DIFFERENTIATION POTENTIAL OF ADIPOSE DERIVED STEM CELLS (ADSCs) WHEN CO-CULTURED WITH SMOOTH MUSCLE CELLS (SMCs) AND THE ROLE OF LOW INTENSITY LASER IRRADIATION (LILI)

A Thesis presented to the

Faculty of Health Sciences, University of Johannesburg

as fulfilment for the Doctoral degree in

Biomedical Technology

by

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DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Technology at the University of Johannesburg. It has not been submitted before for any degree or examination in any other University.

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25th day of May 2015.

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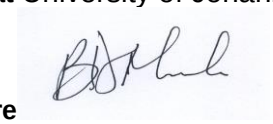
Signature  **Print name** BERNARD DANDENAULT MVULA

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ABSTRACT

Stem cells are defined as undifferentiated cells that can proliferate and have the capacity of both self-renewal and differentiation to one or more types of specialised cells (Bishop *et al.,* 2002). The two types of stem cells are embryonic and adult stem cells. Adult stem cells have been isolated from adipose tissue in abundance and with ease (Mvula *et al.*, 2010) and these cells have been differentiated into smooth muscle cells (SMCs) with the enhancement of low intensity laser irradiation and the growth factors (de Villiers *et al.*, 2011). Smooth muscles play an important role in diseases like cancer, hypertension, asthma and others (Rodriguez *et al.*, 2006). Studies have shown that low intensity laser irradiation (LILI) can increase proliferation of cells, cellular attachment, differentiation and production of transforming growth factor-beta 1 (TGF-β1) in cells indicating that *in vitro* LILI can modulate the activity of cells and tissues (Khadra *et al*., 2005). Further studies have also discovered that LILI enhances wound healing (Fiszerman and Markmann, 2000). LILI has been successfully used for pain attenuation and to induce wound healing in non-healing defects (Hawkins and Abrahamse, 2005). LILI has been shown to increase viability and proliferation of adipose derived stem cells (ADSCs) (Mvula *et al.*, 2008 and Mvula *et al.,* 2010).

Growth factors such as retinoic acids (RA) have been shown to have major influences on cells. They are involved specifically in apoptosis, cell proliferation, differentiation and maturation (Duong and Rochette, 2011; Gudas and Wagner, 2011). Co-culturing is used to achieve several cellular processes including proliferation, differentiation and migration (Kim *et al*., 2012). When two types of cells are cultured together, they are exposed to a number of complex environmental factors such as cytokines, extracellular matrix components, cell interactions, mechanical stimuli, signalling transcriptional pathways and transcriptional factors such as growth factors.

These factors are able to affect migration, proliferation and differentiation of one cell type into another (Zhang *et al*., 2012).

The aim of this study was to investigate the differentiation potential of ADSCs when co-cultured with (SMCs) and to determine the role of LILI on the co-cultured cells. Short and long term biological effects were monitored on these cells following exposure to LILI and addition of growth factors. The study used commercial and isolated human ADSCs and SMCs (SKUT-1) cells. After growing cells to semiconfluency for ADSCs and confluency for SMCs, they were co-cultured in a ratio of 1:1 using the established methods supplemented with and without growth factors (TGF-β1and RA) and then exposed to LILI. The cellular morphology, viability and proliferation activities of the irradiated cells were then assessed using direct inverted and differential interference contrast microscopy (DIC), trypan blue test, adenosine triphosphate luminescence, optical density analysis, and carboxyfluorescein diacetate succinimdyl ester (CFSE) methods. In particular the expression of the specific markers of both ADSCs, β1 Integrin (CD29) and Thy-1 (CD90) and SMCs, Myosin Heavy Chain (MHC) were investigated through immunoflourescent microscopy and flow cytometric analysis. Up and down regulation of genes involved in the human mesenchymal stem cell array were analysed through Reverse Transcriptase Polymerase Chain Reaction (RTPCR).

ADSCs were characterised by the expression of their specific markers (CD29 and CD90) which appeared as green fluorescences within the cytoplasm of the cells. MHC which characterise SMCs appeared as red fluorescences within the nuclei of the cells. Morphologically, both ADSCs and SMCs displayed the same fibroblast-like shapes. Through DIC, ADSCs appeared to be more elongated than SMCs and when stained with CFDSE, ADSCs were larger in sizes than SMCs. Cell viability and proliferation increased in the co-

cultures that were exposed to LILI as compared to those that were not progressively over time post irradiation but there was a decrease in viability and proliferation in the co-cultures that were supplemented with growth factors as compared to those that did not have the growth factors. Trypan blue analysis showed a significant decrease in the co-cultured with TGF-β1 as compared to the co-cultures without growth factors (P<0.05) at 24 h, 48 h and 1 wk. ATP luminescence significantly decreased in the co-cultures with growth factors as compared to those not supplemented with growth factors at 24 h, 72 h and 1 wk (P<0.001) and 48 h (P<0.05). Optical density results revealed significant decreases in co-cultures that were supplemented with growth factors as compared to those that were not at 24h and 1 wk with P values of less than 0.05 and at 48 h and 72 h with P values of less than 0.001.

Expression of CD29 and CD90 decreased in the co-cultures over the period of 1 wk while the co-cultures increased the expression of MHC over the same period of time as revealed through flow cytometric analysis. After co-culturing for 24 h, flow cytometry results showed two distinct cell types but after 1 wk, there was only one population of cells that appeared on flow cytometry indicative of differentiation into SMCs. PCR array analysis revealed a down-regulation of catenin beta1 (CTNNB1) but up-regulation of CD29, CD90, SMAD and TGF-β1 and TGF-β3 at 24 h post irradiation. However at 1 wk post irradiation in the co-cultures that were irradiated and supplemented wth TGF-β1, CD29, CD90 and SMAD, were all not expressed, again indicative of ADSCs differentiating into SMCs.

The study concluded that ADSCs could differentiate into SMCs in a co-culture environment when exposed to LILI and growth factors in a short period of time. This method once well established, would be beneficial in differentiating

stem cells into required specialised cells to be used in treatment of several degenerative diseases including those affecting smooth muscle tissues.

However, this study did not analyse SMC genes through RT PCR that were expressed to confirm SMC differentiation. It is therefore suggested that future studies should include SMC gene analysis for confirmation of SMC differentiation. Smooth muscle contractility functional test should also be analysed. Clinical studies could also be evaluated to determine the effectiveness of the differentiated SMCs.

RESEARCH OUTPUTS EMANATING FROM THIS STUDY:

The research outcome of this study has been presented at the following conferences and workshops.

- The 56TH South African Institute of Physics annual conference (University of Pretoria), 2011, South Africa (Oral Presentation).
- The 58TH South African Institute of Physics annual conference (University of Zululand, Richards Bay), 2013, South Africa (Oral Presentation).
- Post-graduate annual conference, Bunting Road Campus, University of Johannesburg, 2013, South Africa (Oral Presentation).

The following publications were produced from this study.

- Mvula B. and Abrahamse H. (2011) Adipose derived stem cells (ADSCs) and low intensity laser irradiation (LILI): Potential use in regenerative medicine, *56th Annual SAIP proceedings*, 707-710.
- Mvula B. and Abrahamse H. (2014)) Low Intensity Laser Irradiation (LILI) in Combination with the Growth Factors in a Co-culture System Supports the Differentiation of Mesenchymal Stem Cells, *58th Annual SAIP Proceedings*, In Press.
- Mvula B. and Abrahamse H. (2013) The role of adipose derived stem cells, smooth muscle cells and low intensity laser irradiation (LILI) in tissue engineering and regenerative medicine, *Central European Journal of Biology*, **8(4)**: 331-336.
- Mvula B. and Abrahamse H. (2014) Low intensity laser irradiation and growth factors influence differentiation of adipose derived stem cells into smooth muscle cells in a co-culture environment over a period of 72 hours, *International Journal of Photoenergy*, DOI 10.1155/2014/598793.
- Mvula B. and Abrahamse H. (2014) Differentiation potential of adipose derived stem cells when co-cultured with smooth muscle cells and the role of

low intensity laser irradiation, *international Journal of Molecular Sciences*, Submitted.

- Mvula B. and Abrahamse H. (2014) Gene expression of mesenchymal stem cell array in the co-cultures of adipose derived stem cells (ADSCs) and smooth muscle cells (SMCs) and the role of low intensity laser irradiation (LILI) and transforming growth factor beta1 (TGF-β1). In draft.

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DEDICATION

To God Be Glory, Honour and Praise

In memory of my late father, Phillip Mvula (1940-2005)

and

my late mother, Modester Vwemu (1938-1994)

I dedicate this thesis to my wife, Annie and my son, Bernard Junior

TABLE OF CONTENTS

CHAPTER THREE – METHODOLOGY

CHAPTER FOUR – RESULTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND SYMBOLS

ADSCs	Adipose derived stem cells
APC	Allophycocyanin
ATCC	American Tissue Culture Collection
ATP	Adenosine Triphosphate
BMSC	Bone Marrow Derived Stem Cells
bFGF	Basic Fibroblast growth Factor
BSA	Bovine Serum Albumin
Ca^{+2}	Calcium ions
CC	Co-culture
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFDASE	Carboxyflourescein Diacetate Succinimdyl Ester
Ct	Threshold Cycle Value
DAPI	4'-6 Diamidino-2- phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ELB	Erythrocyte Lysis Buffer
ES	Embryonic Stem Cells
FBS	Foetal Bovine Serum
FITC	Fluorescein
gDNA	Genomic Deoxyribonucleic Acid
HBSS	Hank's Balanced Salt Solution
LILI	Low intensity laser irradiation
mAb	Mouse Anti-human Antibody
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RA	Retinoic Acid
RLU	Relative light unit(s)

RNA	Ribonucleic Acid
RT PCR	Reverse Transcriptase Polymerse Chain Reaction
SA	South Africa
SMCs	Smooth muscle cells
TGF-β1	Transforming Growth Factor Beta 1
Thy-1	Thymocyte-1
μg/ml	Micrograms per millilitre
mm	Millimetre
U/ml	Units per millilitre
μl	Microlitre
U/mg	Units per milligram
rpm	Revolutions per minute
xg	Gravitational Force
cm	Centimetre
nm	Nanometre
μm	Micrometre
mm^2	Millimetre squared
cm^2	Centimetre squared
Min	Minute/s
h	Hour/s
wk	Week
sec	Second/s
mMol/L	Millimoles per litre
W	Watts
W/cm^2	Watts per centimetre squared
^0C	Degrees Celsius
CO_2	Carbon Dioxide
H_2O	Water
ng/ml	Nanogram per millilitre
nM	Nanomole

nM/ml	Nanomole per millilitre
mM	Millimole
J/cm^2	Joules per centimetre squared
%	Percent
π	Pi
λ	Wavelength
mW	Milliwatt
mW/cm^2	Milliwatts per centimetre squared
g	Grams
IgG	Immunoglobulin
H_2SO_4	Sulphuric Acid
M	Mole
V	Volt/s
β	Beta
α	Alpha
A	Absorbance
P	Probability
=	Equal
<	Less than
>	More than
n	Sample number
r^2	Beam spot size
He-Ne	Helium Neon
μg/μl	Micrograms per microlitre
μg/ml	Micrograms per millilitre
μg	Micrograms
ml	Millilitre
mg	Milligram
mg/ml	Milligram per millilitre
mol/ml	Mole per millilitre

CHAPTER 1

INTRODUCTORY CHAPTER

1.1 Introduction

Stem cells can proliferate and have two main characteristics of self-renewal and differentiation into specialised cells. Stem cells play a major role in basic biological processes *in vivo,* including the development of an organism and tissue maintenance and repair. They have also been indicated as playing a role in cancer (Tsai, 2004). Great progress has been achieved in the field of stem cell research from their isolation and culture, to being used in genomic studies, drug discovery and cell-based therapy. Laser radiation at different intensities has been shown to inhibit as well as stimulate cellular processes (Moore *et al*., 2005). Studies on LILI and stem cells have shown that low intensity lasers can change the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration (Gasparyan *et al*., 2004). Furthermore, LILI has also been shown to promote the proliferation of rat mesenchymal bone marrow and cardiac stem cells *in vitro* (Tuby *et al*., 2007). In order for stem cells to be used for treatment of regenerative diseases, they have to be produced in large numbers and differentiate into the required cell type. One method for differentiating them is co-culturing with the required cell type, exposing to LILI and adding the growth factors.

1.2 Problem Statement

Traumatic injury and degenerative diseases are a major problem in South Africa and worldwide. In 1988, 24.5% of all the deaths in South Africa were due to chronic diseases of life style which are a type of degenerative diseases (Steyn *et al*.,1992). Degenerative diseases accounted for 60% of all deaths in USA in the late 1990s (National Centre for Health Statistics). Adult stem cells hold great promise for use in tissue repair and regeneration as a novel therapeutic option. Due to the shortage of organ donors, tremendous advances have been made in tissue engineering to

treat damaged heart tissues and blood vessels by replacing them with stem cells (Ngan *et al*., 2007). Parkinson's disease, stroke and multiple sclerosis are thought to be caused by a damage or loss of neurons and glial cells. It is now possible to culture these types of cells from stem cells in culture which can in turn be used to possibly treat the above diseases in human patients through transplantation (Lindvall and Kokaia, 2006). However, in order to be effective, certain criteria for stem cells used in tissue engineering need to be met such as the abundance of cells to be harvested, the procedure for harvesting should be performed with minimal invasion, the cells should be able to differentiate into multiple specialised cells and be transplanted safely and effectively. Since low intensity laser has been found to increase ATP and proliferation of cells, therefore it could be used to increase the production of large numbers of stem cells and with the addition of different growth factors, differentiation of the stem cells into different specialised cells would be possible.

1.3 Aim

The aim of the study was to investigate the differentiation potential of ADSCs when co-cultured with SMCs and the role of LILI on the differentiation potential of these ADSCs in presence of the growth factors. The project aimed to:

- Monitor short and long term effects on co-cultures of ADSCs and SMCs by assessing cellular morphology, viability, proliferation and expression of CD29, CD90 and MHC.
- Determine the effect of LILI and growth factors have on proliferation and differentiation of ADSCs in a co-culture environment at a mechanistic level.
- Determine the regulation of genes expressed in the human mesenchymal stem cell array in the co-culture of ADSCs and SMCs.
- Evaluate the effect of co-culture system for differentiation of ADSCs into SMCs.

CHAPTER 2

LITERATURE REVIEW

2.1 Stem Cells

Stem cells are primal cells found in all multi-cellular organisms that are able to renew themselves through mitotic cell division and can differentiate into a variety of specialised cell types. Stem cells can be described according to their differentiation potential as totipotent, pluripotent, multipotent and unipotent. Totipotent cells have the potential to become any type of cells in the adult body and of the extra-embryonic tissues including the placenta. Pluripotent stem cells are cells with the potential to become any differentiated cells in the body except those of the placenta. Multipotent stem cells can be differentiated into a limited number of cell types while unipotent stem cells can only be differentiated into one type of cell type of the body (John Kimball, 2006). Stem cells have characteristics of self-renewal, extensive proliferative potential and an ability to give rise to one or more differentiated cell types (Spradling *et al*., 2001). One strategy by which stem cells can accomplish these two roles is by asymmetric cell division, whereby each stem cell divides to generate one daughter cell with a stem cell fate (self-renewal) and another that differentiates along a specialised cell lineage (figure 1). Symmetric divisions are defined as the production of daughter cells that are destined to acquire the same fate (either stem cells only or differentiated cells only) (Morrison and Kimble, 2006). Stem cells divide asymmetrically to maintain their number in the tissue, while at the same time generating cells committed to becoming differentiated tissues and organs (Serakinci and Keith, 2006). It is believed that the molecular distinction between symmetric and asymmetric divisions lies in differential segregation of cell membrane proteins between the daughter cells (Kyoto University, 2006). There are two types of stem cells namely embryonic and adult stem cells.

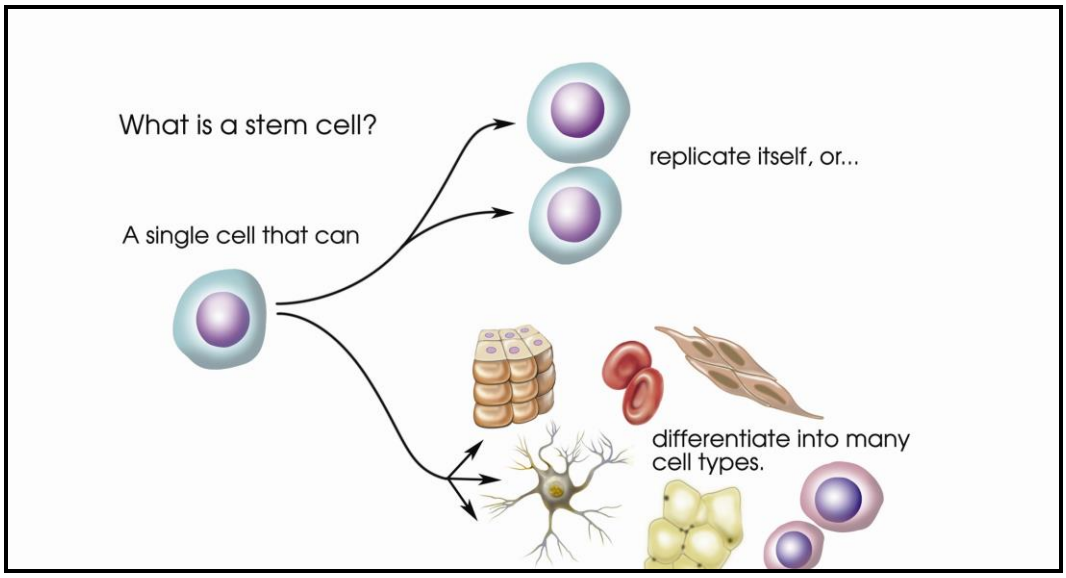

Figure 1. **Stem cell can either self-renew or differentiate into different specialised cells (http://www.nationalacadamies.org/stemcells, 25/10/2013).**

Embryonic stem cells originate from the blastocyst and are able to differentiate into all different cells of the body (figure 2).

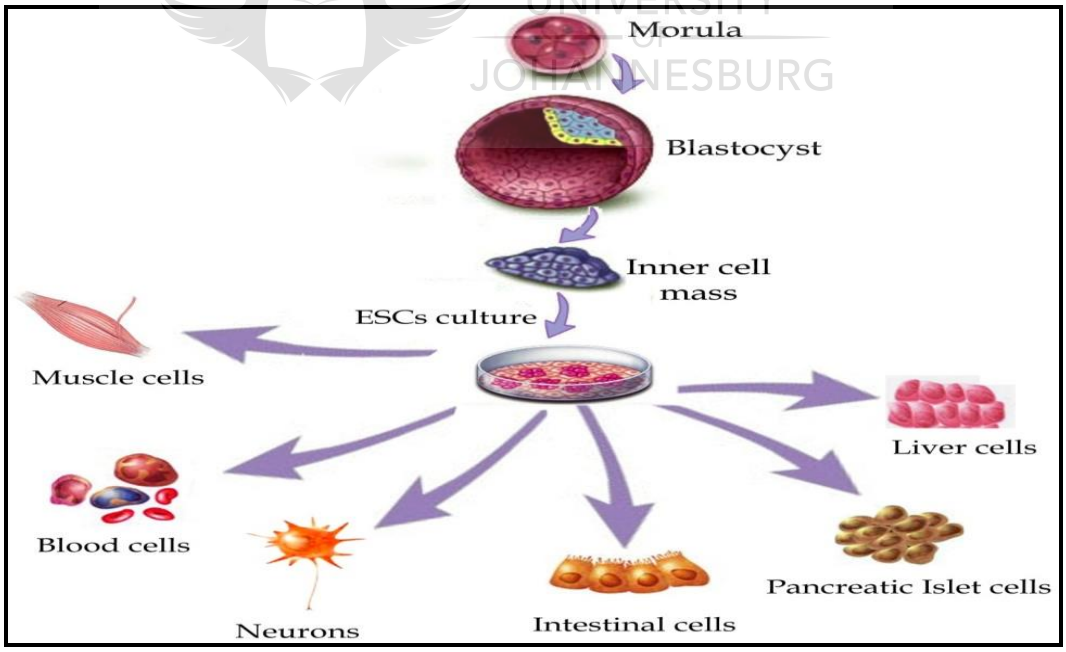

Figure 2. **Human embryonic stem cell differentiation into different types of specialised cells like liver, muscle, blood, neurons, intestinal and pancreatic islet cells (Meregalli *et al*., 2011).**

2.1.1 Adult stem cells

Adult stem cells are undifferentiated cells found throughout the body that divide to replace dying cells and regenerate damaged tissues, also known as somatic stem cells, they can be found in individuals throughout their lifespan. Adult stem cells have a limited capacity for cellular differentiation but can renew themselves and differentiate to produce most of the specialised cell types of an organism tissue or organ (Gardner, 2002). Adult stem cells, like embryonic stem cells, have pluripotent potential and can differentiate into cells derived from all three germ layers. Unlike embryonic stem cells, the use of adult stem cells in research and therapy is not as controversial because the production of adult stem cells does not require the destruction of an embryo. Based on their extensive differentiation potential and, in some cases, the relative ease of their isolation, adult stem cells are an appropriate tool for clinical development (Serakinci and Keith, 2006). Among the advances in adult stem cell therapy are the treatment of a variety of human conditions, ranging from blindness to spinal cord injury (Kyoto University, 2006). In recent years, the concept of adult stem cells has changed to include the theory that stem cells reside in many adult tissues and that these unique reservoirs of adult stem cells are not only responsible for the normal reparative and regenerative processes but are also considered to be a prime target for genetic and epigenetic changes culminating in many abnormal conditions including cancer (Kyoto University, 2006; Chaudhary and Roninson, 1991). Some examples of adult stem cells that are currently used in research are haematopoietic, mammary, dermal, adipose derived, neural and olfactory stem cells (figure 3).

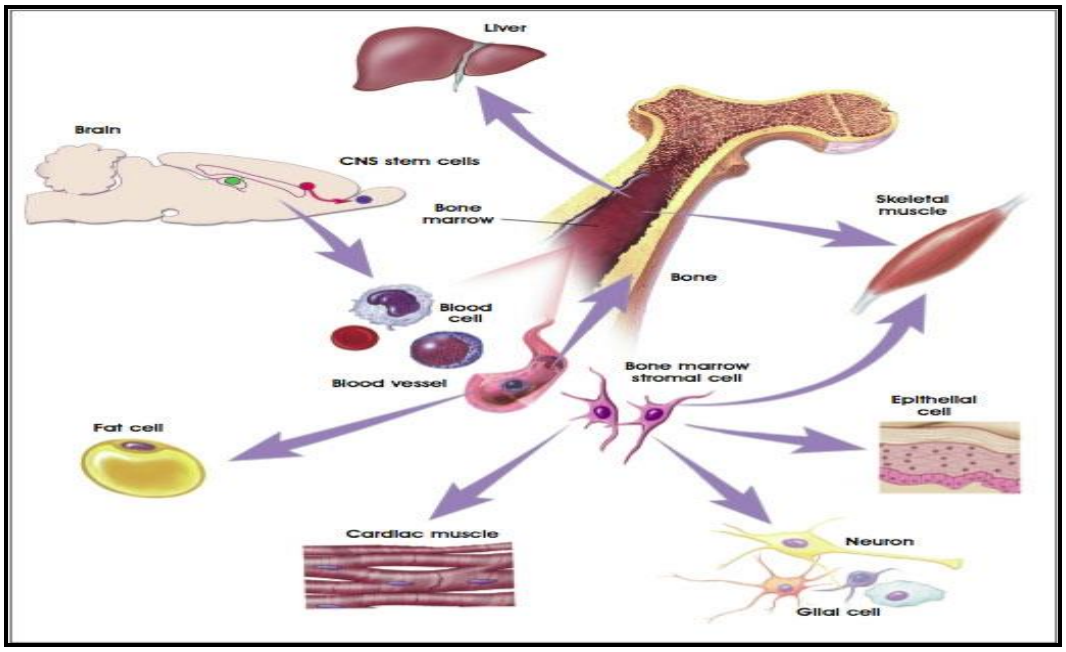

Figure 3. Common functional cells (brain, cardiac muscle, neurons, skeletal muscle, bone, fat and liver) derived from central nervous system stem cells and body stem cells (Terese *et al*., 2001).

2.1.2 Adipose derived stem cells

It has recently been shown that adipose tissue is an abundant and easily accessible source of stem cells (ADSCs, adipose-derived stromal cells), resembling the mesenchymal stem cells (MSCs) obtained from adult bone marrow (Peroni *et al.*, 2008). The initial method of isolation was engineered by Rodbell, 1966(1), 1966(2) and Rodbell and Jones, 1966. They minced rat fat pads and this was washed extensively to remove hematopoietic cells and then incubated the tissue parts with collagenase. Due to their plasticity (figure 4) and easy harvest, ADSCs are preferred to be used in tissue engineering than bone marrow derived cells (Strem and Hedrick, 2005; Strem *et al.*, 2005). These cells have a doubling period of 2 to 4 days depending on the medium conditions and passage number (Mitchell *et al*., 2006). They could be passaged for a long time but studies have shown that after 4 months ADSCs developed malignant transformation (Rubio *et al*., 2005).

Gonthos *et al*., 2001, did examine the expressions of cell surface antigens on the ADSCs. These cells generally are characterised for being positive

for CD13, CD29, CD44, CD49d, CD90 and CD105 and negative for CD14, CD31, CD45 and CD144 (Mitchell *et al*., 2006; Yoshimura *et al*., 2006). Characterisation has found out that these cells are mesenchymal stem cells by expressing mesenchymal stem cell markers (CD29 and CD90) and not expressing the haematopoietic marker like CD45 (Zahran *et al*., 2012). Mesenchymal stem cells have been defined by the International Society for Cellular Therapy by a number of criteria. First, the cells must adhere to plastic, then the cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts. Finally, the cells must express the specific markers (CD90, CD105 and CD73) and not the haematopoietic makers like CD45, CD34, CD19, CD79, CD11b and CD14 (Dominici *et al*., 2006).

Human ADSCs have been reported to have differentiated into smooth muscle cells over a period of time *in vivo* as well as *in vitro* under smooth muscle environment (Zhang *et al*., 2011 and Marra *et al*., 2011). These ADSCs have been induced into SMCs *in vitro* to be used in tissue engineering (Yang *et al*., 2008). Recently, scientists differentiated these derived stem cells into functional neurons by using bFGF and forskolin (Jang *et al*., 2010). These cells increased in viability and proliferation when exposed to LILI alone as well as in combination with the growth factors (Mvula *et al*., 2008; 2010). Studies have shown also that ADSCs could differentiate into cardiomytes (Van Dijk *et al*., 2008) and their effects on animals have produced no major effects (Vet-Stem Regenerative Veterinary Medicine, 2008). Recently, these cells have been suggested that they could have a major therapeutic effects in patients who have pelvic or abdominal cancer and have developed radiation-induced intestinal injuries (Chang *et al*., 2013).

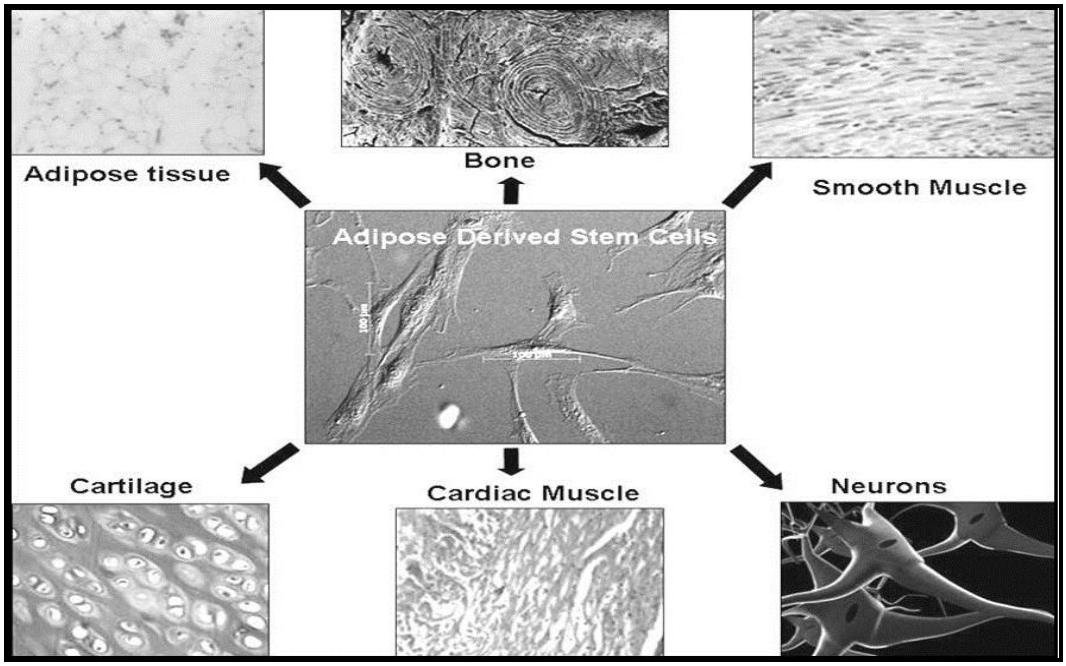

Figure 4. Different types of cells differentiating from Adipose derived stem cells that could be used in tissue engineering (Zuk *et al.,* 2001).

Scientists have suggested a number of mechanisms through which ADSCs could be used in repairing degenerative tissues. These cells could be delivered to the injured tissues where they would secrete growth factors and cytokines. They could also stimulate a stem cell niche and promote the required lineage differentiation. These transplanted stem cells could provide antioxidant chemicals, heat shock proteins and radical scavengers to the ischaemic sites. Mitochondria could also be delivered to the damaged areas, providing aerobic metabolism and finally they themselves could differentiate into the required lineage (Gimble *et al*., 2007 and Spees *et al*., 2006). Recent studies have also demonstrated that stem cells from adipose tissue could promote angiogenesis, which is the formation of blood vessels. This could be done through a particular mechanism such as depending on the plasmin system (Kachgal and Putnam, 2011).

2.2 Smooth Muscle Cells

Smooth muscles play important roles in diseases like cancer, hypertension, asthma and others and are active components of intestinal, reproductive, cardiovascular and urinary systems in the regenerative medicine (Rodriguez *et al.*, 2006 and Sinha *et al.*, 2006). SMCs can react to stimuli for tissue specific actions to occur and this may be due to origins of different progenitor populations (Firth and Yuan, 2013). Studies have shown that ADSCs have been differentiated into SMCs (de Villiers *et al.*, 2011). Transforming growth factors (TGF-β1), RA, platelet derived growth factor and ascorbic acid are some of the growth factors that enhance smooth muscle differentiation (Rodriguez *et al.*, 2006: Yang *et al.*, 1999: Narita *et al.*, 2008). SKUT-1 cells are human uterine mixed leiomyosarcoma cells. These cells are SMCs derived from the uterus (Steynberg *et al*., 2012).

TGF-β has been shown to be an integral for the up-regulation of SMC markers, smooth muscle 22α, calponin-1 and MHC in ADSCs differentiation into SMCs. In neural crest cells, the pathway involves the binding of the complement 32 (RGC-32) which is a response gene to polyomovirus enhance element (PEA3) and SBE leading to SMC gene transcription (Huang *et al*., 2011) while in ADSCs, angiotensin 11 up-regulates the transcription of SMC genes through induction of TGF-β and activation of the MEK/ERK/Smad2 pathway (Kim *et al*., 2008). Recent studies have revealed the involvement of TGF-β dependent upregulation of ADAM12 (a metalloprotease) found in lipid rafts in adipose cells. The disruption of ADAM12/lipid raft complex attenuated the differentiation of ADSCs to SMCs (Kim *et al*., 2012).

2.3 Co-culturing

Co-culturing is used to achieve several cellular processes including proliferation, differentiation and migration (Kim *et al*., 2012). When two types of cells are cultured together, they are exposed to a number of

complex environmental factors such as cytokines, extracellular matrix components, cell interactions, mechanical stimuli, signalling transcriptional pathways and transcriptional factors such as growth factors. These factors are able to affect migration, proliferation and differentiation of one cell type into another (Zhang *et al*., 2012). Studies on co-culturing have indicated that osteoblasts stimulated the proliferation of endothelial cells when co-cultured directly due to the production of vascular endothelial growth factor and reciprocally osteoblasts proliferation also increased (Wang *et al*., 1997. Recent studies have shown that ADSCs differentiated into SMCs when injected into SMCs environment *in vivo* as well as when exposed to SMCs *in vitro* (Zhang *et al*., 2012). Melanocyte proliferation and migration were also increased in the study done by Kim *et al* in 2012 when co-cultured with ADSCs and this was suggested to be due to the stem cell factor, basic fibroblast growth factor and other growth factors for melanocytes that were produced by ADSCs. In another recent study (Zuo *et al*., 2013), bone marrow mesenchymal stem cells differentiated into articular chondrocytes when co-cultured directly. Articular chondrocytes induced and promoted differentiation of bone marrow mesenchymal stem cells into chondrocytes while at the same time bone marrow stem cells stimulated and supported chondrocytes to express specific proteins. Chondrocytes have been found to produce soluble factors that induce differentiation of mesenchymal stem cells in co-culture *in vitro* as well as *in vivo* (Hwang *et al*., 2007). Direct co-culturing method of human nucleus pulposus cells of the intervertebral disc with mesenchymal stem cells resulted in generating large number of mesenchymal stem cells differentiating into nucleus pulposus cells after co-culturing for a period of 7 days (Richardson *et al*., 2006).

2.4 Growth Factors

Growth factors are proteins that stimulate cellular growth, proliferation and differentiation. They regulate a wide range of cellular processes (Goustin *et al*., 1986). Growth factors such as retinoic acids (RA) have been shown

to have major influences on cells and these influences are specifically on apoptosis, cell proliferation, differentiation and maturation (Duong and Rochette, 2011; Gudas and Wagner, 2011). RA has been discovered to stabilise stem cell cultures when supplemented with foetal bovine serum or bovine serum albumin in the dark (Sharow *et al*., 2012). Human pluripotent stem cells have been directed to neuronal differentiation in a non-cell-autonomous way by RA (Tong and Andrews, 2010).

Transforming growth factor-β (TGF-β) is a family of growth factors that include β1, β2, β3, bone morphogenetic proteins (BMPs), actin/inhibin and cytokines such as nodal. It plays important roles in regulating biological processes such as apoptosis, cell growth, differentiation, migration, angiogenesis, tumor invasion and metastasis as well as embryonic development (Zhang, 2009; Rahimi and Leof, 2007). It is able to promote or inhibit cell growth depending on the cell type. TGF-β promotes fibroblasts, osteoblasts and other mesenchymal cell growths while inhibiting epithelial and neuroectodermal cells. It produces inflammatory cytokines and in the concluding stage of the inflammation it shows immunosuppressive action. Depending on the cell type and stimulation context, carcinogenesis can be suppressed or promoted (Akhurst, 2002). A scientific report indicated that TGF-β induced differentiation of smooth muscle from a neural crest stem cell line (Chen and Lechleider, 2004). TGF-β3 promoted the differentiation of ADSCs into cartilage cells in a pellet culture system (Hashemibeni *et al*., 2008). This growth factor played a role in differentiation of human embryonic stem cell-derived mesenchymal cells to smooth muscle cells in a time and dosage dependent way. It actually induced the differentiation of Smad and serum response factor/myocardin. It regulated the expression of myocardin through many pathways such as Smad2/3, PI3K and p38 MAPK (Guo *et al*., 2012). Studies have also shown that TGF-β1 can promote the expression of smooth muscle differentiation genes by inhibiting *Notch3* pathway and activating *Hes1* pathway in fibroblasts (Kennard *et al*., 2007).

However, TGF-β1 up modulated the CD34 antigen in hematopoietic stem cells before the s phase and maintained its high levels which also suggests that TGF-β1 is a major physiological factor in maintenance of the stem cell reserve (Batard *et al*., 2000).

Studies have shown that smooth muscle differentiation of ADSCs do induce smooth muscle gene expression. Smooth muscle cell specific alpha actin (ASMA), calponin, smoothelin and MHC increased in expression when ADSCs differentiated into SMCs (Rodriguez *et al*., 2006). MHC and smoothelin are highly restricted to SMCs and can only be detected in SMCs (Miano JM, 2002). In their study in investigating the regulation of SMC marker during differentiation of ADSCs into SMCs, Lee *et al*., 2006 found out that α-smooth muscle actin (α-SMA) significantly increased when the cells were treated with TGF-β1 and slightly increased when treated with RA.

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2.5 Regenerative Medicine

Tissue engineering and regenerative medicine is a multi-disciplinary science that has evolved in parallel with novel biotechnological advances. It is a combination of biomaterials, growth factors and stem cells to repair organs (Butler *et al*., 2000). Adult stem cells hold great promise for use in tissue repair and regeneration as a new therapeutic option (Huh *et al*., 2000). This can be done by culturing the cells and differentiating them into the required lineage *in vitro* and then introducing the differentiated cells into the failing organs. Plastic and regenerative surgeons are constantly burdened with the challenge of replacing lost soft tissue. More than 6.2 million individuals received reconstructive plastic surgery procedures in 2002, approximately 70% of them as a result of tumour removal (Choi *et al*., 2006). Elective cosmetic procedures also require the placement of soft tissue implants to restore or improve tissue contour for the purpose of enhancing anaesthetic appearance. Conventional soft tissue-grafting procedures have had some clinical success for soft tissue augmentation and reconstruction. However, the

need for secondary surgical procedures to harvest autologous tissues and an average of 40-60% reduction in graft volume over time are considered setbacks of current autologous fat transplantation procedures. It should be possible to overcome these problems with tissue-engineered soft tissue grafts generated from the patient's own adult stem cells (Choi *et al*., 2006).

