EFFECT OF LOW LEVEL LASER IRRADIATION ON HUMAN ADULT ADIPOSE DERIVED STEM CELLS – AN *IN VITRO* STUDY

A dissertation submitted to the Faculty of Health Sciences, University of Johannesburg, in fulfillment of the requirement for the degree of

Master of Technology: Biomedical Technology

by

Bernard Dandenault Mvula

(Student number: 200582701)

Supervisor: _____ 12 August 2008

Prof. H. Abrahamse Date

Co-Supervisor: _____ 12 August 2008

Dr. T.J. Moore Date

Johannesburg, 2008

DECLARATION

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

_____
B.D. Mvula

_____ day of _____ 2008

ABSTRACT

Stem cells are defined as undifferentiated cells that can proliferate indefinitely and have the capacity of both self-renewal and differentiation to one or more types of specialised cells. Traumatic tissue injury and age-related degenerative diseases are a major problem in South Africa and worldwide. Stem cells could be used for tissue engineering and reconstructive surgery. In treating these conditions, the main principle of stem cell therapy is the replacement of damaged and dead cells in injured tissues and organs with new healthy ones expanded *in vitro* from stem cells (Orlic *et al*., 2002). These cells can be isolated from adipose tissue in significant numbers and exhibit stable growth and proliferation kinetics in culture and could be differentiated into bone, fat, cartilage and muscle when treated with established lineage-specific factors (Zuk *et al*., 2002).

Low Level Laser Therapy (LLLT) is currently applied in the treatment of numerous diseases and pathological conditions (Gasparyan *et al*., 2004). LLLT produces positive effects on irradiated cells and tissues such as proliferation of cells, capillary growth and adenosine triphosphate (ATP) activation (Schindl *et al*., 1998). Low level laser radiation at different intensities has been shown to stimulate as well as to inhibit cellular processes (Moore *et al*., 2005). Epidermal growth factor (EGF) is a growth factor that plays important roles in the regulation of cell growth, proliferation and differentiation. This study investigated the effect of low level laser radiation alone as well as in combination with EGF on adult adipose derived stem cells (ADSCs) isolated from human adipose tissue.

ADSCs were isolated from human adipose tissue through collagenase digestion and cultured in DMEM-F12 containing 10% FBS and antibiotics and incubated at 37°C in a humidified atmosphere of 5% CO_2 (Zuk *et al*., 2001).

Semi-confluent monolayers of ADSCs were exposed to low level laser at 5 J/cm^2 using 636 nm diode laser with a power density of 12.1 mW/cm^2 at room temperature in the dark. Cell morphology was monitored at 0, 24 and 48 h using an inverted light inverted microscope. Cell viability was evaluated at 0, 24 and 48 h using the Trypan Blue exclusion test and an adenosine triphosphate (ATP) luminescence assay. bFGF (basic fibroblast growth factor) indirect ELISA and optical density assays were used to monitor cell proliferation at 0, 24 and 48 h post irradiation. In addition the expressions of stem cell markers, β1-integrin and Thy-1, were monitored by immunocytochemical live cell surface labelling and Western blot analysis. Cells were incubated with EGF to enhance proliferation and differentiation and the cell morphology, viability and proliferation were monitored as well as the expressions of stem cell markers, β1-integrin and Thy-1.

Morphology of the cells was not altered by irradiating them with 5 J/cm^2 using diode laser at 0, 24 and 48 h. Cell viability and proliferation showed an increase at 24 and 48 h post irradiation. At 0 h, there was no significant difference between irradiated and non-irradiated cells in cell viability and proliferation. There was an increase in the expression of β1-integrin and Thy-1 after irradiation as shown by Western blot analysis and immunocytochemical live cell surface labelling. Cell viability and proliferation showed a significant increase at all time points post irradiation with the addition of EGF. There was no noticeable change in cellular morphology at any time point.

