of collagen and elastin. Elastin deposition in TESG with high seeding density of e-hADMSCs was observed to be significantly elevated than collagen, favouring stable TESG. Error bars*

*represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.8. Effect of overexpressed secretome in rabbit wound healing

4.8.1. Characteristics of rabbit ADMSCs

Combinatorial effect of hADMSC over-expressed secretome in the presence of transplanted cells, rabbit ADMSC were used. Isolated rabbit ADMSCs (rADMSCs) from multiple allogenic donor tissues showed typical plastic adherent property with spindle shaped morphology as shown in Figure 31. Before transplantation rADMSCs were tagged with PKH26 to track transplanted cells upon termination.

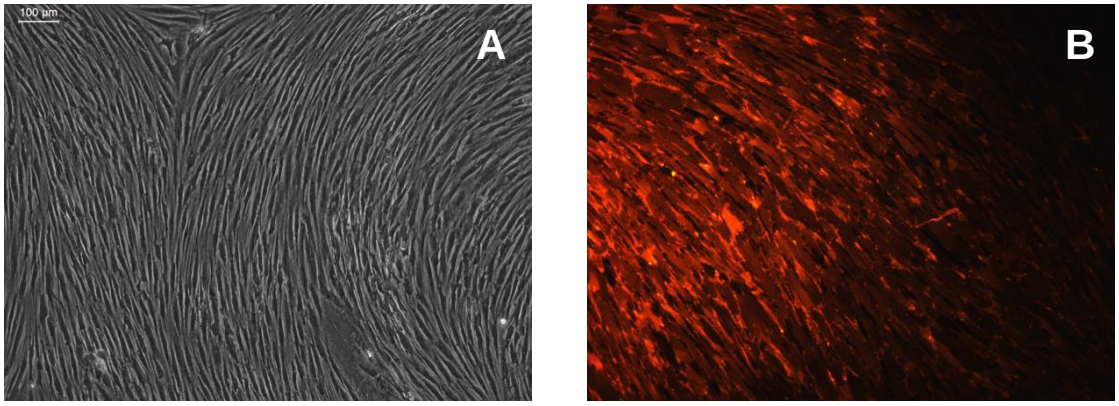


Figure 31: Isolation and culture of rADMSCs*. (A) Micrograph of isolated and cultured hADMSCs showing typical spindle shape morphology (20x) (B) rADMSCs labelled with PKH26 were observed by fluorescence microscopy (10x).*

4.8.2. Ex vivo visualization of transplanted rADMSCs by using IVIS®.

Fluorescence detection determined that transplanted rADMSCs migrated to distant sites: but, when transplanted along with the s-e-hADMSCs, the cells were retained for 28 days at the injury site Figure 32.

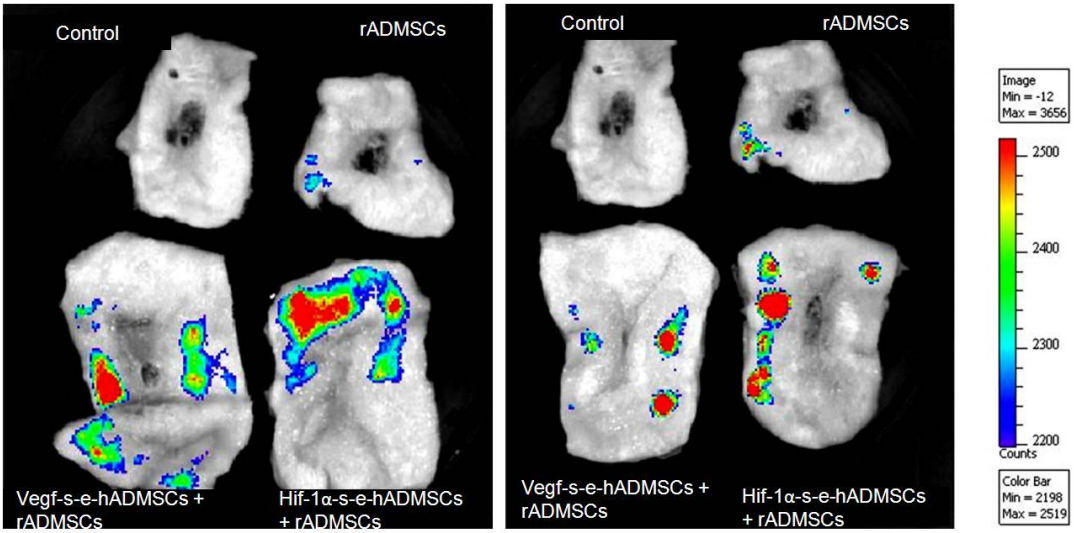


Figure 32: Transplanted cells tracked using fluorochrome images. *Representative images of skin explants from control and experimental groups analyzed with IVIS® for the presence of PKH26 labelled cells obtained by manual alignment of the spectrum. The colour scale bar shows the range of strongest to weakest signal. The intensity is strongest for the red colour points. The darker the spot, the stronger the signal.*

4.8.3. Visual observation of surface wound healing in rabbits.

Fig. 33 shows the photographs (4cm x 4cm) of postoperative wound healing in control and treatment groups for 5, 10, 15 and 28 days.

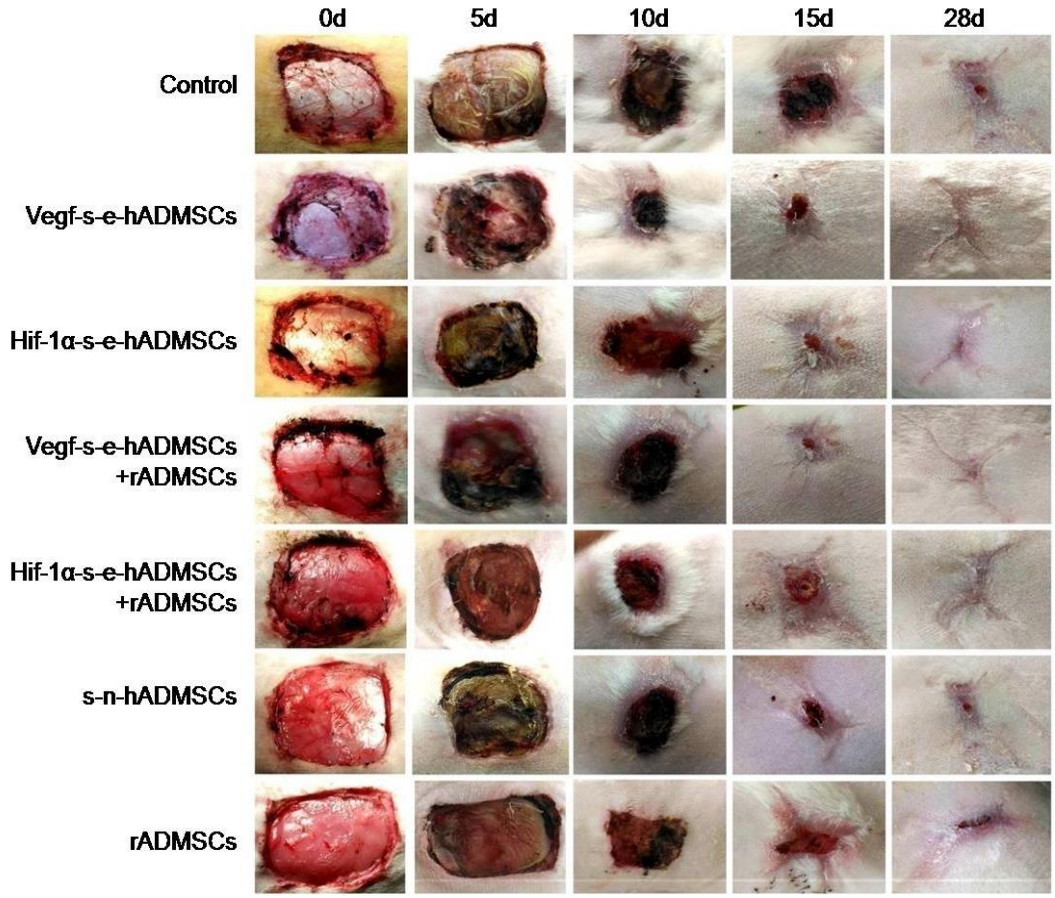


Figure 33: Representative images demonstrating progression of Wound Closure*: Seven different full thickness excision wound groups in the study of the same dimension (4cmx4cm) were gross imaged on 0d, 5d, 10d, 15d and upon termination on 28d. Comparative analysis showed potential of Vegf-s-e-hADMSCs to accelerate the rate of excision wound closure in vivo by 10d. Complete wound closure was observed in s-e-hADMSCs groups, both independently and in combination with rADMSCs at 28d, (n=3).*

Significantly larger wound areas achieved better closure in wounds treated with s-e-hADMSCs than with saline (control grou p). On day 10, the percentage of wound

closure was 47% for Vegf-s-e-hADMSCs-treated wounds vs. 19% for control wounds as quantified in Figure 34.