Parkinson's disease, stroke and multiple sclerosis are thought to be caused by a loss of neurons and glial cells. These cells can now be generated from stem cells in culture and can be used to treat the above diseases in human patients through transplantation (Lindvall and Kokaia, 2006). Clinical trials for the regeneration of soft tissue, craniofacial tissue and cardiovascular tissue have enrolled a number of patients.

ADSCs are multipotent and could be used in a number of treatment applications (Gimble *et al*., 2007). Scientists have postulated a number of mechanisms through which ADSCs could be used in tissue engineering and regenerative medicine. These cells could secrete cytokines and growth factors and stimulate recovery in a paracrine way. They would also stimulate the recruitment of stem cells from the stem cell niche to the site enhancing differentiation of a particular required lineage (Kachgal and Putnam 2011). ADSCs would be differentiated along a particular lineage and applied to a particular damaged organ (Gimble *et al*., 2007). These stem cells might provide antioxidant, free radical scavangers and heat shock proteins to an ischemic site there by recovering the cells. Some studies have suggested that ADSCs could deliver new mitochondria to the damaged area thereby promoting aerobic metabolism (Spees *et al*., 2006). Breast reconstruction with ADSCs trials have been reported by Yoshimura and colleages (2010). These cells have also been used to stimulate bone repair in calvarial defects (Lendeckel *et al*., 2004). ADSCs have been used to heal chronic fistulas in Crohns disease (Garcia-Olmo *et al*., 2008) and hold great promise for the treatment of cardiovascular diseases (Bai *et al*., 2010). Some studies have shown that neurotrophic factors have been

expressed by ADSCs and cells isolated from abdominal fat layer suggesting that these cells could be used *in vivo* generation of nerves in future (Kalbermatten *et al*., 2011).

The paracrine mechanisms which enhances cardiac function through transplantation of stem cells is not fully known but some mechanisms postulated by scientists suggest that these cells produce molecules that reduce apoptosis, improve perfusion and increase angiogenesis to the ischaemic sites (Crisostomo *et al*., 2007).

The effective treatment for liver failure is liver transplantation but has been associated with donor shortages and high costs. Regenerative medicine and tissue engineering could provide an alternative to this treatment (Ogawa and Miyagawa, 2009). Hepatic differentiation of human embryonic stem cells was successfully induced by adding bFGF, TGFβ1, Activin-A, Bone morphogenic protein4, hepatocyte growth factor, epidermal growth factor, β nerve growth factor and Retinoic Acid (Schuldiner *et al*., 2000).

Another type of disease that could benefit from this treatment is Type 1 diabetic mellitus which results from the autoimmune mediated destruction of langerhans cells in the pancreas (Shapiro *et al*., 2006). However, studies have shown that isolated ADSCs when autotransplanted would be a possible tool for the cure of this disease (Couri *et al*., 2009).

A co-culture system is believed to provide physical, structural and molecular factors that induce differentiation of cells. Functional implants may be established for transplantation and replacement of degenerated tissues. The other possibility would be the investigation of developmental processes and understanding the stem cell behavior in the environment and finally avoiding the usage of biomaterials to avoid immuno-rejection (Yue *et al*., 2013). But there is need for methods for the cell-based tissue

engineering to be perfected. Table 1 shows some developments that have occurred in stem cell research.

Table 1. Current stem cell research focus areas, stem cell sources and developments (Moore, 2007).

Focus area	Stem Cell Source	Developments	References
Degenerative neurological disorders, e.g. Parkinson's, Alzheimer's, Huntington's, Strokes, Spinal cord or brain injuries.	Embryonic stem cells (ES) and adult stem cells (ASCs) from bone, skin, from bone marrow-bone marrow derived mesenchymal stem cells (BMSC), adipose tissue and foetal brain.	*In vitro* differentiation and production of neurons. Transplants into experimental animals poor and require further investigation.	Zuk *et al*., 2002 Fernandes *et al*., 2004 Lindvall and Kokaia, 2006 Toma *et al*., 2001 Strem *et al*., 2005 Woodbury *et al*., 2000 Miller, 2006
Haematopoietic disorders, e.g. haemoglobinopathies, leukaemia's, lymphomas.	ES cells and ASC's BMSC, umbilical cord blood adipose tissue.	BMSC's routinely used for numerous haematopoietic disorders Genetic modification of BMSC's is also currently used in clinical and experimental procedures.	Strem *et al*., 2005 Bordignon, 2006 Rubistein, 2006 Chang and Kan, 2006 Hallemeier *et al*., 2006
Cardiovascular disorders, e.g. Myocardial infarction, Damaged heart valves, muscle, blood vessels.	ES cells and ASC's from heart muscle BMSC's, adipose tissue.	*In vitro* differentiation and manipulation of stem cells into cardiac and supporting tissues. Transplantation studies require further investigation.	Zuk *et al*., 2006 Strem *et al*., 2005 Srivastava and Ivey, 2006 Yamada *et al*., 2006 Wang *et al*., 2006
Reproductive disorders, e.g. Infertility.	ES cells and ASC's from ovary, testis.	*In vitro* manipulation and differentiation of stem cells into cartilage, bone and muscle.	Hutt and Albertini, 2006 Kubota and Brinster, 2006
Musculo-Skeletal disorders, e.g. bone fractures and defects, osteoarthritis, muscular dystrophy.	ES cells and ASC's from muscle, BMSC's and adipose tissue.	*In vitro* manipulation and differentiation of stem cells into cartilage, bone and muscle.	Zuk *et al*., 2002 Toma *et al*., 2001 Strem *et al*., 2005 Wang *et al*., 2006 Abderrahim-Ferkoune *et al*., 2004 Rodriguez *et al*, 2006
Metabolic disorders, e.g. diabetes.	ES cells and ASC's fro m pancreas, BMSC's a nd adipose tissue.	*In vitro* manipulation and differentiation of stem cells into cells with a pancreatic endo crine phenotype. Trans plantation studies require further investigation.	Timper *et al*., 2006 Noguchi, 2007

2.6 Lasers

Laser is an acronym for Light Amplification by Stimulated Emission of Radiation. It is monochromatic, coherent and directional. It was first developed in 1959 and in 1963 Leon Goldman was the first physician to use it on human skin (Alster and BettenCourt, 1998). Daniel Choy performed the first applications of laser in cardiology in 1979 and 1983 at Toulouse University (Choy, 2014). In 1967, Endre Mester in Semmelweis University Budapest, Hungary, discovered laser biostimulation when he exposed laser light to mice (Mester *et al*., 1967).

Laser can be defined as any device that can be made to produce or amplify electromagnetic radiation in the wavelength range from 180 nm to 1 mm primarily by the process of controlled stimulated emission. Lasers are now used in medicine and surgery. Lasers are also used in spectroscopy, for the cutting of steel and other metals and in bar code readers and in laser pointers. The two types of lasers of medical importance are namely low intensity lasers and high intensity lasers.

The power of lasers is measured in watts (w) or milliwatts (mW). The higher power output, the higher power density. Power density or light intensity is the light output power per unit area of the target being illuminated by the laser light. It is measured in watts per square centimetre (w/cm^2) or milliwatts per square centimetre (mW/cm^2). Power density is of significance both in laser surgery and laser therapy (Tuner and Hode, 2002). The transmission of laser radiation in tissues is related to its wavelength. The effects that optical radiation may have on tissue can be separated into categories depending on the portion of the spectrum (wavelength) that is incident on the tissue and the intensity (power density) of radiation. Wavelength is expressed in nanometres (nm) (Basford, 1995). Wavelength is the distant between two peaks of a wave. The symbol of wavelength is λ (lambda) (Peavy, 2002).

A light source that emits light at a constant intensity is known as continuous wave emission whereas pulsed light has varying intensity. When a laser is pulsed, the laser light power varies between the pulse peak output power and zero so the average power is used to calculate the dose while continuous wave lasers use the output power (Hawkins and Abrahamse, 2007). Infrared laser radiation shows a higher penetration into tissues than the laser light of the red region of the visible spectrum and therefore, the latter has proved useful in treatment of skin and mucosal disorders (Koutna *et al*., 2003). The two types of lasers of medical importance are namely low intensity lasers and high intensity lasers.

2.6.1 Low intensity laser irradiation (LILI)

LILI, a form of phototherapy, involves the application of monochromatic light over biological tissue to elicit a biomodulative effect within that tissue. LILI is now accepted in many countries and is used in medical and dental practices. LILI can have both a photobiostimulative and a photobioinhibitive effect within the irradiated tissue, each of which can be used in a number of therapeutic applications. LILI is not thermic (that is it does not produce heat) (Matic *et al*., 2003) and uses monochromatic light in the range of 630 to 905 nm (Stadler *et al*., 2004). It is a visible light in the electromagnetic spectrum (figure 5). Some of the therapeutic lasers are Helium-Neon (HeNe) and diode lasers.

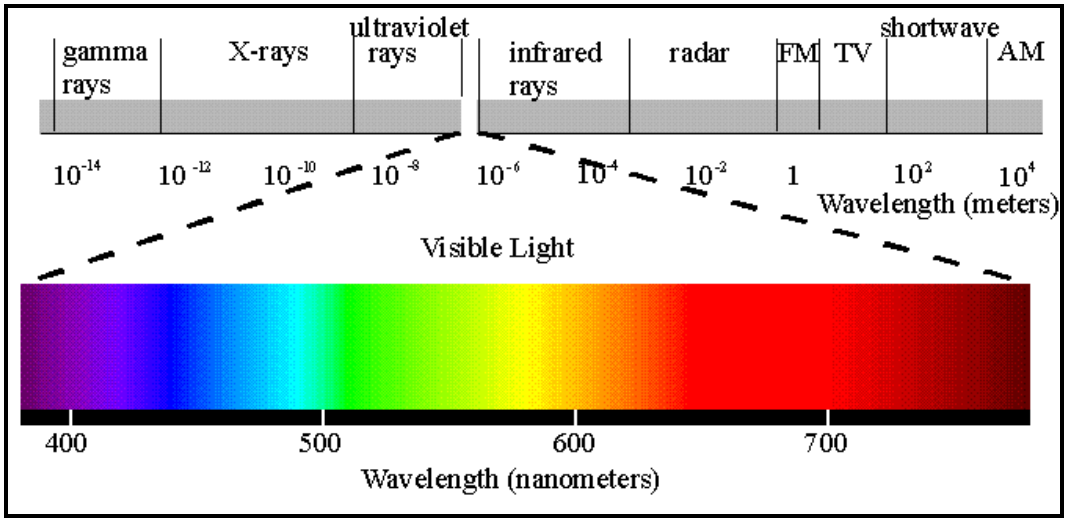

Figure 5. Electromagnetic spectrum showing visible light between the wavelength of 380 nm to 750 nm (http://bro3400.nicerweb.com 03/10/2014).

Diode lasers are solid state devices and are not all that different from laser emitting diodes (LED). The first diode lasers were developed quite early in the history of lasers but it was not until early 1980s that they became widely available (Goldwasser, 1997). Diode lasers emit light with a shorter coherence length than HeNe (632.8 nm) lasers and the biological effects of light from a 633 nm diode laser are less obvious than those from a HeNe laser. A diode laser can obtain similar results by using a higher power output and higher doses (Tuner and Hode, 2002; Ohshiro and Calderhead, 1988).

LILI can accelerate wound and burn healing and improve conditions of patients after myocardial infarction and stroke. Low intensity laser can help in the treatment of diabetic angiopathy and neuropathy and reduce atherosclerotic plaque formation by increasing micro-circulation (blood circulation) (Gasparyan *et al*., 2004).

Wound healing is the universal response to the inflammation that follows injury. It consists of a series of consecutive but overlapping events which

include cell proliferation, migration and extracellular matrix deposition (Darby and Hewitson, 2007). Growth factors and proteases play an important role in regulating the above events (Cullen *et al*., 2002). LILI has been shown to enhance the release of the growth factors and stimulate cell proliferation at certain wavelength and fluences (Yu *et al*., 2001).

Studies have shown that LILI produces positive effects on irradiated cells and tissues. Examples of these effects are cell proliferation and motility, augmented formation of mRNA (messenger ribonucleic acid) and protein secretion, stimulation of calcium influx and mitosis rate, activation of inactive enzymes like ATPase (Adenosine triphosphatase) and photosensitised formation of reactive oxygen species (Schindl *et al*., 1998). LILI stimulates capillary growth, granulation tissue formation and alters cytokine production. Altered keratinocyte motility and fibroblast movement have also been shown following low level laser irradiation (Basford, 1995). These effects aid in treatment of disorders like acute or chronic tissue hypoxia, destruction of tissues, as well as altered cell metabolism (Gasparyan *et al*., 2004).

The effects of LILI on wound healing are attributed to increased cell proliferation. It is thought that when LILI is targeted at the cells, photons are absorbed in the cytochromes and porphyrins within the mitochondria. Singlet oxygen is then produced and there is a formation of proton gradients across the mitochondrial membrane resulting in an increased ATP and DNA production (AU Eells *et al*., 2003). Laser irradiation was also found to induce synthesis of cell cycle regulatory proteins in satellite cells from muscles due to activation of early cell cycle regulatory genes, mitogen activated protein kinase and extracellular signal regulated kinase cascades (Ben-Dov *et al*., 1999). It has also been shown that application of low intensity laser irradiation to the infarcted heart results in the

increase of antioxidants in the blood (Oron *et a*l., 2001), heat shock protein (Yaakobi *et al*., 2001), inducible nitric oxide content (Tuby *et al*., 2006) and angiogenesis (Mirsky *et al*., 2002 and Stein *et al*., 2005).

LILI has been shown to promote the maturation of human osteoblasts *in vitro* (Stein *et al*., 2005) and increase proliferation of lymphocytes (Sadler *et al*., 2000) and articular chondrocytes in culture (Jia *et al*., 2004). LILI has also been shown to stimulate proliferation of certain cells (HeLa cells) *in vitro* (Koutna *et al*., 2003). In contrast to the above effects, some investigators have found damaging or even destructive action of low intensity lasers. For instance, He-Ne laser irradiation has produced degenerative effects on bovine oocytes (Ocana-Quero *et al*., 1997). However, the true effect of LILI on cell proliferation is controversial because of conflicting reports on the effects of visible light on cells in culture (Pinheiro *et al.,* 2002). This emphasises the need of more cellular research into laser biology. Since cell proliferation is one of the basic manifestations of any living organism, an insight into factors affecting cell proliferation in response to laser irradiation may be important in terms of the therapeutic applications of lasers.

Studies on LILI and stem cells have shown that low intensity lasers can alter the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration (Gasparyan *et al*., 2004). It has been shown that 5 J/cm^2 of laser irradiation at a wavelength of 635 nm positively affects ADSCs by increasing cellular proliferation, viability, and expression of β1-Integrin (Mvula *et al*., 2008) and Thy-1, and LILI in combination with EGF enhanced the proliferation of ADSCs (Mvula *et al*., 2010). Low intensity laser improves the growth of stem cells when cultured under conditions of less nutrients (Eduardo *et al*., 2008). Studies carried out on rats revealed the promoting of recovery of atrophied gastrocnemius skeletal muscles when subjected to LILI (Nakano *et al*., 2009). Using

diode LILI, scientists have reduced lipopolysaccharide-induced bone marrow inflammation (Huang *et al*., 2012) and this low intensity laser has also been discovered to facilitate fibronectin and collagen type 1 turnover during tooth movement (Kim *et al*., 2010). Studies done by Roland *et al* in 2013 found that LILI stimulated the transcription of genes involved in the chain of electron transport at a wavelength of 660 nm at 5 J/cm^2. The genes involved in the metabolism of energy and oxidative phosphorylation were upregulated thereby stimulating ATP production increase. Peridontal ligament cells were found to increase in proliferation when exposed to a wavelength of 670 nm laser diode with an output of 500 mW at an energy density of 5 and 10 J/cm^2 and this resulted in decreased inflammation (Huang *et al*., 2012). Recent studies done by Soleimani *et al*., 2012, found that LILI promoted proliferation of human bone marrow stem cells and their differentiation into neurons and osteoblasts though this was dosage dependent. They found out that at a wavelength of 810 nm and a range of 2-6 J/cm^2, LILI enhanced differentiation of these bone marrow stem cells into neurons and osteoblasts. There was also increased proliferation of the stem cells. Other studies on LILI have also found out that it could increase differentiation of mesenchymal stem cells into myogenes and osteogenes (Hou Jian-feng *et al*., 2008; Abramovitch-Gottlib *et al*., 2005). LILI also increases growth factor secretion (Hou Jian-feng *et al*., 2008).

CHAPTER 3

METHODOLOGY

This research study was executed according to the flow diagram in appendix A. All items (chemicals and reagents) used in the project with the names of the supplying companies are listed in Appendix E and solutions in Appendix F.

3.1 Cell Culture

Primary ADSCs were isolated from human adipose tissue of the consenting donors undergoing abdominoplasty. The Research Ethics Committee of the Faculty of Health Sciences of the University of Johannesburg granted the ethical approval (Approval Number 01/06) Appendix B. Commercial ADSC line (Stempro Adipose Derived Stem Cells/ Adipose-Derived Mesenchymal Stem Cells, R7788-115, Invitrogen, SA) and a SMC cell line (SKUT-1) from ATCC (American Type Culture Collection-HTB-114), were also purchased (ATCC certificate analysis, Appendix C).

3.1.1 Isolation of adipose derived stem cells

Adipose tissues from consenting donors undergoing abdominoplasty were used to isolate ADSCs. The adipose tissue was obtained from donors attending the plastic surgery of Dr. Doucas (and associates) at the Linksfield Park Clinic, Sandringham, Johannesburg. All donors received consent forms and information about the donation of the tissues (Appendix D). Donated tissues, surgically excised dermal and adipose tissues were personally collected and transported as biological specimens in a sealed polystyrene containers from the Linksfield Park Clinic, Sandringham, Johannesburg to the Laser Research Centre Laboratory, Doornfontein Campus, University of Johannesburg, by Prof H. Abrahamse of the Laser Research Centre. The Research Ethics Committee of the Faculty of Health Sciences of the University of Johannesburg granted the Ethical approval (Approval Number 01/06).

The tissue was either processed immediately or stored overnight in Hanks Balanced Salt Solution (HBSS) containing 10, 000 units of Pen/Strep and 250 µg/ml of fungizone in a ratio of 1:10 to avoid contamination. The tissue was minced into 3-5 mm pieces and 100 ml of the minced tissue was placed in a collagenase type-1 solution at a concentration of 600 U/ml containing 5 M Calcium Chloride, 240 U/mg collagenase type-1 and HBSS. The mixture was incubated in a shaking incubator (Labcon, Instrulab, SA) at 70 rpm for 80 min at 37^0C in Falcon centrifuge tubes. After incubation, equal volume of complete medium (Dulbecco's Modified Eagle's Medium – DMEM) containing 10% Fetal bovine serum (FBS), 0.1 % Penicillin/Streptomycin and 1 µg/ml Fungizone were added and mixed in 50 ml falcon tubes.

The mixture was then centrifuged at 650 xg for 5 min at 20^0C in a Hereus megafuge 16R centrifuge (Thermo Scientific, SA, figure 6) and then the oil layer was removed. The infranatant was removed and resuspended and filtered using a BD Falcon cell strainer filter (40 µm in size). The suspension was then centrifuged at 650 x g for 5 min at 20^0C and supernatant removed. DMEM with antibiotics was added to the pellets and centrifuged at 650 x g for 5 min. The supernatant was removed and the pellets resuspended in erythrocyte lysis buffer (ELB) for 10 min at room temperature to lyse the red blood cells. The solution was then centrifuged at 650 xg for 5 min and the pellets resuspended in complete DMEM medium in 75 cm^2 culture flasks (Corning Incorporated, USA). The cells were incubated at 37^0C in 85% humidity of 5% carbon dioxide (CO_2) in a Hera cell 150 incubator (Thermo Scientific, SA, figure 7). After 24 h of incubation, cells were observed using inverted microscopy.

Figure 6. Hereus megafuge 16R centrifuge used to spin cell suspensions.

More stocks of the cells were made by storing 1 x 10^6 cells in 1 ml of the freezing medium, Biofreeze (Biochrom, Biocombiotech, SA) in cryogenic vials (Corning Incorporated, USA) and immediately keeping them at -20^0C until the mixture is frozen and then transferring them (vials) into liquid nitrogen at -196^0C.

3.1.2 Co-culturing

After isolation ADSCs were cultured in DMEM F12 with 10% FBS, 0.1% penicillin/streptomycin and 1µg/ml fungizone incubated at 37^{0}C in an atmosphere of 5% CO_2 and 85% humidity in a HERA CELL 150 incubator (Heraeus, Separation Scientific, SA) figure 6. SMCs of a commercial cell line SKUT-1 and Adipose Commercial Stem Cells, Stempro Human Adipose Derived Stem Cells (cADSCs) were also cultured in the same medium with similar conditions as isolated ADSCs. After reaching semi-confluency for ADSCs and confuency for SKUT-1, both ADSCs and SKUT-1 cells were co-cultured directly in 3.4 cm^2 diameter dishes in a 1:1 ratio with and without the growth factors in MCDB 131 medium with 2% FBS, 0.1% penicillin/streptomycin and 1µg/ml fungizone incubated at 37^{0}C in an atmosphere of 5% CO_2. The co-cultures were divided into 6 groups:

Group 1 were the co-cultures without the growth factors and were not exposed to LILI (CC), group 2 were the co-cultures without growth factors but were exposed to LILI (CC+LILI), group 3 were the co-cultures supplemented with the growth factor, retinoic acid (RA) and not exposed to LILI (CC+RA). Group 4 were the co-cultures supplemented with RA and exposed to LILI (CC+RA+LILI), group 5 were the co-cultures supplemented with a growth factor, transforming growth factor beta 1 (TGF-β1) but not exposed to LILI (CC+TGF-β1) and the last group 6 were the co-cultures with TGF-β1 and were exposed to LILI (CC+TGF-β1+LILI). RA was added to the co-cultures at a concentration of 0.1 µM and TGF-β1 at 1 ng/ml. These concentrations were optimised in the laboratory.

Figure 7. **A Hera Cell 150 incubator where cells were incubated for culturing (A). Flasks containing attached cells are incubated in 5% CO_2 and 85% humidity at 37^{0}C (B).**

3.2 Laser Irradiation

The co-cultures of group 2, 4 and 6 were exposed to diode laser (Oriel, LTIO00-PLT20, Oriel Corporation, SA) at 5 J/cm^2 with a wavelength of 636 nm in the dark. The medium was removed and the cells were washed with HBSS for three times and then the complete medium was added to the plates. LILI was then delivered to the plates without the lids in the dark via

the optical fibre as described in Mvula *et al*., 2008. The beam covered the entire area of 3.4 cm^2 of the small plate. Irradiation was performed once and the co-cultures were re-incubated at 37^0C in a humidified atmosphere after which various assays were performed at 24 h, 48 h, 72 h, and 1 wk post irradiation. The laser parameters are shown in table 1. The co-cultures which were not irradiated were used as controls and kept under the same conditions as the irradiated ones. Figure 8 shows the 636 nm diode laser.

Figure 8. The diode laser with wavelength of 636 nm which was used to irradiate the co-cultures with 5 J/cm^2.

Table 2. Laser parameters.

Laser Parameters	
Wavelength (nm)	636
Wave Emission	Continuous wave
Power output (mW)	85
Spot size (cm^2)	9.08
Output density (mW/cm^2)	9.3
Irradiation duration	9 mins 10 s
Fluence (J/cm^2)	5

3.3 Cell Morphology

Cellular morphology was measured by direct inverted light microscope (Olympus CKX41, 5K09553, Olympus Corporation, Japan, figure 9) and live cell image microscope (Axio Observer Z1, 3834000513, Carl Zeiss, Germany) which observed differential interference contrast microscopy and fluorescence microscopy.

Figure 9. Inverted light microscope (Olympus CKX41). The equipment was used to observe cell morphology.

Differential interference contrast microscopy (DIC), also known as Nomarski interference contrast (NIC) or Nomarski microscopy, is an optical microscopy illumination technique used to enhance the contrast in unstained, transparent samples. DIC works on the principle of interferometry to gain information about the optical density of the sample, to see otherwise invisible features. A relatively complex lighting scheme produces an image with the object appearing black to white on a grey background. This image is similar to that obtained by phase contrast microscopy but without the bright diffraction halo (Murphy, 2001; Salmon

and Tran, 1998). Carl Zeiss Axio Observer Z1 fluorescence microscope (Carl Zeiss, 195-041872, Randburg, SA) was used for observation of the images in this procedure.

Cell morphology of individual cells was assessed by culturing the cells on heat sterilised coverslips and after confluency, the cells were stained with carboxyfluorescein diacetate succinimydyl ester (5(6)-CFDA,SE) and observed using the flouroscence microscopy.

CFSE passively diffuses into cells. It is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. The dye–protein adducts that form in labeled cells are retained by the cells throughout development and meiosis, and can be used for *in vivo* tracing (Browner-Fraser 1985). The label is inherited by daughter cells after either cell division or cell fusion, and is not transferred to adjacent cells in a population (Hasbold *et al*, 1996; Lyons and Parish, 1994; Nose and Takeichi, 1986). Cells on the coverslips were washed three times with HBSS and then stained with CFSE at a concentration of 10 µM for 10 min at 37^0C. After staining, the cells were washed three times with HBSS and fixed with 3.7% paraformaldehyde for 10 min and then washed two times with HBSS. The coverslips were mounted on slides with 0.1M propyl gallate and viewed through a Carl Zeiss, Axio Observer ZI (a fluorescence microscope).

3.4 Cellular Responses

Trypan blue staining and Adenosine triphosphate luminescence analysis were used to measure cell viability while Optical density was used to

measure cell proliferation. Immunofluorescence and flow cytometry analyses were used to characterise the cells through expression of the markers as well as to monitor the expression of the specific markers over a period of time after irradiation.

For viability and proliferation analysis, the co-cultures in small plates were washed three times with HBSS. Next 500 µl of Triple Express (Invitrogen) was added to the co-cultures and incubated at 37^0C for 5 min to detach the cells from the plates. After incubation, 500 µl of HBSS was added and centrifuged at 650 xg for 5 min at 20 ^{0}C. The supernatant was removed and the pellets suspended in complete medium ready for analysis.

3.4.1 Cell viability

3.4.1.1 Trypan blue staining

Trypan blue is a blue dye that penetrates the damaged cell membrane. Live cells remain colourless as they do not take up this dye while the dead cells stain blue as they absorb the dye.

Cell suspension in HBSS (10 µl) was mixed with equal volume of trypan blue reagent (10 µl) and incubated at room temperature for 5 min. Cells were counted on a Haemocytometer with Neubauer rulings with a light microscope (Olympus CKX41). The cell viability percentage was calculated by dividing the number of translucent cells (viable ones) by the total number of cells and multiply by 100.

3.4.1.2 Adenosine triphosphate luminescence

Adenosine triphosphate (ATP) luminescence was used to assess cellular viability. Cellular ATP was measured using the Cell Titer-Glo luminescence cell viability assay. The assay provides a method for determining the number of metabolically active cells through the quantification of ATP.

Cell suspension in complete medium (50 µl) was mixed with an equal volume of Glo reagent (1 ml buffer and 0.007 g substrate) in an opaque 96 well plate (OptiPlate^{TM}-96) and incubated in the dark at room temperature and read in Relative Light Unit (RLU) in a Perkin Elmer, Victor^3 (Perkin Elmer, 172809, Separation Scientific, SA, figure 10). The actual readings of the co-cultures were obtained by substracting the readings of the blank medium (without cells) from the initial readings of the co-cultures.

Figure 10. Perkin Elmer, Victor^3. The equipment used to read samples for ATP luminescence and Optical density analysis. ATP luminescence was read in Reading Lights Units while the readings for Optical density was absorbance at 540 nm.

3.4.2 Cell proliferation

Proliferation of the co-cultures were analysed by using optical density (OD) where the absorbance of one hundred microliter of the cell suspension in complete medium in a clear 96 well plate (Corning) well was read at A_{540} nm in a Perkin Elmer, Victor^3 (Perkin Elmer, Separation Scientific, SA). Complete medium without cells was used as a blank sample.

3.5 Immunofluorescence

Immunofluorescence assays were used to observe cell characterisation through the expression of the specific markers as well as monitoring the expression of the specific markers of the cells over a period of time post-irradiation.

Cells cultured on heat sterilised coverslips were grown to semi-confluency, rinsed three times with phosphate buffered saline (PBS) bovine serum albumin (BSA–0.1% w/v) and azide (0.01% w/v) and then incubated with anti-β1 Integrin, CD29 mouse anti-human monoclonal antibody (mAb), fluorescencein (FITC) conjugated and CD90/Thy-1 mouse anti-human mAb, fluorescein (FITC) conjugated as for ADSCs and Myosin Heavy Chain mouse anti-human mAb, Allophycocyanin (APC) conjugated as for SMCs in the ratio 1:200 in PBS/BSA for 30 min on ice. For SMCs, they were incubated with 1% TRITON-X100 to permeabilise them prior to antibody incubation. Then the cells were washed three times with PBS/BSA/azide. The cells were then fixed with 3.7% paraformaldehyde for 10 min and then washed two times with PBS/BSA/azide and then once with water before being stained with 4-6 Diamidino-2-Phenylinole (DAPI) at a concentration of 0.1 µg/ml. The cells were then mounted on slides with 0.1M propyl gallate and viewed through a Carl Zeiss, Axio Observer Z1 (a fluorescence microscope, figure 11).

Figure 11. Expression of the markers through immunofluorescence was observed by the Carl Zeiss, Axio Observer Z1. The microscope was also used to get direct interference contrast images of the co- cultures.

3.6 Flow Cytometry

Flow cytometry quantitatively analysed the expression of the specific markers over a period of time after irradiation. Co-cultures were rinsed three times with HBSS. The cells were then removed from the 3.4 cm^2 diameter plates by adding 500 µl of tripLe Express and incubated at 37^0C for 5 min. The cells were then washed in HBSS once and twice in PBS/BSA/azide. All the washing was done at 650 xg for 5 min at 20^0C. Permeability was done by incubating the cell suspension in 1% triton x-100. Cells were resuspended in PBS/BSA/azide at a concentration of 1 x 10^6 cells/ml. Anti-human CD29, anti-human CD90 and anti-human Myosin Heavy Chain were then added and incubated for 30 min on ice at a ratio of 1:250. The cells were washed three times with PBS/BSA/azide and then fixed for 10 min in 3.7% paraformaldehyde. The suspension was washed twice and the pellet was suspended in PBS, ready for reading with the BD FacsAria^{TM111} flow cytometer (BD Biosciences, 22300099, Scientific Group Biosciences, Woodmead, SA, figure 12).

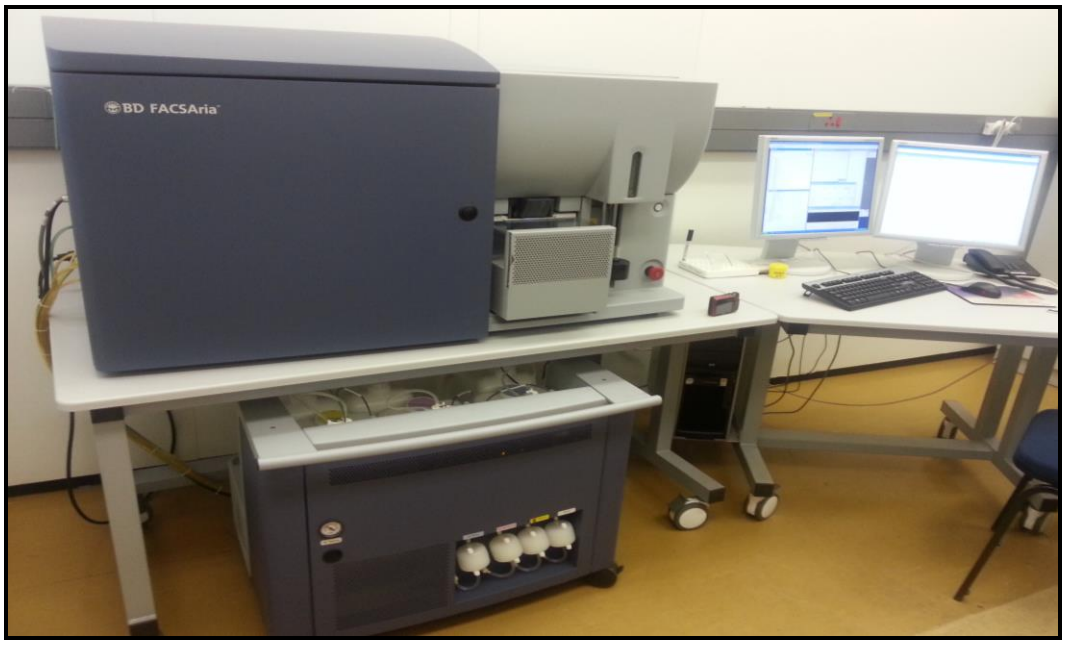

Figure 12. BD FacsAria^{TM111} was used to analyse the quantitative expression of markers through flow cytometry.

3.7 Microarray Polymerase Chain Reaction

Gene expression profiling or microarray analysis has enabled the measurement of thousands of genes in a single RNA sample. There are a variety of microarray platforms that have been developed to accomplish this and the basic idea for each is simple: a glass slide or membrane is spotted or arrayed with DNA fragments or oligonucleotides that represent specific gene coding regions. Purified RNA is then fluorescently or radioactively labeled and hybridized to the slide/membrane. Hybridization may also be done simultaneously with reference RNA to facilitate comparison of data across multiple experiments. After thorough washing, the raw data is obtained by laser scanning or autoradiographic imaging. At this point, the data may then be entered into a database and analyzed by a number of statistical methods (Schena *et al.*, 1995 and Hegde *et al.*, 2000).

3.7.1 Real-time Reverse Transcriptase Polymerase Chain Reaction

Gene expression analysis was performed using real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The technology

monitors the amplification of gene products during cycles in real-time. Gene expression analysis is performed due to the real-time RT-PCR selectivity and multigene profiling capacity of microarray. SYBR Green is a dye used to detect any double stranded DNA molecule and binds to their nitrogenous bases leading to a high fluorescent signal in the presence of double stranded DNA (Bustin *et al.*, 2005; Wang *et al*., 2006). Four groups (1, 2, 5 and 6) at both 24 h and 1 wk were analysed. Group 1 (co-cultures not supplemented with the growth factors and not exposed to LILI) was used as a control.

Complementary DNA (cDNA) is the template used for RT-PCR and is synthesised from RNA. The RNeasy kit (Qiagen, 74104) with QIAshredder homogenisers (Qiagen, 79654) was utilised to extract RNA from the the co-cultures due to the specific binding properties of a silica-membrane and the speed of microspin technology. The use of a high salt buffer allows the extraction of up to 100 µg of RNA longer than 200 bases bound to the RNeasy Silica-membrane.

Nucleic acids extraction depends on the properties of the Silica-materials. Silica-membranes are cation rich components such as sodium and form cation bridges. They break the hydrogen bonds between the water molecules in solution under high salt concentration (pH<7). The cation bridges in the Silica-membranes attract negatively charged oxygen in the backbone phosphate groups of nucleic acids. Nucleic acids thus become tightly bound to the Silica-membranes and during extraction washing processes remove all unbound substances and contaminants. Purified nucleic acids are eluted under low ionic concentration of pH>7.0. (Lai *et al*., 2007).

3.7.2 Extraction of ribonucleic acid (RNA)

RNA extraction was performed using the RNeasy kit (Qiagen, 74104) with QIAshredder homogenisers (Qiagen, 79654) on treated co-cultures, which

were incubated for 30 min prior to RNA extraction. Cells were trypsinised, the supernatants discarded, and cells washed with PBS to remove all traces of culture medium. A volume of 600 µl of RLT buffer was added to the cells, which were mixed by vortexing. RLT buffer inactivates RNase to guarantee purification of intact RNA. Reagents and cell suspensions were loaded on the QIAcube (Qiagen, ETI-9017715-E, Switzerland, figure 13) and RNA isolation was performed. Thirty microliters of eluted RNA was collected and kept on ice.

Figure 13. QiaCube instrument used to extract RNA from the co-cultures. It took a total of 23 mins to extract RNA.

Alternatively, RNA was extracted manually. After cells were harvested, 600 µl of RLT buffer was added to lyse them. The lysate was pipette into a Qiashredder spin column and placed into a 2 ml tube and centrifuged for 2 min at 13000 rpm and then 600 µl of 70% alcohol was added to the lysate. A 700 µl volume of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged at 8000 x g or 10000 rpm for 15 s and the flow through was discarded. Another 700 µl of Buffer RW1 was added to the spin column and spun for 8000 xg or 10000 rpm for 15 s

and then the flow through was discarded. RPE Buffer in the volume of 500 µl was added to the spin column and spun for 8000 xg or 10000 rpm for 15 s and the flow through was discarded. This was done twice. RPE is supplied as a concentrate and before use alcohol is added. The collection tube was then discarded and then replaced with the new tube. 40 µl of the RNase-free water was added to the spin column membraneand centrifuged at 8000 xg or 10000 rpm for I min to elute RNA. To yield more RNA, the eluate from the previuos step was added to the spin column and spun again at 8000 xg or 10000 rpm for 1 min.