Low level laser irradiation of human ADSC's at 636 nm with 5 J/cm^2 and 12.1 mW/cm^2 increased the viability and proliferation of these cells *in vitro*. Furthermore, low level laser irradiation appeared to increase the expression of stem cell markers, β1-integrin and Thy-1. In addition, laser irradiation did not alter the morphology of the cultured cells. The addition of EGF to the

cells also increased their viability and proliferation as well the expression of the markers, β1-integrin and Thy-1. The study showed that laser irradiation stimulates two important cellular responses namely cell viability and proliferation which indicates that ADSCs may be suitable for tissue engineering and future cell differentiation studies.

ACKNOWLEDGEMENTS

- Prof. H. Abrahamse, supervisor and head of Laser Research Group.
- Dr. T.J. Moore, co-supervisor.
- Dr. Nicolette Houreld
- Dr. Denise Hawkins
- The University of Johannesburg, National Laser Centre, National Research Foundation and Council for Scientific and Industrial Research and Medical Research Council for their financial support.
- To the University of Johannesburg and the African Laser Centre for the bursaries.
- Staff at the National Laser Centre for their assistance, guidance and setting up of the lasers.
- Prof. Alan Widgerow and Dr. Russell Walton, Linksfield Clinic and University of the Witwatersrand.
- Colleagues at Laser Research Group, University of Johannesburg, Doornfontein Campus: Tebogo, Alwin, Innocent, Sello, Palesa, Jennifer and Cherie.
- The technical staff: University of Johannesburg, Doornfontein Campus, Linka, Alastair, Mandla, Dineo, Tebogo and Ludzani.
- The staff at Radiology Department, University of Johannesburg, Doornfontein Campus.
- Friends: Kondwani Banda, Mr. Mseteka, Khwima Mkandawire, Cypriano Mkinga, John and Moses Nkhata and all my brothers, sisters and relatives.

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 dissertation to my wife, Annie and my son, Bernard Junior

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

KDa	Kilodalton
Da	Dalton
CD	Cluster of differentiation
μg/ml	Micrograms per millilitre
mm	Millimetre
U/ml	Units per millilitre
μl	Microlitre
U/mg	Units per milligram
rpm	Revolutions per minute
cm	Centimetre
nm	Nanometre
μm	Micrometre
mm^2	Millimetre squared
cm^2	Centimetre squared
Min	Minute/s
h	Hour/s
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
mW	Milliwatt
mW/cm2	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 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. Stem cells play a large 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). Remarkable 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 LLLT and stem cells have shown that low level lasers can change the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration (Gasparyan *et al*., 2004). Furthermore, low level laser irradiation has also been shown to promote the proliferation of rat mesenchymal bone marrow and cardiac stem cells *in vitro* (Tuby *et al*., 2007).

1.2 Problem Statement

Traumatic injury and age-related degenerative diseases are a major problem in South Africa and worldwide. 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 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). Since low level laser has been found to increase ATP and proliferation of cells, it could be used to increase

the generation of large numbers of stem cells and with the addition of different growth factors, differentiation of the stem cells into different cell types necessary to be used in tissue engineering and reconstructive surgery could be enhanced. 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, harvesting should be performed by a minimally invasive procedure, be able to be differentiated into multiple cell lineages and be transplanted safely and effectively.

1.3 Aim

Since there has been no research on ADSCs and LLLT this study focused on monitoring the effect of low level laser irradiation on human ADSCs. Cell morphology, viability, proliferation and expression of the markers were evaluated using different methods. This study also investigated the effect of EGF on ADSCs alone as well as in combination with low level laser irradiation with regards to proliferation as well as differentiation. The research also aimed at differentiating the ADSCs into skin (dermal) fibroblast cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Stem Cells

Stem cells are primal cells found in all multi-cellular organisms that retain the ability to renew themselves through mitotic cell division and can differentiate into a wide range of specialised cell types. Stem cells can be described according to their developmental 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 (Kimball, 2006) (Figure 1).