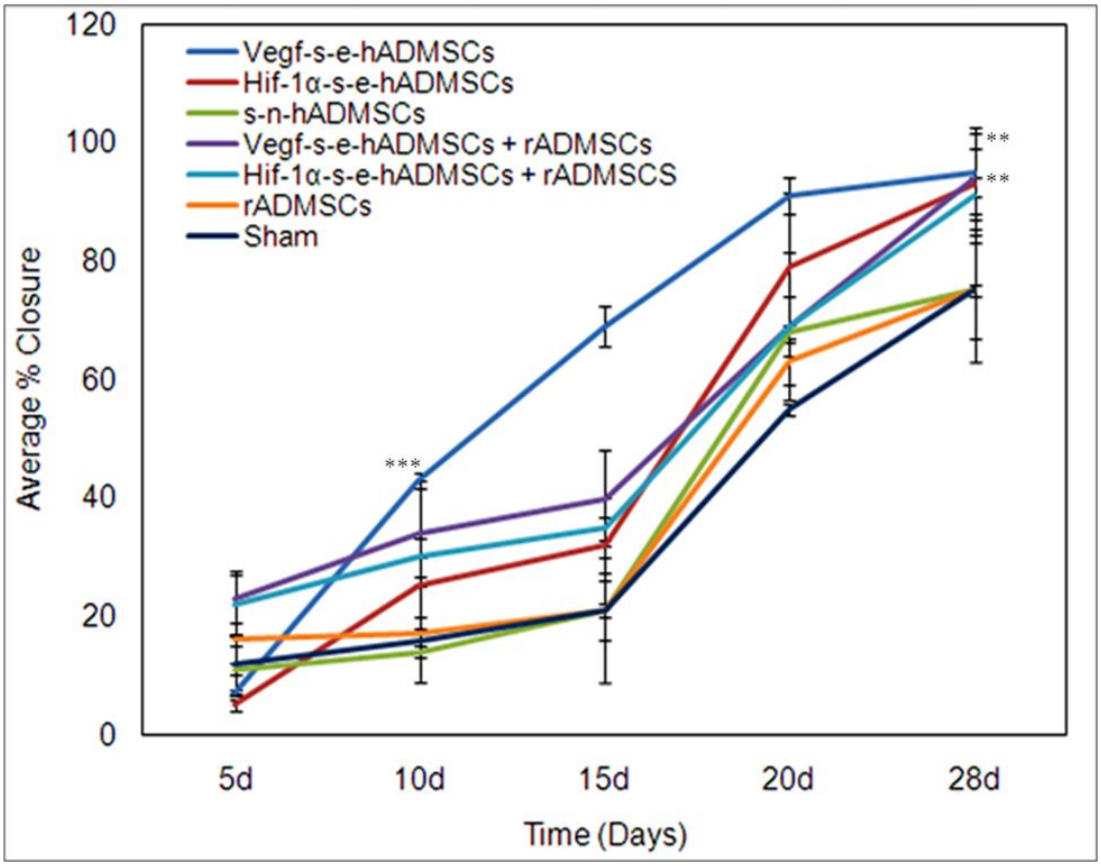


Figure 34: Graphical representation of wounds closure. *The wound area measured during the animal evaluation at 0d, 5d, 10d, 15d and upon termination on 28d was computed and analyzed. Wound closure demonstrates the effect of seven different types of application highlighting potential of s-e-hADMSCs, both independently and in combination with rADMSCs. Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs non electroporated hADMSCs, n=3.*

4.8.4. Microscopic observations of H&E stained sections

Histological analysis of the wounds of all respective groups of treatment and control were performed by H&E staining, as shown in Figure 35(A), (B) and (C).The healing rate groups treated with s-e-hADMSCS, independently and in combination with rADMSCs was higher compared to other groups.28 days after wound creation reconstruction of epidermis or epithelialization and angiogenesis was evident in tissue samples of the groups treated with s-e-hADMSCS, independently and in combination with rADMSCs groups. Granulation tissues were mature. Fibroblasts were low and collagen fibers deposition was high. Also the inflammatory cells were scant in wound area.

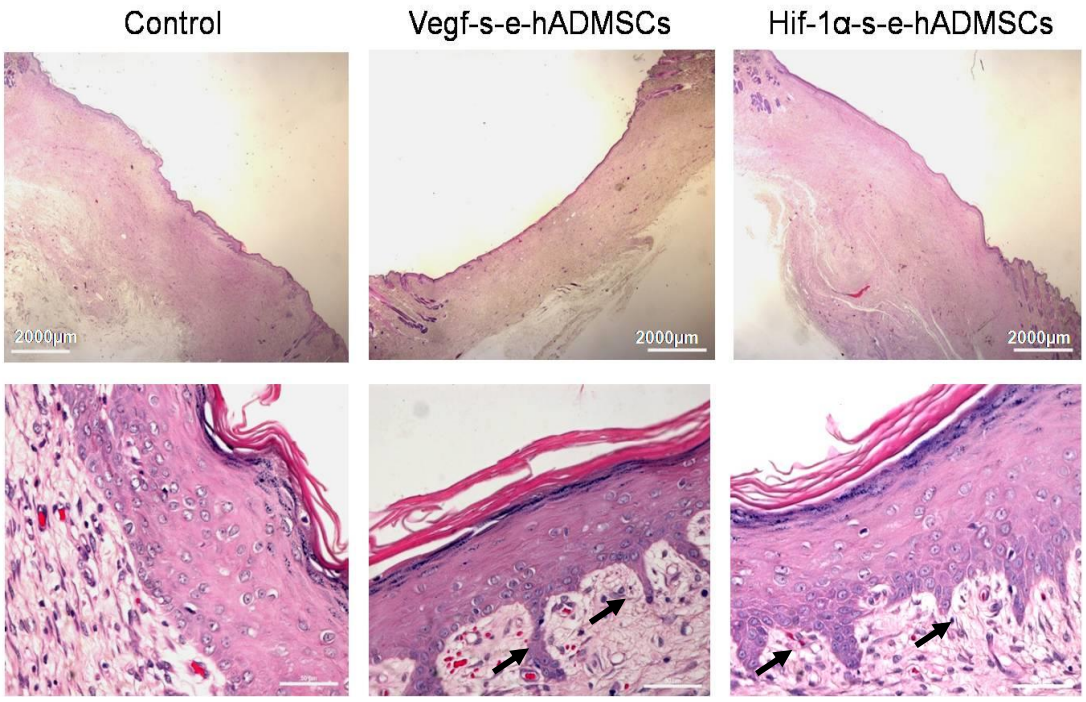


Figure 35(A): Representative images of H&E stained tissue sections. *Photomicrograph of histopathological section of wound tissue 28 day after creation of wound (H&E) in large area full thickness rabbit wound groups without treatment (Control) and with treatment (Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs. As evident in figures, no-treatment samples showing incomplete epidermis, poor vascularization and no rete pegs at the same time. Treated groups have well organized epidermis with developed rete pegs (black arrows) and neo-vascularized dermis layers at day 28 of treatment. Top panel images at Scale bar = 2000µm, bottom panel images at scale bar = 500µm, respectively, (n=3).*