3.7.3 RNA quantification

RNA was quantified using the Quant-iT™ RNA Assay kit (Invitrogen, Q32852) on the Qubit™ fluorometer (Invitrogen, 45256-239,Turner BioSystems), figure 14). Thin-walled, clear 0.5 ml optical-grade PCR tubes were used to quantify the RNA. The working solution of the assay was prepared by diluting Quant-iT™ RNA reagent in Quant-iT™ RNA buffer in a 1:200 ratio and stored at room temperature. A volume of 190 µl working solution was loaded into two tubes for standards (1 and 2) and 10 µl of each Quant-iT™ standard was added to the appropriate tube and mixed. For isolated RNA, 199 µl working solution and 1 µl of the eluted RNA sample was mixed. The mixtures were incubated at room temperature for 2 min and then measured in the Qubit™ fluorometer. The Qubit™ fluorometer was calibrated using the two standard tubes and then RNA sample concentration (µg/ml) was calculated by the instrument once the volume of sample (1 µl) was selected. The total concentration of RNA isolated was calculated by multiplying the concentration of the RNA (µg/ml) by the total eluted volume (30 µl).

Figure 14. Qubit flourometer was used to quantify RNA from the co-cultures.

3.7.4 RNA purity

RNA purity was evaluated by obtaining the ratio of the absorbance value at 260 nm over the value at 280 nm using the Biomate 3 spectrophotometer (Thermo Spectronic, USA, 335904P, figure 15). One microliter RNA was added to 99 µl buffer AE (Qiagen, 19077) in a quartz cuvette to quantify nucleic acids. The buffer consists of 10 mM Tris-Cl and 0.5 mM EDTA; pH 9.0.

The ratio $A_{260}/_{280}$ nm is an important indicator of the quality of the RNA and it is used to evaluate the degree of purity. Due to the aromatic rings, proteins absorb light at 280 nm. A normal RNA sample should have a ratio between 1.8 to 2 and less than the above indicates protein contamination.

Figure 15. Biomate 3 Spectrometer which was used to purify RNA and cDNA from the co-cultures.

3.7.5 Complementary DNA (cDNA) synthesis

The flow of genetic information is usually from DNA to RNA through transcription and then RNA to protein through translation of the transcript except in reverse transcription where a reaction is catalysed by a RNA dependent DNA polymerase, known as reverse transcriptase. Reverse transcriptases have since been utilised to convert RNA into its cDNA counterpart.

QuantiTect Reverse Transcriptase used in this study had a high affinity for RNA and the ability to synthesise cDNA from 10 pg RNA. A volume of 30 ng of RNA was used for RNA reverse transcription into cDNA using the SABiosciences RT First Strand kit (330401). The PCR Array system used yields results with as little as 25 ng or as much as 5 µg total RNA per array. A genomic DNA (gDNA) elimination reaction mixture was done on ice and consisted of gDNA buffer, template RNA and RNase free water (Table 3).

Table 3. **Genomic DNA elimination reaction and various components.**

Component	Volume/reaction	Final concentration
gDNA wipeout buffer	2 µl	1X
Template RNA	Variable (30 ng)	
RNase free water	Variable	
Final volume	**14µl**	**-**

gDNA elimination tubes were incubated for 8 min at 42˚C and placed on ice. A final volume of 20 µl of reverse transcription reaction was prepared on ice by adding Quantiscript Reverse Transcriptase, QuantiscriptRT Buffer, RT Primer Mix and 14 µl of the gDNA elimination reaction according to Table 4. The reverse transcription reaction tubes were kept on ice, mixed and incubated for 15 min at 42˚C. The reverse transcription reaction tubes were then incubated for 3 min at 95˚C to inactivate Quantiscript Reverse Transcriptase. The cDNA was stored at -20˚C and/or used as a template in the real-time PCR array.

3.7.6 cDNA purity

cDNA purity was analysed being used for real-time PCR by the ratio between the absorbance values at 260 and 280 nm ($A_{260/280}$ nm). One microliter cDNA was added to 99 µl buffer AE (Qiagen, 19077) in a quartz cuvette and read in the Biomate 3 spectrophotometer (figure 12). Ratios between 1.82 and 1.94 were obtained and used for gene expression analysis (Appendix F).

3.7.7 Gene expression

The synthesised cDNA was used for real-time PCR to study the expression of a number of genes in Human Mesenchymal Stem Cells PCR Array (SABiosciences) using the Stratagene Mx3000p® (Agilent Technologies, DE 94300411, Germany, figure 16). The RT² Profiler PCR Array profiles the expression of 84 genes (table 5). The genes are involved in mesenchymal stem cell (MSC) maintenance and differentiation

(table 4). The RT^2 Profiler PCR Array System takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of many genes simultaneously.

Table 4. Reverse transcription reaction and various components.

Component	Volume/reaction
Reverse-Transcription master mix	
Quantiscript Reverse Transcriptase	1µl
Quantiscript RT Buffer	4µl
RT Primer Mix	1µl
Template RNA	
gDNA elimination reaction	14µl
Final volume	**20 µl**

The PCR plate has 96 wells with 12 columns (1 to 12) and 8 rows (A to H). The first 84 genes (wells A1 to G12) are for the human mesenchymal stem cell array and the remaining 12 consist of 5 housekeeping genes (wells H1 to H5) inserted for normalisation of the PCR Array data, 1 gDNA control gene (well H6) for gDNA contamination, 3 Reverse Transcription Controls (RTC, wells H7 to H9) and 3 Positive PCR Control genes (PPC, wells H10 to H12) for assessment of the efficiency of the primer set provided in the kit and of a predispensed artificial DNA sequence, respectively (Figure 17).

Table 5 **Human Mesenchymal Stem Cells PCR Array functional gene grouping profiler.**

	Gene subunits
Stemness Markers:	FGF2 (bFGF), INS, LIF, POU5F1 (OCT4), SOX2, TERT, WNT3A, ZFP42.
MSC Specific Markers:	ALCAM, ANPEP, BMP2, CASP3, CD44, ENG, ERBB2 (HER2), FUT4, FZD9, ITGA6, ITGAV, KDR, MCAM, NGFR, NT5E, PDGFRB, PROM1, THY1, VCAMI.
Other Genes Associated with MSC:	ANXA5, BDNF, BGLAP (Osteocalcin), BMP7, COL1A1, CSF (GM-CSF), CSF3 (GCSF), CTNNB1, EGF, FUT1, GTF3A, HGF (Scatter Factor), ICAM1, IFNG, IGF1, IL10, IL1B, IL6, ITGB1, KITLG, MMP2, NES, NUDT6, PIGS, PTPRC, SLC17A5, TGFB3, TNF, VEGFA, VIM, VWF.
MSC Adipogenesis Differentiation:	PPARG, RHOA, RUNX2.
MSC Chrondrogenesis Differentiation:	ABCB1 (MDR1), BMP2, BMP4, BMP6, GDF5 (CDMP-1), GDF6, GDF7, HAT1, ITGAX, KAT2B, SOX9, TGFB1.
MSC Myogenesis Differentiation:	ACTA2, JAG1, NOTCH1.
MSC Tenogenesis Differentiation:	BMP2, GDF15 (PLAB), SMAD4, TGFB1.

The cDNA was thawed on ice and 92 µl of RNase-DNase free water was added to make a final volume of 111 µl. An experimental cocktail was prepared and the SABiosciences RT^2 qPCR master mix, the diluted cDNA mixture and RNase-DNase free water were mixed in a 50 ml RNase-DNase free tube according to the manufacturer protocol (Table 6). The SABiosciences RT^2 qPCR Master mix is specifically designed for the Stratagene Mx3000p® and consists of a RT^2 SYBR Green (detected dye) and ROX (reference dye) qPCR components.

Figure 16. Stratagene Mx3000p® used to analyse the expression of the genes from the cDNA of the co-cultures.

The 96 well PCR array plate was loaded by adding 25 µl of the experimental cocktail to each well. The PCR array plate was sealed with the optical thin walled 8-cap strips and kept on ice. The plate was centrifuged for 1 min at room temperature at 1,000 xg using a Heraeus Labofuge 400 centrifuge (Thermo Scientific) to remove all air bubbles. The Stratagene Mx3000p® was programmed as follows: 1 cycle, 10 min at 95˚C to activate the HotStart DNA polymerase, 40 cycles of 15 sec and 1 min at 95˚C and 60˚C respectively (the annealing step). During the annealing step of each cycle, SYBR Green intercalates and detects double stranded DNA from every well.

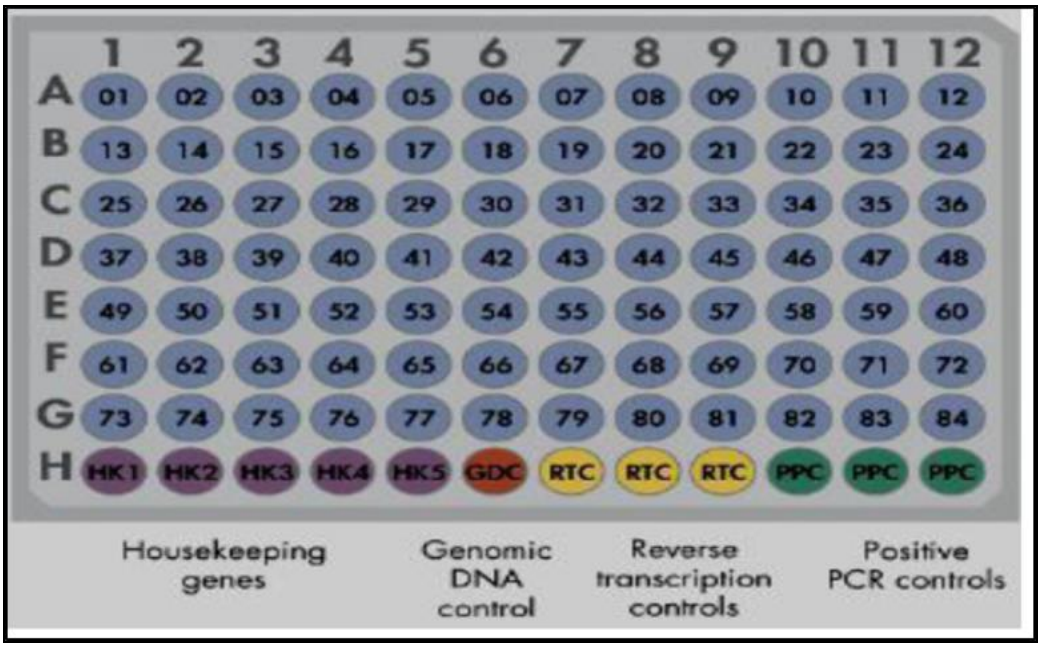

Figure 17. **Layout of the catalogued 96 well PCR Array. Wells A1 through G12 contain the 84 genes of the human mesenchymal stem cell array. Wells H1 through H5 contain housekeeping genes (HK1-HK5) used for normalising the PCR array data. Well H6 to H12 contain controls namely genomic DNA contamination (GDC), Reverse Transcription Controls (RTC), and Positive PCR Control (PPC).**

The amplification process involves the stage that the target DNA sequence will be amplified through a thermal process of repeated heating (for DNA melting) and cooling (for enzyme dependent replication) steps. The DNA melting step unwinds the double stranded helical molecule into two single stranded DNA molecules. Two single stranded DNA molecules are used as a template by DNA polymerase for the amplification of the target DNA sequence in the cooling step. SYBR Green is not specific and detects any other double stranded DNA such as the target DNA in each well, primer dimers, contaminant DNA or products from mis-annealed primers.

Table 6. **Experimental cocktail preparation for RT-PCR application.**

Plate format:	**96 well**
2x SABiosciences RT^2 qPCR Master mix	1,350 µl
Diluted First Strand cDNA Synthesis Reaction	102 µl
PCR Water	1,248 µl
Final Volume	**2,700 µl**

The melting curve analysis after the PCR process evaluates the dissociation of the helical molecule. During the heating step, hydrogen bonds between the nitrogenous bases are destabilised and the double stranded DNA starts to dissociate. As the dissociation occurs, the molecule becomes hyperchromic due to the increase in the absorbance intensity. The temperature at which 50% of hydrogen bonds are dissociated is called the melting point and is dependent on the G-C content of nucleic acid molecules and used for identification purposes. Therefore, the identification of a single peak in each well indicates the amplification of one specific gene. The software melting curve program of the Stratagene Mx3000p® was run immediately after the cycling program and the instrument was pre-programmed as follows: 95˚C, 1 min; 65˚C, 2 min (optics off) and 65 to 95˚C at 2˚C per min (optics on). A melting curve with one peak for each well in the plate at temperatures greater than 80˚C had to be obtained.

3.7.8 Real-time PCR array data analysis

A loaded PCR array plate was placed on the real-time thermal cycler and the program was run. The equipment software was used to calculate the threshold cycle (C_t) value for each well. All C_t values equal to or greater than 35 were considered as absence of amplicon (negative). A gDNA control well (well H6) with a C_t value greater than 35 indicates that there was no gDNA contamination. A positive C_t value (C_t<35) indicates gDNA contamination. PCR control wells (PPC, wells H10 to H12) with a positive C_t value indicated successful amplification and the C_t value should be 20±2 (should not vary by more than 2 cycles between PCR Arrays being compared). Larger differences in average C_t PPC values between plates and wells on the same plate indicates different amounts of starting template or the presence of PCR amplification inhibitors in each sample, which then requires purification steps. An average C_t PPC value above 22 indicates a problem with the cycling condition or sensitivity of the instrument. The software used the average C_t values of all 5

housekeeping genes to normalise the 84 genes studied; the average C_t value of all 5 of the housekeeping genes was deducted from the gene C_t value.

The C_t values were exported to a blank Excel spread sheet for use with the SABioscience PCR array Data Analysis Template available from the SABioscience website with the suitable pathway focused genes (330231-PAHS-082Z-A-12). The PCR Array Data Analysis software automatically performed the calculations and interpretation of the control wells upon including C_t data from the real-time instrument. The results are presented in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included). The Students t-test is used to calculate the *p* value and the significant difference of gene expression. Fold-change ($2^{(\Delta\Delta C_t)}$) was calculated by dividing the normalised gene expression ($2^{(\Delta C_t)}$) of the test sample by the normalised gene expression ($2^{(\Delta C_t)}$) of the control sample. Fold-change values greater than one indicate a positive or an up-regulation, and the fold-regulation is equal to the fold-change, while fold-change values less than one indicate a negative or down-regulation. The fold-regulation is the negative inverse of the fold-change. The *p* values were calculated based on the Student's t-test of the replicate $2^{(\Delta C_t)}$ values for each gene in the control group and treatment groups. A value of $p<0.05$ was considered significant. A diagram summarising the PCR array analysis is shown in figure 18.

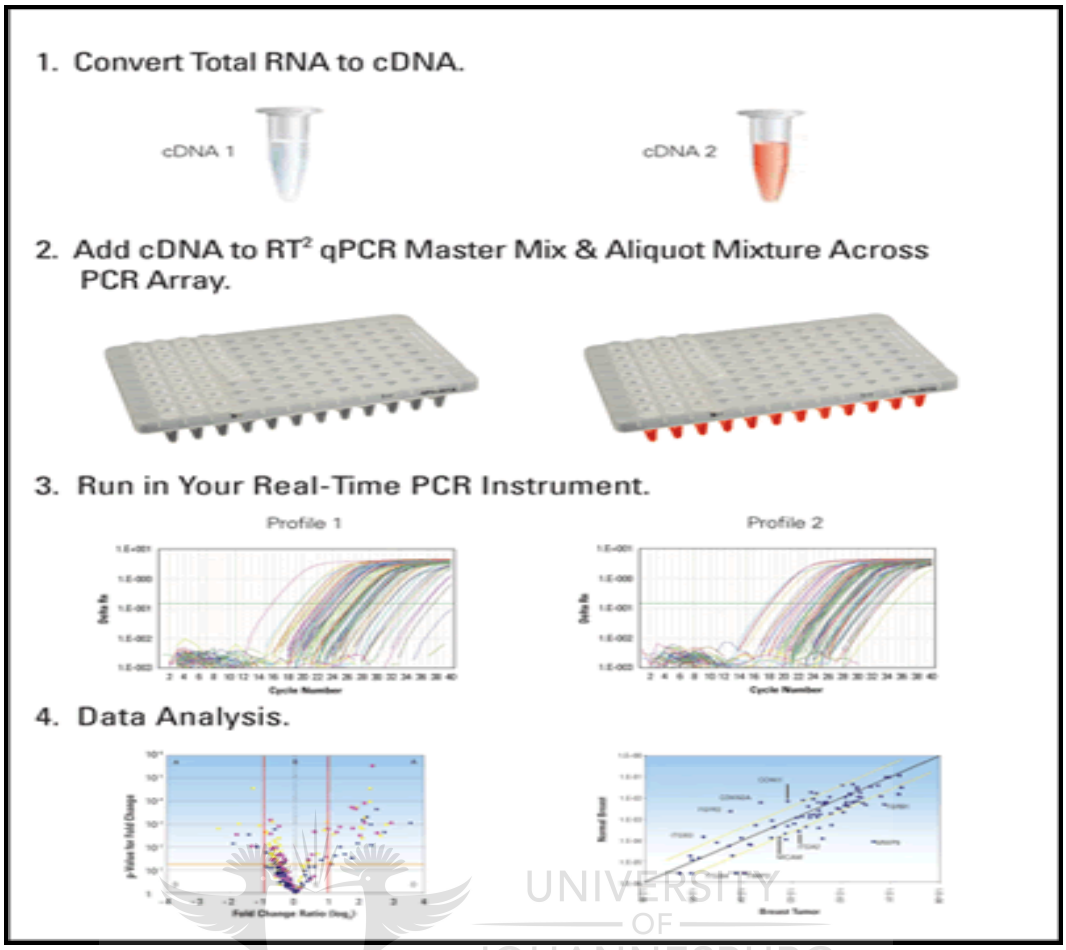

Figure 18. **A diagram summarising PCR microarray analysis. http://www.sabiosciences.com. 26/06/2014**

3.8 Statistical Analysis

All experiments were performed 6 times (*n=6*), assays in duplicate and the data was analysed with a sigma plot 11.0 software. Students t-test was used to determine the differences between the groups for each independent variable. Statistical significances comparing the groups are shown in the table and graphs as stars * where *P*<0.05 (*), *P*<0.001 (**) and *P*<0.005 (***).

CHAPTER 4

RESULTS

ADSCs and SKUT-1 cells were both cultured in DMEM F1 with 10% FBS and antibiotics separately. After reaching semiconfluency for ADSCs and confluency for SKUT-1 cells, both cells were co-cultured in a differential medium, MCDB 131 with 2% FBS and antibiotics. The co-cultures were divided into six groups. Group 1 (CC) was a co-culture of two types of cells which were not exposed to irradiation and were not grown in the presence of the growth factors. Group 2 (CC+LILI) was a co-culture that was exposed to irradiation. Groups 3 (CC+RA) was a co-culture supplemented with RA but not exposed to irradiation, group 4 (CC+RA+LILI) was a co-culture supplemented with RA and exposed to irradiation, group 5 (CC+TGF-β1) was a co-culture supplemented with TGF-β1 but not exposed to irradiation and group 6 (CC+TGF-β1+LILI) was a co-culture supplemented with retinoic acid and exposed to irradiation. Irradiation with 5 J/cm^2 using 636 nm diode laser (Appendix A). Cell characterisation, morphology, viability, proliferation and expression of the markers were evaluated at different time points. Regulations of different genes were also monitored.

4.1 Cell Characterisation

Cells were characterised through expression of the specific markers. ADSCs expressed CD29 and CD90 as seen in figure 19A and 19B respectively. As cell surface markers they appeared as green fluorescence within the cytoplasm of the cells surrounding the nuclei. Skut-1 cells expressed a smooth muscle cell marker, myosin heavy chain, which is an internuclear marker, appeared as red fluorescence within the nuclei of the cells (figure 19C).

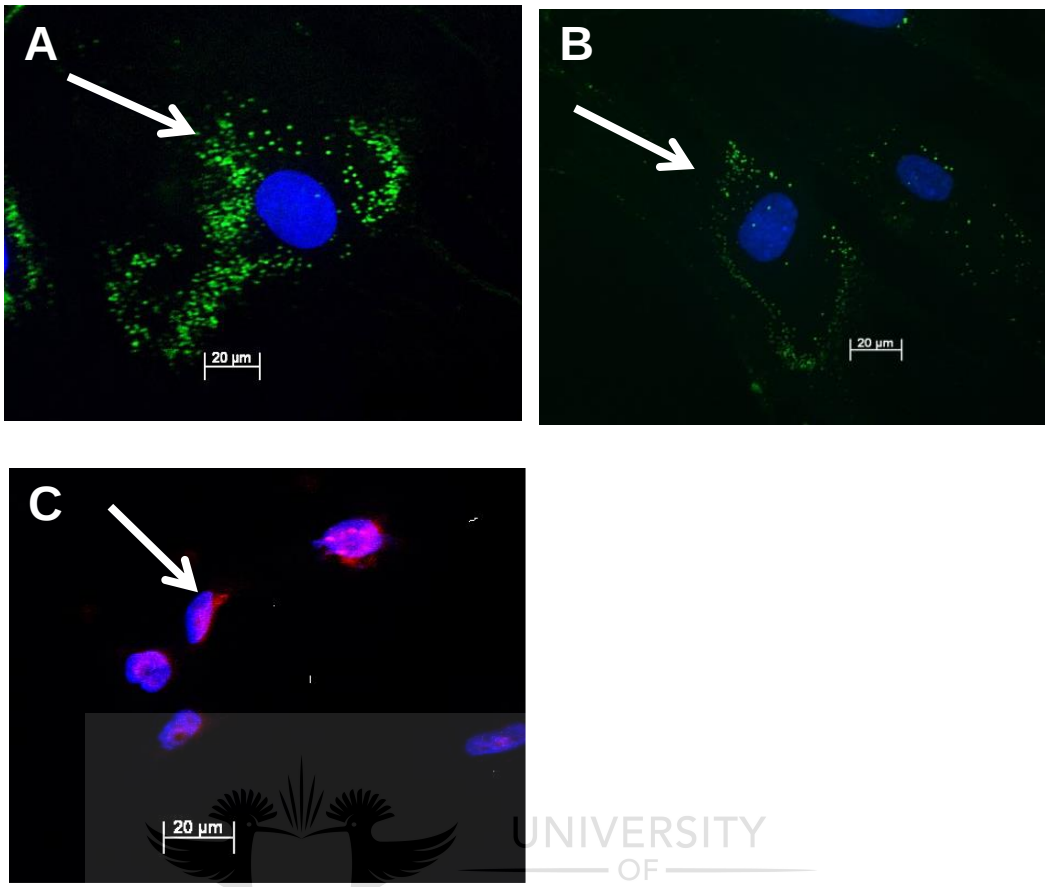

Figure 19. **The expression of the markers. ADSCs expressing CD90 (A) and CD29 (B) shown as green fluorescence and SKUT-1 cells expressing MHC (C) seen as red fluorescence within the nuclei. The nuclei of the cells were stained with DAPI. MHC are localised within the nuclei.**

The expression of the markers as observed through fluorescence microscopy was also seen in the co-culture when stained with CD29, 24 h later. ADSCs in the co-culture had green fluorescence around the nuclei but SKUT-1 cells did not express CD29 (figure 20A). One week after irradiation, cells in the co-cultures expressed MHC marker as seen in figure 20B.

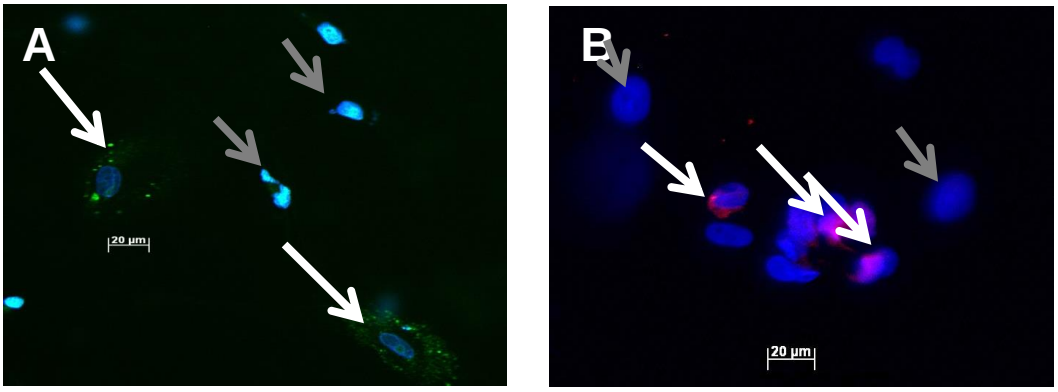

Figure 20. Expression of the markers in a co-culture. In figure A, expression of the ADSC marker (CD29) is shown by white arrows and grey arrows are showing SKUT-1 cells which did not express CD29 after 24 h. In figure B, cells expressed MHC after 1 wk as shown by white arrows and grey arrows are showing cells which did not express MHC. The nuclei appear blue stained with DAPI.

4.2 Cell Morphology

Morphologically when stained with CFDSE, both ADSCs and SKUT-1 cells displayed a more fibroblastic-like shapes, which is a monolayer of elongated cells but the ADSCs were larger in sizes than the SKUT-1 cells (Figure 21A and 21B). Both types of cells appeared green under a fluorescence microscope.

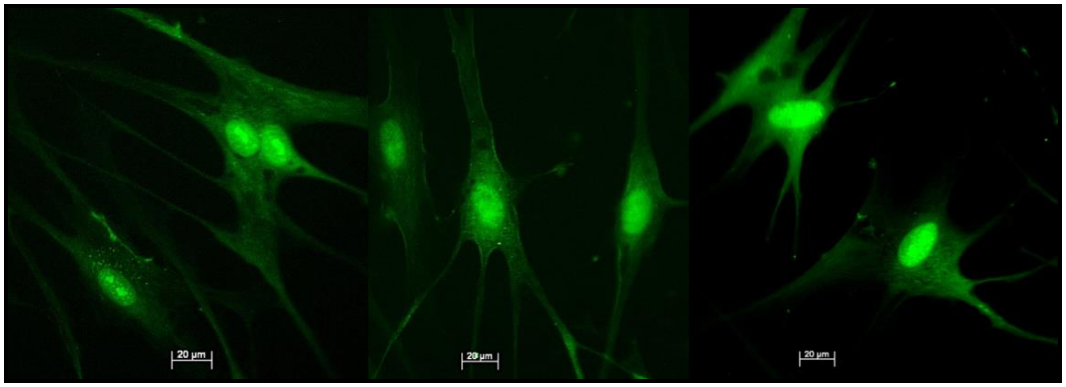

Figure 21A. Morphology of the ADSCs seen under the fluorescence microscopy stained with CFDSE.

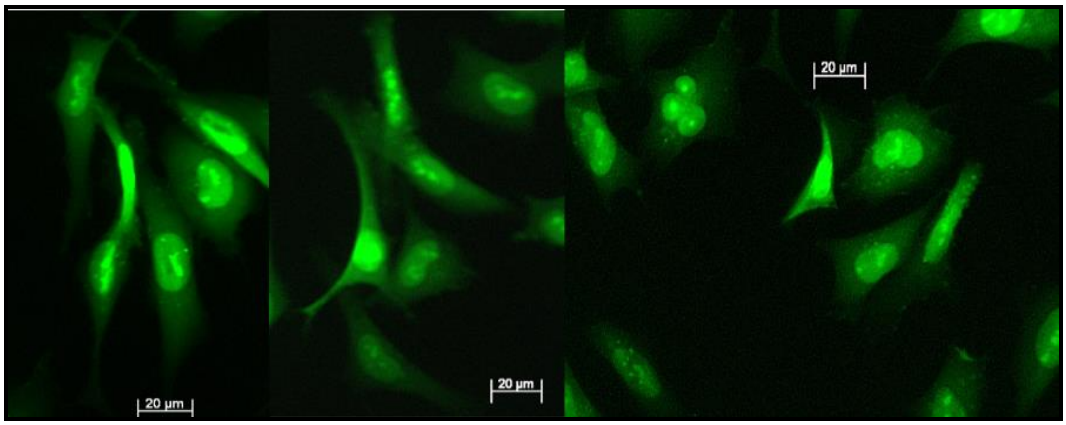

Figure 21B. Morphology of the SKUT-1 cells. The cells were stained with CFDSE and viewed under a fluorescent microscope.

In differential contrast interference micrographs, ADSCs appeared to be more elongated than SKUT-1 (Figure 22).

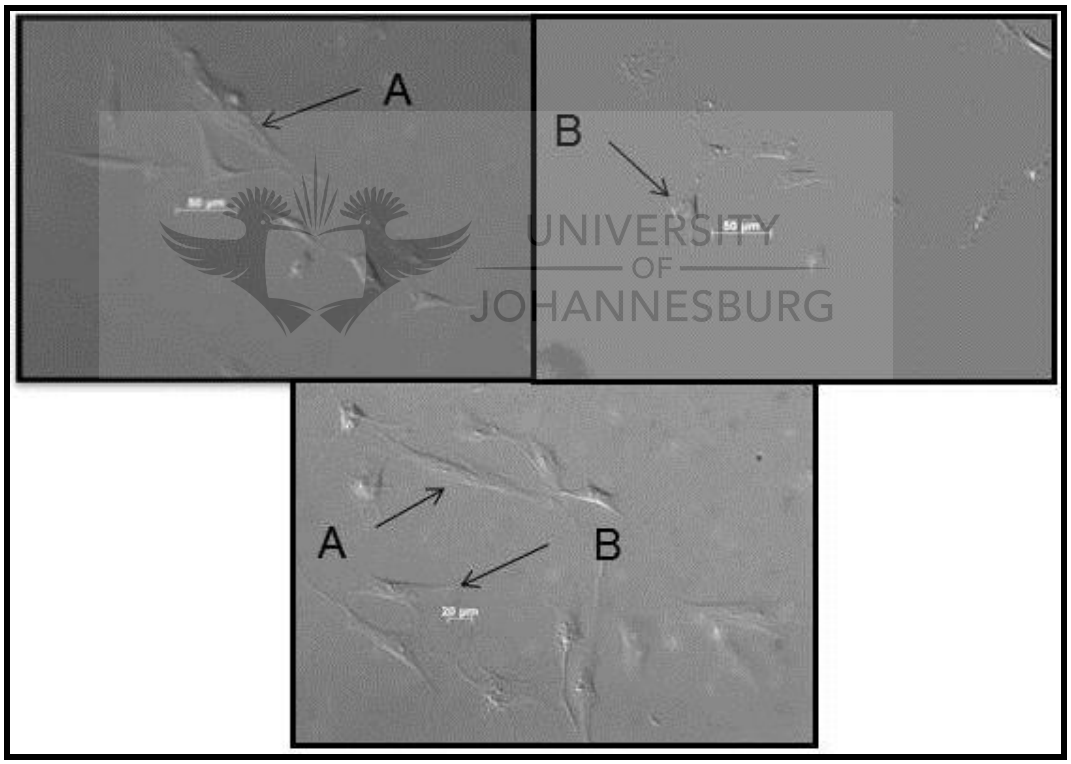

Figure 22. The morphology of co-cultured cells. Both ADSCs and SKUT-1 cells were co-cultured on heat sterilised coverslips in a small plate (3.4 cm^2). ADSCs are shown by the arrow with letter A and letter B shows the SKUT-1 cells. The micrographs were taken through differential interference contrast microscopy 24 h after co-culturing.

4.3 Cell Viability

Trypan blue and ATP luminescence assays were used to investigate the viability of the cells in the co-cultures at different time point post-irradiation.

The assays were done on six groups namely: co-cultured without the growth factors and not exposed to irradiation (CC), co-cultured without growth factors but exposed to irradiation (CC+LILI), co-cultured with RA but not exposed to irradiation (CC+RA), co-cultured with RA and exposed to irradiation (CC+RA+LILI), co-cultured with TGF-β1 and not exposed to irradiation (CC+TGF-β1) and co-cultured with TGF-β1 and exposed to LILI (CC+TGF-β1+LILI).

At 24 h post irradiation, there was a decrease in cell viability percentage in the groups that were grown in the presence of the growth factors as compared to the control group (CC) but the decrease was significant in CC+RA and CC+TGF-β1 groups (P<0.05). Groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI) showed increases in cell viability as compared to their respective control groups (CC, CC+RA and CC+TGF-β1). But the increase was significant in CC+TGF-β1+LILI group as compared to CC+TGF-β1 group (P<0.05). At 48 h post irradiation, the co-cultures that were grown in the presence of the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI) showed decreases in cell viability as compared to the control group (CC) and the decreases were significant in CC+RA and CC+TGF-β1 groups (P<0.05), however the groups that were irradiated showed increases in cell viability as compared to their respective controls and this was significant in CC+RA+LILI group as compared to CC+RA group (P<0.05). At 72 h after irradiation, the co-cultures that were grown in the presence of the growth factors showed decreases in viability as compared to those that had no growth factors but the significant decrease was seen in co-cultures of CC+RA group (P<0.05) as compared to the control group (CC). However all the co-cultured groups that were irradiated showed increases in cell viability as compared to their respective control groups and this was significantly observed in CC+RA+LILI group as compared to CC+RA group (P<0.05). Cell viability was also shown to decrease in the groups that were grown in the presence of the growth factors at 1 wk after irradiation and the decrease

was significant in CC+TGF-β1 group as compared to CC group (P<0.05). There were increases in viability in the groups that were irradiated as compared to the non-irradiated ones at this time post-irradiation though the increases were not significant (table 7).

Table 7. Trypan blue assay showing percentage viability of different groups of co-cultures cells at different time post irradiation. There was an increase in viability percentage of the co-cultured cells that were irradiated. The increase was more profound in the co-cultured cells that were not supplemented with the growth factors. The star* indicate significant decrease in viability of the co-cultures as compared to the control group (CC) where P<0.05 (*).

Time Post Irradiation	CC	CC+LILI	CC+RA	CC+RA+LILI	CC+TGF-β1	CC+TGF-β1+LILI
24 h	74.5±2.7	72.8±3.2	66.3*±2.4	68.2*±3.1	65.4*±2.9	71±2.4
48 h	77.3±2.0	80.8±3.1	70.5*±3.0	76.3±2.5	72*±2.1	75.3±3.0
72 h	75±2.8	78.5±3.3	70*±3.6	76.8±2.2	73±3.0	73.7±2.5
1 wk	72±3.0	74.1±2.6	67.9*±3.4	69.3±2.2	65.2*±2.1	70±2.8

ATP luminescence at 24 h after irradiation, showed significant decreases in the co-cultures that were supplemented with the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that were not (CC and CC+LILI). CC+RA and CC+TGF-β1 groups significantly decreased in viability as compared to CC group with P values of less than 0.001 and 0.0005 respectively. While those of CC+RA+LILI and CC+TGF-β1+LILI groups significantly decreased in viability as compared to CC+LILI group with P values of less than 0.001. There were increases in viability in co-cultures that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1 groups) as compared to their respective controls (CC, CC+RA and CC+TGF-β1 groups) however the increases were not significant (figure 23).

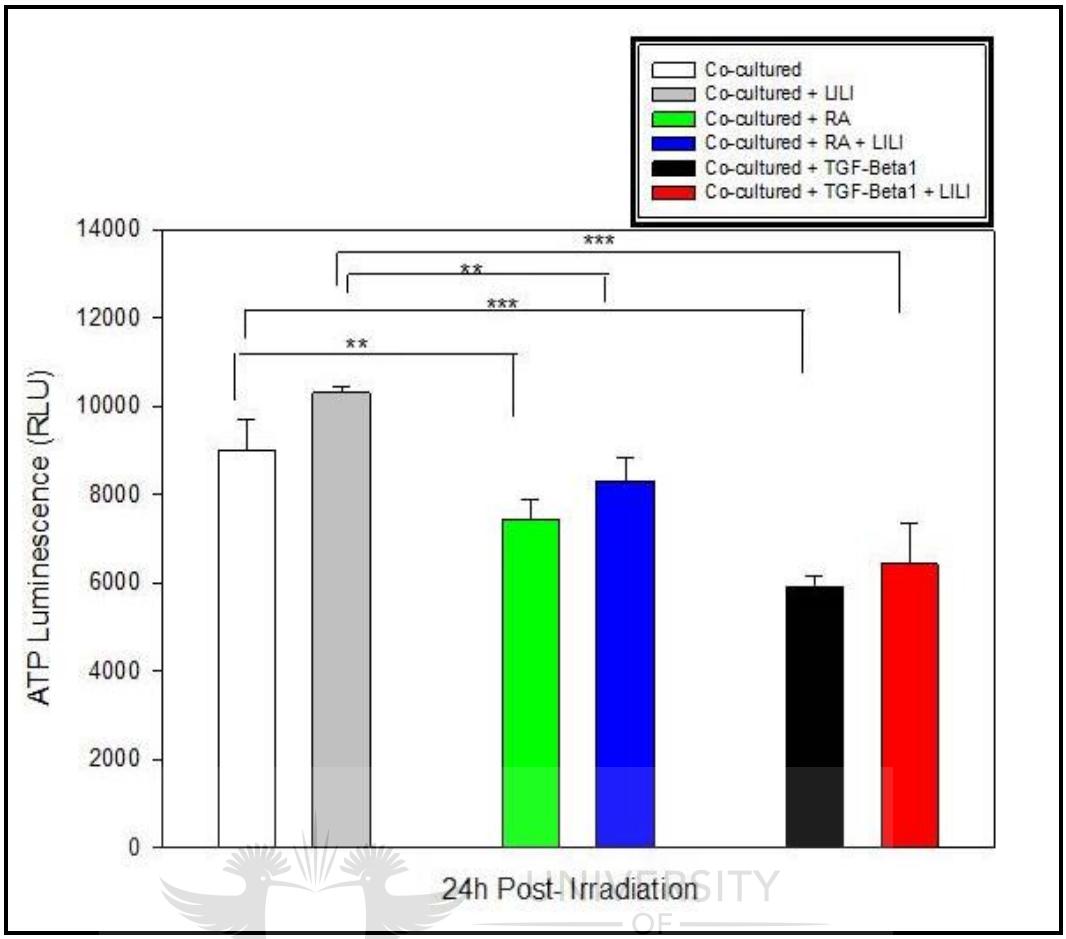

Figure 23. **ATP luminescence assay was used to determine cell viability of the co-cultured cells of the different six groups at 24 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were grown in the presence of the growth factors. ATP luminescence decreased in the groups that had growth factors however the groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI) increased in ATP luminescence as compared to their respective controls (CC, CC+RA and CC+TGF-β1) *P* value <0.05.**

At 48 h post irradiation, ATP luminescence decreased in the co-cultures that were supplemented with the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that did not have the growth factors (CC and CC+LILI groups). Co-cultures of CC+RA group had significant decreases as compared to CC group (P<0.05), while the co-cultures of CC+TGF-β1 group had significant decreases as compared to those of CC group (P<0.0005). The co-cultures of CC+RA+LILI and CC+TGF-β1+LILI groups were significantly decreased as compared to those of CC+LILI group (P<0.05). The groups that were

irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) showed increases in ATP luminescence as compared to those that were not irradiated (CC, CC+RA and CC+TGF-β1 groups) respectively, but the increases were significant in CC+TGF-β1+LILI group as compared to CC+TGF-β1 group (*P*<0.001) as seen in figure 24.