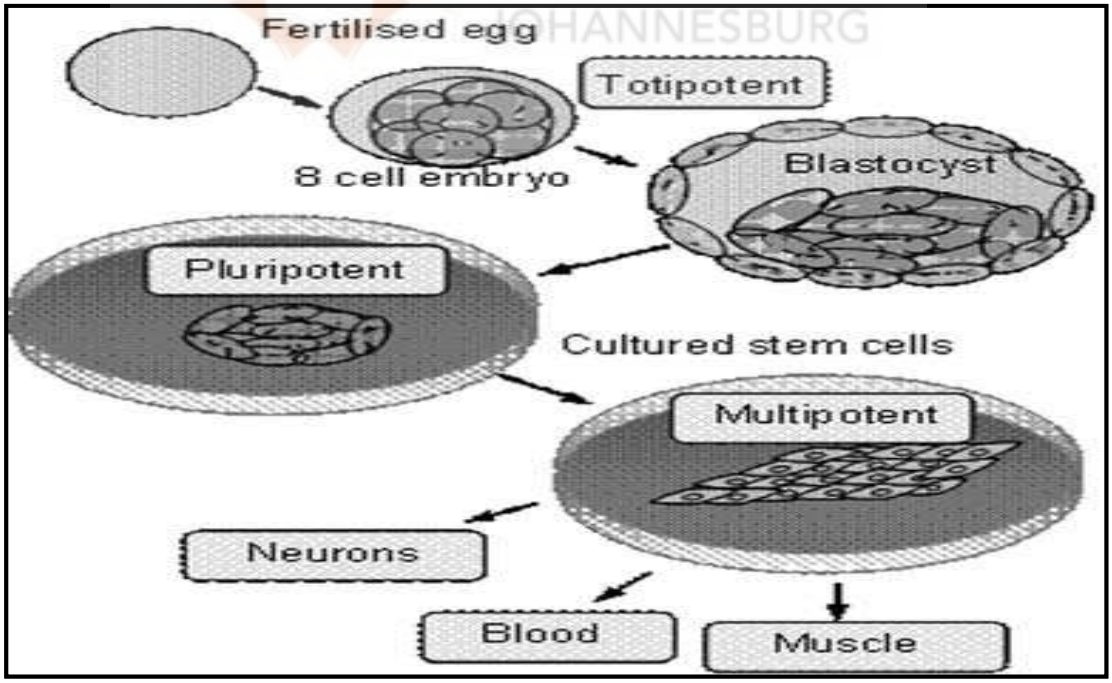


Figure 1. Pluripotent cells from the inner cell mass of the bastocyst cultured *in vitro*. Clones of these cells are then subcultured to form multipotent stem cells lines that can be stimulated to differentiate into numerous tissue lineages (Moore 2007).

Research in the field of human stem cells evolved from findings by Canadian scientists Ernest A. McCulloch and James E. Till in the 1960s. They discovered the presence of these self-renewing cells in the bone marrow of mice (Becker *et al*., 1963 and Siminovitch *et al*., 1963). Stem cells are broadly categorised into the following groups, namely embryonic, adult and umbilical cord blood stem cells. In this study, adult stem cells were used.

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. Symmetric divisions are defined as the generation 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 giving rise to cells committed to becoming differentiated tissues and organs (Serakinci and Keith, 2006) as seen in Figure 2. 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).

Stem cell self-renewal, proliferation and differentiation are regulated by the niche. These “niches” are anatomical locations that regulate how stem cells play roles in tissue generation, maintenance and repair. The niche saves stem cells from depletion while at same time protecting the host from over proliferation of the stem cells. The stem cell niche includes the surrounding cells that form the main component as well as the extra-cellular matrix and the physiochemical nature of the environment. Some of the physiochemical

nature of environments includes pH, calcium concentration and metabolites like adenosine triphosphate (ATP) (Scadden, 2006).