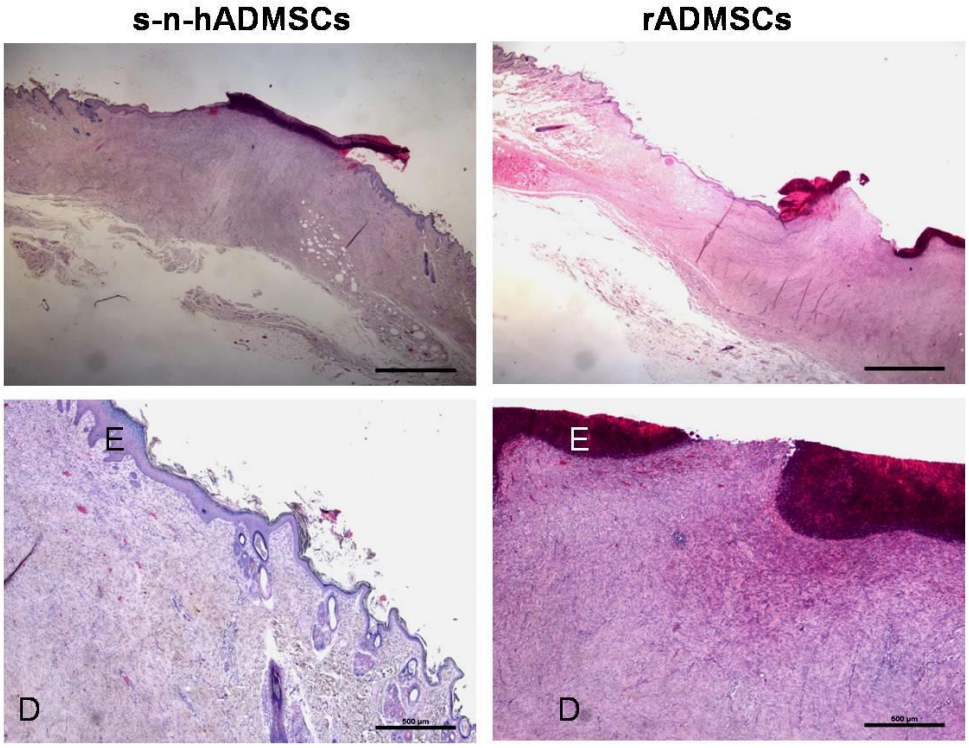


Figure 35(B): Representative images of H&E stained tissue sections. *Photomicrograph of histopathological section of wound tissue 28 day after creation of wound (H&E) in large area full thickness rabbit wound groups with treated with s-n-hADMSCs and rADMSCs respectively. As evident in figures, both groups display poor*

epithelisation and incomplete healing. Top panel images at Scale bar = 2000µm, bottom panel images at scale bar = 500µm, respectively, (n=3).

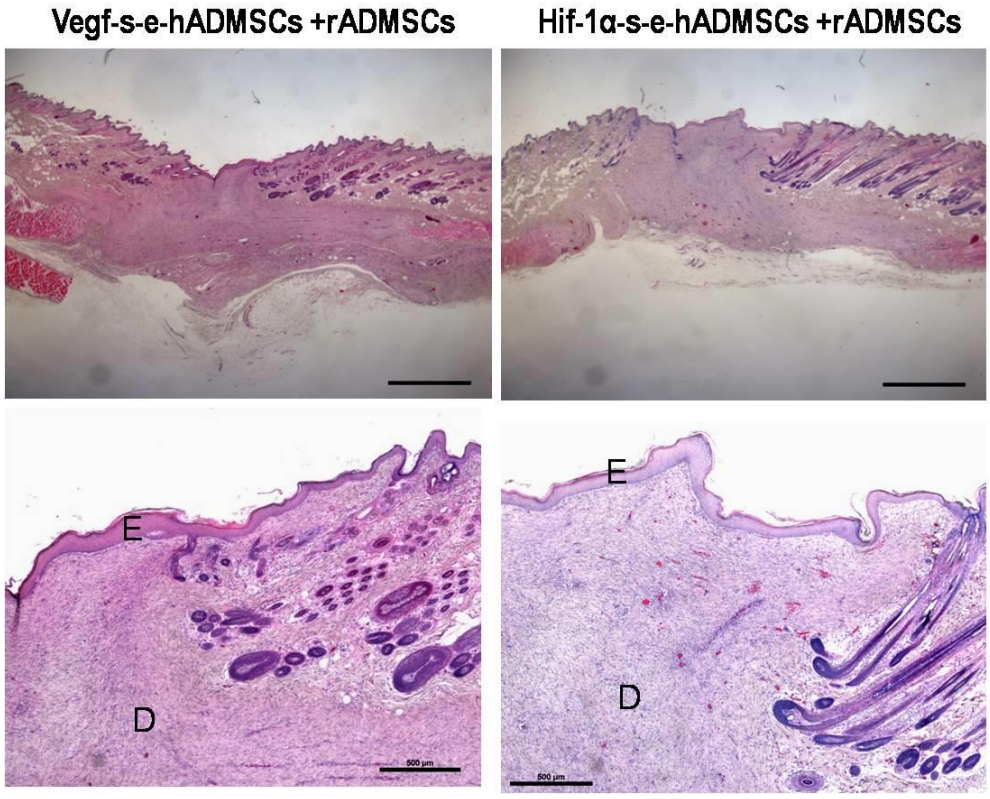


Figure 35(C): Representative images of H&E stained tissue sections. *Photomicrograph of histopathological section of wound tissue 28 day after creation of wound (H&E) in large area full thickness rabbit wound groups with treated with (Vegf-s-e-hADMSCs + rADMSCs) and (Hif-1α-s-e-hADMSCs +rADMSCs) respectively. As evident in figures, both groups display complete epithelisation and healing. Top panel images at Scale bar = 2000µm, bottom panel images at scale bar = 500µm, respectively, (n=3).*

4.8.5. Microscopic Analysis of Collagen stained sections

Granulation tissue was examined by Sirius Red staining for mature collagen. Thick, high-density collagen fibers stained bright red were observed in secretome-treated wounds and combination groups (secretome + rADMSCs) compared with the control saline-treated wounds, where the staining shows disorganized collagen fibres (Figure 36). The percentage of mature collagen was determined by measuring the red pixelated areas using ImageJ software. The area of mature collagen was significantly larger in secretome-treated wounds than in control (Figure 37)