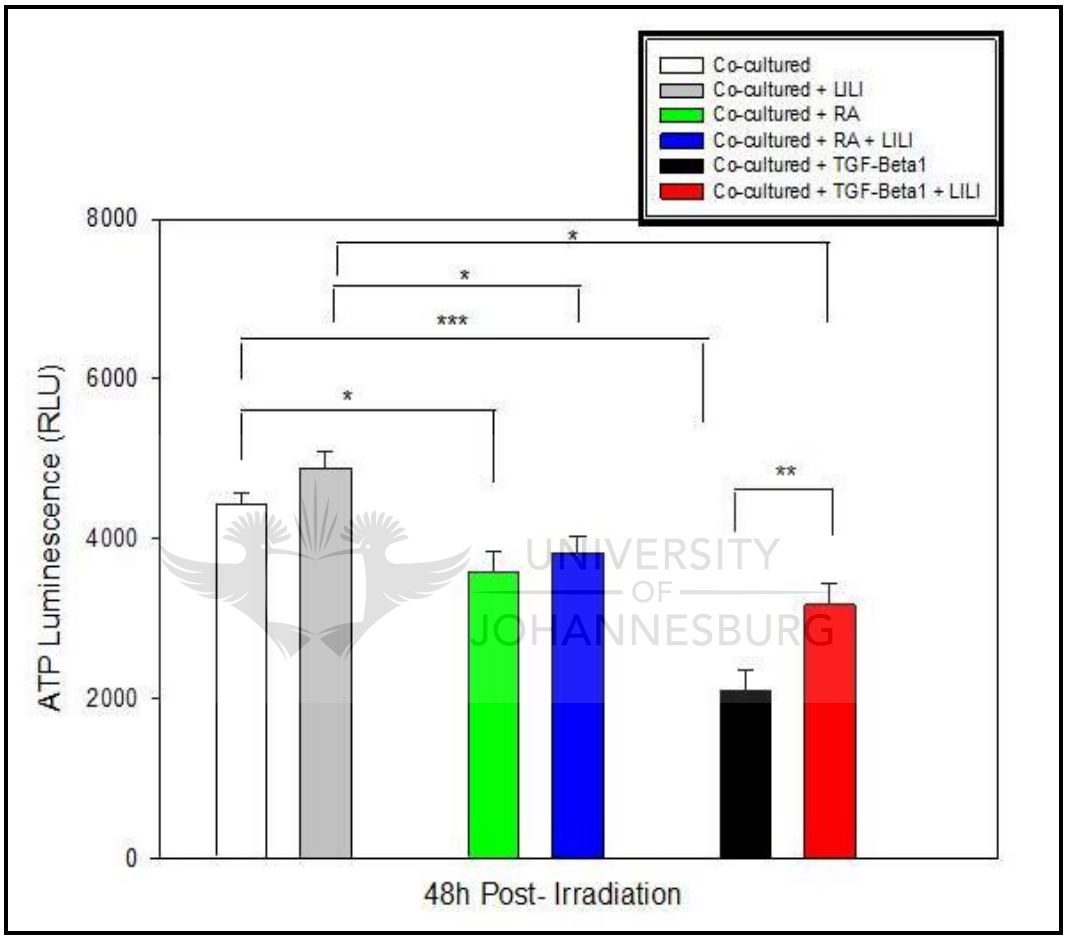

Figure 24. **ATP luminescence assay was used to determine cell viability of the co-cultured cells of the different six groups at 48 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were grown in the presence of the growth factors. ATP luminescence decreased in the groups that were grown in the presence of the growth factors however the groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILgroups) increased ATP luminescence as compared to their respective controls (CC, CC+RA and CC+TGF-β1) P value <0.05.**

ATP luminescence at 72 h post irradiation decreased in co-cultures that were supplemented with the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that

were not (CC and CC+LILI groups). CC+RA and CC+TGF-β1 groups had significant decreases as compared to CC group (P<0.0005) while CC+TGF-β1 and CC+TGF-β1 groups had significant decreases as compared to CC+LILI group (P<0.001). At this time point post irradiation, ATP luminescence increased in co-cultures that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) as compared to those that were not irradiated (CC, CC+RA, and CC+TGF-β1) respectively. The increase was significant in CC+RA+LILI group as compared to CC+RA group (P<0.001) and CC+TGF-β1+LILI group as compared to CC+TGF-β1 group with P values of less than 0.001 (figure 25).

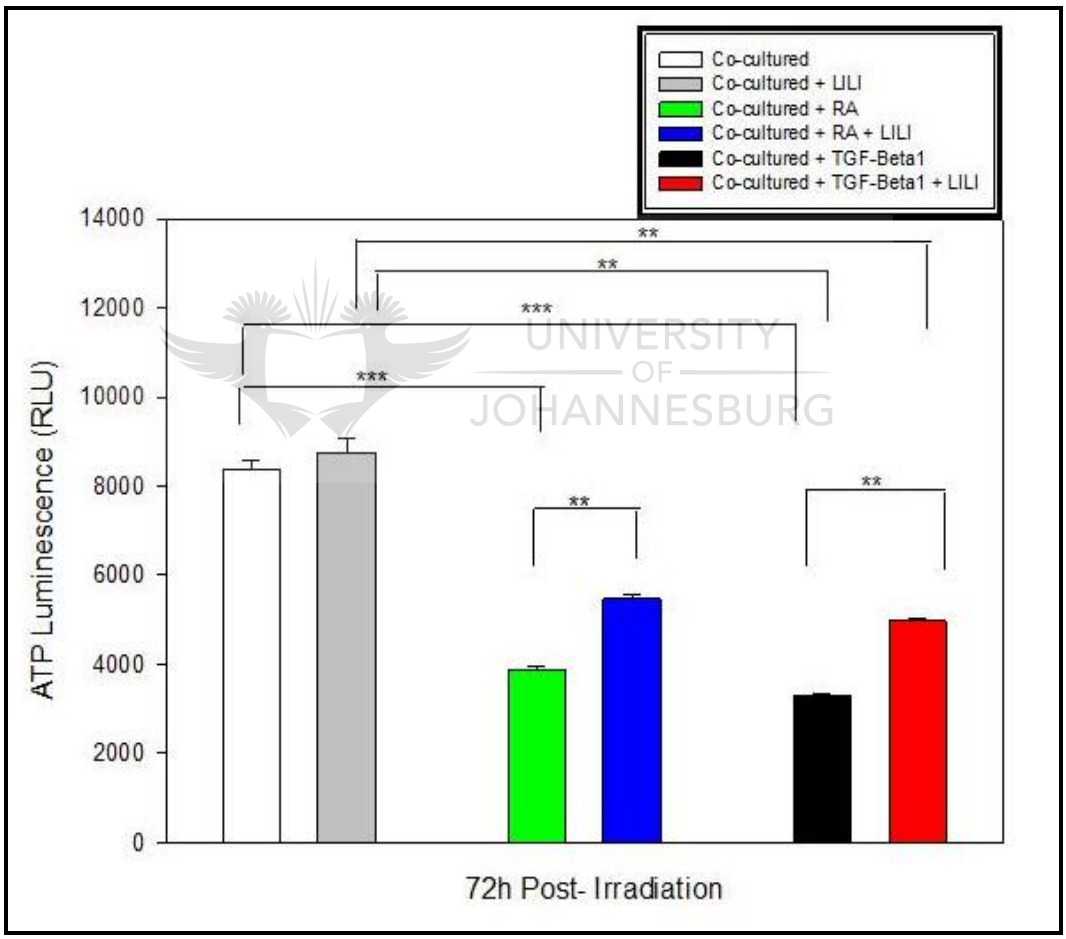

Figure 25. **ATP luminescence assay was used to determine cell viability of the co-cultured cells of the different six groups at 72 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were supplemented with growth factors. ATP luminescence decreased in the groups that had growth factors however the groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI) increased in ATP luminescence as compared to their respective controls (CC, CC+RA and CC+TGF-β1) *P* value <0.05.**

In the co-cultures incubated with growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups), ATP luminescence decreased at 1 wk post irradiation as compared to CC and CC+LILI groups, which did not have the growth factors. The decrease was significant in CC+RA and CC+TGF-β1 groups as compared to CC group (P<0.0005) while CC+RA+LILI and CC+TGF-β1+LILI groups decreased significantly as compared to CC group (P<0.001). The co-cultures which were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) showed increases in ATP luminescence as compared to those that were not irradiated (CC, CC+RA and CC+TGF-β1 groups) respectively and the increase was significant in the co-cultures of CC+TGF-β1+LILI group as compared to those of CC+TGF-β1 group (P<0.05) as seen in figure 26.

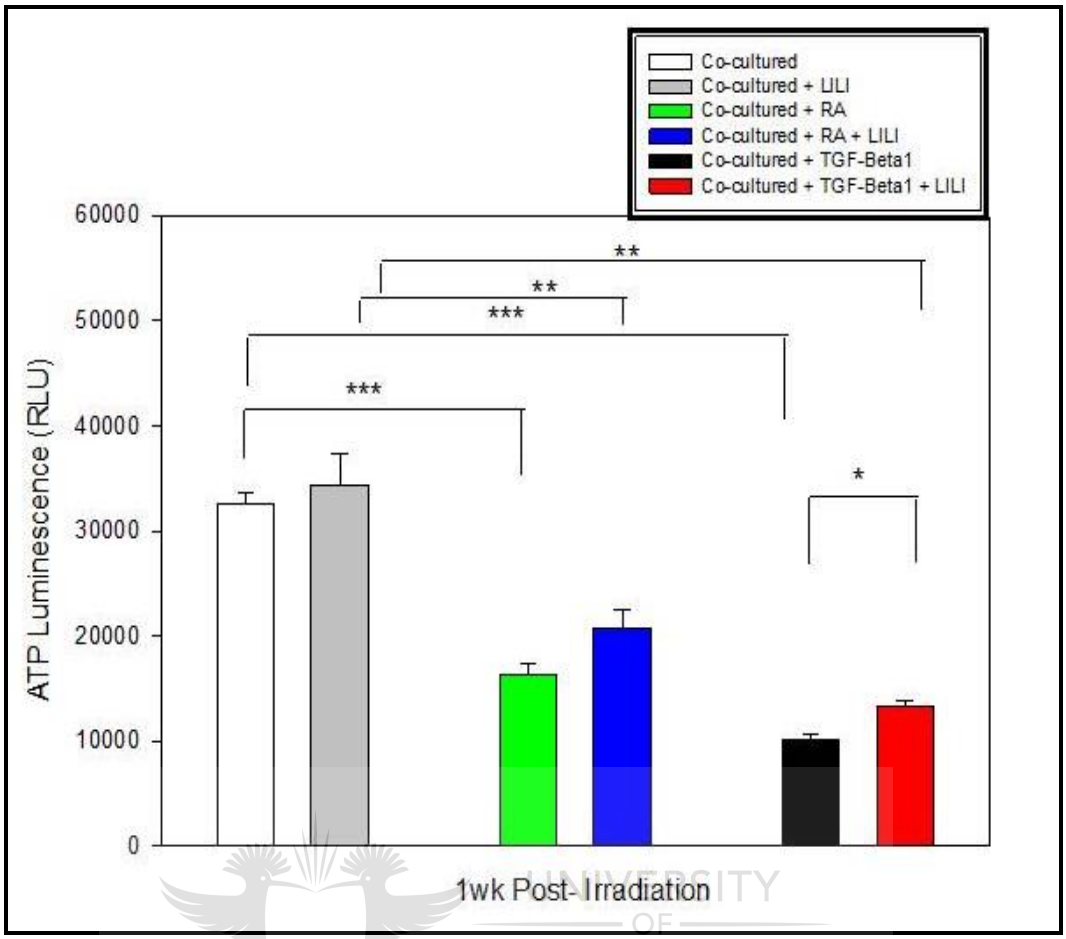

Figure 26. **Cell viability as assessed by ATP luminescence 1 week post-irradiation. There was a decrease in viability in groups that had growth factors as compared to those without growth factors with *P* value of less than 0.05. However the groups that were irradiated increased in cell viability as compared to those that were not irradiated (*P* value <0.05).**

4.4 Cell Proliferation

Cell proliferation at different time points (24 h, 48 h, 72 h and 1 wk) after irradiation showed significant changes in the co-cultures of different groups as analysed by optical density assay.

Optical density results at 24 h post irradiation showed decreased proliferation in the co-cultures that were grown in the presence of the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that had no growth factors (CC and CC+LILI groups). CC+RA and CC+TGF-β1 groups decreased significantly as compared to CC group with *P* values of less than 0.05 and 0.001

respectively while CC+RA+LILI and CC+TGF-β1+LILI groups decreased significantly as compared to CC+LILI group (*P*<0.05). There were increases in proliferation in groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) as compared to the groups that were not irradiated (CC, CC+RA and CC+TGF-β1 groups) respectively and this was significantly observed in CC+TGF-β1+LILI group as compared to CC+TGF-β1 group with *P* value of less than 0.05 (figure 27).

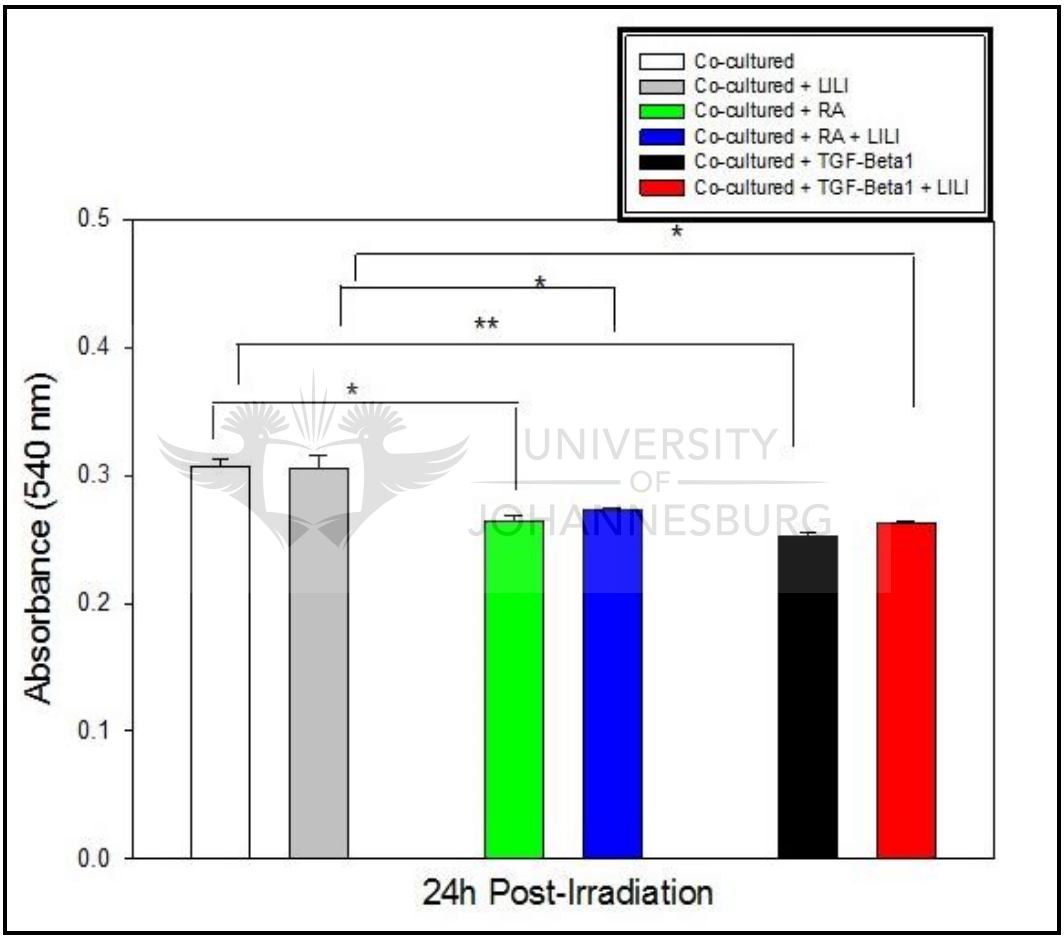

Figure 27. **Optical density assay was used to determine cell proliferation of the co-cultured cells of ADSCs and SKUT-1 of the different six groups at 24 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI were supplemented with growth factors. Cell proliferation decreased in the groups that had growth factors as compared with to those that did not have (*P*<0.05).**

At 48 h after irradiation, the co-cultures showed decreases in proliferation

in the groups that were supplemented with the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to CC and CC+LILI groups which were not. The decreases were significant in CC+TGF-β1 and CC+TGF-β1+LILI groups as compared to CC and CC+LILI group respectively with the *P* values of less than 0.001. The co-cultures that were irradiated (group 2, 4 and 6) increased in proliferation as compared to the non-irradiated ones (CC, CC+RA and CC+TGF-β1 groups) respectively but there were no significant increases observed as seen in figure 28.

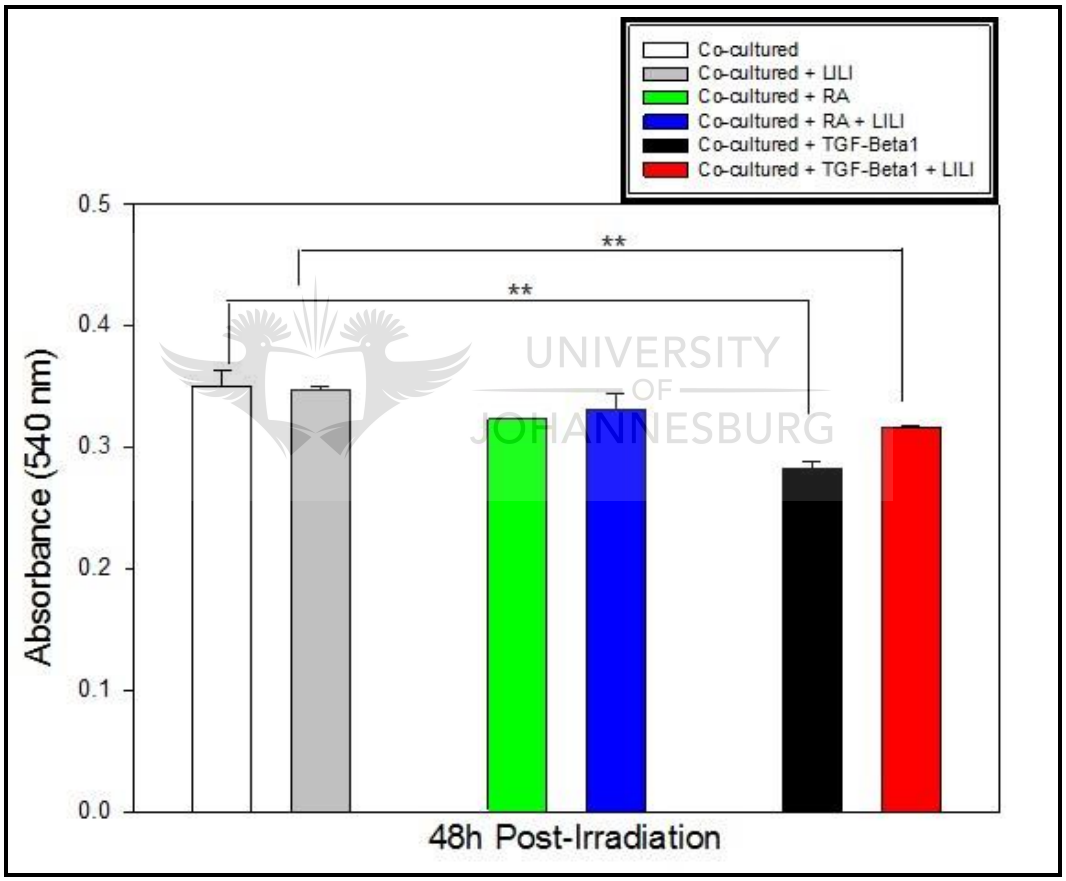

Figure 28. **Optical density assay was used to determine cell proliferation of the co-cultured cells of ADSCs and SKUT-1 of the different six groups at 48 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were supplemented with growth factors. Cell proliferation decreased in groups that were supplemented with growth factors. The decrease was significant in CC+TGF-β1 and CC+TGF-β1+LILI groups as compared to CC and CC+LILI groups respectively (*P*<0.05).**

Optical density results at 72 h after irradiation showed that the co-cultures decreased in proliferation in the groups that were grown in the presence of the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to CC and CC+LILI groups which had no growth factors and these decreases were significantly observed in CC+RA, CC+TGF-β1 and CC+TGF-β1+LILI groups as compared to their control groups (CC for CC+RA and CC+TGF-β1; CC+LILI for CC+RA+LILI and CC+TGF-β1+LILI) respectively (P<0.001). There were increases in proliferation in the co-cultures that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) as compared to those that were not irradiated (CC, CC+RA and CC+TGF-β1 groups) respectively and the increases were significantly observed in CC+RA+LILI and CC+TGF-β1+LILI groups as compared to CC+RA and CC+TGF-β1 groups respectively with P values of less than 0.05 (figure 29).

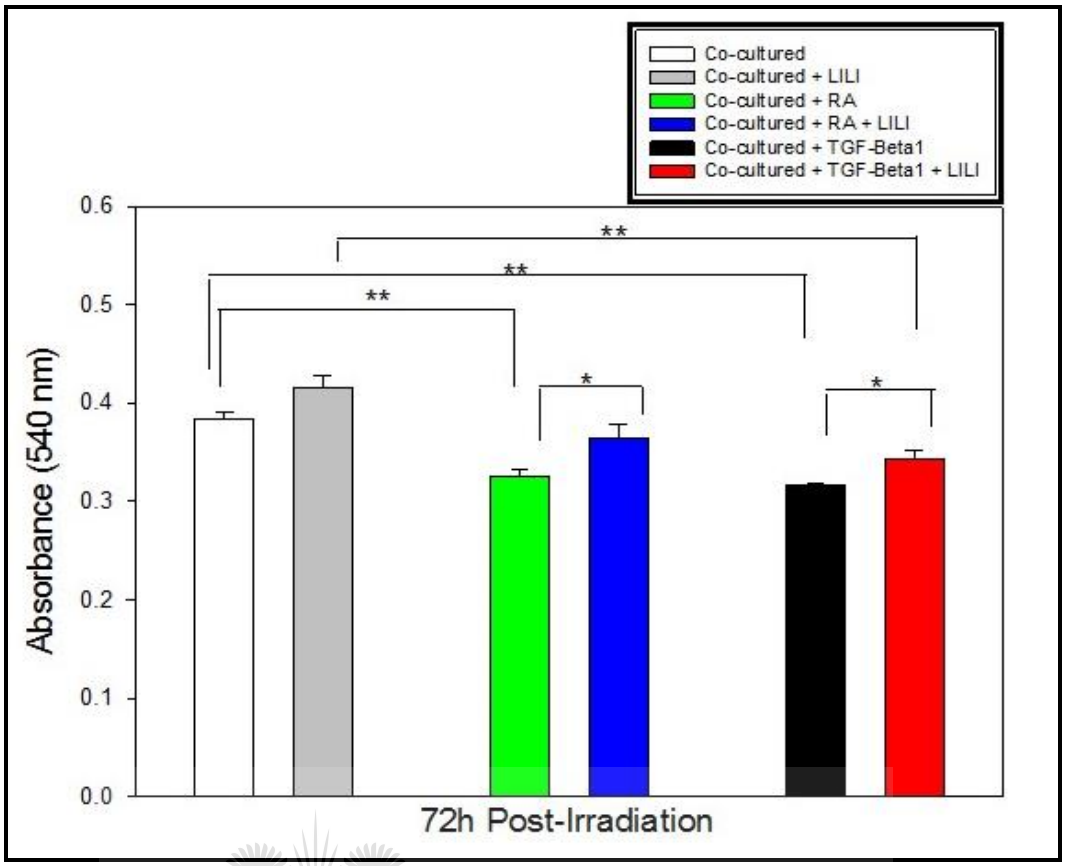

Figure 29. **Optical density assay was used to determine cell proliferation of the co-cultured cells of ADSCs and SKUT-1 of the different six groups at 72 h. CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups were irradiated at 636 nm with 5 J/cm^2. CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were supplemented with growth factors. Cell proliferation decreased in groups that were supplemented with growth factors as compared to those that were not (CC and CC+LILI groups) *P*<0.05. However, cell proliferation increased in the groups that were irradiated as compared to non-irradiation groups (P<0.05).**

Cell proliferation as analysed by optical density at 1 wk after irradiation decreased in the groups that were supplemented with the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that were not (CC and CC+LILI groups). CC+RA and CC+TGFβ1 groups significantly decreased as compared to CC group with *P* values of less than 0.05 while CC+RA+LILI and CC+TGF-β1 groups decreased significantly as compared to CC+LILI group (*P*<0.05). There were increases observed in proliferation in groups that were irradiated (CC+LILI, CC+RA+LILI and CC+TGF-β1+LILI groups) as compared to those that were not irradiated (CC+ CC+RA and CC+TGF-β1 groups)

respectively but the increases were not significant as seen in figure 30 below.

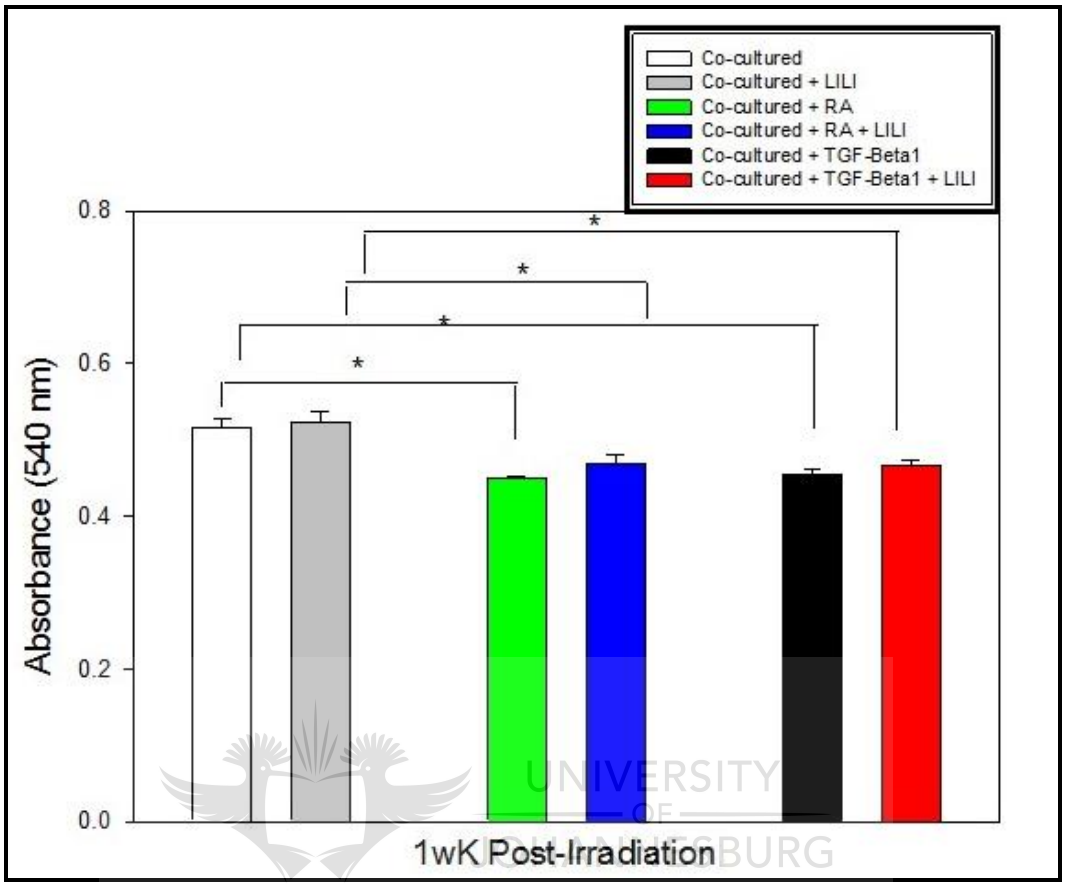

Figure 30. **Cell proliferation as assessed by OD 1 wk post-irradiation. There was a significant decrease in proliferation in the groups that were supplemented with the growth factors as compared to those that were not (*P*<0.05), however the proliferation increased in groups that were irradiated as compared to those that were not exposed to LILI.**

4.5 Expression of the Markers

Quantitative expression of the specific markers were determined through flow cytometry. CD29 (β1 Integrin) and CD90 (Thy-1), expressed by the ADSCs and Myosin heavy chain, expressed by SKUT-1 cells, were the markers used for the analysis. The results were expressed as mean percentage of events.

The expression of CD29 did not show much change at 24 and 48h post-irradiation in all the groups, however at 72h the expression of CD29 decreased in the groups that were grown in the presence of the growth

factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups) as compared to those that were not (CC and CC+LILI groups). The decrease was significant in CC+TGF-β1+LILI group as compared to CC group (P<0.05). The decrease in CD29 expression was also observed in the groups that were grown in the presence of the growth factors as compared to those that were not at 1 wk after irradiation. CC+RA, CC+RA+LILI and CC+TGF-β1 groups decreased in the expression of CD29 significantly with P values of less than 0.05 as compared to CC group. The marker further decreased in expression in C+TGF-β1+LILI group as compared to CC group (P<0.001). At all time points after irradiation, there were increases in expression of the marker in CC+LILI group (groups which were irradiated) as compared to CC group (not irradiated) though the increases were not significant as seen in table 8.

Flow cytometry determination of CD90 expression in the co-cultures showed an increase in the expression in all the co-cultures of CC+LILI group (irradiated) as compared to those of CC group (not irradiated) at all time points. However, there were decreases in the expression of the marker in groups that were grown in the presence of the growth factors (CC+RA, CC+RA+LILI and CC+TGF-β1 groups) as compared to the groups that were not (CC and CC+LILI groups). At 72 h post irradiation, the decreases were significant in CC+TGF-β1 and CC+TGF-β1+LILI groups as compared to CC group with P values less than 0.05 and 0.001 respectively. At 1 wk post irradiation, the decreases were significant in CC+RA, CC+TGF-β1 and CC+TGF-β1+LILI groups as compared to CC group with P values of less than 0.05, 0.001 and 0.0005 respectively (table 8).

MHC expression through flow cytometry showed increases in the group that was irradiated (CC+LILI group) as compared to the group that was not (CC+LILI group) at all time points though the increases were not significant. The increase was more at 1 wk. MHC expression increased in

the groups that were grown in the presence of the growth factors (CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI) at 72 h and 1 wk and the increase was significant at 1 wk in CC+TGF-β1+LILI group as compared to CC group (P<0.05) as seen in table 8.

Table 8. **Flow cytometry results showing the expression of the markers indicated as the mean percentage of events. There was a significant decrease in the expression of ADSCs markers after 1 wk in the co-cultures that were grown in the presence of the growth factors (P<0.05), however the expression of the SMC marker showed an increase over the same period of time (1 wk). The stars* indicate significant decreases in the expression of the ADSC markers (CD29 and CD90) as compared to their controls (CC) while the∞ sign indicates an increase in the expression of the MHC marker as compared to the control (CC) where P<0.05 (*), P<0.001 (**) and P<0.005 (***).**

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Time Post Irradiation	CC	CC+LILI	CC+RA	CC+RA+LILI	CC+TGF-β1	CC+TGF-β1+LILI
CD29 (β1 Integrin)						
24 h	58.93±2.1	59.70±3.2	59.70±2.1	61.00±2.0	69.40±1.8	53.18±4.0
48 h	56.93±2.3	58.75±2.0	52.50±2.6	55.50±2.5	55.00±2.0	56.30±3.2
72 h	49.25±3.5	50.50±2.3	42.75±3.5	41.25±3.0	42.50±2.0	38.00*±3.3
1 wk	55.46±3.1	56.45±1.9	39.50*±2.2	38.00*±2.2	37.25*±2.1	35.25**±2.4
CD90 (Thy1)						
24 h	56.23±2.0	61.75±2.2	52.85±1.8	56.00±2.5	54.05±3.0	55.10±2.2
48 h	49.18±2.8	52.25±3.2	47.05±2.1	48.25±2.7	46.25±3.4	47.00±3.4
72 h	43.00±1.9	45.75±3.4	39.75±3.1	35.75±2.0	31.25*±2.0	24.50**±3.4
1 wk	53.00±3.0	56.00±4.0	39.00*±3.2	31.25±3.6	31.00**±1.9	26.00***±3.3
Heavy Myosin Chain						
24 h	71.70±2.0	73.25±1.9	69.18±2.1	68.63±2.2	65.73±1.8	67.85±2.3
48 h	52.50±2.2	55.25±3.7	43.00±3.3	48.50±3.6	55.25±3.4	58.25±3.1
72 h	72.53±3.2	78.50±2.3	76.38±3.2	81.20±2.5	73.23±2.0	80.55±3.0
1 wk	74.50±3.4	79.75±3.1	73.00±3.0	82.60±3.8	79.50±3.4	87.20∞±3.2

The two types of cells were identified 24 h after co-culturing them through flow cytometry. Skut-1 cells were first stained with CFDSE 72 h before co-culturing. Flow cytometry showed two distinct population of cells (figure

31A). However after 1 wk, flow cytometry could not distinguish the populations of the two types of cells in a co-culture as seen in figure 31B.

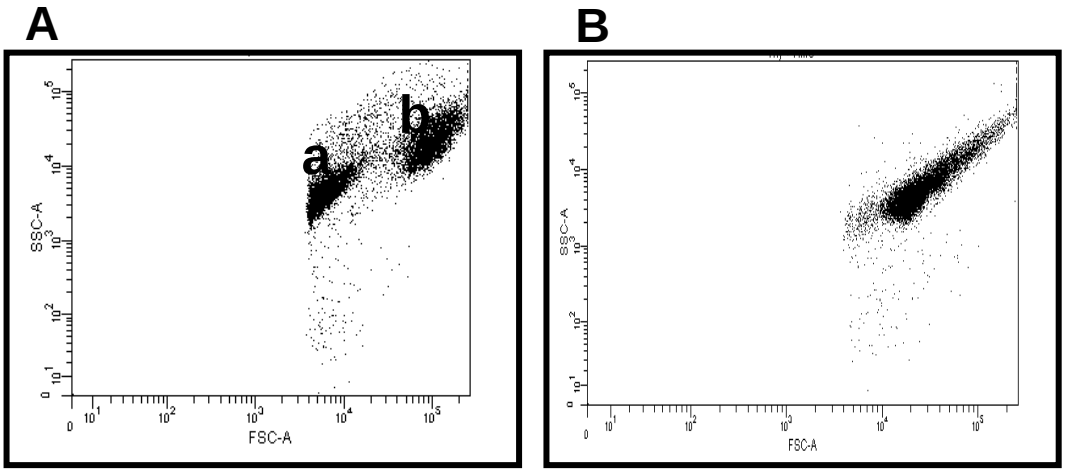

Figure 31. **The flow cytometry results showing the two distinct type of cells in a co-culture 24 h after co-culturing in a ratio of 1:1 as seen in A. SKUT-1 (a) and ADSCs (b). At 1 wk post-irradiation and co-culturing, flow cytometry results showed no two distinct populations of the cells (B).**

4.6 RT PCR Microarrays

PCR microarrays analysis for the human mesenchymal stem array was done on four groups (CC, CC+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups). CC group was used as a control. CC+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups were analysed at both 24 h and 1 wk post irradiation. The results of CC+TGF-β1+LILI group at 24 h were not obtained due to malfunction of the machine. The *P* values of all the genes in the array at both time post irradiation in all the groups are listed in the appendix F.

At 24 h post irradiation, most genes were up-regulated in the co-cultures that were irradiated. Few genes were down-regulated and amongst the few was catenin beta1 (CTNNB1). The down-regulation was significant with *P* value of less than 0.005. All the genes that we were interested in were all up-regulated. CD29, CD90 and SMAD family member 4 (SMAD4) were not significantly up-regulated while TGF-β1, TGF-β111 and bone morphogenetic protein (BMP 2,4,6 and 7) had *P* values of less than 0.05 (figure 32).