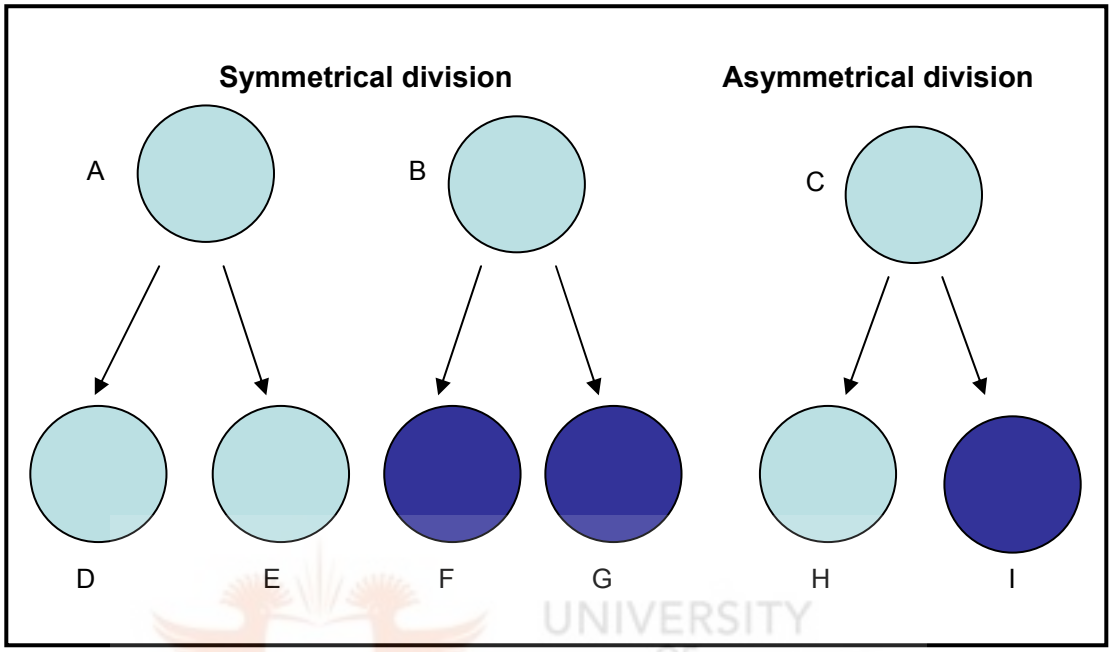


Figure 2. Stem cells A and B undergoing symmetrical divisions, with stem cell A dividing into two identical daughter cells (D and E) and stem cell B dividing into two specialised cells (F and G). Stem cell C undergoing asymmetrical division, dividing into a daughter cell (H) and a specialised cell (I) (Adapted from Morrison and Kimble, 2006).

Stem cells have been isolated from several tissues including the central nervous system, bone marrow, retinal tissue and skeletal muscle. These stem cells are called mesenchymal stem cells and are multi-potent cells that can differentiate into several cell lineages. All of these stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. For example, neural stem cells are biased to generate neurons and glia, bone marrow to generate mesodermal cells, and haematopoietic stem cells to generate blood cells (Figure 3). However, several recent transplant studies indicate that at least a small part of the stem cell population can give rise to cells of a different lineage *in vivo* (Toma *et al.,* 2001). Due to their

ability to differentiate into various cell phenotypes, mesenchymal stem cells are of importance for therapeutic applications.