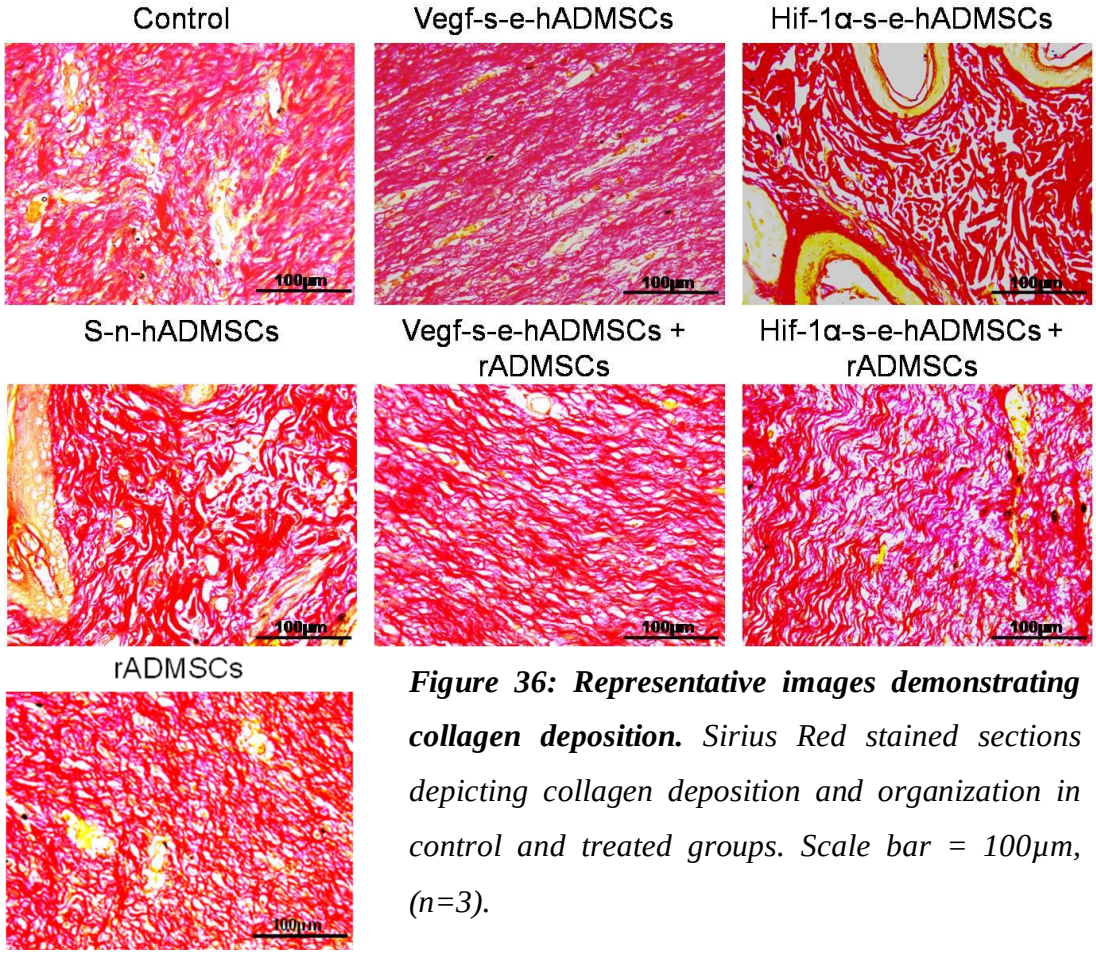


Figure 36: Representative images demonstrating collagen deposition. *Sirius Red stained sections depicting collagen deposition and organization in control and treated groups. Scale bar = 100µm, (n=3).*

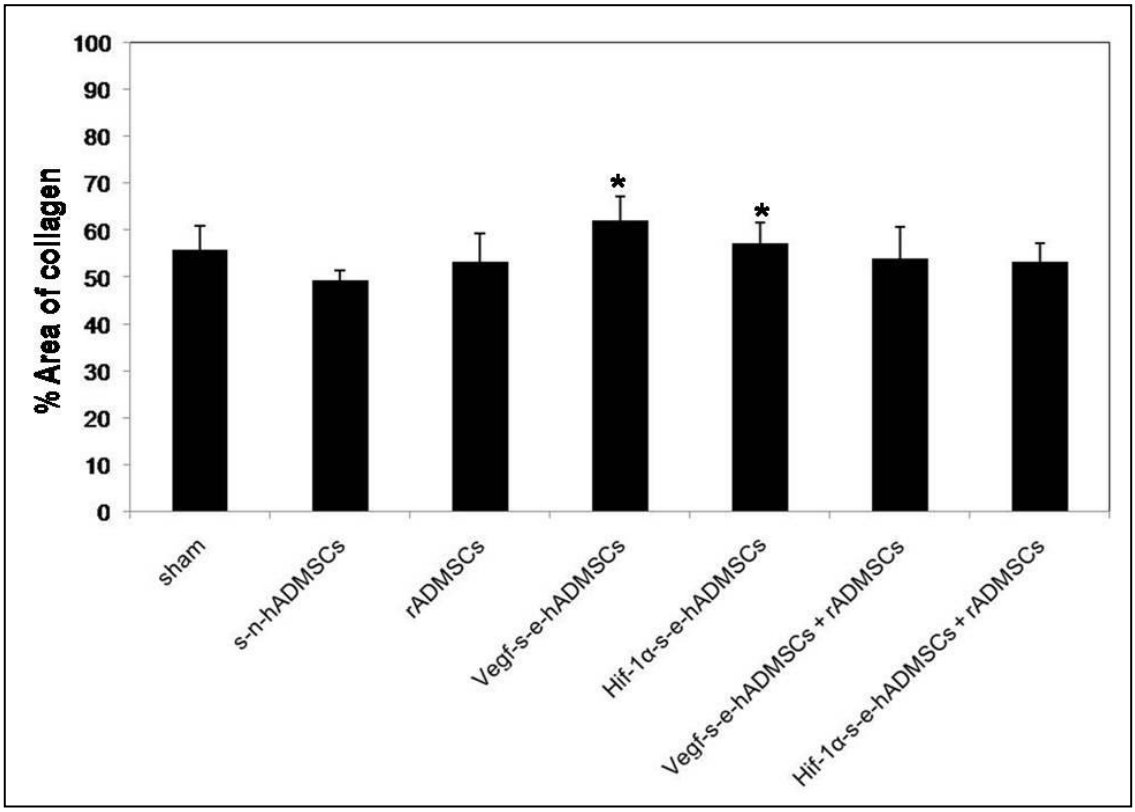


Figure 37: Comparison of percentage area of collagen: *Sirius Red staining, Percentage area occupied by positively stained collagen fibers in relation to the total repair area in rabbit wounds, 28 d post treatments. Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs show significant effect in deposition of collagen. (n=3, Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs sham)*

4.8.6. Effect of treatment on vascularization of regenerated skin

To visualize the newly formed capillary vessels in granulation tissue, the sections were stained with an antibody against endothelial marker CD31. A more extensive capillary network with wider vessels was observed in the microphotographs of wounds treated

with s-e-hADMSCs and in combination (s-e-hADMSCs + rADMSCs) when compared with those of control saline-treated wounds (Figure

38). Quantification of capillary vessels demonstrated a significantly greater number of capillaries in the s-e-hADMSCs treated group compared with the control groups, Figure 39.