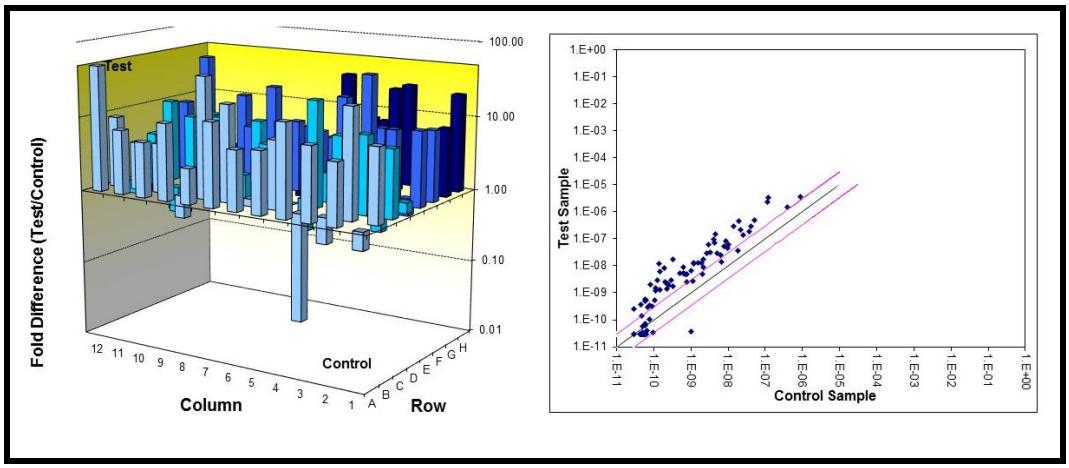

Figure 32. **PCR microarray assay showing up and down-regulation of different genes of the human mesenchymal stem cell array in the co-cultures that were irradiated only at 24 h post irradiation. Most genes were up-regulated however CTNNB1 was significantly down-regulated (*P*<0.05).**

In the co-cultures that were added with TGF-β1 and analysed at 24 h post irradiation, CTNNB1 gene was significantly down-regulated having *P* value of less than 0.005. CD29, CD90, SMAD4, BMP, TGF-β1 and TGF-β111 were all up-regulated though the up-regulations were not significant except for TGF-β1 and TGF-β111 with *P* values of less than 0.05 (figure 33).

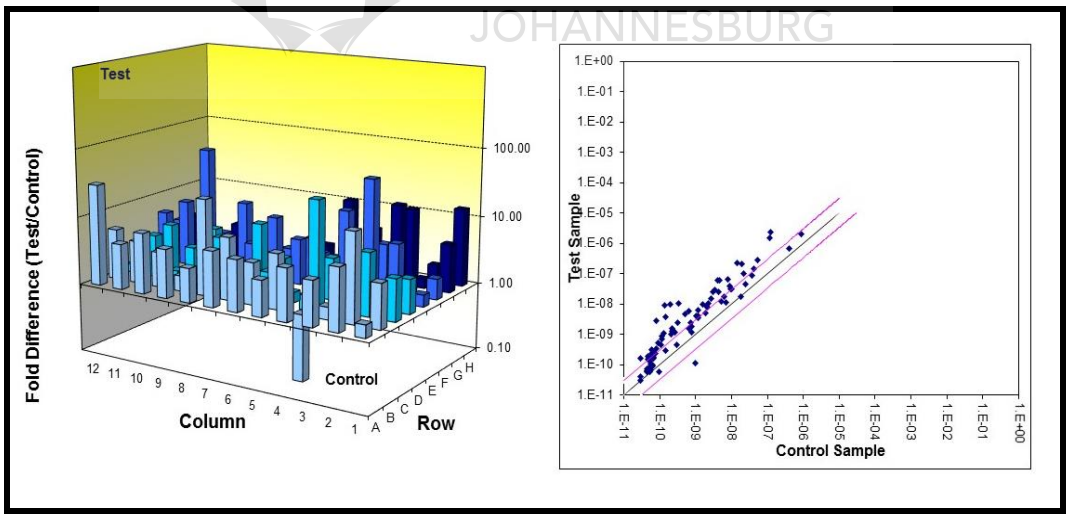

Figure 33. **PCR microarray assay showing up and down-regulation of different genes in the human mesenchymal stem cell array in the co-cultures that were added only with TGF-β1 only at 24 h post irradiation. The genes at this time point were mostly up-regulated except for the gene CTNNB1 which was significantly down-regulated (*P*<0.05).**

At I wk after irradiation, in the co-cultures that were irradiated, most genes were down-regulated significantly including CD29, CD90, SMAD4, BMP and TGF-β1 and 111. However vimentin (VIM), beta-2- macroglobulin (B2M) and actin beta (ACTB) were significantly up-regulated with *P* values of less than 0.05 (figure 34).

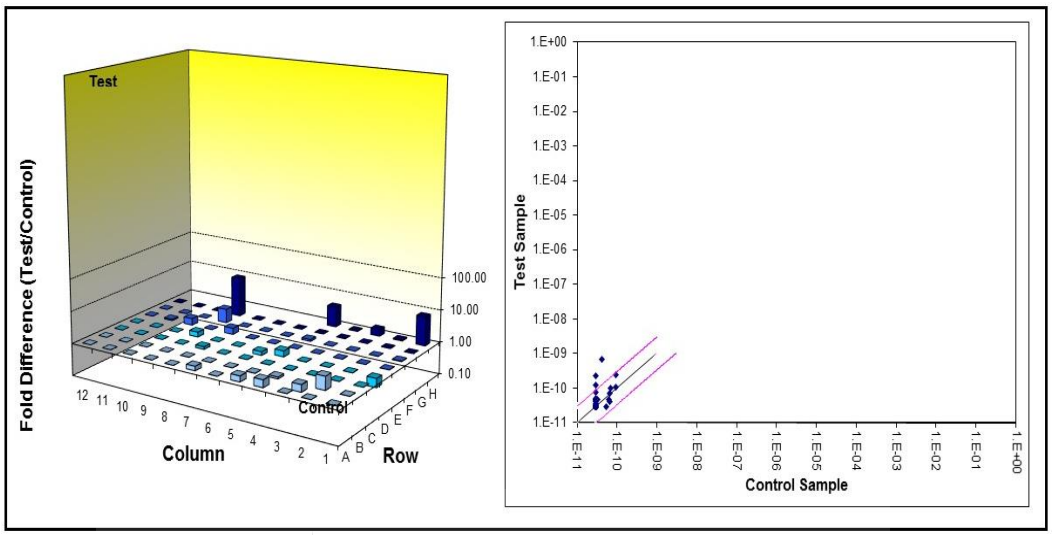

Figure 34. **PCR microarray assay showing up and down-regulation of different genes of the human mesenchymal stem cell array in the co-cultures that were irradiated only at 1 wk post irradiation. VIM, B2M and ACTB were up-regulated, however, most genes were not expressed at all.**

In the co-cultures that were added with TGF-β1, 1 wk post irradiation, vascular endothelial growth factor A (VEGFA), zinc finger protein 42 homolog (ZFP42), POU class 5 homeobox 1, collagen type 1 alpha (COL1A1) and fibroblast growth factor 10 were significantly up-regulated with *P* values of less than 0.05. However CTNNB1, integrin alpha 6 (CD49F), integrin alpha V (CD51) and peroxisome proliferation activated receptor gamma (PPARG) were all significantly down-regulated with *P* values of less than 0.05 (figure 35).

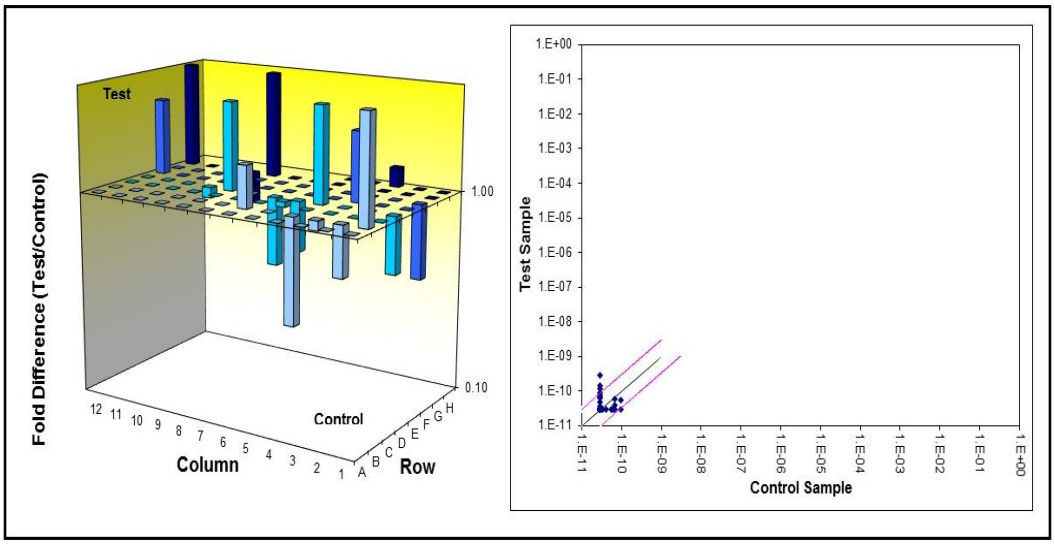

Figure 35. **PCR microarray assay showing up and down-regulation of different genes of the human mesenchymal stem cell array in the co-cultures that were added with TGF-β1 only at 1 wk post irradiation.**

There were no genes in the co-cultures that were added with TGF-β1 and were irradiated and analysed at 1 wk after irradiation that showed significant up-regulation and down-regulation as seen in figure 36.

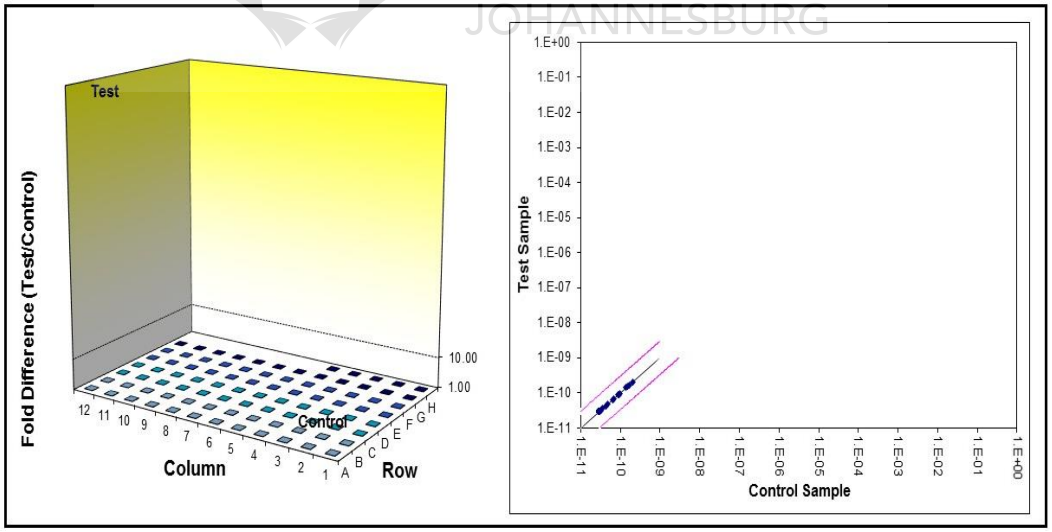

Figure 36. **PCR microarray assay showing up and down-regulation of different genes in the human mesenchymal stem cell array in the co-cultures that were irradiated and added with TGF-β1 at 1 wk post irradiation. There was no gene which was up-regulated or down-regulated.**

The up and down regulations of the genes in the mesenchymal stem cell array are summarised in the table below with *P* and fold change values (table 9).

Table 9. Gene expression profiling. *P*/fold change values at different time points for different groups. Fold change values of above 1.00 signifies up-regulation and less than 1.00 down-regulation of genes, X means no gene expression.

Genes	24 h CC + LILI	24 h CC+TGF-β1	1 wk CC + LILI	1 wk CC+TGF-β1	1 wk CC+TGF-β1 +LILI
ABCB1	0.373/0.58	0.668/1.53	X	X	X
ALCAM	0.018/6.60	0.008/7.89	X	X	X
ANPEP	0.002/9.54	0.067/4.49	X	X	X
ANXA5	0.004/17.03	0.285/5.72	0.116/1.69	X	X
BDNF	0.005/6.85	0.137/3.34	0.116/1.50	X	1.000/1.00
BGLAP	0.001/6.35	0.001/5.60	X	X	X
BMP2	0.023/13.24	0.317/6.42	0.373/1.37	X	X
BMP4	0.065/3.00	0.056/3.16	X	X	X
BMP6	0.057/10.68	0.523/5.16	X	X	1.000/1.00
BMP7	0.318/5.50	0.111/7.66	X	X	X
CASP3	0.162/7.29	0.540/4.63	X	X	X
CD44	0.001/51.15	0.001/30.20	X	X	X
COLIA1	0.011/9.62	0.256/4.38	X	0.001/4.10	X
CSF2	0.001/27.73	0.002/19.25	0.017/2.56	0.149/0.57	1.000/1.00
CSF3	0.282/0.45	0.735/1.51	0.297/0.60	0.520/0.90	1.000/1.00
CTNNB1	0.333/0.04	0.744/0.11	0.668/1.14	0.019/0.31	1.000/1.00
EGF	0.027/8.09	0.085/6.31	X	X	X
ENG	0.729/1.25	0.127/4.07	X	0.373/1.61	X
ERBB2	0.003/19.65	0.142/8.15	X	X	X
FGF10	0.002/42.22	0.020/25.16	X	X	1.000/1.00
FGF2	0.373/0.50	0.718/1.68	X	X	X
FUT1	0.006/4.57	0.037/3.25	X	X	X
FUT4	0.532/4.33	0.559/4.13	X	X	1.000/1.00
FZD9	0.433/ 8.88	0.001/5.46	X	X	X
GDF15	0.001/7.85	0.017/4.01	X	X	X
GDF5	0.292/10.79	0.559/8.00	X	X	X
GDF6	0.002/9.51	0.998/1.38	X	X	X
GDF7	0.001/24.76	0.001/33.36	X	X	X

GTF3A	0.210/1.00	0.116/1.41	X	0.373/1.06	X
HAT1	0.001/5.64	0.101/2.37	X	X	X
HDAC1	0.015/2.01	0.589/0.91	X	X	X
HDF	0.009/10.85	0.136/5.72	0.373/1.21	X	1.000/1.00
HNF1A	0.748/1.13	0.852/1.12	X	X	X
ICAM1	0.373/0.46	0.947/1.39	X	X	X
IFNG	0.270/5.00	0.424/4.14	X	X	X
IGF1	0.373/0.68	0.978/1.32	X	X	X
IL10	0.805/1.38	0.311/3.12	0.116/0.53	0.116/0.53	X
IL1B	0.373/0.51	0.891/1.16	X	X	X
IL6	0.274/4.28	0.791/2.54	X	X	X
INS	0.025/2.45	0.002/4.03	X	0.061/2.96	1.000/1.00
ITGA6	0.371/0.38	0.461/0.60	0.081/1.51	0.004/0.57	1.000/1.00
ITGAV	0.001/9.85	0.436/3.02	0.060/0.77	0.003/0.47	1.000/1.00
ITGAX	0.003/9.19	0.004/8.46	X	X	X
ITGB1	0.373/0.56	0.788/1.51	X	0.115/2.73	X
JAG1	0.004/9.67	0.053/5.53	0.441/1.42	0.356/0.89	X
KDR	0.004/8.26	0.515/2.51	X	X	X
KITLG	0.028/12.41	0.549/4.90	X	X	X
LIF	0.429/0.63	0.694/2.44	X	X	X
MCAM	0.001/10.01	0.789/1.48	X	X	X
MITF	0.105/9.49	0.295/6.87	X	X	X
MMP2	0.001/45.05	0.001/50.80	X	0.116/2.19	X
NES	0.001/21.51	0.002/15.82	X	X	X
NGFR	0.010/6.93	0.217/3.37	X	X	X
NOTCH1	0.005/8.67	0.102/4.66	X	X	X
NT5E	0.011/23.43	0.399/8.63	X	X	X
NUDT6	0.014/6.25	0.271/3.06	0.373/1.55	X	X
KAT2B	0.024/3.21	0.680/1.47	X	X	1.000/1.00
PDGFRB	0.001/87.43	0.001/63.70	0.058/1.61	X	X
PIGS	0.209/10.80	0.330/9.17	0.373/1.09	X	1.000/1.00
POU5F1	0.975/3.52	0.394/5.55	X	0.022/2.37	X
PPARG	0.012/8.90	0.933/1.99	0.369/1.04	0.001/0.44	X
PROM1	0.373/0.59	0.776/1.40	X	X	X
PTK2	0.026/8.02	0.197/4.79	X	X	X
PTPRC	0.373/0.60	0.813/1.34	X	X	X
RHOA	0.024/5.02	0.470/2.31	0.373/1.13	X	X
RUNX2	0.002/6.48	0.023/3.94	X	X	X

SLC17A5	0.006/6.32	0.364/2.40	X	X	X
SMAD4	0.228/4.17	0.321/3.72	X	X	X
SMURF1	0.006/13.90	0.035/9.23	0.116/2.68	X	X
SMURF2	0.125/4.02	0.388/2.84	X	X	X
SOX2	0.379/0.66	0.454/1.60	X	X	X
SOX9	0.865/2.32	0.149/3.51	X	X	X
TBX5	0.007/8.15	0.063/5.05	X	X	X
TERT	0.373/0.66	0.676/1.36	X	X	X
TGFB1	0.002/23.26	0.016/14.83	X	X	X
TGFB3	0.038/8.88	0.200/5.70	X	X	X
THY1	0.001/31.85	0.068/13.55	X	X	X
TNF	0.373/0.57	0.264/2.54	X	X	X
VCAM1	0.373/0.54	0.485/1.92	X	X	X
VEGFA	0.179/1.99	0.389/1.66	X	0.023/9.76	X
VIM	0.029/4.19	0.329/2.34	0.049/16.34	0.373/0.71	1.000/1.00
VWF	0.421/0.67	0.164/3.10	X	X	X
WNT3A	0.352/1.00	0.453/1.00	X	X	X
ZFP42	0.621/1.05	0.257/3.40	X	0.001/5.03	X

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CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

ADSCs when cultured in the flasks formed multilayers and that adhered to the surface of the flasks and appeared like fibroblast-like cells when stained with CFDSE and observed through fluorescence microscopy and also when viewed in the flasks with inverted light microscopy. This is in constistent with Mvula *et al*., 2008 who also isolated and studied the effects of LILI on ADSCs. SMCs (SKUT-1) displayed also fibroblast-like cell appearance that adhered to the surface of the flasks but when viewed directly under a light inverted microscope and also when stained with CFDSE and viewed under a fluorescence microscope. This result agrees with studies done by Steynberg and colleagues in 2012 who also cultured SKUT-1 cells to assess qualitatively smooth muscle cells propagated on 2D and 3D polycarprolactone polymers through scanning electron microscopy. ADSCs appeared to be more elongated than SKUT-1 in a co-culture monolayer through a differential contrast microscopy. In their recent studies on ADSCs, Hu and colleagues (2013), found out that most primary ADSCs attached to the surface of the plates and showed a fibroblast-like or spindle-like shapes with stretched out pseudopods within 24h and proliferated rapidly within 5 – 7 days. This agrees with the results of this study.

ADSCs and SMCs were cultured separately and characterised using their distinctive markers. ADSCs expressed the cell surface markers (CD29 and CD90) and SMCs expressed MHC marker. This agrees with previous studies on characterisation of ADSCs and SMCs (Mitchell *et al*., 2006; de Villiers *et al*., 2011 and Yoshimira *et al*., 2006). Zahran and colleagues in 2012 in their studies on isolation and characterisation of ADSCs, found that these cells expressed mesenchymal markers (CD29, CD90) and not haematopoetic marker (CD45). This finding agrees with the one of the criteria put forward by the Mesenchymal and Tissue Stem Cell Committee

in identifying mesenchymal stem cells (Dominici *et al*., 2006) which says that a cell is classified as a mesenchymal if it expresses markers that include CD90 (Zahran *et al*., 2012). MHC was found to be expressed by the SMCs. Studies have found out that MHC isoforms are vital markers in studying human vascular smooth muscle cell differentiation and other cellular mechanisms in atherosclerosis (Aikawa *et al*., 1993). MHC gene encodes a protein that expresses the smooth muscle cell lineage (Madsen *et al*., 1998). In 1994 studies done by Miano and colleagues on embryonic development in mouse found that MHC was a highly specific marker for SMCs.

There are many co-culturing methods that are being applied for many purposes including differentiation and proliferation. In the past ten years studies have shown that endothelial cells could differentiate into osteoblasts and this had been influenced by direct contact of these two cells (endothelial cells and osteoblasts) and not necessarily due to paracrine effects (Grellier *et al*., 2009). The main problem with direct co-culture is to differentiate between the two types of cells in a mixture if they have similar phenotypic appearances. The present study carried out a direct co-culturing method for the ADSCs and SMCs which had similar morphological appearances.

After co-culturing for 1 wk, flow cytometry results could not show two distinct population of the two types of the cells. This could be due to the fact that one population of cells (ADSCs) may have been differentiating into other (SMCs). Direct co-culturing has proved to increase differentiation of cells due to the differentiation and growth factors secreted by the cells to be differentiated into. In the study done by Zuo *et al* (2013) bone marrow mesenchymal stem cells differentiated into chondrocytes in a direct co-culture system however static co-culture system has resulted in decreased viability and proliferation over a long period of time (Toogood *et al*., 1980). The viability and proliferation results in this study agree with

what scientists have demonstrated in recent years. LILI increased cellular growth after culturing cells under conditions of less nutrients (Eduardo *et al*., 2008). It has increased cellular viability, proliferation, collagen production and the release of growth factors in ADSCs and other cells (Mvula *et al*., 2008 and Eells *et al*., 2004). In this study, the co-cultures that were irradiated increased in viability and proliferation as compared to those that were not exposed to LILI. But the co-cultures with added growth factors had decreased viability and proliferation. This is due to the fact that at higher cell density, cell proliferation is inhibited while differentiation is promoted (Zhang *et al*., 2010).

Growth factors such as RA and TGF-β play vital roles in many cells including ADSCs and SMCs. RA has had many effects on cells including apoptosis, proliferation, differentiation as well as maturation (Duong and Rochette-Egly, 2011 and Gudas and Wagner, 2011) while TGF-β1 plays vital roles in migration, angiogenesis, proliferation, differentiation, embryonic development and metastasis (Zhang *et al*; 2012; Rahimi and Leof, 2007). Proliferation results of this study showed that addition of growth factors resulted in the decrease of the co-cultures at all time points after irradiation. This could be explained in the context that when cells are differentiating, proliferation is stalled in the G1 phase of the cell cycle. There has been neural differentiation from embryonic stem cells promoted by RA. This growth factor was found to have inhibitory effects on fibroblast growth factor signalling which prevented self-renewal of stem cells and increased their differentiation into neurons (Stavridis *et al*., 2010). In another study done by Tong and Andrews, RA was found to be one of the mediators for neuronal differentiation from human pluripotent stem cell lines after a long exposure and intercellular communication (Tong and Andres, 2010). Studies have shown that TGF-β can proliferate or inhibit growth of cultured SMCs (Topouzis and Majesky, 1996) and TGF-β1 has been shown to promote differentiation of stem cells into SMCs (Stowers *et al*., 2013). TGF-β3 was found to promote differentiation of ADSCs into

cartilage (Hashemibeni *et al*., 2008). In this study, the addition of growth factors decreased viability and proliferation of the co-cultures. This could be explained by the fact that after a period of 1 wk in culture, the cells may have overgrown and therefore decreased in viability and proliferation.

LILI, a form of phototherapy has been shown to produce a number of effects on cells. The results of this study indicate that the groups which were exposed to low intensity laser did have different results other than those that were not irradiated. Cell viability showed increases in all groups that were irradiated as compared to those that were not exposed to irradiation at all time points. This result is in agreement with Mvula *et al*., 2008, who found that ADSCs increased in cell viability after being exposed to LILI. Cell proliferation through optical density analysis also showed increases in all the groups that were exposed to laser as compared to their control groups. This is also in agreement with studies done by Mvula *et al*., 2010. Their study showed increased cell proliferation in groups that were irradiated at 636 nm with 5 J/cm^2 using diode laser. There have been a lot of studies LILI and stem cells. Studies on LILI and stem cells have shown that low intensity lasers can alter the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration (Gasparyan *et al*., 2004). It has been shown that 5 J/cm^2 of laser irradiation at a wavelength of 635 nm positively affects ADSCs by increasing cellular proliferation, viability, and expression of β1-Integrin (Mvula *et al*., 2008) and Thy-1, and LILI in combination with EGF enhanced the proliferation of ADSCs (Mvula *et al*., 2010). Low intensity laser improves the growth of stem cells when cultured under conditions of less nutrients (Eduardo *et al*., 2008). Studies carried out on rats revealed the promoting of recovery of atrophied gastrocnemius skeletal muscles when subjected to LILI (Nakano *et al*., 2009). Using diode LILI, scientists have reduced lipopolysaccharide-induced bone marrow inflammation (Huang *et al*., 2012) and this low intensity laser has also been discovered to facilitate fibronectin and collagen type 1 turnover during tooth

movement (Kim *et al*., 2010). Studies done by Roland *et al* in 2013 found that LILI stimulated the transcription of genes involved in the chain of electron transport at a wavelength of 660 nm at 5 J/cm^2. The genes involved in the metabolism of energy and oxidative phosphorylation were up-regulated thereby stimulating ATP production increase. Periodontal ligament cells were found to increase in proliferation when exposed to a wavelength of 670 nm laser diode with an output of 500 mW at an energy density of 5 and 10 J/cm^2 and this resulted in decreased inflammation (Huang *et al*., 2012). Recent studies done by Soleimani *et al*., 2012, found that LILI promoted proliferation of human bone marrow stem cells and their differentiation into neurons and osteoblasts though this was dosage dependent. They found out that at a wavelength of 810 nm and a range of 2-6 J/cm^2, LILI enhanced differentiation of these bone marrow stem cells into neurons and osteoblasts. There was also increased proliferation of the stem cells. Other studies on LILI have also found out that it could increase differentiation of mesenchymal stem cells into myogenic and osteogenic phenotypes (Hou Jian-feng *et al*., 2008; Abramovitch-Gottlib *et al*., 2005). LILI also increases growth factor secretion (Hou Jian-feng *et al*., 2008).

Flow cytometry results showed decreases of the expression of the CD29 and CD90 markers (ADSCs markers) of the co-cultures over a period of 1 wk in all the six groups. For CD29, the decrease was significantly observed in co-cultures of CC+TGF-β1+LILI group at 72 h post irradiation as well as in co-cultures of CC+RA, CC+RA+LILI, CC+TGF-β1 and CC+TGF-β1+LILI groups at 1 wk. However the expression of the SMCs marker, the MHC, showed increases over the same time period in all groups. The increase was significantly observed in co-cultures of CC+TGF-β1+LILI group which were grown in the presence of a growth factor, TGF-β1, and was irradiated. This result agrees with the study done by Rodriguez *et al* in 2006, who found out that MHC, α-SMA, calponin and smoothelin were expressed during ADSCs differentiation into SMCs. MHC

is only expressed in SMCs (Miano, 2002). This would probably indicate a possible differentiation of the ADSCs into SMCs.

The PCR microarray results show down-regulation of most of the genes in the human mesenchymal stem cell array over the period of 1 wk. At 24 h post irradiation, most genes were up-regulated except few including CTNNB1. CTNNB1 is a protein that regulates cell to cell adhesion and gene transcription (MacDonald *et al*., 2009). It also maintains stem cell pluripotency and self-renewal (Sokol, 2011). Cell proliferation is promoted when there are high levels of CTNNB1 by activating cyclin D1 and c-myc which control the G1 to S phase transition in the cell cycle (Kaldis and Pagano, 2009). This study showed that there was down-regulation of CTNNB1 in all the groups at both time points. Down-regulation of the CTNNB1 gene meant that the stem cell renewal was not occurring. This could have been due to the fact that ADSCs were differentiating into SMCs.

At 24 h SMAD was up-regulated. The protein for this gene transduces extracellular TGF-β from cell membrane to the nucleus where they activate TGF-β transcription genes (Attisan and Wrana, 2002). TGF-β and SMAD play vital roles in the differentiation of ADSCs into SMCs. It has been proposed that TGF-β superfamily binds to a TGF-β11 receptor (serine/threonine kinase) which catalyses the phosphorylation of TGF-β1 receptor. This causes the phosphorylation of Smad2 and Smad3 in the cytoplasm and together with Smad4, they translocate to the nucleus. TGF-β signaling can also induce P13K which activates ROCK to enhance Smad2/Smad3 to differentiate ADSCs to SMCs. TGF-β can also enhance Smad phosphorylation through ERK pathway to differentiate ADSCs into SMCs (figure 37, Kim *et al*., 2008). This study found out that at 24 h post irradiation both these genes (SMAD and TGF-β) were up-regulated but were down-regulated at 1 wk. At 24 h differentiation of the ADSCs had been initiated by the interaction of TGF-β and SMAD and after 1 wk most

ADSCs had been differentiated into that is the reason SMCs and why both genes were either down-regulated or not expressed at all.

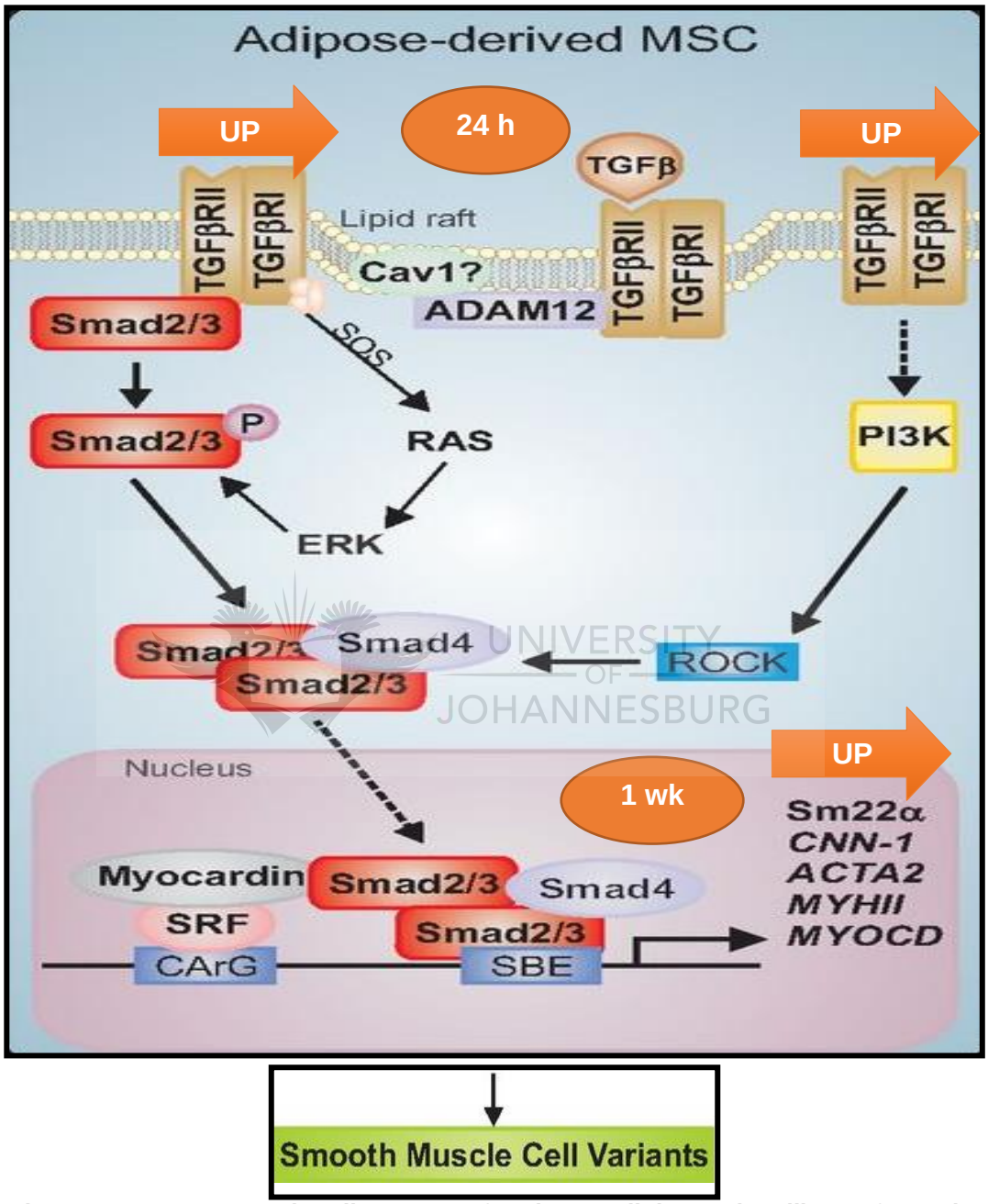


Figure 37. A proposed diagram of the cellular signalling for the differentiation of the ADSCs into SMCs. Initially there is an interaction between TGF-β and SMAD (adopted from Kim *et al*., 2008).

At 1 wk in the co-cultures that were irradiated, very few genes were up-regulated as compared to the co-cultures that were supplemented with TGF-β1. At the same time post irradiation, in the co-cultures that were

irradiated and supplemented with TGF-β1, no genes in the array including CD29 (integrin beta1-ITGB1) and CD90 (Thy-1-THY1) were up-regulated or down-regulated. These genes including TGFB1 (transforming growth factor β1) did up-regulate at 24 h (figure 38). These results agree with those produced through flow cytometry analysis which revealed decreased expression of CD29 and CD90 by the co-cultured cells at 1 wk post irradiation. It could be suggested that LILI enhanced differentiation of ADSCs into SMCs. This is in agreement with the results of many studies done on cells that involved LILI. These studies found out that LILI could enhance differentiation of ADSCs (de Villiers et al., 2011) and proliferation of ADSCs (Mvula *et al*., 2008)

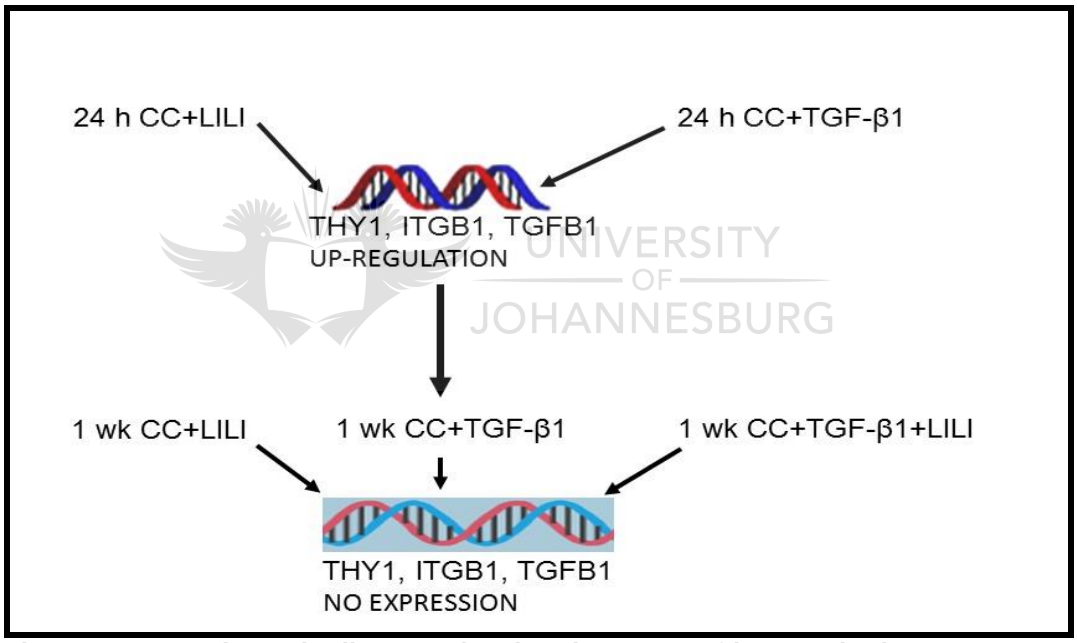


Figure 38. A schematic diagram showing the genes of interest in the array.THY1 (Thy-1), ITGB1 (β1 Integrin) and TGFB1 (Transforming growth factor β1 up-regulated at 24 h but were not expressed in the co-cultures at 1 wk.

5.2. Conclusion

Adipose derived stem cells can potentially play a major role in treating several degenerative diseases, however they have to be produced in large numbers and differentiate into the required cell lineage like smooth muscle cells. ADSCs have been differentiated into SMCs after addition of growth factors and exposure to LILI. In this study:

- Cell viability and proliferation increased in the co-cultures that were irradiated as compared to those that were not.
- Cell viability and proliferation decreased in the co-cultures that were supplemented with the growth factors.
- Expression of the markers for ADSCs decreased with time in the co-cultures that were irradiated and supplemented with growth factors while the SMC marker increased over the same period of time.
- There was no expression of genes in the human mesenchymal stem cell array after 1 wk in the co-cultures that were irradiated and supplemented with TGF-β1.

The above results indicate that ADSCs were differentiating into SMCs. However, most studies done on ADSCs revealed that differentiating into other cells could take longer period of time. This study has revealed that co-culturing, addition of the growth factor and LILI could enhance differentiation of ADSCs into smooth muscle cells in a short period of time. This would be a significant method in differentiating ADSCs into SMCs and other cells that would be required for therapeutic use in tissue engineering and regenerative medicine.

RECOMMENDATIONS FOR THE FUTURE STUDIES

This study did not analyse the genes that are expressed in smooth muscle cells through RT PCR. The contractile functionality test for smooth muscle was also not analysed confirm that the cells generated after 1 wk were SMCs. Due to high costs for materials and reagents for RT PCR, not all the co-cultured groups including those supplemented with RA were analysed for the gene expression through RT PCR in the array at all time points. Future studies must include RT PCR of mesenchymal stem cell array of all the co-cultured groups. It is therefore also suggested that future studies on the differentiation of ADSCs into SMCs should include RT PCR analysis of the genes that are expressed by SMCs such as smoothelin, α-actin and MHC. The functionality test should also be carried

out throughout the differentiation period. Future studies must also involve the evaluation of the differentiated cells in clinical trials in order to determine the effectiveness of the differentiated cells for therapeutic use in regenerative medicine.