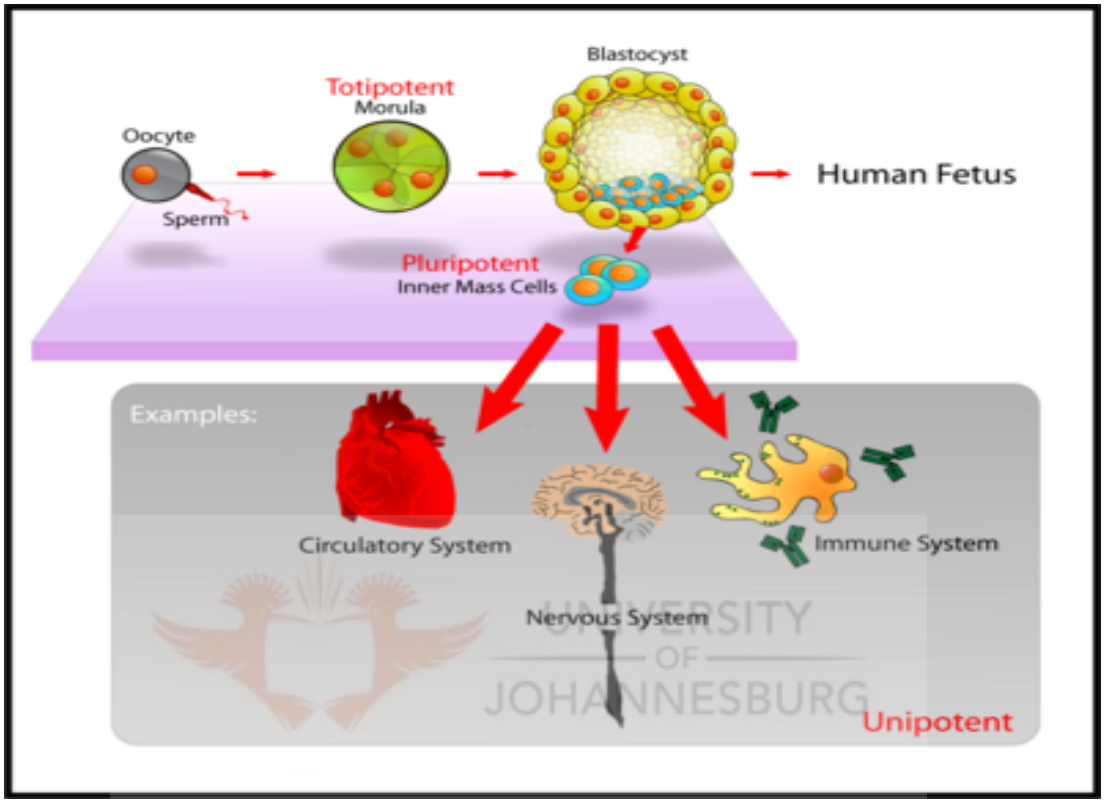


Figure 3. Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta (Wikipedia, Stem cells).

Numerous studies have been performed using implantation of mesenchymal stem cells in order to repopulate ischaemic or injured organs such as the heart and peripheral nervous system. A sufficient number of cells is necessary in order to obtain beneficial effect of the mesenchymal stem cells on the implanted organ. Culturing mesenchymal stem cells to the required quantity before reaching the stage of overdifferentiation is a time-dependent procedure. Moreover, getting the cells into the injured or ischemic organ in the right quantity and within a short time interval after trauma may be a crucial factor in achieving an optimal functional improvement of the organ

post-implantation (Tuby *et al*., 2007). For example, while some studies have reported beneficial effects of stem cell implantation on the infarcted myocardium of experimental animals, some did not reveal any improvement in heart function after mesenchymal stem cell implantation and failed to show differentiation of mesenchymal stem cells into cardiomyocytes (Grinnemo *et al*., 2006).

The mechanisms that stem cells use to accomplish self-renewal promise fundamental insight into the origin and design of multi-cellular organisms. Pathways such as notch, wnt and β1-Integrin are co-ordinated for the stem cells self-renewal to be accomplished (Campos *et al*., 2005). These pathways make it possible to repair damage and extend the life of an organism beyond that of component cells, and probably preceded the evolution of complex metazoans (Spradling *et al*., 2001). Successful long term restoration of continuously self-renewing tissues, such as skin, depends on the use of self-renewing stem cells (Bianco and Robey, 2001).