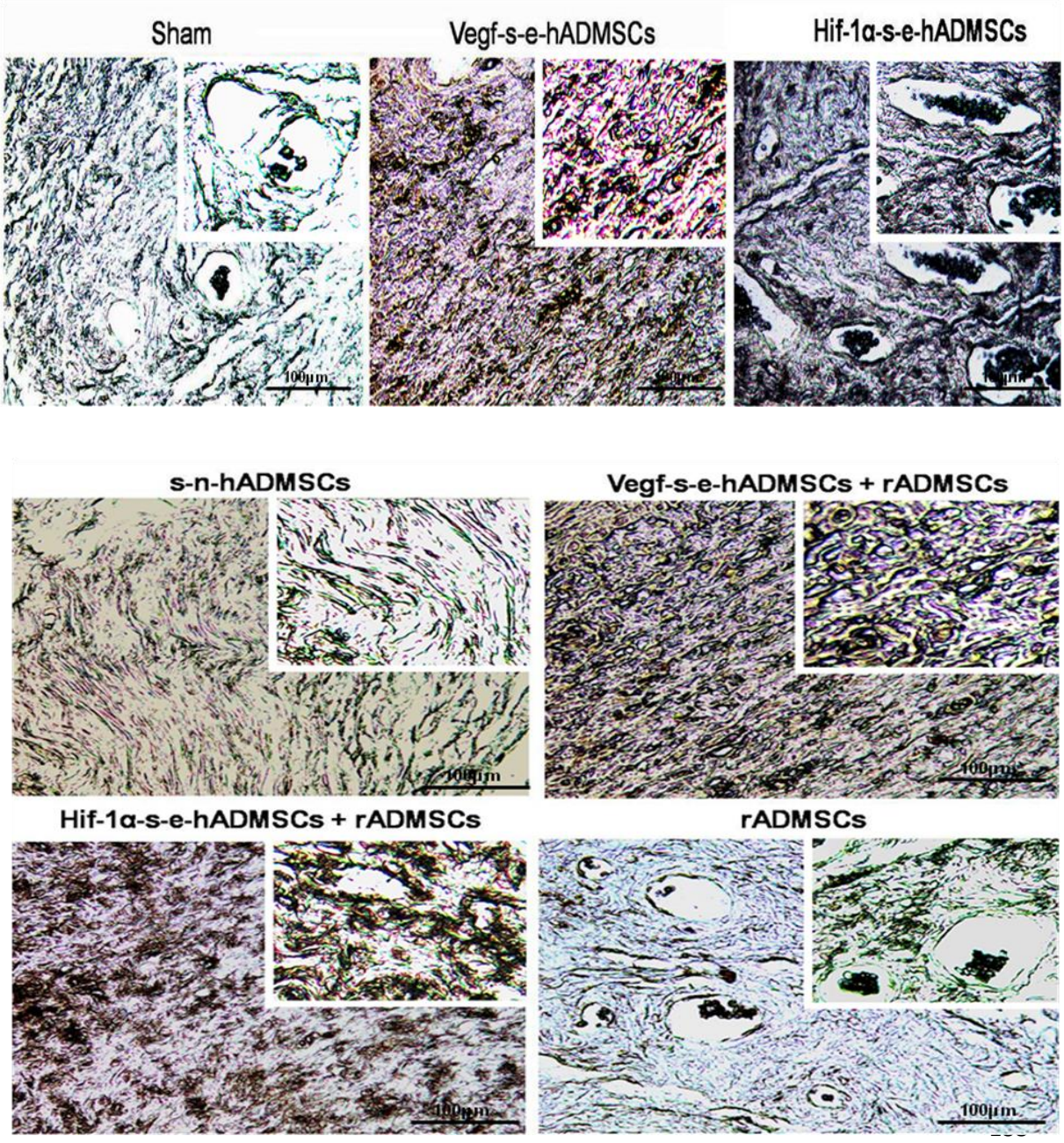


Figure 38: Blood vessel staining in rabbit skin wounds. *CD-31 (PECAM-1) is expressed by endothelial cells and CD-31 immunostaining is commonly used to identify blood vessels (indicated by brown staining). Representative images are shown of the center of the excision wounds after 28d of healing. Scale bar = 2000µm, n =3.*

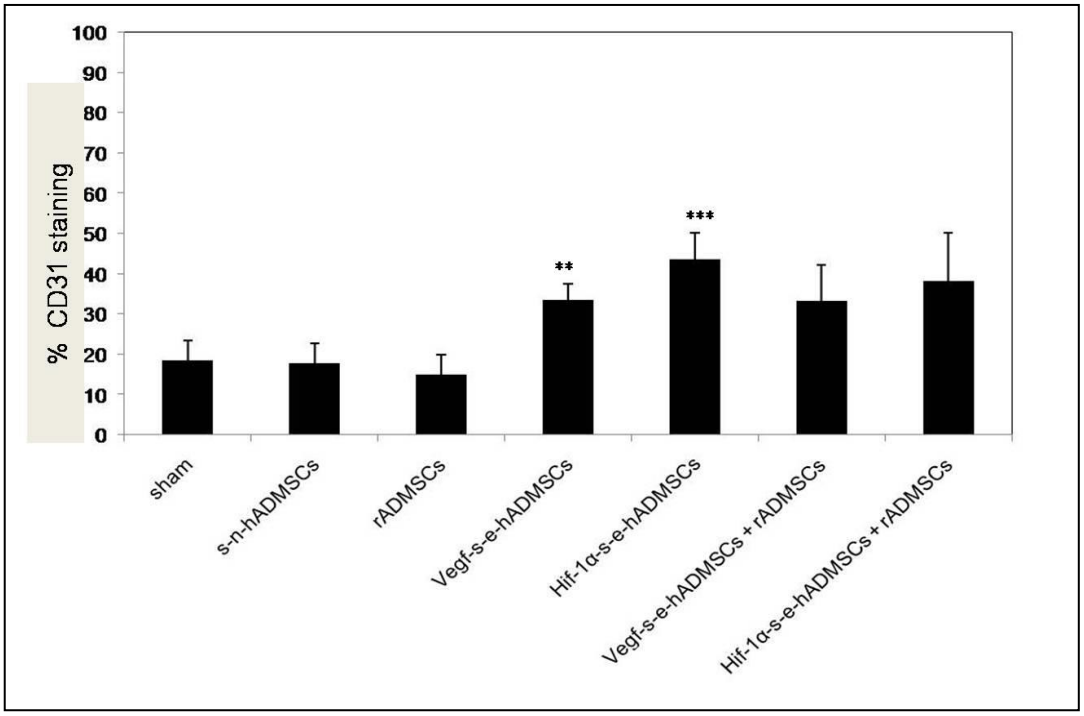


Figure 39: Blood vessel staining in rabbit skin wounds. *Percentage area of CD31 (DAB Staining, % positively stained area) in rabbit wounds, 28d post treatment. Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs show significant angiogenic potential in terms of CD31 expression in comparison with sham, (n=3). Error bars represent standard deviation, *** (P<0.001); ** (P<0.01); and * (P<0.05) vs. sham.*

4.8.4.4. Semi-quantitative analysis of histological parameters

Semi-quantitative assessment of the regenerated epithelial layer features, inflammatory infiltrate extent and collagen deposition in treated and control wounds, post 28 days, is reported in Table 6. In our study, the best and worst re-epithelization rates were noted after transplantation.

Groups	Amount of granulation tissue	Inflammatory infiltrate	Collagen fiber orientation	Pattern of collagen	Amount of early collagen	Amount of mature collagen	Total Score
Sham (control)	2	4	3	2	2	2	**15**
Vegf-s-e-hADMSCs	4	4	4	4	3	3	**22**
Hif-1α-s-e-hADMSCs	4	4	3	4	2	4	**21**
Vegf-s-e-hADMSCs + rADMSCs	4	4	4	4	4	3	**23**
Hif-1α-s-e-hADMSCs + rADMSCs	4	4	3	4	3	3	**21**
s-n-ADMSCs	3	4	3	2	2	4	**18**
rADMSCs	3	4	3	2	2	2	**16**

Table 6: Healing scores analysis*: Using this healing score, it is concluded that test groups in the study were correlated with accelerated wound healing in comparison to the sham group (n=3)*

The healing score analysis establishes healing status graded as good for groups treated with s-e-hADMSCs, both independently and in combination with rADMSCs where as all other groups were graded fair.

CHAPTER 5: DISCUSSION

This chapter discusses the results of the study, which is illustrated in chapter 4. The observations found are discussed in the light of published literature and the study hypothesis developed.

5.1 Transfected hADMSCs in vitro angiogenic response

Chief requirements for establishment of vasculature have been attained through experimental evidences emphasizing EC proliferation, migration and maturation. Angiogenesis is tightly coordinated and regulated by GFs both in physiology and pathology. In conditions such as ischemia and inflammation, GF therapy is often opted to account their depletion. MSCs are well established to regulate tissue homeostasis in regenerative medicine through its reservoir of growth factors for therapeutic product delivery and is in clinical development (Julianto and Rindastuti, 2016), (D'souza et al., 2015a). MSC on genetic manipulation, serve as programmed molecule transmitters, addressing better bioavailability of therapeutic molecules in addition to native immune-modulatory factors that improve effective and safe delivery in chronic cutaneous wounds (Briquez et al., 2015). Over expressing GFs in MSCs, using techniques that may contribute to immunogenicity and toxicity has been reported(Keeler et al., 2017). Effective delivery of AF through validated and safe methods is of demand. Future translational potential of the study outcome was envisaged

when the problem was selected. Therefore, the first part of this study explored angiogenic potential of genetically engineered hADMSCs through (i) a clinically safe and effective method for delivery of two potent AF (VEGF-A and Hif-1α), (ii) benefits upon combined delivery (Vegf-s-e-hADMSCs and Hif-1α-s-e-hADMSCs) on *in vitro* angiogenic response.

The present study exhibits non-comparative strength of Neon® transfection system for bioengineering hADMSCs based on recent reports on its comparative superiority amongst commonly used non-viral transfection methods of primary cells/ lines(Covello et al., 2014; Liang et al., 2015; May et al., 2017; Montoya and Ansel, 2017; Moore et al., 2010; Zhao et al., 2016). Use of neon® transfection system seemed feasible; exhibiting ~ 47% to 50% transfection efficiency consistently, with acceptable transfection-associated cell viability loss to <30% ("ISO 10993-5:2009 Biological Evaluation of Medical Devices. Part 5: Tests for In Vitro Cytotoxicity. International Organization for Standardization; Geneva, Switzerland: 2009", n.d.). This determined efficiency was established to be sufficient and effective enough for primary cell transfection based on similar reports on *in vivo* application (Halim et al., 2014; May et al., 2017). Concerns of long term GF production and undesired angiogenesis during translation has emphasized the preference of transient over stable expression (Zalups and Lash, 1996). In this context, this study reports safety of Neon® transfection system based on maintenance of hADMSCs' morphology (Beegle et al., 2016), stemness, acceptable cell viability and effective transient AF delivery, post-transfection.