REFERENCES

- Abderrahim-Ferkoune, A., Bezy, O., Astri-Roques, S., Elabd, C., Ailhaud, G. and Amri, E. (2003) Transdifferentiation of preadipose cells into smooth muscle-like cells: a role of aortic carboxypeptidase-like protein*, Experimental Cell Research,* **293**: 219-228.
- Abramovitch-Gottlib, L., Gross, T., Naveh, D., Geresh, S., Rosenwaks, S., Bar, I. and Vago, R. (2005) Low-level laser irradiation stimulates osteogenic phenotype of mesenchymal stem cells seeded on a three dimensional biomatrix, *Lasers in Medical Sciences*, **20**: 138-146.
- Akhurst, R. J. (2002) TGF-beta Antagonists: Why suppress a tumor suppressor, *Journal of Clinical Investigation*, **109**: 1533-1536.
- Aikawa, M., Sivam, P.N., Kuro-o, M., Kimura, K., Nakahara, K., Takewaki, S., Ueda, M., Yamaguchi, H., Yazaki, Y., Priasmy, M. (2993) Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis, *Circulation Research*, **73(6)**: 1000-1012.
- Alster, T.S. and BettenCourt, M.S. (1998) Review of cutaneous lasers and their applications, *Current Concepts*, **91(9)**: 806-812.
- Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N. and Patel, H.R. (2005) Basic principles of real time quantitative PCR, *Expert Review of Molecular Diagnosis*, **5(2)**: 209-219.
- Attisan, L. and Wrana, J.L. (2002) Signal transduction by TGF-Beta superfamily, *Science*, **296(5573)**: 1646-1647.
- Au Eells, J.T., Henry, M.M., Summerfelt, P., Wong-Riley,M.T.T., Buchmann, E.V., Kane, M., Whelan, N.T. and Whelan, H.T. (2003) Therapeutic photomodulation for methanol-induced retinal toxicity, *Proceedings of the National Academy of Sciences*, **100(6)**: 3439-3444.

- Basford, J.R. (1995) Low intensity laser therapy: still not an established clinical tool, *Lasers in Surgery and Medicine*, **16**: 331-342.
- Batard, P., Monier, M., Fortunel, N., Ducos, K., Sansilvestri-Morel, P., Phan, T., Hatzfeld, A. and Hatzfeld, J.A. (2000) TGF-β1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation, *Journal of Cell Science*, **113**: 383-390.
- Ben-Dov, N., Shefer, G., Irintchev, A., Wernig, A., Oron, U. and Halevy, O. (1999) Low-energy laser irradiation affects satellite cell proliferation and differentiation *in vitro, Biochemistry Biophysics Activation*, **1448**: 372-381.
- Bishop, A.E., Buttery, L.D.K., Polak, J.M. (2002) Embryonic stem cells, *Journal of Pathology*, **197**: 424-429.
- Bordignon, C. (2006) Stem cell therapies for blood diseases, *Nature*, **441**: 1100-1102.
- Bronner-Fraser, M (1985) Alterations in nueral crest cell migration by a monoclonal antibody that affects cell division, *Journal of Cell Biology*, **101**: 610-617.
- Butler, D. L., Goldstein, S. A. And Guilak, F. (2000) Functional tissue engineering: The role of biomechanics, *Journal of Biomechanical Engineering*, **122**: 570-575.
- Bustin, S.A., Benes, V., Nolan, T. and Pfaffl, M.W. (2005) Quantitative real-time RT-PCR: a perspective, *Journal of Molecular Endocrinology*, **34**: 597-601.
- Catherine Twomey, Nationalacadamies, Understanding stem cells: An overview of the science and issues from nationalacadamies, http://www.nationalacadamiies.org/stemcells, 25/10/2013
- Chang, P., Qu, Y., Liu, Y., Cui, S., Zhu, D., Wang, H. and Jin, X. (2013) Multi-therapeutic effects of human adipose-derived mesenchymal stem cells on radiation-induced intestinal injury, *Cell Death and Disease***, 4**, e685; doi:10.1038/cddis.2013.178.

- Chang, J.C., Ye, L. and Kan, Y.W. (2006) Correction of the sickle cell mutation in embryonic stem cells, *Proceedings of the National Academy of Sciences*, **103**: 1036-1040.
- Chaudhary, P.M. and Roninson, I.B. (1991) Expression and activity of P-glycoprotein, a multi-drug efflux pump, in human hematopoetic stem cells, *Cell*, **66(1)**: 85-94.
- Chen, S. and Lechleider, R. J. (2004) Transforming growth factor-β-Induced differentiation of smooth muscle from a neural crest stem cell line, *Circulation Research*, **94**: 1195-1202.
- Choi, S. S., Cha, S. M., Lee, Y.Y., Kwon, S. W., Park, C. J. and Kim , M. (2006) Adipogenic differentiation of adipose tissue derived adult stem cells in nude mouse, *Biochemical Biophysics Research Communication,* **345(2):** 631-637.
- Choy, D.S.J. (2014) History of lasers in medicine, *Photomedicine and Laser Surgery*, **32(3)**: 119-120.
- Couri, C.E., Oliveira, M.C., Stracieri, A.B., Moraes, D.A., Pieroni, F. and Barros, G.M., Madeira, M.I., Malmegrin, K.C., Foss-Freitas, M.C., Samoes, B.P., Martinez, E.Z., Foss, M.C. Burt, R.K. and Voltarelli, J.C. (2009) C-peptide levels and insulin independence following autologous non-myeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetic mellitus, *Journal of the American Medical Association*, **301**: 1573-1579.
- Crisostomo PR, Wang M, Markel TA, Lahm T, Abarbanell AM, Herrmann JL and Meldrum DR (2007) Stem cell mechanisms and paracrine effects: potential in cardiac surgery, *Shock*, **28(40):** 375-383.
- Darby, I.A. and Hewitson, T.D. (2007) Fibroblast differentiation in wound healing and fibrosis, *International Review of Cytology*, **257**: 143-179.
- de Villiers, J.A., Houreld, N.N. and Abrahamse, H. (2011) Influence of low intensity irradiation on isolated human adipose derived stem cells over 72hrs and their differentiation potential into smooth

muscle cells using retinoic acid, *Stem Cell Reviews and Reports*, **7(4)**: 869-882.

- Dominici, M., Le Blac, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J. and Horwitz, E.M. (2006) Minimum criteria for defining multipotent mesenchymal stromal cells. The international society for therapy position statement, *Cytotherapy*, **8(4)**: 315-317.
- Duong, V. and Rochette-Egly, C. (2011) The molecular physiology of nuclear retinoic acid receptor. From health to disease, *Biochimica et Biophysica Acta*, **1812**: 1023-1031.
- Eells, J.T., Wong-Riley, M.T.T., VerHoeve, J., Henry, M., Buchman, E.V., Kane, M.P., Gould, L.J., Jett, M., Hodgson, B.D., Margolis, D. and Whelan, H.T. (2004) Mitochondrial signal transduction in accelerated wound and retinal healing by near-infrared light therapy, *Mitochondrion*, **4:** 559-567.
- Eduardo, F.P., Bueno, D.F., de Freitas, P.M., Marques, M.M., Passos-Bueno, M.R., Eduardo, C.P. and Zatz, M. (2008) Stem cell proliferation under low intensity laser irradiation: A preliminary study, *Lasers in Surgery and Medicine*, **40(6)**: 433-438.
- Fernandes, K.J.L., McKenzie, I.A., Mill, P., Smith, K.M., Akhavan, M., Barnabe-Heider, F., Biernaskie, J., Juneck, A., Kobayashi, N.R., Toma, G.J., Kaplan, D.R., Labosky, P.A., Rafuse, V., Hui, C. and Miller, F.D. (2004) A dermal niche for multipotent adult skin-derived precursor cells, *Nature Cell Biology*, **6**: 1082-1092.
- Firth, A.L. and Yuan, J.X.J. (2013) Human models for smooth muscle cell differentiation. Focus on a novel in vitro model system for smooth muscle differentiation from human embryonic stem cell-derived mesenchymal cells, *American Journal of Physiological Cell Physiology*, **304**: C287-C288.
- Fiszerman, R. and Markmann, C.A. (2000) Effects of low level laser therapy in chronic diabetic foot ulcers, *Diabetes.*

- Gardner, R.L. (2002) Stem cells: potency, plasticity and perception, *Journal of Anatomy*, **3**: 277-282.
- Gasparyan, L., Brill, G. and Makela, A. (2004) Influence of low level laser radiation on migration of stem cells, *Laser Florence*, 1-7.
- Gimble, J.M., Katz, A.J. and Bunnell, B.A. (2007) Adipose-derived stem cells for regenerative medicine, *Circulation Research*, **100**: 1249-1260.
- Gonthos, S., Franklin, D.M., Leddy, H.A., Robey, P.G., Storms, R.W. and Gimble, J.M. (2001) Surface protein characterisation of human adipose tissue-derived stromal cells, *Journal of Cellular Physiology*, **189(1)**: 54-63.
- Goldwasser, S.M. (1997) Lasers: safety, diode lasers, helium neon lasers, drive, information, parts, *A Practical Giude for Experimenters and Hobbyists*, *Science Electronics.FAQ*, Version 2.62.
- Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986) Growth factors and cancer, *Cancer Research*, **45**: 1015-1029.
- Gudas, L.J. and Wagner, J.A. (2011) Retinoids regulate stem cell differentiation, *Journal of Cellular Physiology*, **226:** 322-330.
- Guo, X., Stice, S.L., Boyd, N.L. and Chen, S. (2013) A novel in vitro model system for smooth muscle differentiation from human embryonic stem cell-derived mesenchymal cells, *American Journal of Physiology of Cell Physiology*, **304**: C289-C298.
- Hallemeier, C.L., Girgis, M.D., Blum, W.G., Brown, R.A., Khoury, H.J., Devine, S.M., Vij, R., Lin, H.S., DiPersio, J.H. and Adkins, D.R. (2006) Long-term remissions in patients with myelodysplastic syndrome and secondary acute myelogenous leukaemia undergoing allogeneic transplantation following a reduced intensity conditioning regimen of 550 cGy total body irradiation and cyclophosphamide, *Biology of Blood and Marrow Transplantation*, **12**: 749-757.

- Hawkins, D. and Abrahamse, H. (2007) Phototherapy- a treatment modality for wound healing and pain relief, *African Journal of Biological Research*, **10**: 99-109.
- Hawkins, D., and Abrahamse, H. (2005) Biological effects of helium-neon laser irradiation on normal and wounded human skin fibroblasts, *Photomedicine Laser Surgery*, **23(3)**: 251-259.
- Hasbold, J., Lyons, A.B., Kehry, M.R. and Hodgkin, P.D. (1996) Cell Division number regulates IgG1 and IgE switching of B Cells following stimulation by CD40 ligand and IL-4, *Journal of Experimental Medicine*, **184**: 277-281.63.
- Hashemibeni, B., Ravazi, S., Esfandiary, E., Karbasi, S., Mardani, M., Nasresfahani, M. (2008) Induction of Chondrogenic Differentiation of human adipose-derived stem cells with TGF-β3 in pellet culture system, *Iranian Journal of Basic Medical Sciences,* **11(1)**:10-17.
- Hegde,P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard. R., Hughes, J.E., Snesrud, E., Lee, N. and Quackenbush, J. (2000) A concise guide to cDNA microarray analysis, *Biotechniques,* **3**: 548-554.
- Hou, J., Zhang, H., Yuan, X., Li, J., Wei, Y. and Hu, S. (2008) In vitro effects of low-level laser irradiation for bone marrow mesenchymal stem cells: proliferation, growth factors secretion and myogenic differentiation, *Lasers in Surgery and Medicine*, **40**: 726-733.
- Huang, W.Y., Xie, W., Guo, X., Li, F., Jose, P.A. and Chen, S.Y. (2011) Smad2 and PEA3 cooperatively regulate transcription of response gene to complement 32 in TGF-β-induced smooth muscle cell differentiation of neural crest cells, *American Journal of Physiological Cell Physiology*, **301**: C499-C506.
- Huang, T.H., Lu, Y.C. and Kato, C.T. (2012) Low-level diode laser therapy reduces lipopolysaccharide (LPS)-Induced bone cell inflammation, *Lasers in Medical Science*, **27**:621-622.

- Huang, T.H., Liu, S.L., Chen, C.L., Shie, M.Y. and Kao, C.T. (2013) Low-level laser effects on simulated orthodontic tension side peridontal ligament cells, *Photomedicine and Laser Surgery*, **31(2)**: 72-77.
- Human mesenchymal stem cell PCR array, http://www.sabiosciences.com. 26/06/2014.
- Hu, L., Hu, J., Zhao, J., liu, J., Quyang, W., Yang, C., Gong, N., Du, L., Khanal, A. and Chen, L. (2013) Side-by-side comparison of the biological characteristics of human umbilical cord and adipose tissue-derived mesenchymal stem cells, *Biomedical Research International*, doi 10.1155/2013/438243.
- Huh, C. H., Kim, S. Y., Cho, S. J., Kim, D. S., Lee, W. H., Kwon, S. B., Na, J. I. And Park, K. C. (2000) Effects of mesenchymal stem cells in the reconstruction of skin equivalents, *Journal of Dermatological Science*, **46(30):** 217-220.
- Hutt, K.J. and Albertini, D.F. (2006) Clinical applications and limitations of current ovarian stem cell research: A review, *Journal of Experimental Clinical Assistant Reproduction*, **3**: 6.
- Hwang, N.S., Varghese, S., Puleo, C. and Zhang, Z. (2007) Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells, *Journal of Cell Physiology*, **212**: 281-284.
- Jang, S., Cho, H., Cho, Y., Park, J. and Jeong, H. (2010) Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin, *BMC Cell Biology*, **11**:25.
- Jia, Y.L. and Guo, Z.Y. (2004) Effect of low power He-Ne laser irradiation on rabbit articular chondrocytes *in vitro*, *Laser Surgery and Medicine*, **34**: 323-328.
- John W. Kimball (2006) Stem cells, http://users.rcn.com/jkimball.ma .ultranet/BiologyPages/S/Stem_Cells.htm l-17K (Accessed 17/08/2007).

- Kachgal, S. and Putnam, A.J. (2011) Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via disctinct cytokine and protease expression mechanisms, *Angiogenesis*, **14**: 47-59.
- Kalbermatten, D.F., Shaakxs, D., Kingham, P.J. and Wiberg, M. (2011) Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat, *Cell Tissue Research*, **344**: 251-260.
- Kaldis, P. and Pagano, M. (2009) Wnt signalling in axon guidance, *Trends in Nuerosciences*, **27(9)**: 528-532.
- Kennard, S., Liu, H. and Lilly, B. (2008) Transforming growth factor-*β* (TGF-β1) down-regulates notch3 in fibroblasts to promote smooth muscle gene expression, *The Journal of Biological Chemistry*, **283(3)**: 1324-1333.
- Khadra, M., Lyngstadans, S.P., Haanaes, H.R., Mustafa, K. (2005) Effects of laser therapy on attachment, proliferation and differentiation of human osteoblasts-like cells cultured on titanium implant material, *Biomaterials*, **26(170)**: 3503-3509.
- Kim, J., Park, C.D., Lee, J.H., Lee, C.H., Do, B.R. and Lee, A.Y. (2012) Co-culture of melanocytes with adipose-derived stem cells as a potential substitute for co-culture with keratinocytes, *Acta Dermato-Venereologica*, **92**: 16-23.
- Kim, Y.D., Kim, S.S., Kim, S.J., Kwon, D.W., Jeon, E.S. and Son, W.S. (2010) Low level laser Irradiation facilitates fibronectin and collagen type 1 turnover during tooth movement, *Lasers in medical Science*, **25**:25-31.
- Kim, Y.M., Kim, J., Heo, S.C., Shin, S.H., Do, E.K.,Suh, D.S., Kim, K.H., Yoon, M.S., Lee, T.G and Kim J.H. (2012) Proteomic identification of ADAM12 as a regulator for TGF-β1-induced differentiation of human mesenchymal stem cells to smooth muscle cells, *Public Library of Science One*, **7**: e40820.

- Kim, Y.M., Jeon, E.S., Kim, M.R., Jho, S.K., Ryu, S.W. and Kim, J.H. (2008) Angiotensin II-induced differentiation of adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. *International Journal of Biochemistry and Cellular Biology*, **40:** 2482–2491.
- Koutna, M., Janisch, R. and Veselska, R. (2003) Effects of low–power laser irradiation on cell proliferation, *Scripta Medica (BRNO)*, **76(3)**: 163-172.
- Kubota, H. and Brinster, R.L. (2006) Technology insight: *In vitro* culture of spermatogonial stem cells and their potential therapeutic uses, *Nature of Clinical Practical Endocrinology Metabolism*, **2**: 99-108.
- Kyoto University, 2006, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, http://www.cell.com/content/article/abstract?uid=PIIS0092867406009767& highlight (Accessed 24/02/07).
- Lai, Y.L., Chung, Y.K., Tan, H.C., Yap, H.F., Yap, G., Ooi, E.E., Ng, L.C. (2007) Cost-effective real-time reverse transcriptase PCR (RT-PCR) to screen for Dengue virus followed by rapid single-tube multiplex RT-PCR for serotyping of the virus, *Journal of Clinical Microbiology*, **45(3)**: 935-941.
- Lee, W.C., Rubin, J.P. and Marra, K.G. (2006) Regulation of alpha-smooth muscle actin protein expression in adipose-derived stem cells, *Cell Tissue Organs*, **183**: 80-86.
- Li, B., Zheng, Y.W., Sano, Y. and Tamaguchi, H. (2011) Evidence for mesenchymal-epithelial transition associated with mouse hepatic stem cell differentiation, *Public Library of Science*, DOI 1371/ journal.pone.0017092.
- Lindvall, O. And Kokaia, Z. (2006) Stem Cells for the treatment of neurological disorder, *Nature*, **441**: 1094-1096.

- Lyons, A.B. and Parish, C.R. (1994) Determination of lymphocyte division by flow cytometry, *Journal of Immunological Methods*, **171**: 131-137.
- MacDonald, B.T., Tamai, K. and He, X. (2009) Wnt/beta-Catenin signaling: Compnents, mechanisms and diseases, *Developmental Cell*, **17(1)**: 9-26.
- Madsen, C.S., Regan, C.P., Hungerford, J.E., White, S.L., Manabe, I. and Owens, J.K. (1998) Smooth muscle–specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5'-flanking and first intronic DNA sequence, *Circulation Research*, **82**: 908-917.
- Marra, K.G., Brayfield, C.A. and Rubin, J.P. (2011) Adipose stem cell differentiation into smooth muscle cells, *Methods of Molecular Biology*, **702**: 261-268.
- Matic, M., Lazetic, B., Polljacki, M., Duran, V. and Ivkov-Simic, M. (2003) Low level laser irradiation and its effects on repair processes in the skin, *Medicinski Pregled* , **56(3-4)**: 137-141.
- Meregalli, M., Farini, A. and Torente, Y. (2011) Stem cell therapy for neuromuscular diseases, Chap: 17, *Stem Cells in Clinic and Research*, ED: Ali Gholamrezanezhad, **ISBN**: 978-953-307-797-0, CC BY-NC-SA, **pp**. 437-468, InTech, http://www.intechopen.com/books/stem-cells-in-clinic-and-research/stem-cell-therapy-for-neuromuscular-diseases.
- Mester, E., Szende, B. and Tota, J.G. (1967) Effect of laser on hair growth of mice, *Kiserl Orvostud*, **19**: 628-631.
- Miano, J.M., Cserjesi, P., Ligon, K.L., Periasamy, M. and Olson, E.N. (1994) Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis, *Circulation Research*, **75**: 803-812.
- Miano, J.M. (2002) Mammalian smooth muscle differentiation: orgins, markers, and transcriptional control, *Results and Problems in Cell Differentiation*, **38**: 39-59.

- Miller, R.H. (2006) The promise of stem cells for neural repair, *Brain Research*, **1091**: 258-264.
- Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.M., Goh, B., Kilroy, G., Wu, X. and Gimble, J.M., (2006) The immunophenotype of human adipose derived cells: temporal changes in stromal- and stem cell-associated markers, *Stem Cells*, **24:**376-385.
- Mirsky, N., Shoshany, Y., Maltz, L. and Oron, U. (2002) Promotion of angiogenesis by low energy laser irradiation, *Antioxidant Redox Signal*, **4**: 785-790.
- Moore, P., Ridgway, T.D., Higbee, R.G., Howard, E.W. and Lucroy, M.D. (2005) Efect of wavelength on low-intensity laser irradiation stimulated cell proliferation *in vitro*, *Lasers in Surgery and Medicine*, **36**: 8-12.
- Morrison, S.J. and Kimble, J. (2006) Asymmetric and symmetric stem cell divisions in development and cancer, *Nature*, **441**: 1068-1074.
- Murphy, D. (2001) Differential interference contrast (DIC) microscopy and modulation contrast microscopy, *Fundamentals of Light Microscopy and Digital Imaging*, Wiley-Liss, New York, **pp**. 153–168.
- Mvula, B., Mathope, T., Moore, T. and Abrahamse, H. (2008) The effect of low level laser therapy on adult human adipose derived stem cells, *Lasers in Medical Science*, **23(3)**: 277-282.
- Mvula B., Moore T. and Abrahamse H. (2010) The effect of low level laser irradiation and epidermal growth factor (EGF) on human adult adipose derived stem cells, *Lasers in Medical Science*, **25**: 33-39.
- Nakano, J., Kataoka, H., Sakamoto, J., Origuch, T., Okita, M. and Yoshimura, T. (2009) Low-level laser irradiation promotes the recovery of atrophied gastrocnemius skeletal muscle in rats, *Experimental Physiology*, **94(9)**: 1005-1015.

- Narita, Y., Yamawaki, A., Kagami, H., Ueda,M., & Ueda, Y. (2008) Effects of transforming growth factor-beta 1 and ascorbic acid on differentiation of human bone marrow-derived mesenchymal stem cells into smooth muscle cell lineage, *Cell and Tissue Research*, **333**: 449-459..
- National Centre for Health Statics, National office of vital statistics, USA, National vital statistics system mortality, *National Vital Statistics Reports* Vol 48(11).
- Ngan, F., Randall, J.L. and Song, L. (2007) Chemical and physical regulation of stem cells and progenitor cells: Potential for cardiovascular tissue engineering, *Tissue Engineering*, **13(8)**: 1809-1823.
- Noguchi, H. (2007) Stem cells for the treatment of diabetes, *Endocrinology Journal*, **54(1)**: 7-16.
- Nose, A. and Takeichi, M. (1986) A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos, *Journal Of cell Biology*, **103**: 2649-2658.
- Ocana-Quero, J.M., Gomez-Villamondos, R., Moreno-Millan, M. and Santisteban-Valenzuela, J.M. (1997) Detrimental effect of low power helium-neon laser irradiation on *in vitro* meiotic maturation of immature bovine oocytes, *Lasers Life Science*, **7**: 157-166.
- Ohshiro, T. and Calderhead, R.G. (1988) *Low level laser therapy: A Practical Introduction*, Wiley and Sons, New York and Brisbane, **pp**: 17, 28, 29, 30, 33, 34.
- Ogawa, S. and Miyagawa, S. Potentials of regenerative medicine for liver disease, *Surgery Today*, **39**: 1019-1025.
- Oron, U., Yaakabi, T., Oron, A., Mordechovitz, D., Shofti, R., Hayam, G., Dror, U., Geptein, L., Wolf, T., Haudenschild, C. and Ben Haim, S.A. (2001) Low level laser irradiation reduces formation of scar tissue following myocardial infarction in dogs, *Circulation*, **103**: 296-301.

- Oron, U., Yaakabi, T., Oron, A., Hayam, G., Geptein, L., Wolf, T., Rubin, O. and Ben-Haim, S.A. (2001) Attenuation of the formation of scar tissue in rats and dogs post myocardial infarction by low energy laser irradiation, *Laser Surgery Medicine,* **28**: 204-211.
- Peavy, G.M. (2002) Lasers and laser-tissue interaction, *Veterinary Clinical Small Animal*, **32**: 517-534.
- Peroni, D., Scambi, I., Pasini, A., Lisi, V., Bifari, F., Krampera, M., Rigotti, G., Sbarbati, A. and Galie, M. (2008) Stem molecular signature of adipose-derived stromal cells, *Experimental Cell Research*, **314**: 603-615.
- Phillips, H.L. and Terrberry, J.E. (1957) Counting actively metabolizing tissue cultured cells*, Experimental Cell Research*, **13**: 341-347.
- Pinheiro, A.L.B., Nascimento, S.C., Veira, A.L.B., Brugnera, A., Zanin, F.A., Rolim, D.D.S. and Da Silva, P.S. (2002) Effects of low level laser therapy on malignant cells: *in vitro* study, *Journal of Clinical Laser Medicine and Surgery*, **20(1)**: 23-26.
- Promega, 2005, Cell Titer-Glo™ luminescent, cell viability assay, Product information, Fact sheet # G757rev02.
- Rahimi, R. A. and Leof, E. B. (2007) TGF-beta Signaling: A tale of two responses, *Journal of Cellular Biochemistry*, **102**: 593-608.
- Richardson, S.M., Walker, R.V., Parker, S., Rhodes, N.P., Hunt, J.A., Freemont, A.J. and Hoyland, J.A. (2006) Intervertebral disc cell-mediated mesenchymal stem cell differentiation, *Stem Cells*, **24**: 707-716.
- Robinson, P., Hyun, W., Orfao, A. and Robinovitch, P. (1998) *Current Protocols in Cytometry*, New York, NY: John Willey and Sons, **pp**: 9.11.1-9.11.9.
- Rodbell, M. (1966) Metabolism of isolated fat cells. II. The similar effects of phospholipase c (Clostridium Perfringes Alpha Toxin) and of insulin on glucose and aminoacid metabolism, *Journal of Biological Chemistry*, **241**: 130-139.

- Rodbell, M. (1966) Metabolism of isolated fat cells. IV. Regulation of release of protein by lipolytic hormones and insulin, *Journal of Biological Chemistry*, **241**: 3909-3917.
- Rodbell, M. and Jones, A.B. (1966) Metabolism of Isolated fat cells. 3. The similar inhibitory action of phospholipase c (Clostridium Perfringes Alpha Toxin) and of insulin on lipolysis stimulated by lipolytic hormones and theophylline, *Journal of Biological Chemistry*, **241**: 140-142.
- Rodriguez, L.V., Alfonso, Z., Zhang, R., Leung, J., Wu, B. and Ignarro, L.J. (2006) Clonogenic multipotent stem cells from human adipose tissue differentiate into functional smooth muscle cells, *Proceedings of the National Academy of Sciences*, **103(32)**: 12167-12172.
- Roland, T.M., Houreld, N.N. and Abrahamse, H. (2013) Low-Intensity laser Irradiation at 660 nm stimulates transcription of genes Involved in the electron transport chain, *Photomedicine and Laser Surgery*, **31(2)**: 47-53.
- Rubio, D., Garcia-Castro, J., Martin, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C. and Bernard, A. (2005) Spontaneous human adult stem cell transformation, *Cancer Resraerch*, **65**: 3035-3039.
- Rubinstein, P. (2006) Why cord blood? *Human Immunology*, **67**: 398-404.
- Sadler, I., Evans, R., Kolb, B., Naim, J.O., Narayan, V., Buehner, N. and Lanzafame, R.J. (2000) *In vitro* effects of low-level laser irradiation at 660 nm on peripheral blood lymphocytes, *Lasers in Medical Science*, **3**: 255-261.
- Salmon, E. and Tran, P. (1998) High-resolution video-enhanced differential interference contrast (VE-DIC) light microscope., *Video Microscopy*, Sluder, G. and Wolf, D. (eds), Academic Press, New York**, pp**: 153–184.

- Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science,* 270: 368-371.
- Schindl, A., Schindl, M., Schon, H., Knobler, R., Havelec, L. and Schindl, L. (1998) Low intensity laser irradiation improves skin circulation in patients with diabetic microangiopathy, *Emerging Treatments and Technology*, **21(4)**: 580-584.
- Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A. and Benvenisty, N. (2000) Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells, *Proceedings of the National Academy of Sciences*, **97**: 11307-11312.
- Serakinci, N. and Keith, W.N. (2006) Therapeutic potential of adult stem cells, *European Journal of cancer*, **42(9)**: 1243-1246.
- Shapiro, A.M., Ricordi, C., Hering, B.J., Auchinclos, H., Lindblad, R., Robertson, R.P., Secchi, A., Brendel, M.D., Berney, T., Brennan, D.C., Cagliero, E., Alejandro, R., Ryan, E.A., DiMercurio, B., Morel, P., Polonsky, K.S., Reems, J.A., Bretzel, R.G., Bertuzzi, F., Froud, T., Kandaswamy, R., Sunderland, D.E., Eisenbarth, G., Segal, M., Preiksaitis, J., Korbutt, G.S., Barton F.B., Viviano, L., Seyfert-Margolis, V., Bluestone, J. and Lakey, J.R. (2006) International trial of the Edmonton protocol for islet transplantation, *The New England Journal of Medicine*, **355**: 1318-1330.
- Sharow, K. A., Temkin, B. and Asson-Batres, M. A. (2012) Retinoic acid stability in stem cell cultures, *International Journal of Developmental Biology*, **56**: 273-278.
- Sinha, S., Wamhoff, B.R., Hoofnagle, M.H., Thomas, J., Neppi, R.L., Deering, T., Helmke, B.P., Bowles, D.K., Somlyo, A.V. and Owens, G.K. (2006). Assessment of contractility of purified smooth muscle cells derived from embryonic stem cells, *Stem Cells*, **24**: 1678-1688.

- Sokol, S.Y. (2011) Maintaining embryonic stem cell pluripotency with wnt signalling, *Development*, **138(20)**: 4341-4350.
- Soleimani, M., Abbasnia, E., Fathi, M., Sahraei, H., Fathi, Y. and Kaka, G. (2012) The effects of low-level laser irradiation on differentiation of human bone marrow mesenchymal stem cells into neurons and osteoblasts – an *in vitro* study, *Lasers in Medical Sciences*, **27**: 423-430.
- Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001) Stem cells find their niche, *Nature*, **414**: 98-104.
- Spees, J.L., Olson, S.D., Whitney, M.J. and Prockop, D.J. (2006) Mitochondrial transfer between cells can rescue aerobic respiration, *Proceedings of the National Academy of Sciences USA*, **103**: 1283-1288.
- Srivastava, D. and Ivey, K.N. (2006) Potential of stem-cell-based therapies for heart disease, *Nature*, **441**: 1097-1099.
- Stavridis, M.P., Collins, B.J. and Storey, K.G. (2010) Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation, *Developmental Stem Cell*, **137:** 881-890.
- Steynberg, T., Visagie, M., Mqoco, T., Idicula, A., Moolman, S., Richter, W. and Joubert, A. (2012) Qualitative assessment of smooth muscle cells propagated on 2D and 3D polycaprolactone ploymers via scanning electron microscope, *Biomedical Research*, **23(2)**: 191-198.
- Stein, A., Benayahu, D., Maltz, L. and Oron, U. (2005) Low level laser irradiation promotes proliferation and differentiation of human osteoblasts *in vitro, Photomedicine and Laser Surgery*, **23(2)**: 161-166.
- Steven, M. Carr, (2005) *Principle of RT- PCR.*
- Steyn, K., Fourie, J. and Bradshaw, D. (1992) The impact of chronic diseases of life style and their major risk factors on mortality in South Africa, *South African Medical Journal*, **82(4)**: 227-231.

- Stowers, R.S., Drinnan, C.T., Chung, E. and Saqqs, L.J. (2013) Mesenchymal stem cell response to TGF-β1 in both 2D and 3D environments, *Biomaterial Science*, **1:** 860-869.
- Strem, B.M., and Hedrick, M.H. (2005) The growing Importance of fat in regenerative medicine, *Trends Biotechnology*, **23**: 64-66.
- Strem, B. M., Hicok, K. C., Zhu, M., Wulur, I.,Alfonso, Z., Schreiber, R.E. (2005) Multipotential differentiation of adipose tissue-derived stem cells. *Keio Journal of Medicine*, **54(3)**: 132-141.
- Terese, W., Kibiuk, L. and Duckwall, C. (2001) The adult stem cell, In Stem Cell Information, http://stem cells.nih.gov/info/scireport/pages/chapter4.aspx. Accessed 29/10/2013.
- Thomas, M. (2013) Stem cells and eternal life, exposing the truth, http://www.exposingthetruth.co/stem-cells-eternal-lif, 28/10/2013.
- Timper, K., Eberhardt, M., Linscheid, P., Christ-Crain, M., Keller, U., Muller, B. and Zulewski, H. (2006) Human adipose tissue derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells, *Biochemical and Biophysical Research Communications*, **341(4)**: 1135-1140.
- Toogood, I.R.G., Dexter, T.M., Allen, T.D., Suda, T. and Lajtha, L.G. (1980) The development of liquid culture system for the growth of human bone marrow, *Leukaemia Research*, **4(5):** 449-461.
- Toma, G.J., Akhavan, M., Fernandes, K.J.L., Barnabe-Heider, F., Sadikots, A., Kaplan, D.R. and Miller, F.D. (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin, *Nature Cell Biology*, **3**: 778-784.
- Topouzis, S. and Majesky, M.W. (1996) Smooth muscle lineage diversity in the chick embryo: two types of aortic smooth muscle cell differ in growth and receptor-mediated transcriptional responses to transforming growth factor-*β*, *Developmental Biology*, **178:** 430-445.

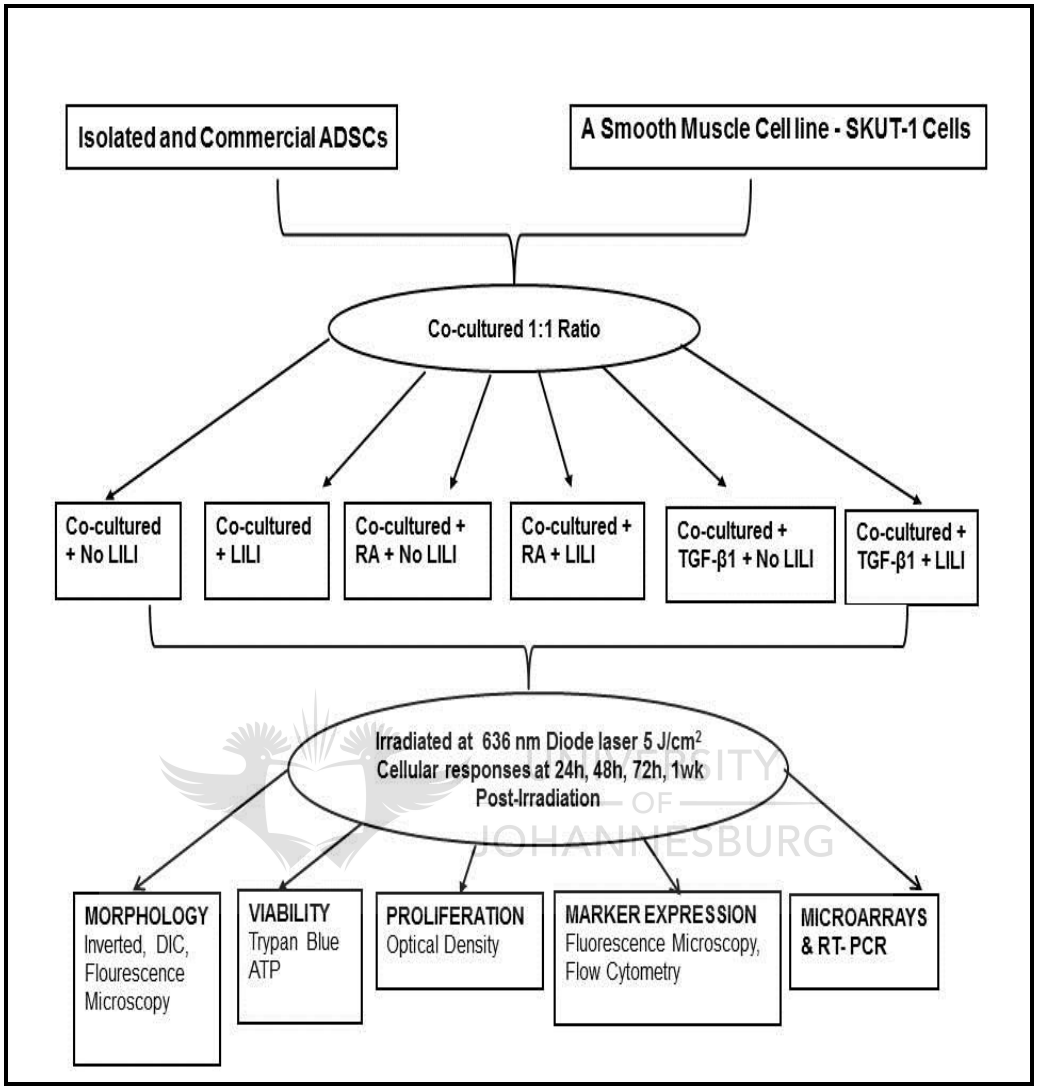
- Tong, P.D. and Andrews, P.W. (2010) Retinoic acid directs neuronal differentiation of human pluripotent stem cell lines in a non-cell-autonomous manner, *Differentiation*, **80(1)**: 20-30.
- Tsai, R.Y.L. (2004) A molecular view of stem cell and cancer cell self-renewal, *Introductory Journal of Biochemistry and Cell Biology*, **36**: 684-694.
- Tuby, H., Maltz, L. and Oron, U. (2006) Modulation of VEGF and iNOS in the rat heart by low energy laser irradiation are associated with cardioprotection and enhanced angiogenesis, *Laser in Surgery and Medicine*, **38**: 682-688.
- Tunér, J. and Hode, L. (2002) *Laser Therapy-Clinical Practice and Scientific Background*, Prima Books, AB Grängesberg, Sweden, Chapter 1, Some basic laser physics. **pp**. 8, 12, 16, 17, 21, 22 (ISBN: 91-631-1344-9).
- Van Dijk,, A., Niessen H.W.M., Zandieh Doulabi, B., Visser, F. C., van Milligen, F.J. (2008) Differentiation of human adipose-derived stem cells towards ardiomyocytes is facilitated by laminin, *Cell Tissue Research,* **334**: 457-467.
- Vet Stem Regenerative Veterinary Medicine (2008) http://www.marketwire.com/pressrelease/vet-stem-announces-milestone-of-8000-animals- t reated-wi th-vet -stem-cel l -therapy-1611912.htm.
- Wang, D.S., Miura, M., Demura, H. and Sato, K. (1997) Anabolic effects of 1,25-dihydroxyvitamin D3 on oesteoblasts are enhanced by vascular endothelial growth factor produced by osteoblasts and by growth factors produced by endothelial cells, *Endocrinology*, **138**: 2953-2962.
- Wang, L., Zhang, Z.G., Zhang, R.L., Jiao, Z.X., Wang, Y., Pourabdollah-Nejad, D.S., Letourneau, Y., Gregg, S.R. and Chopp, M. (2006) Neurogenin 1 mediates erythropoietin enhanced differentiation of adult neural progenitor cells, *Journal of Cerebral Blood Flow and Metabolism*, **26**: 556–564.