2.1.1 Embryonic stem cells

Embryonic stem cells are derived from the epiblast tissue of the inner cell mass of a blastocyst. A blastocyst is an early stage embryo which is approximately 4 to 5 days old in humans, consisting of 50-150 cells. Embryonic stem cells are pluripotent, that is, they are able to develop into cells from all three types of the germ layers of the developing embryo (Gardner, 2002). Nearly all research to date has taken place using murine or human embryonic stem cells and stem cell lines derived from these. Both have the essential stem characteristics, yet they require very different environments in order to be maintained in an undifferentiated state. Some murine embryonic stem cells require leukemia inhibitory factor (LIF) and are grown on a layer of gelatin (Becker *et al*., 1963) while some human embryonic stem cells are grown on a feeder layer of murine embryonic

fibroblasts and require basic fibroblast growth factor (Siminovitch *et al*., 1963). These culture conditions are required for the embryonic cells to remain in an undifferentiated state (Chambers *et al*., 2003).

Embryonic stem cells are unique in their ability to grow indefinitely in culture while retaining a normal karyotype (Schuldiner *et al*., 2000). Due to their pluripotency, they are a potential source for regenerative medicine and tissue replacement after injury or disease. Thompson and colleagues at the University of Wisconsin reported isolation of primate embryonic stem cells in 1995 and human embryonic stem cells in 1998 (Thompson *et al*., 1995 and Thompson *et al*., 1998). However due to ethical issues many countries do not carry out embryonic stem cell research as it involves the destruction of embryos. Researchers also face difficulties working with embryonic stem cells as these cells have a tendency to produce tumors and malignant carcinomas. Embryonic stem cells also cause some transplant rejection (Wu *et al*., 2007).

2.1.2 Adult stem cells

Adult stem cells are undifferentiated cells found throughout the body that divide to replenish 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 yield most of the specialised cell types of an organisms tissue or organs (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 transformed 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.

2.1.3 Adipose derived stem cells

Adipose tissue is derived from the mesenchyme and contains a supportive stroma that is easily isolated (Zuk *et al*., 2002), and provides an abundant source of multipotent cells (Rodriguez *et al.,* 2006). *In vivo* models of adipogenesis suggest that the mature adipocyte is a terminally differentiated cell, with limited capacity for proliferation and replication. Therefore a stem cell population is responsible for replacing mature adipocytes within the adipose tissue through the life time of an individual (Gimble *et al*., 2007). 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 adipose derived stem cells (ADSCs) (Choi *et al*, 2006).

Studies have shown that mesodermal and ectodermal capacities are detected in processed lipo-aspirates suggesting that adipose tissue represents a

source of adult stem cells (Zuk *et al*., 2002). Investigators from around the world have shown that adipose tissue is a rich source of autologous regenerative cells. These cells have practical benefits over other types of adult stem cells such as haematopoietic stem cells and additionally these stem cells from a patient's own adipose tissue present no risk of rejection or disease transmission and avoid certain ethical concerns. Some of the benefits include the ease of isolation and abundancy of cells during isolation as well as the cells' multilineage differentiation potential (Gimble and Guilak, 2003).

Human ADSCs have been shown to differentiate into bone, cartilage, fat, muscle, and might be able to differentiate into neurons, making them a possible source for future applications in clinical settings (Zuk *et al*., 2001) (Figure 4). ADSCs have also been found to have the potential to differentiate into functional smooth muscle cells (Rodriguez *et al*., 2006).