Maintenance of hADMSCs' stemness aided by paracine/autocrine influence of delivered AF, together with guided endothelial trans-differentiation on hypoxic exposure is highlighted in this study. However, earlier reports, stating hypoxic EC-differentiation of MSC as a negligible factor, for angiogenic potential of MSCs (Beckermann et al., 2008) contradicts the findings of this study. It is hence assumed that ~50% of transfected hADMSCs on over expression of AFs, may have contributed in inducing endothelial lineage commitment of the remaining 50% non engineered hADMSCs through paracrine action upon hypoxic exposure. Moreover, *in vitro* induction of pro-angiogenic signal release by hypoxia pre-conditioned stem cells is reported (Fierro et al., 2015) with comprehensive increase of endogenous regenerative gene expression (Wang et al., 2015). *In vivo* experiments are much required to establish this efficacy. Further, the functional role of hADMSCs' over expressed VEGF-A and HIF-1α in mediating HUVEC responses was evaluated, as recognized for therapeutic angiogenesis (Krock et al., 2011). Potential of HIF-1α in proliferation and migration (Chen et al., 2015; Eul et al., 2005; Hubbi and Semenza, 2015) is highlighted upon comparison of HUVECs treated with Hif-1α-s-e-hADMSCs to Vegf-s-e-hADMSCs and s-n-hADMSCs. However, in view of tube-formation, HUVECs treated with Vegf-s-e-hADMSCs showed distinct orientation, elongated branches and mature tubes than on Hif-1α-s-e-hADMSCs and s-n-hADMSCs treatment. In terms of loops formed, Hif-1α-s-e-hADMSCs treatment stood higher. This acceleration may correlate to better mitogenic activity of HIF-1α, contributing to larger number of cells for participation in loop formation (DeCicco-Skinner et al., 2014). Instigation of EC migration, proliferation,

differentiation, survival, vessel permeability and dilation has been correlated to Flk-1 up-regulation, thereby earning its priority as the predominant receptor in angiogenic signaling(Cébe-Suarez et al., 2006). In this context, though endothelial cell proliferation and migration is significantly higher on treatment with Hif-1α-s-e-hADMSCs; the instigation of lengthier tubes and up-regulation of Flk-1 seemed negligible. The absence of *Flk-1* on Hif-1α-s-e-hADMSCs treatment seemed unclear regardless of the presence of VEGF-A in the Hif-1α-s-e-hADMSCs. An earlier report stating that *Flk-1* expression is not activated by Hif-1α in ECs, rather by the Hif-2α, strikingly correlates to the observation in this study (Elvert et al., 2003). Additionally, in tissue neovascularization with respect to aging, reports on impaired VEGF expression, advocate combinatorial restoration of HIF-1α and VEGF-A ((Elvert et al., 2003) . Addition of Vegf-s-e-hADMSCs, to overcome the lacuna of Hif-1α-s-e-hADMSCs to independently establish *Flk-1* expression, strongly highlights combinatorial delivery for faster tissue restoration.

The investigations in this part of the study thus confirmed angiogenic superiority of combinatorial delivery using hADMSCs, based on well established *in vitro* HUVEC response assays. However, the choice of transiently expressing either/both of the two AF in hADMSCs for efficient vascularization in terms of wound type, size and closure time, is to be assessed and determined in appropriate *in vivo* models. This part of the study precisely points out the possible potential of incorporating transient yet sustained delivery of GFs in the field of tissue engineered products/skin substitutes that focus on faster healing times, meaning overall decreased treatment period(Murphy and

Evans, 2012). Again, determining quantity of engineered hADMSCs for efficient delivery and vascularization based on wound size and type is left for further investigation.

5.2. Application in Tissue Engineered Skin Grafts

In the last decade, skin tissue engineering has evolved dramatically with promising approaches to manage difficult wounds. However, impaired vascularization within tissues remains a reconstructive challenge (Frueh et al., 2018) During early stages of graft transplantation, the exposure to relatively long hypoxic and ischemic period after surgery often leads to its degeneration and necrosis.(Chen et al., 2017) While bio-engineered products such as natural substitutes - human allograft, Oasis wound matrix®; synthetic substitutes - Biobrane™; permanent skin substitutes - Epicel®, and Integra® have been commercially available for years, major problems still exist with poor blood supply, material sources, manufacturing techniques, material compatibility, and therapeutic effects (Halim et al., 2010). A new strategy with better therapeutic effects emphasizing vascularization is critical. Improving vascularization of dermal substitutes by means of angiogenic or prevascularization approaches have been attempted (Frueh et al., 2017). Modification of structural and physicochemical properties of dermal scaffolds, biological scaffold activation with growth factor-releasing systems or gene vectors, and generation of prevascularized skin substitutes by seeding scaffolds with

vessel-forming cells have been explored (Frueh et al., 2017). These conventional approaches have also been supplemented by emerging strategies, such as transplantation of adipose tissue-derived microvascular fragments (Tremolada et al., 2016b) and endothelial progenitor cells (EPCs) for the regeneration of new blood vessels (Dai et al., 2018). Previous studies indicate that the angiogenesis mechanisms of ASCs that function in tissue repair and wound healing cannot be solely ascribed to the differentiation of ASCs into endothelial lineages, but also involve paracrine effects mediated by the secretion of numerous cytokines and growth factors, like VEGF, HGF, bFGF, and others (Dash et al., 2018; Kokai et al., 2014). Additionally, studies have also demonstrated that MSCs can promote the proliferation, migration, and collagen secretion of fibroblasts through a paracrine mechanism independently of the promotion of angiogenesis (Chen et al., 2014). Number of *in vitro* strategies has been used to drive the secretome towards a more desirable pro-angiogenic stimulator including addition of growth factors and hypoxic preconditioning. However, the efficacy of these approaches has not been extensively assessed in preclinical models. Moreover, the requirement of repeated administration or control release systems often limits current approaches (Liu et al., 2013). Skin substitutes seeded with cells producing growth factor on a continuous basis could hence provide an attractive alternative (Spiekstra et al., 2007).

The second part of the present study thus attempted vascularization of in house generated tissue engineered skin graft (TESG) (Nair et al., 2014a) by incorporating previously described bioengineered hADMSCs showing angiogenic

potential (Ajit et al., 2019). Incorporated Hif-1α-e-hADMSCs and Vegf-e-hADMSCs at both densities of 5,000cells/cm^2 and 10,000cells/cm^2 showed adequate proliferation, confirming the TESG's ideal micro-environment (Dai et al., 2016). It is reported that early GF delivery approaches for tissue regeneration, such as direct injection or systematic local supplementation, resulted in low availability of bioactive GFs due to their rapid degradation *in vivo* (De Witte et al., 2018). Hence, common strategy to improve scaffold vascularization is the stimulation of the angiogenic host tissue response at the implantation site by incorporation of angiogenic growth factors into the implants. In this study, detection of sustained AF release by the engineered hADMSCs (e-hADMSCs) incorporated within the TESG, for a period of 20d, addresses the challenge of possibly attaining targeted/local cellular production of AFs. Moreover, in comparison to the release pattern of angiogenic factors (VEGF-A and Hif-1α) independent of the effect of scaffolds as described in the earlier Chapter 4, section 4.4., their detection at longer durations may be assumed to be a result of their binding to the scaffolds surface with sustained release to the surrounding tissue, supporting angiogenic outcome upon *in vivo* transplantation (Saberianpour et al., 2018). Additionally, the e-hADMSCs irrespective of the over expressed AFs release, is a reservoir of numerous pro-angiogenic GFs that favour repair and regeneration. This bears the advantage that the angiogenic process is stimulated much more efficiently than by the use of one growth factor alone.