- Woodbury, D., Schwarz, E.J., Prockop, D.J. and Black, I.B. (200) Adult rat and human bone marrow stromal cells differentiate into neurons, *Journal of Neuroscience Research*, **61**: 364-370.
- Yaakobi, T., Shoshani, Y., Levkovitz, S., Rubin, O., Ben-Haim, S.A. and Oron, U. (2001) Long term effect of low energy laser irradiation on infarction and reperfusion injury in the rat heart, *Journal of Applied Physiology*, **90**: 2411-2419.
- Yamada,Y., Wang, X.D., Yokoyama, S., Fukuda, N. and Takakura, N. (2006) Cardiac progenitor cells in brown adipose tissue repaired damaged myocardium, *Biochemical and Biophysical Research Communications*, **342**: 662-670.
- Yang, Y., Yin, S., Cui, L., Li, H., Wu, Y. and Cao, Y. (2008) Experiment of adipose derived stem cells induced into smooth muscle cells, *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, **22(4)**: 481-486.
- Yang, Y., Relan, N. K., Przyywara, D. A., & Schugar, L. (1999) Embryonic mesenchymal cell share the potential for smooth muscle differentiation: Myogenesis is controlled by the cell's shape, *Development*, **126:** 3027–3034.
- Yoshimura, K., Shigeura, T., Matsumoto, D., Sato, T., Takaki, Y., Aiba-Kojima, E., Sato, K., Inoue, K., Nagase, T., Koshima, I. and Gonda, K. (2006) Characterisation of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates, *Journal of Cellular Physiology*, **208(1)**: 64-76.
- Yue, F., Shirasawa, S., Ichikawa, H., Yoshie, S., Mogi, A., Masuda, S., Nagai, M., Yokohama, T., Daihachiro, T. and Yokohama, K. (2013) Induce differentiation of embryonic stem cells by co-culture system, *Intech*, http://dx.doi.org/10.5772/56000.
- Yu, W., Naim, J.O. and Lunzafame, R.J. (1997) Effects of photostimulation on wound healing in diabetic mice, *Lasers in Surgery and Medicine*, **20(1)**: 56-63.

- Zahran, F., Salam, M., Loffy, A. and El-Deen, I.M. (2012) Isolation and characterisation of adipose tissue-derived stem cells: An *in vitro* study, *Basic Research Journal of Medicine and Clinical Sciences*, **1(5)**: 88-94.
- Zhang, R., Ignarro, L. J., Rodriguez, L. V., Wu, B., Zuk, P., Rao, N. and Jack, G. S. (2012) Nuclear fusion-independent smooth muscle differentiation of human adipose derived stem cells induced by a smooth muscle environment, *Stem Cells*, **30(3)**: 481-490.
- Zhang, Y. E. (2009) Non-smad pathways in TGF-beta signaling, *Cell Research*, **19**: 128-139.
- Zhang, Y., Schedle, A., Matejka, M., Rausch-fan, X. and Andrukhov, O. (2010) The proliferation and differentiation of osteoblasts in co-culture with human umbilical vein endothelial cells: An improved analysis using flourscence-activated cell sorting, *Cellular and Molecular Biology Letters*, **15**: 517-529.
- Zuk, P.A., Zhu, M., Mizuno, H., Huang, J.I., Chaudhari, S., Lorenz, H.P., Benhaim, P. and Hedrick, M.H. (2001) Multilineage cells derived from human adipose tissue, a putative source of stem cells for tissue engineering, *Tissue Engineering*, **7(2)**: 211-216.
- Zuo, Q., Cui, W., Liu, F., Wang, Q., Chen, Z. and Fan, W. (2013) Co-cultivated mesenchymal stem cells support chondrocytic differentiation of articular chondroctyes, *International Orthopaedics*, **37(4):** 747-752.

APPENDIX A

METHODOLOGY



APPENDIX B

FACULTY OF HEALTH SCIENCES

ACADEMIC ETHICS COMMITTEE

29 March 2006

Clearance Reference Number: 01/06

TITLE OF RESEARCH PROJECT: "A study to evaluate the effect of Low Level Laser Therapy (LLLT) on Dermal and Adipose Stem Cells".

RESEARCHER: **Dr T J Moore**

SUPERVISOR: **Prof H Abrahamse**

The Committee of Academic Ethics of the Faculty of Health Sciences evaluated the research proposal and consent letters of the above research project and confirms that it complies with the approved Ethical Research Standards of the University of Johannesburg.

The study supervisor and researcher demonstrated their intent to comply with the approved Ethical Research Standards during conduct of the research project.

Yours sincerely

PROF J Ferreira
CHAIRPERSON: FACULTY ACADEMIC ETHICS COMMITTEE

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APPENDIX C

CONSENT FORM

INFORMATION AND CONSENT FORM

DONATION OF EXCESS TISSUE

Dear Sir/Madam,

You are invited to donate any tissue left over from your surgery to the research project entitled: DIFFERENTIATION POTENTIAL OF ADIPOSE DERIVED STEM CELLS (ADSCs) WHEN CO-CULTURED WITH SMOOTH MUSCLE CELLS (SMCs) AND THE ROLE OF LOW INTENSITY LASER IRRADIATION (LILI).

Please take a few moments to read the attached information and consent form (including the terms and conditions thereof). Please inform your doctor or surgeon of your decision whether or not to participate in this research venture.

Should you have any questions that your doctor or surgeon are unable to answer, please do not hesitate to ask him/her to forward those particular questions onto the Laser Research Group, at the University of Johannesburg. We will make a speedy response to any such questions.

We hope that you will be able to assist us in our research efforts with your tissue donation.

Kind regards,

Prof H. Abrahamse
Laser Research Centre
University of Johannesburg

AUTHORISATION FOR DONATION OF EXCESS TISSUE

LASER RESEARCH GROUP

UNIVERSITY OF JOHANNESBURG

TITLE: DIFFERENTIATION POTENTIAL OF ADIPOSE DERIVED STEM CELLS (ADSCs) WHEN CO-CULTURED WITH SMOOTH MUSCLE CELLS (SMCs) AND THE ROLE OF LOW INTENSITY LASER IRRADIATION (LILI).

Principal Investigator: Prof H. Abrahamse

Tissue Donation

You are invited to donate any tissue left over from your surgery to a research project (s). The tissue collected will be used by researchers to investigate the Effects of Low Level Laser Therapy on Wound Healing.

In order to decide whether or not you wish to donate your tissue, you should know enough about its risks and benefits to make an informed decision. This form gives you information about the research and how the tissue is used. Once you understand the process, you will be asked if you wish to participate; if so, you will be asked to sign a form prior to surgery.

What happens to the tissue?

Tissue that is removed during surgery is sent to the Laser Research Group at the University of Johannesburg.

In addition to the donated tissue, only the following information will be required: Site of tissue removal, date of your surgery, date of birth, race, and gender.

The tissue will then be used to make primary cell cultures, which can then be used to study the Effects of Low Level Laser Therapy on Wound Healing. The tissue and any by-products, will *not* be used for cloning studies.

Any left-over tissue will be discarded.

Risks and Inconveniences

There are no known risks associated with donating your tissue for research. You will not be required to give any more tissue than that which will be taken during your surgery. If you choose not to donate your tissue, it will be discarded.

Benefits

You will not receive any direct benefit from donating your tissue to the research project. We hope that the information gained from the research studies will increase our knowledge of human health and disease, and that this information will lead to better treatments.

Economic Considerations

You will *not* receive any payments for donating your tissue to the research bank. Your tissue will only be used for research and will not be sold. The information we get from your sample may help to develop new products in the future, but you will not get paid.

Confidentiality

All identifiable information that is obtained in connection with your tissue will remain confidential. The researchers will only have the following information: site of tissue removal, date of your surgery, date of birth, race, and gender. Dr Doucas will document your donation on your medical record. When the results of the research are published or discussed in conferences, no personal information, other than the details mentioned above, will be included.

Voluntary Participation and Withdrawal

You are free to choose not to donate your tissue to research, however if you do become a donor, you will no longer have any rights to the tissue once it has been donated – Your permission will never expire.

If you choose not to donate it will not harm your relationship with your own doctors.

Questions

We have used some technical terms in this form. Please feel free to ask about anything you don't understand and to consider this donation and the consent form carefully – as long as you feel is necessary – before you make a decision.

Privacy Rights

All reasonable efforts will be made to protect the confidentiality of your tissue donation, which may be shared with others to support this research.

By agreeing to donate tissue, you give permission for the researchers to use that tissue (including the above mentioned details of the tissue) and any findings/results from the use of that tissue in research to be published.

You have a right to refuse to donate.

If you do not agree to donate, your tissue will not be used for research purposes, and will be discarded.

Authorisation

I have read (or someone has read to me) the Authorisation for Donation of Excess Tissue and have decided to donate my tissue to the Laser Research Centre, University of Johannesburg. Its general purposes, the

particulars of my involvement and possible hazards and inconveniences have been explained to my satisfaction. By signing below, I give permission for the described uses and disclosures of information. My signature also indicates that I have received a copy of the consent/authorisation form. I do not give up any of my legal rights by signing this form.

TICK ONE:

___ I wish to donate my leftover tissue to the Laser Research Group for research.

____ I do not wish to donate my leftover tissue to the Laser Research Group for research.

Details of donated tissue: (to be filled in by surgeon removing tissue)

Site: _____

Reference code: _____

Date of tissue removal: _____

Donor date of birth: _____

Gender: _____

Race: _____

Signature of Subject

_____ _____

Date

Print Name of Subject

_____

_____

Signature of Person Obtaining Consent Date

Tissue collected by _____

Signature_____

_____

Date

APPENDIX D

MATERIALS, COMPANIES, COUNTRIES AND CATALOGUE NUMBERS

Item	Company/Country	Catalogue number
DMEM F12	Sigma, Sigma-Aldrich, SA	D8062
HBSS	Sigma, Sigma-Aldrich, SA	H9394
Penicillin/Streptomycin	Sigma, Sigma-Aldrich, SA	P4333
Fungizone	Sigma, Sigma-Aldrich, SA	A2942
Collagenase Type-1	Gibco, Lifetechnologies, SA	17100-017
40 µM cell strainer	BD Biosciences, USA	352340
SKUT-1 cells	ATCC, USA	HTB-114
Stempro Human Adipose Derived Stem cells	Celtic Molecular Diagnostics, Invitrogen, SA	R7788-115
Adipose-Derived Mesenchymal Stem cells Normal Human	ATCC, USA	PCS-500-011
Biofreeze	Biocombiotech, SA	F2270
2 ml Cryogenic vials	Corning Incorporated, Scientific Group, SA	430489
Liquid Nitrogen	Afrox, SA	
EDTA	Merck, SA	223 60 20 EM
FBS	Biochrom, Biocombiotech, SA	S0615
75 cm^2 culture flasks	Corning Incorporation, Scientific Group, SA	431080
Cell culture dishes (3.4 cm^2)	Corning Incorporation, Scientific Group, SA	430165
50 ml Falcon tubes	Biocombiotech, SA	50050
MCDB 131	Gibco, Lifetechnologies, SA	10372-019
Retinoic Acid	Sigma, Sigma-Aldrich, SA	R2605
TGF-β1	Invitrogen, Lifetechnologies, SA	PHG9204
CFDA-SE	Invitrogen, Lifetechnologies, SA	C1157
Paraformaldehyde	Sigma, Sigma-Aldrich, SA	P6148

Propyl gallate	Sigma, Sigma-Aldrich, SA	02370
Triple Expless	Gibco, Lifetechnologies, SA	1260-028
Trypan Blue reagent	Invitrogen, Lifetechnologies, SA	T10282
ATP Glo-reagent	Promega, Amersham Biosciences, SA	G7573
OptiPlate^{TM}-96 well plates	PerkinElmer, USA	6005290
96 Well Plate Corning	Corning Incorporated, Scientific Group, SA	3598
Bovine Serum Albumin	Sigma, Sigma-Aldrich, SA	A9056
Azide	Analar, Sigma-Aldrich, SA	103692K
DAPI	Invitrogen, Lifetechnologies, SA	D1306
Anti-CD29 FITC	Invitrogen, Lifetechnologies, SA	CD2901
Anti-human CD90 FITC	Invitrogen, Lifetechnologies, SA	A15761
Anti-human Myosin Heavy Chain, Allophycocyanin	R&D Systems, Whitehead Scientific, SA	ABC J0110121
Triton X-100	USB Corporation, USA	22686
RNeasy Mini Kit	Whitehead Scientific, SA	74104
Quant-iT^{TM} RNA Assay Kit	Invirogen, SA	Q32852
SABiosciences RT^2 First Strand Kit	Whitehead Scientific, SA	330401
SABiosciences RT2 qPCR SYBR Green/Rox MasterMix-12	Whitehead Scientific, SA	330522
SABiosciences Mesenchymal Stem Cells PCR Array	Whitehead Scientific, SA	330231-PAHS-082Z-A-12

APPENDIX E

SOLUTIONS, MEDIUM AND CHEMICALS

Cell Isolation and Culture				
E1	Collagenase type-1 solution (600 U/ml-100 ml)	Collagenase type-1	240 U/mg	250mg
		HBSS		99.8 ml
		Calcium Chloride		200 µl
E2	DMEM F12 complete medium (100 ml)	DMEM F12		88.6 ml
		FBS	10%	10 ml
		Penicillin/Streptomycin	0.1%	1 ml
		Fungizone	1 µg/ml	400 µl
E3	Erythrocyte lysis buffer (500 ml)	NHCl		4.1 g
		$KHCO_3$		0.5 g
		EDTA		0.019 g
		Distilled H_2O		500 ml
Cell Differentiation				
E4	Complete MCDB 131 medium with RA (50 ml)	DMEM F12		48.79 ml
		RA	0.1 µM	10 µl
		FBS	1%	500 µl
		Penicillin/Streptomycin	0.1%	500 µl
		Fungizone	1 µg/ml	200 µl
E5	Complete MCDB 131 medium with TGF-β1 (50 ml)	MCDB 131		48.775 ml
		FBS	1%	500 µl
		TGF-β1	1 ng/ml	25 µl
		Penicillin/Streptomycin	0.1%	500 µl
		Fungizone		200 µl
E6	Complete MCDB 131 medium (50 ml)	MCDB 131		48.800 ml
		FBS	1%	500 µl
		Penicillin/Streptomycin	0.1%	500 µl
		Fungizone		200 µl
Immunofluorescence and Flow Cytometry				
E7	Anti-CD29 FITC (1 ml) 1:200	Antibody		1 µl
		PBS BSA/azide		199 µl
E8	Anti-human CD90 FITC (1 ml) 1:200	Antibody		1 µl
		PBS BSA/azide		199 µl
E9	Anti-human Myosin	Antibody		1 µl

	Heavy Chain (1ml) 1:200			
		PBS BSA/azide		199 µl
E10	PBS buffer (200 ml)	PBS tablets		1
		Distilled H_2O		200 ml
E11	PBS BSA/azide buffer (100 ml)	PBS		100 ml
		BSA	0.1%	0.1 g
		Azide	0.01%	0.01 g
E12	Paraformaldehyde 3.7% (100 ml)	Paraformaldehyde powder		3.7 g
		PBS BSA/azide buffer		100 ml
E13	Propyl gallate 0.1M (100 ml)	Propyl gallate powder		2.122g
		PBS BSA/azide buffer		100 ml
E14	CFDA,SE 10 µM (4.5 ml)	CFDA,SE powder		25 mg
		PBS BSA/azide buffer		4.5 ml

APPENDIX F

LASER PARAMETER CALCULATIONS

F1. Power Density

$$\frac{(mW \times 4)}{\pi(r)^2} = \frac{110 \times 4}{36.3168} = 12.1 \text{ mW/cm}^2$$

$$\text{In W/cm}^2 = \frac{12.1}{1000} = 0.0121 \text{ W/cm}^2$$

F2. Duration of Exposure

$$\frac{J/cm^2}{W/cm^2} = \text{Time (s)}$$

$$\frac{\text{Time (s)}}{60} = \text{time (min)}$$

Time (s) – (time (min) X 60) = Time (s)

For example:

$$\frac{J/cm^2}{W/cm^2} = \frac{5}{0.0121} = 413.22 \text{ sec}$$

$$\frac{413.22}{60} = 6 \text{ min}$$

6 X 60 = 360 sec

413-360 = 53 sec

Total duration of exposure = 6 min 53 sec

Research Article

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Research Article

Low Intensity Laser Irradiation and Growth Factors Influence Differentiation of Adipose Derived Stem Cells into Smooth Muscle Cells in a Coculture Environment over a Period of 72 Hours

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Stem cells have the ability to self-renew and differentiate into several specialised cells. Low intensity laser irradiation (LILI) has been shown to have positive effects on cells including adipose derived stem cells (ADSCs). Growth factors such as retinoic acid and transforming growth factor (TGF-β1) play significant roles in the differentiation of cells. This study aimed at investigating the role of LILI and growth factors on differentiation of adipose derived stem cells cocultured with smooth muscle cells (SMCs). The study used isolated human adipose derived stem cells and smooth muscle commercial cells (SKUT-1). The cells were cocultured directly in the ratio 1 : 1 using the established methods with and without growth factors (retinoic acid and TGF-β1) and then exposed to LILI at a wavelength of 636 nm with 5 J/cm^2 using a diode laser. The cellular proliferation and expression of the both cell type markers were assessed using optical density and flow cytometry at 24 h and 72 h. The study showed that LILI increased the proliferation of cocultured cells. The expression of the smooth muscle cell markers increased in the coculture groups that were exposed to LILI in the presence of growth factors while those of the ADSCs decreased.

1. Introduction

Stem cell treatment is becoming a promising therapy for many degenerative diseases [1]. One source of these cells is adipose tissue [2]. Bone marrow stem cells were commonly used in scientific and clinical applications but, due to their limited number, differentiation potential limits with age [3], and an invasive isolation procedure which may cause complications and death, ADSCs are now the preferred source [4]. Adipose derived stem cells (ADSCs) can be harvested from adipose tissue with ease and in abundance. These cells are easily cultured and maintain their mesenchymal stem cell pluripotency after many passages [5]. ADSCs are able to self-renew and differentiate into several lineages [6, 7]. Studies have shown that ADSCs could be differentiated into smooth muscle cells in the presence of the growth factors [8, 9]. These cells have also been differentiated into adipocytes, osteocytes, and neurons upon exposure to growth factors [10].

Smooth muscle cells form smooth muscle tissues. These tissues are major components of systems like cardiovascular, reproductive, urinary, and intestinal systems. Smooth muscle cells play a major role in diseases like cancer, asthma, arteriosclerosis, and hypertension since they constitute the main layer of smooth muscle tissues [9, 11]. Gastrointestinal smooth muscle diseases represent a major health problem affecting 2 million individuals every year [12]. Smooth muscle cell regeneration is required in the gastrointestinal tract as defects commonly occur [13].

Low intensity laser irradiation has shown different effects on several biological systems. It induces increased ATP production in mitochondria [14], elevation in collagen production in fibroblasts [15], and muscle regeneration processes

following injury [16]. LILI has shown to increase viability and proliferation of human fibroblast cells cultured in media with high glucose levels [17]. Cellular viability and proliferation have also been increased in ADSCs when exposed to LILI [18]. It has been shown to improve dental pulp stem cells when cultured in low nutritional conditions [19].

Growth factors are polypeptides that affect a number of cellular processes such as proliferation and differentiation both *in vivo* and *in vitro* [20]. Studies on retinoic acid have shown that it has several effects on cells including apoptosis, proliferation, differentiation, and maturation [21, 22]. Another growth factor, TGF-β1, plays a vital role in migration, angiogenesis, differentiation, proliferation, metastasis, and embryonic development [23, 24].

Beta1 integrin (CD29) is a protein that is encoded in humans by ITGB1 gene [25]. It is associated with a late antigen receptor. It is expressed by ADSCs as a cell surface marker [26]. Thymocyte differentiation antigen 1 (Thy-1 CD90) is used as a marker for a variety of mesenchymal stem cells [27]. Both CD29 and CD90 are expressed by ADSCs and have been confirmed as mesenchymal stem cell markers [28]. Myosin heavy chain is a cytoplasmic protein and a major component of SMCs [29]. It is a specific marker for smooth muscle differentiation. The expression of MHC is restricted to smooth muscle tissues [30].

The aim of the study was to investigate the role played by LILI and growth factors on differentiation of ADSCs when cocultured with smooth muscle cells monitored over a period of 72 h.

2. Materials and Methods

ADSCs were isolated from adipose tissue voluntarily donated by individuals undergoing abdominoplasty (Academic Ethics approval number 01/06). The isolation was done through collagenase digestion method as described in Mvula et al., 2010 [31]. After isolation, these were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D8062, SIgMA-Aldrich, Kempton Park, SA) with 10% foetal bovine serum (FBS) (Biochrom, S0615, Biocom biotech, Centurion, SA), 0.1% penicillin/streptomycin (Sigma, P4333, SIGMA-Aldrich, Kempton Park, SA), and 1 μg/mL fungizone (Sigma, A2942, SIGMA-Aldrich, Kempton Park, SA) incubated at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in a HERA CELL 150 (Heraeus, 44857, Separation Scientific, Honeydew, SA). A smooth muscle commercial cell line, SKUT-1, purchased from ATCC was also cultured in the same medium with similar conditions as ADSCs.

After reaching semiconfluency for ADSCs and confluency for SKUT-1, both cell types were cocultured directly in 3.4 cm^2 diameter dishes in a ratio 1 : 1 with and without the growth factors in MCDB 131 medium (Gibco, 10372-019, Life technologies, Roosevelt, SA) with 2% FBS, 0.1% penicillin/streptomycin, and 1 μg/mL fungizone incubated at 37°C in an atmosphere of 5% carbon dioxide (CO_2). The cocultures were divided into 6 groups. Group 1 were cocultures without the growth factors and were not exposed to LILI (CC); group 2 were cocultures without growth factors but were exposed to LILI (CC + LILI); group 3 were cocultures with the growth factor retinoic acid (RA) (Sigma, R2605, Sigma-Aldrich, Kempton Park, SA) and not exposed to LILI (CC + RA). Group 4 were with RA and exposed to LILI (CC + RA + LILI); group 5 were cocultures with a growth factor, transforming growth factor beta 1 (TGF-β1) (Invitrogen, PHG 9204, Life technologies, Roosevelt Park, SA) but not exposed to LILI (CC + TGF-β1) and the last is group 6 which had TGF-β1 and were exposed to LILI (CC + TGF-β1 + LILI). RA was added to the cocultures at a concentration of 0.1 μM and TGF-β1 at 1 ng/mL.

Table 1: Laser parameters.

Wavelength (nm)	636
Wave emission	Continuous wave
Power output (mW)	85
Spot size (cm^2)	9.08
Output density (mW/cm^2)	9.3
Irradiation duration	9 mins 10 s
Fluence (J/cm^2)	5

The cocultures of groups 2, 4, and 6 were exposed to diode laser (Oriel, Orroyo Instruments, LTIO00-PLT20, NLC, Pretoria, SA) at 5 J/cm^2 with a wavelength of 636 nm in the dark. The medium was removed and 1 mL of Hanks Balanced Salt Solution (HBSS) was added to the plates. Low laser irradiation was then delivered to the plate via the optical fibre as described previously [18, 31]. Cocultures which were not irradiated were used as controls and kept under the same conditions as the irradiated ones. The laser parameters are shown in Table 1.

Proliferation of the cocultures was analysed using optical density (OD) where the absorbance of one hundred microliters of the cell suspension was read at A_{540} nm in a Perkin Elmer, Victor^3 (Perkin Elmer, 1420, Separation Scientific, Honeydew, SA).

Cocultures were rinsed three times with Hanks Balanced Salt Saline (HBSS) (SIGMA, H9394, Sigma-Aldrich, Kempton Park, SA). The cells were then removed from the small plates by adding 500 μL of Triple Express (Life technologies 1260-028) and incubated at 37°C for 5 min. The cells were then washed in HBSS once and twice in PBS/BSA/azide. Antihuman CD29 (β1 integrin), antimouse CD90 (Thy-1), and antihuman myosin heavy chain (MHC) were then added and incubated for 30 min on ice. The cells were washed three times with PBS/BSA/azide and then fixed for 10 min in 3.7% formalin. Permeability was done by incubating the cell suspension in 1% triton x-100. The suspension was washed twice and the pellet was suspended in PBS, ready for reading with the BD FacsAria^{TM111} machine (BD Biosciences, 22300099, Scientific Group Biosciences, Woodmead, SA).

Sigma plot 11.0 software was used to analyse all experiments which were performed 6 times and assays in duplicates. Determination of the differences between the groups for each independent variable was done by using Student's *t*-test. Statistical significances comparing the groups are shown as

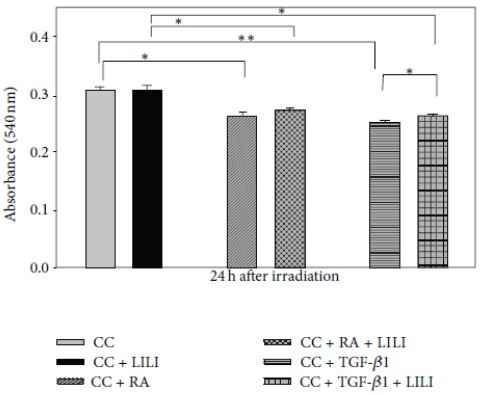

FIGURE 1: Cellular proliferation as assessed by optical density at 24 h after irradiation. Groups that were irradiated increased in proliferation as compared to those that were not exposed to irradiation. A decrease in proliferation was observed in groups that had growth factors as compared to those without growth factors.

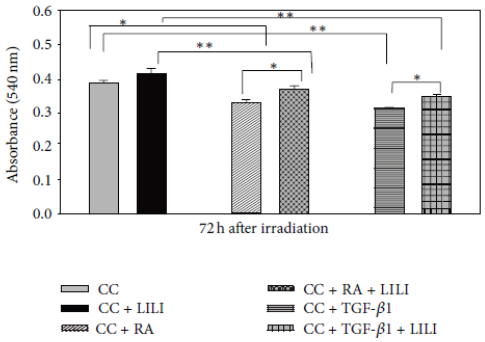

FIGURE 2: Cellular proliferation as assessed by optical density at 72 h after irradiation. Groups that were irradiated increased in proliferation as compared to those that were not exposed to irradiation but there was a decrease in the groups with growth factors as compared to those without growth factors.

star *, where $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) in the table and figures.

3. Results

The optical density results which measured cellular proliferation showed increases in cocultures that were irradiated as compared to their respective control groups and this was significantly observed in the groups that had TGF-β1 growth factor at 24 h ($P < 0.05$). The cocultures which were grown in the presence of growth factors had decreases in cellular proliferation. At 24 h, the proliferation significantly decreased in cocultures that were grown in the presence of RA and TGF-β1 as compared to those without growth factors (CC and CC + LILI) as seen in Figure 1.

Proliferation of the cells, as observed through optical density analysis at 72 h, showed a similar trend to that at 24 h. The cocultures that were grown in the presence of growth factors decreased in proliferation as compared to the cocultures that had no growth factors. The decrease was significant in groups with RA and TGF-β1 as compared to the groups without growth factors (CC and CC + LILI). The groups that were irradiated increased in proliferation compared to those that were not irradiated and this was significant in groups 1, 2, 3, 4, 5, and 6 (Figure 2).

The expression of the markers for both ADSCs (CD29 and CD90) and SMCs (MHC) was analysed through flow cytometry. CD29 expression decreased in the cocultures that were grown in the presence of growth factors at both 24 h and 72 h and the decrease was significant ($P < 0.05$) in the cocultures that had TGF-β1 and were irradiated (CC + TGF-β1 + LILI) at 72 h as compared to the control group (CC). At both 24 h and 72 h, CD90 expression decreased statistically significant in the cocultures that had TGF-β1 and were irradiated (CC + TGF-β1 and CC + TGF-β1 + LILI) as compared to the control group (CC) at 72 h with P values of less than 0.05 and 0.001, respectively. SMC marker, myosin heavy chain, showed an increase in the cocultures at 72 h though the increase was not statistically significant (Table 2). However, this may change if the expression of markers was followed for an additional extended period.

4. Discussion

Stem cells could be used for treatment of several diseases such as Parkinson's, stroke, diabetes, traumatic injury, and multiple sclerosis diseases. These diseases are caused by either loss or damage of the cells in the organs or tissues [32]. Stem cells have to be differentiated into cells which are required to repair or replace the lost or damaged cells. ADSCs have been shown to have a high plasticity capability. They have been able to differentiate into smooth muscle, neuron, bone, cartilage, and fat cells [9, 33]. Differentiating ADSCs into SMCs would assist in the treatment of diseases that affect diseases in the cardiovascular, intestinal, urinary, and reproductive systems [11, 34].

Studies on coculturing of cells have proved that differentiation can be increased due to the secretion of growth factors of the cells that will be differentiated into. Previous studies have shown that LILI can increase cell viability and proliferation [18, 25]. Growth factors have shown to have many effects on cells that include proliferation and differentiation and they play major roles in these processes [20–24]. The results in the present study showed a decrease in the proliferation of ADSCs and an increase in the proliferation of SMCs. This was observed through flow cytometry analysis; however, this could not be distinguished in the optical density analysis of the cocultures.

Table 2: Flow cytometric results for the expression of the markers for both ADSCs and SKUT-1 cells. CD29 and Thy-1 expressions decreased significantly at 72 h in cocultures that had TGF-β1 and were irradiated at 72 h. $^{*}P < 0.05$ and $^{**}P < 0.01$.

	CC	CC + LILI	CC + RA	CC + RA + LILI	CC + TGF-β1	CC + TGF-β1 + LILI
CD29 (β1 integrin)						
24 h	58.93	59.70	59.70	61.00	69.40	53.18
72 h	49.25	50.50	42.75	41.25	42.50	38.00*
CD90 (Thy-1)						
24 h	56.23	61.75	52.85	56.00	54.05	55.10
72 h	43.00	45.75	39.75	35.75	31.25*	24.50**
Myosin heavy chain						
24 h	71.70	73.25	69.18	68.63	65.73	67.85
72 h	72.53	78.50	76.38	81.20	73.23	80.55

In this study LILI increased the proliferation of cocultured cells; however, in the cocultures that had growth factors, proliferation decreased as compared to the cocultures without growth factors. This could have been due to the fact that proliferation of ADSCs was halted while differentiation into SMCs was initiated. Flow cytometry results concurred with this observation since a decrease in expression of the ADSCs markers in the cocultures was observed while those of the SMCs increased. This once again supports the argument that proliferation was inhibited since ADSCs were preparing for differentiation.

5. Conclusion

This study, in agreement with other studies done previously, found that LILI increases cell proliferation. LILI in combination with growth factors could differentiate ADSCs into SMCs. The study recommends that further investigations, especially, analysing the regulation of different genes involved in the differentiation of ADSCs into SMCs, are necessary to confirm differentiation. Once differentiation is confirmed, LILI and growth factors, such as RA and TGF-β1, would play major roles in the established direct coculturing method for the differentiation of stem cells into SMCs and this would be very beneficial in the stem cell therapy for many degenerative diseases which involves smooth muscle cells. However, significant further research and investigation are required to realise the clinical potential for cell therapy of ADSC differentiation into SMCs and the contributory role that LILI may have in this process.

Conflict of Interests

There is no conflict of interests regarding the publication of this paper.

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References

[1] M. F. Taha, "Cell based-gene delivery approaches for the treatment of spinal cord injury and neurodegenerative disorders," *Current Stem Cell Research and Therapy*, vol. 5, no. 1, pp. 23–36, 2010.

[2] H. Nakagami, R. Morishita, K. Maeda, Y. Kikuchi, T. Ogihara, and Y. Kaneda, "Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy," *Journal of Atherosclerosis and Thrombosis*, vol. 13, no. 2, pp. 77–81, 2006.

[3] S. M. Mueller and J. Glowacki, "Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges," *Journal of Cellular Biochemistry*, vol. 82, no. 4, pp. 583–590, 2001.

[4] S. Y. Lee, M. Miwa, Y. Sakai et al., "*In vitro* multipotentiality and characterization of human unfractured traumatic hemarthrosis-derived progenitor cells: a potential cell source for tissue repair," *Journal of Cellular Physiology*, vol. 210, no. 3, pp. 561–566, 2007.

[5] P. A. Zuk, M. Zhu, P. Ashjian et al., "Human adipose tissue is a source of multipotent stem cells," *Molecular Biology of the Cell*, vol. 13, no. 12, pp. 4279–4295, 2002.

[6] S. Jang, H.-H. Cho, Y.-B. Cho, J.-S. Park, and H.-S. Jeong, "Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin," *BMC Cell Biology*, vol. 11, article no. 25, 2010.

[7] T. Huang, D. He, G. Kleiner, and J. Kuluz, "Neuron-like differentiation of adipose-derived stem cells from infant piglets *in vitro*," *Journal of Spinal Cord Medicine*, vol. 30, no. 1, pp. S35–S40, 2007.

[8] J. A. de Villiers, N. N. Houreld, and H. Abrahamse, "Influence of low intensity laser irradiation on isolated human adipose derived stem cells over 72hrs and their differentiation potential into smooth muscle cells using retinoic acid," *Stem Cell Reviews and Reports*, vol. 7, no. 4, pp. 869–882, 2011.

[9] L. V. Rodríguez, Z. Alfonso, R. Zhang, J. Leung, B. Wu, and L. J. Ignarro, "Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 32, pp. 12167–12172, 2006.

[10] L. Hu, J. Hu, J. Zhao et al., "Side-by-side comparison of the biological characteristics of human umbilical cord and adipose

tissue-derived mesenchymal stem cells," *BioMed Research International*, vol. 2013, Article ID 438243, 12 pages, 2013.

[11] S. Sinha, B. R. Wamhoff, M. H. Hoofnagle et al., "Assessment of contractility of purified smooth muscle cells derived from embryonic stem cells," *Stem Cells*, vol. 24, no. 7, pp. 1678–1688, 2006.

[12] V. Gabetta, W. Trzyna, C. Phiel, and K. M. McHugh, "Vesicle-associated protein-A is differentially expressed during intestinal smooth muscle cell differentiation," *Developmental Dynamics*, vol. 228, no. 1, pp. 11–20, 2003.

[13] K. M. McHugh, "Molecular analysis of gastrointestinal smooth muscle development," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 23, no. 4, pp. 379–394, 1996.

[14] S. Passarella, E. Casamassima, S. Molinari et al., "Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated *in vitro* by helium-neon laser," *FEBS Letters*, vol. 175, no. 1, pp. 95–99, 1984.

[15] I. B. Kovacs, E. Mester, and P. Gorog, "Stimulation of wound healing with laser beam in the rat," *Experientia*, vol. 30, no. 11, pp. 1275–1276, 1974.

[16] A. C. Amaral, N. A. Parizotto, and T. F. Salvini, "Dose-dependency of low-energy HeNe laser effect in regeneration of skeletal muscle in mice," *Lasers in Medical Science*, vol. 16, no. 1, pp. 44–51, 2001.

[17] M. Esmaeelinejad, M. Bayat, H. Darbandi, M. Bayat, and N. Mosaffa, "The effects of low-level laser irradiation on cellular viability and proliferation of human skin fibroblasts cultured in high glucose mediums," *Lasers in Medical Science*, vol. 29, no. 1, pp. 121–129, 2014.

[18] B. Mvula, T. Mathope, T. Moore, and H. Abrahamse, "The effect of low level laser irradiation on adult human adipose derived stem cells," *Lasers in Medical Science*, vol. 23, no. 3, pp. 277–282, 2008.

[19] F. D. P. Eduarde, D. F. Bueno, P. M. De Freitas et al., "Stem cell proliferation under low intensity laser irradiation: a preliminary study," *Lasers in Surgery and Medicine*, vol. 40, no. 6, pp. 433–438, 2008.

[20] A. S. Goustin, E. B. Leof, G. D. Shipley, and H. L. Moses, "Growth factors and cancer," *Cancer Research*, vol. 46, no. 3, pp. 1015–1029, 1986.

[21] V. Duong and C. Rochette-Egly, "The molecular physiology of nuclear retinoic acid receptors. From health to disease," *Biochimica et Biophysica Acta*, vol. 1812, no. 8, pp. 1023–1031, 2011.