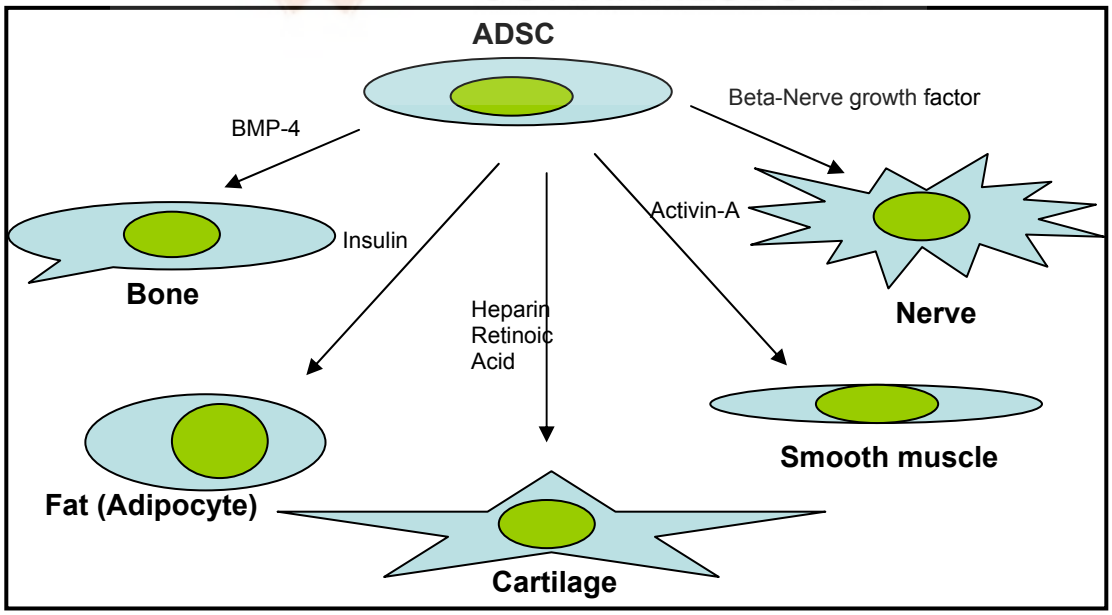


Figure 4. Differentiation of ADSCs into several lineages. The differentiation is possibly stimulated by different growth factors. Each growth factor has its own effect that may result from a directed differentiation.

Investigators have outlined a number of mechanisms through which ADSCs can be used to repair and regenerate tissues. One possibility is that ADSCs delivered to an injured or diseased tissue can secrete cytokines and growth factors that stimulate recovery. ADSCs would modulate the host's stem cell niche by stimulating the recruitment of stem cells to the injured or diseased site and promote their differentiation along the pathway of the required lineage. ADSCs might provide antioxidant chemicals, free radical scavengers, and heat shock proteins at an ischaemic site. As a result, toxic substances released would be removed and thereby promote the recovery of the surviving cells. Studies have shown that transplanted bone marrow derived stem cells can deliver new mitochondria to damaged cells, thereby rescuing aerobic metabolism (Spees *et al*., 2006).

2.2 Tissue Engineering and 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 (Butler *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 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 (Choi *et al*., 2006).

Adult stem cells hold great promise for use in tissue repair and regeneration as a novel therapeutic option (Huh *et al*., 2007). 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).

The mammalian heart contains a population of adult cardiac stem cells able to undergo self-renewal and could replicate and differentiate into smooth muscle cells, cardiomyocytes or endothelial cells *in vivo* or *in vtro.* Following myocardial infarction, cardiac stem cells can migrate and invade the infarcted area leading to a significant replacement of the damaged cells and recovery of the ventricles (Tuby *et al*., 2007). Administration of cardiac stem cells into coronary arteries after infarction has been shown to cause tissue regeneration, involving the formation of new vascular structures, resulting in restoration of myocytes in the infarcted region. It has also been found that cardiac stem cells give rise to new cardiomyocytes in both infarcted and non-infarcted regions (Tuby *et al*., 2007). Table 1 shows current stem cell research focus areas, stem cell sources and developments (Moore, 2007).

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

A stem cell to be used in tissue engineering and regenerative medicine should be found in abundance, be harvested by a minimally invasive procedure, be able to be differentiated along multiple cell lineage pathways and be safely and effectively transplanted to either an autologous or allogeneic host (Gimble, 2003). Compared to other derived stem cells, adipose derived stem cells can be easily harvested by a simple, minimally invasive method and also easily cultured and therefore could be used in tissue engineering and regenerative medicine (Huh *et al*., 2007). Furthermore, a major obstacle that must be resolved before human stem cell therapy can become a reality is the threat of rejection of the transplanted cells if a non-autologous transplant is performed. One way to avoid this would be to use stem cells that are genetically identical to the host, by using stem cells that are derived from the host's own tissues (Kimball, 2006).