Literature findings also indicate that scaffold biomaterials with slight proinflammatory properties may be appropriate for the stimulation of the angiogenic process at the implantation site. For instance, it is reported that poly-L-lactide co-

glycolide (PLGA) scaffolds induce slight inflammation after implantation into the dorsal skin fold chamber of Balb/c mice which is associated with a marked angiogenic host tissue response and a good vascularization of the implants after 14 days(Laschke and Menger, 2012). However, the pivotal problem in vascularizing implanted tissue constructs through *in vivo* angiogenesis is the fact that the development of new blood vessels is a time-consuming process, which can only be accelerated to a limited extent(Tremblay et al., 2005). This concern has led to the need of safe and efficient prevascularization approaches in the field of tissue engineering. The observed detection of significantly up-regulated endothelial markers; CD31, eNOS and *Flk-1* in the TESG incorporated with Vegf-e-hADMSCs, CD31 and eNOS in the TESG incorporated with Hif-1α-e-hADMSCs at higher seeding densities (10,000cells/cm^2) compared to lower seeding densities (5,000cells/cm^2) is an indication towards effective autocrine/paracrine mediated endothelial commitment contributing to pre-vascularization, in correlation to cell number availability. Detection of CD31 at translational level further confirmed the observation. On the other hand, fibroblasts are known to rapidly migrate into wound sites to establish an extracellular matrix that supports dermal vascular repair (Marino et al., 2014). Thus, simultaneous expression of fibroblast specific markers; Fibroblast specific protein-1 and Fibrillin-1 was determined and seen to be significant within TESG incorporated with e-hADMSCs as compared to n-e-hADMSCs at higher seeding densities. We speculate that, in our system, higher seeding density contributed to elevated fibroblast lineage commitment which supported the formation of endothelial committed cells by creating a physiological environment by matrix remodelling,

production and deposition of nonsoluble factors; however, this has not been demonstrated yet.

Fibroblasts are the main population of the dermis, which secretes collagen and elastin and thus provides suppleness, mechanical strength and elasticity to the skin. At 20 days, abundant matrix secretion was observed. Estimation of collagen and elastin proteins within the TESG incorporated with e-hADMSCs established their elevated and significant increase compared to TESG with n-e-hADMSCs, at higher seeding densities. Additionally, the presence of α-SMA indicated angiogenic potential towards micro-vessel stabilization. Furthermore, α-SMA also associates to type-1 pericytes which play a role in regulating vessel tone(Bodnar et al., 2016).

The investigations in this part of the study thus confirmed pre-vascularization potential of e-hADMSCs in in-house developed TESG, favouring co-existence of endothelial, fibroblast, smooth muscle cell lineage commitment. Clearly, the findings highlight that e-hADMSCs within scaffolds can direct viable stem cells in desired fate such as proliferation and differentiation into dermal cell lineages. The TESG is demonstrated to be successful in terms of accelerated cell proliferation, elastin and collagen deposition and neovascularisation in line to earlier reports(Dai et al., 2018). The choice of incorporating either/both of the two AF in e-hADMSCs for efficient pre-vascularization of TESG is left for further investigation and validation in terms of wound type, size and appropriate *in vivo* models. Again, pre-vascularization potential of

e-hADMSCs in other commercially available TESG lacking vasculature is to be explored.

5.3. Application in large full thickness rabbit wounds

In the quest to improve our understanding on the pathophysiology of chronic wounds and its associated therapy, several experimental models are highlighted in the literature. However, the attempt to reproduce a chronic wound with characteristics similar to those found in clinical practice still remains challenging.(Andrade et al., 2017) Mouse and Rat models, although contains the major layers of human skin (epidermis, dermis), there are significant histological and physiological differences of these layers to that of humans.(Wong et al., 2011) These differences in the thinness of epidermis and dermis, dense hair and loose skin elasticity,(Dorsett-Martin, 2004) raises concerns for accuracy in therapy and wound research. Though rabbit skin models have much similarity, the presence of superficial fasciae (or panniculus adiposus) and the panniculus carnosus, a layer of striped muscle responsible for skin-twitching movements which is absent in human skin, may aid faster wound healing (BILLINGHAM and MEDAWAR, 1951). More recently, the pig has been extensively validated as a model for studying human skin because of its anatomical and physiological skin architecture closely resembling that of humans. The epidermis and dermis of the pig is thick, which is also the case in humans (Grada et al., 2018). Unfortunately, pigs have a significant cost disadvantage to smaller animals and the amount of wound contraction varies depending on where the wound is made. Pigs also grow quickly and can become

difficult to handle should the study continue past a few months(Grada et al., 2018) To resolve the complexities and high costs associated with the pig model while maintaining the metabolic relevance to humans, researchers have pioneered the rabbit wound model due to their affordable size for large wound area generation and close similarities to human skin in terms of wound healing (Grada et al., 2018). Thus the present study utilized the rabbit wound model for generation of large area full thickness wounds and to analyze the angiogenic efficiency of s-e-hADMSCs, both independently and in combination with allogenic rabbit ADMSCs. Investigations in the present study showed enhanced homing of transplanted rADMSCs when administered in combination with s-e-hADMSCs in comparison to independent administration of rADMSCs. We assume the cytokines within the s-e-hADMSCs may have favoured stem cell homing. This may be correlated to similar reported findings that mention significant increases in cytokine expression to have promoted BMSC homing upon transplantation (Ziadloo et al., 2012). On gross observation of wound closure, significantly larger wound areas achieved better closure in wounds treated with s-e-hADMSCs than with control group. On day 10, the percentage of wound closure was 47% for Vegf-s-e-hADMSCs-treated wounds vs. 19% for control wounds. As the most common method for histopathology, HE staining is mainly used to assess the fibroblast number, collagen content, angiogenesis and epithelial formation during the skin healing process. In the present study, histological assessment indicated a noteworthy enhancement of wound healing in groups treated with s-e-hADMSCS, independently and in combination with rADMSCs compared to other groups, showing reconstruction of epidermis or epithelialization and evident

angiogenesis. No evident inflammatory reaction (infection, fistula, or fibrous capsule) was observed at either the implanted sites or adjacent sites in all groups, post transplantation. Further, presence of mature collagen was significantly larger in secretome-treated wounds than in control. A more extensive capillary network with wider vessels was observed upon endothelial specific marker; CD31 detection, in the wounds treated with s-e-hADMSCs and in combination (s-e-hADMSCs + rADMSCs) when compared with those of control saline-treated wounds. S-e-hADMSCs treated groups also showed well organized epidermis with developed rete pegs and neo-vascularized dermis layers at 28days of treatment. . Despite a single dose application of s-e-hADMSCs at 0d, it was interesting to observe elevated granulation tissue deposition at 28d, suggesting that tissue repair stimulated by exogenous GFs persisted. This observation falls in line with an earlier report on single dose topical VEGF application in wound healing (Galiano et al., 2004). The in vivo findings show that wounds treated with s-e-hADMSCs shows better wound angiogenesis as compared to allogenic rADMSC transplantation, promising cell free therapy. This finding correlates with recent studies that report secretome from adipose-derived mesenchymal stem cells shows robust effects on cellular processes that promote tissue regeneration (Mitchell et al., 2019). The MSCs-derived cell-free secretome appears to be able to recapitulate many of the properties/effects that have been described for the MSCs themselves. Pro-regenerative effects of MSCs secretome is already known in many different systems, by modulating the immune system, inhibiting cell death and fibrosis, stimulating

vascularization, promoting tissue remodelling, and recruiting other cells (Ferreira et al., 2018).