[22] L. J. Gudas and J. A. Wagner, "Retinoids regulate stem cell differentiation," *Journal of Cellular Physiology*, vol. 226, no. 2, pp. 322–330, 2011.

[23] R. Zhang, G. S. Jack, N. Rao et al., "Nuclear fusion-independent smooth muscle differentiation of human adipose-derived stem cells induced by a smooth muscle environment," *Stem Cells*, vol. 30, no. 3, pp. 481–490, 2012.

[24] R. A. Rahimi and E. B. Leof, "TGF-β signaling: a tale of two responses," *Journal of Cellular Biochemistry*, vol. 102, no. 3, pp. 593–608, 2007.

[25] P. J. Goodfellow, H. A. Nevanlinna, P. Gorman, D. Sheer, G. Lam, and P. N. Goodfellow, "Assignment of the gene encoding the beta-subunit of the human fibronectin receptor (β-FNR) to chromosome 10p11.2," *Annals of Human Genetics*, vol. 53, no. 1, pp. 15–22, 1989.

[26] K. Yoshimura, T. Shigeura, D. Matsumoto et al., "Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates," *Journal of Cellular Physiology*, vol. 208, no. 1, pp. 64–76, 2006.

[27] N. M. Masson, I. S. Currie, J. D. Terrace, O. J. Garden, R. W. Parks, and J. A. Ross, "Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1," *American Journal of Physiology*, vol. 291, no. 1, pp. G45–G54, 2006.

[28] F. Zahran, M. Salam, A. Loffy, and I. M. El-Deen, "Isolation and characterisation of adipose tissue-derived stem cells: an *in vitro* study," *Basic Research Journal of Medicine and Clinical Sciences*, vol. 1, no. 5, pp. 88–94, 2012.

[29] M. M. Nicolas, P. Tamboli, J. A. Gomez, and B. A. Czerniak, "Pleomorphic and dedifferentiated leiomyosarcoma: clinicopathologic and immunohistochemical study of 41 cases," *Human Pathology*, vol. 41, no. 5, pp. 663–671, 2010.

[30] M. Aikawa, Y. Sakomura, M. Ueda et al., "Redifferentiation of smooth muscle cells after coronary angioplasty determined via myosin heavy chain expression," *Circulation*, vol. 96, no. 1, pp. 82–90, 1997.

[31] B. Mvula, T. J. Moore, and H. Abrahamse, "Effect of low-level laser irradiation and epidermal growth factor on adult human adipose-derived stem cells," *Lasers in Medical Science*, vol. 25, no. 1, pp. 33–39, 2010.

[32] K. M. Safford and H. E. Rice, "Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells," *Current Drug Targets*, vol. 6, no. 1, pp. 57–62, 2005.

[33] N. Serakinci and W. N. Keith, "Therapeutic potential of adult stem cells," *European Journal of Cancer*, vol. 42, no. 9, pp. 1243–1246, 2006.

[34] P. Carmeliet, "Mechanisms of angiogenesis and arteriogenesis," *Nature Medicine*, vol. 6, no. 4, pp. 389–395, 2000.

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VERSITA

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The role of adipose derived stem cells, smooth muscle cells and low intensity laser irradiation (LILI) in tissue engineering and regenerative medicine

Mini-Review

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Abstract: Tissue engineering and regenerative medicine has become the treatment of choice for several degenerative diseases. It involves the repairing or replacing of diseased or damaged cells or tissues. Stem cells have a key role to play in this multidisciplinary science because of their capacity to differentiate into several lineages. Adipose derived stem cells (ADSCs) are adult mesenchymal stem cells that are easily harvested and have the capacity to differentiate into cartilage, bone, smooth muscle, fat, liver and nerve cells. ADSCs have been found to differentiate into smooth muscle cells which play major roles in diseases such as asthma, hypertension, cancer and arteriosclerosis. Low Intensity Laser Irradiation (LILI), which involves the application of monochromatic light, has been found to increase viability, proliferation and differentiation in several types of cells including ADSCs. This review discusses the role of ADSCs, smooth muscle cells and LILI in the science of tissue engineering and regenerative medicine.

Keywords: *Adipose Derived Stem Cells • Smooth muscle cells • Low Intensity Laser Irradiation • Tissue engineering and regenerative medicine*

1. Introduction

Tissue engineering and regenerative medicine is a new multidisciplinary science that combines growth factors, biomaterials and stem cells to repair tissues and damaged organs [1]. Myocardial ischemia as well as cardiovascular disease is becoming a major health problem in developed as well as developing countries, being the third cause of death after cancer and violent deaths. By 2007, in France, 32% of the deaths were caused by cardiovascular disease [2]. Since stem cells have the ability to self renew and differentiate into multiple lineages [3], these cells could be used in cell-based therapy for repairing and regenerating various tissues and organs. Many studies have been done involving LILI and stem cells, both *in vitro* and *in vivo*. Results have shown that LILI can produce negative and positive effects on stem cells. Positive results include inhibition of inflammation and increased cell viability and proliferation [4,5]. Due to ease of isolation and plasticity [6], ADSCs hold great promise for tissue engineering and regenerative medicine [7]. These cells have been differentiated into smooth muscle cells with the help of growth factors and LILI [8].

2. Stem cells

Stem cells have the capacity to proliferate and renew themselves. These cells are able to differentiate into several other cell lineages [9,10]. They are cells that are able to provide replacement for particular differentiated cell types [11]. These cells can divide and renew for a long period. Since they are not specialised and are not matured, they do not carry out specialised tissue-specific functions. Stem cells can only differentiate into one specific type of cell [12-15]. This makes stem cells suitable for repairing and replacing cells in patients. The most commonly used stem cell types are embryonic and adult stem cells [11,16]. Due to resistance towards the use of embryonic stem cells [11,17], as it involves the destruction of embryos

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[18-20], current studies mostly involve the use of adult stem cells. The use of stem cells obtained from adult tissues avoids the controversial issues surrounding the ethical use of embryonic stem cells [21].

3. Adipose derived stem cells

Adipose derived stem cells are harvested from adipose tissue and resemble those from bone marrow [22]. Due to their ability to be isolated easily and plasticity, ADSCs are more preferred than bone marrow stem cells [23,24]. Studies have shown that these cells can differentiate into bone, cartilage, fat or muscle, a property that makes them very suitable for tissue engineering [25]. Adipose derived stem cells have been shown to differentiate into cardiomyoctyes in the presence of laminin [26]. They have also been differentiated into smooth muscle cells that are functional [27]. These cells have also shown increased viability and proliferation when exposed to LILI alone [5] and, as well as, in combination with epidermal growth factor (EGF) [28]. Recent studies have demonstrated the functional neural differentiation of human ADSCs [29]. Adipose stem cell treatments have been used in animals without adverse effects [30] and several clinical trials are underway in humans that have shown no adverse effects to date [31]. However, ADSCs are not immortal and their source (adipose tissue), they vary in their metabolic activity, and their differentiation capacity depends on the location of the tissue and the age and gender of the patient [32,33].

4. Smooth muscle cells

Studies have shown that stem cells from human adipose tissue differentiate into functional smooth muscle cells [27]. Smooth muscles are major components of cardiac, reproductive, urinary and intestinal systems. They play a key role in several diseases like asthma, cancer, hypertension and arteriosclerosis [27,34]. Recent studies have shown that mesenchymal derived stem cells can differentiate into smooth muscle cells in the presence of growth factors such as retinoic acid, transforming growth factor (TGF), ascorbic acid and platelet growth factor (PGF). These muscle cells have also been differentiated from ADSCs when exposed to LILI [8,27,34-36].

5. Low Intensity Laser Irradiation

Low intensity laser irradiation, a phototherapy, has been found to induce a biostimulatory effect in several tissues. It involves the application of monochromatic light to tissue [37]. The tissue respond differently to specific wavelengths and dosages [38,39]. Some of the effects of LILI on ADSCs are summarised in Table 1 below [37].

6. Tissue engineering and regenerative medicine

Tissue engineering and regenerative medicine is a novel and interdisciplinary science that involves growth

Study	Laser Parameters	Differentiation Inducer (DI)	Results	References
ADSCs & LILI	5 J/cm² 635 nm	No DI	Increased viability & proliferation	[5]
ADSCs & LILI	5 J/cm² 636 nm	EGF	Increased viability & proliferation	[28]
ADSCs & LILI	10 & 15 J/cm² 830 nm	No DI	Decreased viability & proliferation	[51]
ADSCs	No LILI	Retinoic Acid	Increased viability, proliferation & differentiation	[8]
ADSCs & LILI	5 J/cm² 636 nm	Retinoic Acid	ADSC differentiation into smooth muscle cells	[8]
ADSCs	No LILI	Angiotensin II, Sphingosylphospho-rylcholine, TGF-β1	ADSC differentiation into smooth muscle cells	[52]
ADSCs trans-planted in ischemic mouse limbs	No LILI	No DI	Enhancement of angiogenesis & osteogenesis	[53,54]
ADSCs	No	LILI Heparin	Differentiation into smooth muscle cells	[27]

Table 1. Studies conducted on ADSCs and/or differentiation inducers and/or LILI [37].

factors and stem cells to repair tissues and organs [40-42]. ADSCs are mesenchymal stem cells and have been isolated from adipose tissue through collagenase enzyme digestion and centrifugation [43]. These cells are potential candidates for cell based therapies as they are able to differentiate into several lineages [25,41].

Scientists have postulated a number of mechanisms through which ADSCs could be used in tissue engineering and regenerative medicine. These cells can secrete cytokines and growth factors and stimulate recovery in a paracrine way. They are also able to stimulate the recruitment of stem cells from the stem cell niche, enhancing differentiation of a particular required lineage [44]. ADSCs could be differentiated along a particular lineage and applied to a particular damaged organ [40]. Stem cells might also provide antioxidants, free radical scavengers and heat shock proteins to an ischemic site thereby recovering the cells. Some studies have suggested that ADSCs could deliver new mitochondria to the damaged area thereby promoting aerobic metabolism [45].

ADSCs have been used to heal chronic fistulas in Crohns disease [43]. Recent studies have shown that neurotrophic factors have been expressed by ADSCs and cells isolated from abdominal fat layer suggesting that these cells could be used for *in vivo* generation of nerves in the future [46]. The effective treatment for liver failure is liver transplantation, but this has been associated with donor shortages and high costs. Regenerative medicine and tissue engineering could provide an alternative to this treatment [47]. Hepatic differentiation of human embryonic stem cells was successfully induced by adding bFGF, TGFβ1, Activin-A, bone morphogenic protein4, hepatocyte growth factor, epidermal growth factor, β nerve growth factor and retinoic acid [48], suggesting that there may be a possibility of hepatic differentiation from ADSCs. Another type of disease that could benefit from this treatment is Type 1 diabetic mellitus, which results from the autoimmune mediated destruction of Langerhans cells in the pancreas [49]. However, studies have shown that isolated ADSCs - when auto-transplanted - would be a possible tool for the cure of this disease [50].

Considering the advances demonstrated over the last ten years with respect to stem cell based therapy and the possibilities that the identification of different growth factors and other augmenting agents such as LILI may introduce to enhance the spectrum and uses in regenerative medicine, we are proposing that LILI may in fact have a significant role to play in this process (Figure 1).

7. Conclusions

The success of tissue engineering and regenerative medicine requires a number of factors as discussed in this review. For successful therapeutic use, adipose derived stem cells need to be isolated and proliferated extensively to generate adequate numbers of cells prior to differentiation into the required cell type. Since many degenerative diseases involve dysfunctional smooth muscle cells, differentiation of adipose derived stem cells into smooth muscle cells offer significant therapeutic promise provided proliferation and differentiation can be accomplished successfully. LILI has been found to play a major role in inducing the production of growth factors, cell markers, cytokines, adenosine triphoshate and other factors that play major roles in differentiating stem cells. However, LILI protocols have to be established and standardised in order for its safe and confirmed role in tissue engineering and regenerative medicine.

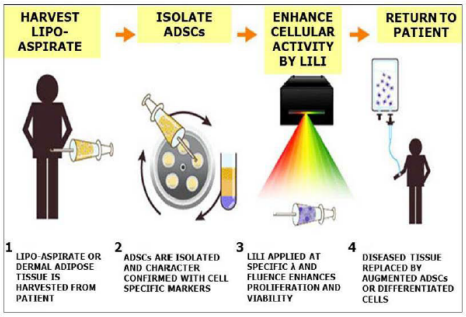

Figure 1. Four stages from harvesting to the use of ADSCs.

References

[1] Butler D.L., Gostein S.A., Guilak, F., Functional Tissue Engineering: The Role of Biomechanics, J. Biomed. Eng., 2000, 122, 570-575

[2] Roche R., Hoareau L., Mounet F., Festy F., Adult Stem Cells for Cardiovascular Diseases: The Adipose Tissue Potential, Expert. Opin. Biol. Th., 2007, 7, 1-8

[3] Jang S., Cho H., Cho Y., Park J., Jeong H., Functional Neural Differentiation of Human Adipose Tissue-Derived Stem Cells using bFGF and Forskolin, BMC Cell Biol., 2010, 11, 25, DOI:10.1186/1471-2121-11-25

[4] Lin F., Josephs S.T., Alexandrescu D.T., Ramos F., Bogin V., Gammill V., et al., Lasers, Stem Cells, and COPD, J. Translat. Med., 2010, 8, 16, DOI:10.1186/1479-5876-8-16

[5] Mvula B., Mathope T., Moore T.J., Abrahamse H., The Effects of Low Level Laser Irradiation on Human Adipose Derived Stem Cells, Laser Med. Sci., 2008, 23, 277-282

[6] Fraser J.K., Wulur I., Alfonso Z., Hedrick M., Fat Tissue: An Underappreciated Source of Stem cells for Biotechnology, Trends Biotech., 2006, 24,150-115

[7] Huh C.H., Kim S.Y., Cho H.J., Kim D.S., Lee W.H., Kwon S.B., et al., Effects of Mesenchymal Stem Cells in the Reconstruction of Skin Equivalents, J. Dermalog. Sci., 2000, 46, 217-220

[8] de Villiers J.A., Houreld N.N., Abrahamse H., Influence of Low Intensity Laser Irradiation on Isolated Human Adipose Derived Stem Cells over 72hrs and Their Differentiation Potential into Smooth Muscle Cells Using Retinoic Acid, Stem Cell Rev. Rep., 2011, 7, 869-882

[9] Spradling A., Drummond-Barbosa D., Kai T., Stem Cells find their Niche, Nature, 414, 98–104

[10] Reya T., Morrison S., Clarke M.F., Weissman I., Stem Cells, Cancer, Cancer Stem Cells, Nature, 2001, 414, 105–111

[11] Ballas C.B., Zielske S.P., Gerson S.L., Adult Bone Marrow Stem Cells for Cell and Gene Therapies: Implications for greater use, J. Cell Biochem. Sup., 2002, 38, 20-28

[12] Ramsay M.A.E., Will Stem Cells Transform Medicine, Proc. Bayl. Univ. Med. Cent., 2002, 15, 135-137

[13] Conrad C., Huss R., Adult Stem Cells Lines in Regenerative Medicine and Reconstructive Surgery, J. Surg. Res., 2005, 124, 201-208

[14] Pelled G.G.T., Aslan H., Gazit Z., Gazit D., Mesenchymal Stem Cells for Bone Gene Therapy and Tissue Engineering, Curr. Pharma. Design, 2002, 8, 1917-1928

[15] Habib N.A., Levicar N., Jiao L., Black G.T., Stem Cell Repair and Regeneration, Imperial College Press, World Scientific Publishing, 2005

[16] Minguell J.J., Erices A., Conget P., Mesenchymal Stem Cells, Exper. Biol. Med., 2001, 226, 507-520

[17] Suh H., Tissue Restoration, Tissue Engineering and Regenerative Medicine, Yonsei Med. J., 2000, 41, 681-684

[18] Kaji E.H., Leiden J.M., Gene and Stem Cell Therapies, J. Amer. Med. Ass., 2001, 285, 545-550

[19] Perry D., Patients' Voices: The Powerful Sound in the Stem Cell Debate, Science, 2000, 287, 1423

[20] Young F.E., A Time for Restraint, Science, 2007, 287, 1424

[21] Gamillion C.T., Burg K.J.L., Stem Cells and Adipose Tissue Engineering, Biomat., 2006, 27, 6052-6063

[22] Peroni D., Scambi I., Pasini A., Lisi V., Bifari F., Krampera M., et al., Stem Molecular Signature of Adipose-Derived Stromal Cells, Exper. Cell Res., 2008, 314, 603-615

[23] Strem B.M., Hedrick M.H., The growing Importance of Fat in Regenerative Medicine, Trends Biotech., 2005, 23, 64-66

[24] Strem B.M., Hicok K.C., Zhu M., Wulur I., Alfonso Z., Schreiber R.E., Multipotential Differentiation of Adipose Tissue-Derived Stem Cells, Keio J. Med., 2005, 54, 132-141

[25] Zuk P.A., Zhu M., Mizuno H., Huang J., Furtell J.W., Kartz A.J., Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies, Tissue Eng., 2001, 7, 211-228

[26] Van Dijk A., Niessen H.W.M., Zandieh Doulabi B., Visser F. C., van Milligen F.J., Differentiation of Human Adipose-Derived Stem Cells towards Cardiomyocytes is Facilitated by Laminin, Cell Tissue Res., 2008, 334, 457-467

[27] Rodriguez L.V., Alfonso Z., Zhang R., Leung J., Wu B., Ignarro L.J., Clonogenic Multipotent Stem Cells in Human Adipose Tissue Differentiate into Functional Smooth Muscle Cells, Proc. Nat. Acad. Sci., 2006, 103, 12167-12172

[28] Mvula B., Moore T., Abrahamse H., Effects of Low-Level Laser Irradiation and Epidermal Growth Factor on Adult Human Adipose-Derived Stem Cells, Laser Med. Sci. 2010, 25, 33-39

[29] Jang S., Cho H., Cho Y., Park J., Jeong H., Functional Neural differentiation of Human

AdiposeTissue-Derived Stem Cells using bFGF and Forskolin, Cell Biol., 2010, 11, 25
[30] Vet-Stem Regenerative Veterinary Medicine, 2008, http://www.marketwire.com/press-release/vet- stem-announces-milestone-of-8000-animals-treated-with-vet-stem-cell-therapy-1611912.htm
[31] Riordan N.H., Ichim T.E., Min W.P., Wang H., Solano H., Lara F., et al., Non-Expanded Adipose Stromal Vascular Fraction Cell Therapy for Multiple Sclerosis, J. Translat. Med., 2009, 7, 29, DOI: 10.1186/1479-5876-7-29
[32] Tholpady S.S., Llull R., Ogle R.C., Rubin J.P., Futrell J. W., Katz A.J., Adipose Tissue: Stem Cells and Beyond, Clin. Plastic Surg., 2006, 33, 55-62
[33] Giorgino F., Laviola L., Eriksson J.W., Regional Differences of Insulin Action in Adipose Tissue: Insights from in vivo and in vitro Studies, Acta Physiol. Scandanavica, 2005, 185, 13-30
[34] Sinha S., Wamhoff B.R., Hoofnagle M.H., Thomas J., Neppi,R.L., Deering T., Assessment of Contractility of Purified Smooth Muscle Cells Derived from Embryonic Stem Cells, Stem Cells, 2006, 24, 1678-1688
[35] Yang Y., Relan N K., Przywara D.A., Schugar L., Embryonic Mesenchymal Cell Share the Potential for Smooth Muscle Differentiation: Myogenesis is Controlled by the Cell, shape, Development, 1999, 126, 3027-3033
[36] Narita Y., Yamawaki A., Kagami H., Ueda M., Ueda Y., Effects of Transforming Growth Factor-Beta1 and Ascorbic Acid on Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells into Smooth Muscle Cell Lineage, Cell Tissue Res., 2008, 333, 449-459
[37] Mvula B., Abrahamse H., Adipose Derived Stem cells and Low Intensity Laser Irradiation: Potential Use in Regenerative Medicine, Proc. South Afric. Inst. Phys., 2011, 707-710
[38] Abrahamse H., The Use of Laser Irradiation To Stimulate Adipose Derived Stem Cell Proliferation and Differentiation for Use in Autologous Grafts, Amer. Inst. Phys., 2009, 1172, 95-100
[39] Renno A.C.M, McDonnell P.A, Parizotto P.A., Laakso E.L., The Effects of Laser Irradiation on Osteobast and Osteosarcoma Cell Prolifertion and Differentiation in Vitro, Photomed. Laser Surg., 2007, 25, 275-280
[40] Gimble J.M., Katz A.J., Bunnell B.A., Adipose-Derived Stem Cells for Regenerative Medicine, Circ. Res., 2007, 100, 1249-1260
[41] Zuk P.A., Zhu M., Mizuno H., Huang J., Furtell J.W., Kartz A.J., et al., Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies, Tissue Eng., 2001, 7, 211-228
[42] Kim J.M., Lee S., Chu K., Jung K., Song E., Kim S., Systemic Transplantation of Human Adipose Stem cells attenuated Cerebral Inflammation and Degeneration in a Hemorrhagic Stroke Model, Brain Res., 2007, 1183, 43-50
[43] Garcia-Olmo D., Garcia-Arranz M., Herrers D., Expanded Adipose-Derived Stem Cells for the Treatment of Complex Perianal Fistula including Crohn,s Disease, Expert Opin. Biol. Th., 2008, 8, 417-1423
[44] Kachgal S., Putnam A.J., Mesenchymal Stem Cells from Adipose and Bone Marrow promote Angiogenesis via Disctinct Cytokine and Protease Expression Mechanisms, Angiogenesis, 2011, 14, 47-59
[45] Spees J.L., Olson S.D., Whitney M.J. Prockop D.J., Mitochondrial Transfer between Cells can Rescue Aerobic Respiration, Proc.Nat. Acad. Sci., USA, 2006, 103, 1283-1288
[46] Kalbermatten D.F., Shaakxs D., Kingham P.J., Wiberg, M., Neurotrophic Activity of Human Adipose Stem Cells Isolated from Deep and Superficial Layers of Abdominal Fat, Cell Tissue Res., 2011, 344, 251-260
[47] Ogawa S., Miyagawa S., Potentials of Regenerative Medicine for Liver Disease, Surg. Today, 2009, 39, 1019-1025
[48] Schuldiner M., Yanuka O., Itskovitz-Eldor J., Melton D.A., Benvenisty N., Effects of Eight Growth Factors on the Differentiation of Cells Derived from Human Embryonic Stem Cells, Proc. Nat. Acad. Sci., 2000, 97, 11307-11312
[49] Shapiro A.M., Ricordi C., Hering B.J., Auchinclos H., Lindblad R., Robertson R.P., et al., International Trial of the Edmonton Protocol for Islet Transplantation, New Eng. J. Med., 2006, 355, 1318-1330
[50] Couri C.E., Oliveira M.C., Stracieri A.B., Moraes D.A., Pieroni F., Barros G.M., et al., C-peptide Levels and Insulin Independence following Autologous Nonmyeloablative Hematopoietic Stem Cell Transplantation in Newly Diagnosed Type 1 Diabetic Mellitus, J. Amer. Med. Assoc., 2009, 301, 1573-1579
[51] Abrahamse H., Houreld N.N., Muller S., Ndhlovu L., Fluence and Wavelength of Low Intensity Laser Irradiation Affect Activity and Proliferation of Human Adipose Derived Stem Cells, Med. Tech. SA, 2010, 24, 9-14
[52] Harris L.J., Abdollahi H., Zhang P., McIlhenny S., Tulenko T.N., DiMuzio P.J., Differentiation of Adult

Stem Cells into Smooth Muscle Cells for Vascular Tissue Engineering, J. Surg. Res., 2011, 168, 306-314

[53] Nakagami H., Maeda K., Morishita R., Iguchi S., Nishikawa T., Takami Y., et al., Novel Autologous Cell Therapy in Ischemic Limb Disease Through Growth Factor Secretion by Cultured Adipose Tissue-Derived Stromal Cells, Arteriosclerosis Thrombosis Vasc. Biol., 2005, 25, 2542-2547

[54] Shoji T., Li M., Mifune Y., Matsumoto T., Kawamoto A., Kwon S., et al., Local Transplantation of Human Multipotent Adipose-Derived Stem Cells Accelerates Fracture Healing via Enhanced Osteogenesis and Angiogenesis, Lab. Investig., 2010, 90, 637-649

Adipose Derived Stem Cells (ADSCs) and Low Intensity Laser Irradiation (LILI): Potential use in Regenerative Medicine

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Abstract. Adipose tissue is highly specialised and a reliable source of adult stem cells. ADSCs can be harvested easily and in large amounts from adipose tissue or lipo-aspirates. Collagenase digestion is one of the procedures used in generating these cells from adipose tissue. ADSCs have been shown to have the differentiation potential to differentiate into other cells including smooth muscle, bone, nerve, heart, cartilage, liver and fat. LILI, a form of phototherapy, involves the application of monochromatic light in the 630 to 905 nm (visible to near infrared) range to biological tissue. Application to a variety of different cell types has been found to enhance cell viability, proliferation and differentiation of ADSCs. The differentiated cells from ADSCs are a major component of cardiovascular, reproductive, urinary, neural and intestinal systems and play a key role in diseases like arteriosclerosis, asthma, hypertension and cancer. These cells could be used in regenerative medicine and tissue engineering. This review discusses ADSCs and LILI and their potential use in regenerative medicine.

1. Adipose Derived Stem Cells

Adult stem cells can be isolated from adipose tissue and lipo-aspirates in significant numbers and exhibit stable growth and proliferative kinetics in culture. These cells are termed ADSCs [1]. ADSCs have been isolated from donors with reduced morbidity, they have been multiplied and handled easily compared to bone marrow cells [2]. In the past decade several studies have provided preclinical data on safety and efficacy of these cells, supporting the use of these cells in future clinical applications [3]. Adipose tissue as a source of these cells allows them to be obtained in large quantities without difficulties and at a minimal risk [4]. White adipose tissue can be obtained in large quantities from human tissue and the stem cells residing in it are easily harvested from the tissue with the ability to differentiate into several cell lineages [5,6].

Cells generated from adipogenic origin have also shown to differentiate not only into osteoblasts, chondrocytes, myocytes, cardiomyocytes, fibroblasts and adipocytes but also into vascular lineages such as endothelial, smooth muscle blood cells [7-11]. This is due to the fact that they can release potent angiogenic factors such as leptin and vascular endothelial growth factor [12,13]. In cell transplantation, ADSCs have been found to promote radiological ossification efficiently, 90% of fractures healed eight weeks after surgery and during this process, blood perfusion enhancement through neovasculatisation was observed [14]. These cells have been shown to restore dystrophin expression of Duchenne skeletal-muscle cells *in vitro* [15]. ADSCs may constitute a potential cell based therapeutic alternative for the treatment of pancreatic ductical adenocarcinoma (PDAC) after being found to strongly inhibit human PDAC cell proliferation both *in vivo* and *in vitro* [16].

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Due to their plasticity and easy harvesting, ADSCs are the preferred stem cells to be used in tissue engineering rather than bone marrow derived stem cells [17,18]. However, ADSCs have certain limiting characteristics due to several factors. First, their capacity is limited in terms of subculturing and secondly, adipose tissue varies in its metabolic activity and as well as its capacity for proliferation and differentiation depending on the location of the depot of tissue and the age and gender of the patient [19,20].

2. Low Intensity Laser Irradiation

LILI, a form of phototherapy, involves the application of monochromatic light to biological tissue to elicit a biomodulative effect within that tissue. LILI is now accepted in many countries and is used in medical and dental practices. It elicits both a photobiostimulative and a photobioinhibitive effect within the irradiated tissue, each of which can be used in a number of therapeutic applications. LILI is not thermic (it does not produce heat) [21], and uses monochromatic light in the 630 to 905 nm range of the electromagnetic spectrum [22]. It stimulates capillary growth, granulation tissue formation and alters cytokine production. Altered keratinocyte motility and fibroblast movement have also been shown following low intensity laser irradiation [23]. These effects aid in the treatment of disorders like acute or chronic tissue hypoxia, destruction of tissues, as well as altered cell metabolism. Studies on LILI and stem cells have shown that low intensity irradiation can alter the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration [24].

It has been shown that 5 J/cm^2 of laser irradiation at a wavelength of 635 nm positively affects ADSCs by increasing cellular proliferation, viability, and expression of β1-Integrin and Thy-1 (established stem cell markers), [25] and LILI in combination with epidermal growth factor (EGF) enhances the proliferation of ADSCs [26]. Several studies have been conducted on ADSCs identifying the effects of LILI at a cellular and molecular level (Table1).

Table 1. Studies conducted on ADSCs and/or differentiation inducers and/or LILI.

Study	Laser Parameters/ Differentiation Inducer (DI)		Results	References
ADSCs & LILI	5 J/cm^2 635 nm	No DI	Increased viability & proliferation	[25]
ADSCs & LILI	5 J/cm^2 636 nm	EGF	Increased viability & proliferation	[26]
ADSCs & LILI	10 & 15 J/cm^2 830 nm	No DI	Decreased viability & proliferation	[37]
ADSCs	No LILI	Retinoic Acid	Increased viability, proliferation & differentiation	[38]
ADSCs & LILI	5 J/cm^2 636 nm	Retinoic Acid	ADSC differentiation into smooth muscle cells	[38]
ADSCs	No LILI	Angiotensin II, Sphingosylphospho-Rylcholine, TGF-β1	ADSC differentiation into smooth muscle cells	[39]
ADSCs trans-planted in ische-mic mouse limbs	No LILI	No DI	Enhancement of angiogenesis & osteogenesis	[40],[14]
ADSCs	No LILI	Heparin	Differentiation into smooth muscle cells	[41]

Studies have also shown that LILI can increase proliferation of cells, cellular attachment, differentiation and production of transformation growth factor beta1 (TGF-β1) in human osteoblasts cells indicating that, *in vitro*, LILI can modulate the activity of cells and tissues [27]. Fiszerman and Markmann, (2000), discovered that LILI enhances wound healing in chronic diabetic foot ulcers [28]. LILI has been successfully used for pain attenuation and to induce wound healing in non-healing defects [29]. In addition, Abrahamse and co-workers, (2010), demonstrated that increasing the fluence and wavelength caused a decrease in ADSC viability and proliferation in a reciprocal manner.

3. Regenerative Medicine

Tissue engineering and regenerative medicine is a multi-disciplinary science that has evolved in parallel with recent biotechnological advances. It combines biomaterials, growth factors and stem cells to repair organs [30]. Adult stem cells hold great promise for use in tissue repair and regeneration as a novel therapeutic option [31]. This can be done by culturing the cells and differentiating them into the required lineage *in vitro* and then introducing the differentiated cells into the failing organs. Plastic and regenerative surgeons are constantly burdened with the challenge of replacing lost soft tissue. More than 6.2 million individuals received reconstructive plastic surgery procedures in 2002, approximately 70% of them as a result of tumour removal [1]. Elective cosmetic procedures also require the placement of soft tissue implants to restore or improve tissue contour for the purpose of enhancing aesthetic appearance. Conventional soft tissue-grafting procedures have had some clinical success for soft tissue augmentation and reconstruction. However, the need for secondary surgical procedures to harvest autologous tissues and an average of 40-60% reduction in graft volume over time are considered drawbacks of current autologous fat transplantation procedures. It should be possible to overcome these problems with tissue-engineered soft tissue grafts generated from the patient's own adult stem cells [1].

Parkinson's disease, stroke and multiple sclerosis are thought to be caused by a loss of neurons and glial cells. These cells can now be generated from stem cells in culture and can be used to treat the above diseases in human patients through transplantation [32]. Clinical trials for the regeneration of soft tissue, craniofacial tissue and cardiovascular tissue have enrolled a number of patients. Breast reconstruction with ADSCs trials have been reported by Yoshimura and colleages [33]. These cells have also been used to stimulate bone repair in calvarial defects [34]. ADSCs have been used to heal chronic fistulas in Crohns disease [35] and hold great promise for the treatment of cardiovascular diseases [36].

4. Conclusion

More cells with increased differentiation potential are required for the treatment of various regenerative diseases. ADSCs have that differentiation potential and exposing them to LILI increases the proliferation rate. Therefore the novelity of using LILI in conjuction with ADSCs could improve tissue regenerative disease treatment by increasing the number of differentiated specialised cells. Much more research has to be conducted to develop standardise procedures for treatments.

5. References

[1] Choi S S, Cha S M, Lee Y Y, Kwon S W, Park C J and Kim M 2006 *Bioch Biophys Res Comm* **345(2)** 631-637
[2] Ogawa R 2006 *Curr Stem Cell Res Th* **1** 13-20
[3] Tobita M, Orbay H and Mizuno H 2011 *Disc Med* **11(57)** 160-170
[4] Fraser J K, Wulur I, Alfonso Z and Hedrick M 2006 *Trends Biotechnol* **24(4)** 150–154
[5] Zuk P A *et al* 2002 *Mol Biol Cell* **13** 4279-4295
[6] Zuk P A, Zhu M, Mizuno H, Huang J I, Chaudhari S, Lorenzo H P, Benhaim P and Hedrick M H 2001 *Tissue Eng* **7(2)** 211-216
[7] Huang J I, Beanes S R, Zhu M, Lorenz H P, Hedrick M H and Benhaim P 2002 *Plast Reconstr*

Surg **109** 1033–1041
[8] Rodriguez A M *et al* 2005 *J Exp Med* **201** 13 97-1405
[9] Lin Y, Luo E, Chen X, Liu L, Qiao J, Yan Z, Li Z, Tang W, Zheng X and Tian W 2009 *J Cell Mol Med* **9** 929-939
[10] Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo J M, Pénicaud L and Casteilla L 2004 *Circ Res* **94** 223–229
[11] Planat-Benard V *et al* 2004 *Circul* **109** 656–663
[12] Bouloumie A, Drexler H C and Lafontan M 1998 *Circ Res* **83** 1059-1066
[13] Sierra-Honigmann M R, Nath A K and Murakami C 1998 *Sci* **281** 1683-1686
[14] Shoji T, Li M, Mifune Y, Matsumoto T, Kawamoto A, Kwon S, Kuroda T, Kuroda R, Kurosaka M and Asahara T 2010 *Lab Inv* **90** 637-649
[15] Viera N M, Brandalise V, Zucconi E, Jazedje T, Secco M, Nunes V A, Strauss B E, Vainzof M and Zatz M 2008 *Biol Cell* **100** 231-241
[16] Cousin B *et al* 2009 *PLoS ONE* **4(7)** e6278
[17] Strem B M and Hedrick M H 2005 *Trends Biotechnol* **23** 64-66
[18] Strem B M, Hicok K C, Zhu M, Wulur I, Alfonso Z, Schreiber R E, Frazer J K and Hedrick M H 2005 *Keo Journal of Medicine* **54** 134-141
[19] Tholpady S S, Llull R, Ogle R C, Rubin J P, Futrll J W and Katz A J 2006 *Clin Plast Surg* **33** 55-62
[20] Giorgino F, Laviola L and Erriksson J W 2005 *Acta Physiol Scand* **183(1)** 13-30
[21] Matic M, Lazetic B, Poljacki M, Duran V and Ivkov-Simic M 2003 *Medicinski Pregled* **56(3-4)** 137-141
[22] Stadler L, Lanzafame R, Oskoui P, Zhang R, Coleman J and Whittaker, M 2004 *Photomed Laser Surg* **22(3)** 227-231
[23] Basford J R 1995 *Lasers Surg Med* **16** 331-342
[24] Gasparyan L, Brill G and Makela A 2004 *Laser Florence* 1-7
[25] Mvula B, Mathope T, Moore T and Abrahamse H 2008 *Lasers Med Sci* **23(3)** 277-282
[26] Mvula B, Moore T and Abrahamse H 2010 *Lasers Med Sci* **25** 33-39
[27] Khadra M, Lyngstadaas S P, Haanaes H R and Mustafa K 2005 *Biomaterials* **26(170)** 3503-3509
[28] Fiszerman R and Markmann C A 2000 *Diabetes* **49** A393
[29] Hawkins D and Abrahamse H 2005 *Photomed Laser Surg* **23(3)** 251- 259
[30] Butler D L, Goldstein S A and Guilak F 2000 *J Biomech Eng* **122** 570-575
[31] Huh C H, Kim S Y, Cho H J, Kim D S, Lee W H, Kwon S B, Na J I and Park K C 2000 *J Derm Sci* **46(30)** 217-220
[32] Lindvall O and Kokaia Z 2006 *Nature* **441** 1094-1096
[33] Yoshimura et al 2010 *Breast J* **16(2)** 169-175
[34] Lendeckel S, Jodicke A, Christophis P, Heidinger K, Wolff J, Fraser J K, Hedrick M H, Berthold L and Howaldt H P 2004 *J Craniomaxillofac Surg* **32(6)** 370-373
[35] Garcia-Olmo D, Garcia-Arranz M and Herreros D 2008 *Expert Opin Biol Ther* **8(9)** 1417-1423
[36] Bai X and Alt E 2010 *Biochem Biophys Res Commun* **401(3)** 321-326
[37] Abrahamse H, Houreld N N, Muller S and Ndhlovu L 2010 *Med Technol SA* **24(2)** 9-14
[38] De Villiers J A, Houreld N N and Abrahamse H 2011 *Stem Cell Rev Rep* March 5 epub ahead of print DOI 10.1007/s12015-011-9244-8
[39] Harris L J, Abdollahi H, Zhang P, McIlhenny S, Tulenko T N and DiMuzio P J 2009 *J Surg Res* DOI: 10.1016/j.jss.2009.08.001 ppg 1-9
[40] Nakagami H *et al* 2005 *Arterioscler Thromb Vasc Biol* **25** 2542-2547
[41] Rodriguez L V, Alfonso Z, Zhang R, Leung J, Wu B and Ignarro L J 2006 *PNAS* **103(32)**12167-12172