Considering MSCs main mechanism of action upon transplantation might be via paracrine signalling, it is surprising that only a few groups have studied angiogenic effect of preconditioned MSCs's secretory profile. Our study on pre-conditioning the secretome through bioengineering hADMSCs with AFs has demonstrated enhanced and accelerated angiogenic potential *in vivo* as determined by elevated CD31 detection at 28d in comparison to s-n-hADMSCs and rADMSCs. The exact composition of e-hADMSCs' secretome has to be further investigated to identify the key molecules responsible for the therapeutic angiogenic potential. The final goal would be the substitution of a cell-free product to achieve the desired therapeutic effect. It may be noted that cell therapies as established so far, notwithstanding its great promise, present several limitations concerning safety, standardization, and practicality of the procedures needed to deliver viable cells to the hostile microenvironment within injured tissues. Hence, use of s-e-hADMSCs for therapeutic angiogenic and wound regeneration as demonstrated in this study seems to be a much realistic option at translation. Further investigations on profiling s-e-hADMSCs may be required. Multiple dose experimentation on larger animal groups is left for investigation to establish comparative potential of s-e-hADMSCs.

5.4. Limitations and Future prospectus of this study

- Comparative studies on multiple dose and single dose effects of s-e-hADMSCs on wound healing may be carried out for establishing efficacy at translation.

- It will be desirable to know the secreted protein and exosome composition of the secretome, when producing bioengineered MSCs for therapeutic use facilitating a cell free therapy.

- Effect of pre-vascularized TESG using e-hADMSCs could be validated in comparison to commercially available skin substitutes, on appropriate *in vivo* models.

- Development of immortalized and bioengineered cell lines could also be explored for cost reduction in terms of large scale AF production and availability at clinical perspective.

- Healing of large wounds in diabetic animal and kinetics of tissue regeneration involving earlier period explant tissues such as on 7d and 14d would be needed to translate the technology for treatment of chronic wounds.

- Effect of secretome for control of inflammation at earlier periods of treatment need to be studied before attempting the treatment in clinics.

CHAPTER 6: SUMMARY AND CONCLUSION

6.1. Summary

The hope of stem cells in providing a foundation for strategies to regenerate diseased tissues and organs has long lived. The ability to 'self-renew' or divide to produce more stem cells and the ability to differentiate in order to give rise to specialized cells have made stem cells an attractive cell source for clinical applications. However, use of stem cells in regenerative therapeutics is hampered by incomplete knowledge of stem cell fate and a lack of technologies that enable translational impact of stem cell biology. In this context, measures on manipulating stem cells to enrich stem cell property and produce desirable behavior upon translation have been widely attempted. There has also been exciting progress in genetically engineering stem cells for delivery of therapeutic molecules using transfection or transduction methods. However, stem cell engineering for clinical applications is still in its infancy and requires further research. There are two main strategies for inducing transgene expression in therapeutic cells: transient and permanent expression. In many cases using cell therapy for the treatment of rapid-onset diseases with a short healing process, transient transgene expression may be a sufficient and optimal approach. Permanent transgene expression is primarily based on the application of viral vectors, and, due to safety concerns, these methods are less opted. There is however need for active, ongoing research toward the development of non-viral

methods that would induce therapeutic benefits with highly efficient transfection, with outstanding safety.

This current study targeted an aspect of chronic wound healing; angiogenesis; for therapeutic intervention using stem cells and bioengineering strategies. The inability of chronic wounds to establish adequate granulation tissue is considered as one of the challenges in wound healing. The action of vascular endothelial growth factor (VEGF), the principal initiator of angiogenesis and Hypoxia induced factor-1α (Hif-1α) is extensively studied in the context of wound healing. Human adipose derived mesenchymal stem cells (hADMSCs) is known to promote paracrine interactions to promote angiogenesis, decrease wound inflammation, regulate extra cellular matrix formation, and enhance regeneration of skin. However, rapid diffusion, poor biostability, requirement of supra-physiological doses which could lead to excessive uncontrolled vasculature and poor transplantation & engraftment, limits independent usage of either growth factor or cell-based therapies, respectively. Hence, a strategy which collectively exploits the potential of these two candidates through bioengineering was studied. Although numerous gene delivery systems are explored, the demand for an ideal delivery mechanism for stem cells with enhanced expression of molecular signals stands significant.

This study explores the use of an advanced electroporation method to improve therapeutic efficiency of hADMSCs through transient over-expression and delivery of potent AFs. The efficiency of the approach was demonstrated both *in vitro* and *in vivo*.

The idea of clubbing growth factor therapy with stem cell therapy using non viral mediated bioengineering turned successful with promising application for accelerated angiogenesis.

The following points highlight the deliverables of the study;

1. Neon® Transfection System was found feasible and effective in bioengineering hADMSCs without compromising stem cell property.

2. Immunochemical assay proved efficient delivery of the AFs.

3. GFP expression and quantified protein release confirmed transiency of the over expression, gradually declining by 14d.

4. Neon® Transfection System was demonstrated to be a translationally safe, non comparable, non viral mediated tool for bioengineering hADMSCs.

5. Bioengineered hADMSCs on exposure to hypoxia, directs endothelial trans-differentiation *in vitro* either by paracrine or autocrine effects.

6. Bioengineered hADMSCs-derived secretome significantly increased HUVECs proliferation, migration and tube formation.

7. The capability of overexpressed VEGF-A and Hif-1α to drive flk-1 mediated angiogenesis was distinguishable such that the former was effective and latter was not.

8. Requirement of combinatorial effect of bioengineered hADMSCs being superior in driving *in vitro* angiogenesis is established.

9. Bioengineered hADMSCs showed significantly higher, directed endothelial and fibroblast lineage commitment, sustained GF release and controlled skin ECM production in TESG favoring pre-vascularization.

10. I*n vivo* studies show s-e-hADMSCs to be superior in the role of an adjunct in cell transplantation.

11. In terms of faster wound contraction, Vegf-s-e-hADMSCs stood superior with accelerated closure by 7d than all other groups in the study.

12. Histological assessment demonstrated complete healing with higher vascularized regions in wounds treated with Vegf-s-e-hADMSCs.

13. Survival and localization of transplanted rADMSCs was enhanced when administered in combination with s-e-hADMSCs in comparison to independent administration of rADMSCs.

14. The advantage of engineered hADMSCs secretome over the non engineered hADMSCs secretome was established in the *in vitro* angiogenic response suggesting that over expressed factors released from the cells contributed immensely.

6.1. Conclusion

The effectiveness of using Neon® transfection system to safely engage hADMSCs as a resource for AFs is advocated based on the study results. The merit of transiently producing functional VEGF-A and Hif-1α through bioengineering of hADMSCs and potential for sustained delivery is highlighted. Advantage of combinatorial delivery of pro-angiogenic factor/s and hADMSCs together for accelerated wound angiogenesis is indicated, *in vitro*. Application of bioengineered hADMSCs on in house generated TESG directed pre-vascularization and *in vivo* transplantation of engineered cells in large area full thickness model wounds validated guided tissue regeneration with accelerated angiogenesis.

The take home message from the study would thus point out the following;

1. Neon® Transfection System is determined to be a suitable tool for efficient and translationally safe engineering of hADMSCs.

2. Secretome of bioengineered hADMSCs delivering AFs does augment angiogenesis through paracrine activity and guided endothelial lineage commitment.

3. The requirement of combinatorial AF therapy is highlighted considering age and faster healing time upon translation.

4. Application of e-hADMSCs in skin grafts does aid controlled release of AFs, neo-vasculature and adequate dermal regeneration.

5. In an *in vivo* perspective, administration of e-secretome alone promises a cell free therapy.

6. Obtaining e-secretome for translational purpose will possibly have greater advantage over use of commercially available, single recombinant GF therapy in terms of functional- and cost- effectiveness.

This study serves as a proof of principle to improve the success of growth factor delivery and cell-based implants in therapeutic angiogenesis. However, to achieve the full therapeutic benefit of the bioengineered hADMSCs, it will be desirable to know the secreted protein and exosome composition of the secretome, facilitating a cell free therapy. Again, the possibility of developing immortalized and bioengineered cell lines could also be explored for cost reduction in terms of large scale AF production and availability at clinical perspective in the long run. It would also be worth exploring better vector promoter systems and combinatorial over expression of multiple genes targeting angiogenesis and regeneration using Neon transfection system, as a welcome addition to the over expression arsenal, for regenerative medicine. It may also be possible to scale up the bioengineering process with larger numbers of cells as the hADMSCs collected by lipoaspiration as demonstrated in this study, has very high proliferation potential and pose less ethical problems.

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