Regenerative medicine and tissue engineering could revolutionise surgical disciplines and is expected to become the gold standard of surgical procedures intended to activate and support the body's natural healing. However, more research is necessary before stem cell based therapy can be widely used.

2.3 Growth Factors

Growth factors are naturally occurring proteins capable of stimulating cellular proliferation. They also regulate a number of processes including tissue morphogenesis, angiogenesis, cell differentiation and neurite outgrowth. Growth factors also play important roles in the maintenance of tissue homeostasis and wound healing (Chen *et al*., 2004). Growth factors are required to enhance proliferation and differentiation of stem cells. Stem cells can be identified by their expression of certain genes and proteins. One such protein expressed on surface membranes of stem cells is β1-integrin.

2.3.1 β1-Integrin

β1-Integrin, also known as CD29, is a 130 kDa transmembrane glycoprotein that forms non-covalent complexes with various integrin alpha subunits (including alpha 1, alpha 2, alpha 3, alpha 4, alpha 5, and alpha 6, also known as CD49a, CD49b, CD49c, CD49d, CD49e, and CD49f respectively) to form the functional receptors that bind to specific extra-cellular matrix proteins. Integrin receptors are involved in the regulation of a variety of important biological functions, including embryonic development, wound repair, homeostasis and prevention of programmed cell death (Hynes, 2002). They are also implicated in abnormal pathological states such as tumor directed angiogenesis, tumor cell growth, and metastasis. These heterodimeric receptors bridge the cytoplasmic actin cytoskeleton with proteins present in the extra-cellular matrix and/or on adjacent cells.

The clustering of integrins on a cell surface leads to the formation of focal contacts. Interactions between integrins and the extra-cellular matrix lead to activation of signal transduction pathways and regulation of gene expression (Brakebusch and Fassler, 2005). β1-integrin has been shown to play a role in maintaining keratinocytes in an undifferentiated state (Jones and Watt, 1993; Jones *et a*l., 1995 and Levy *et al*., 2000). β1-integrin is a known stem cell marker and has previously been shown to be expressed in adipose derived stem cells (Hudson, 2004 and Gimble, 2003), as well as in epidermal stem cells (Lavker and Sun, 2000; Alonso and Fuchs, 2003 and Zhou *et al*, 2004).

2.3.2 Thy-1 (CD90)

Thy-1 is the designation for a major cell surface glycoprotein characteristic to T-cell, as first defined in the mouse and rat. It is a 25-37 kDa heavily N-glycoslylated, glycophosphatidylinositol (GPI) anchored conserved cell surface protein originally discovered as a thymocyte antigen (Ades *et al*., 1979). The Thy-1 gene is located at human chromosome 11q22.3. Among

the cells that express Thy-1 are the thymocytes, neurons, mesenchymal stem cells, haematopoietic stem cells, fibroblasts and myofibroblasts (Kemshead *et al*., 1982; McKenzie and Fabre, 1981). Thy-1 has speculated roles in cell-cell and cell matrix interactions, nerve regeneration, apoptosis, metastasis, inflammation and fibrosis.

Thy-1 is used as a marker for a variety of stem cells including mesenchymal stem cells (Manson *et al*., 2006). Thy-1 can be considered as a surrogate marker for various kinds of stem cells such as haematopoietic stem cells. It is one of the popular combinatorial surface markers for fluorescent-activated cell sorting for stem cells in combination with other markers like CD34 (Manson *et al*., 2006).

2.3.3 Epidermal growth factor (EGF)

Epidermal growth factor (EGF) is a growth factor that plays an important role in the regulation of cell growth, proliferation and differentiation. Human EGF is a 6045 Da protein with 53 amino acid residues and three intramolecular disulfide bonds (Carpenter and Cohen, 1990).

EGF is secreted by platelets, macrophages and monocytes and in salivary, lacrimal and duodenal glands as well as in the kidneys. EGF is part of a complex network of growth factors and receptors that together help to modulate the growth of cells. EGF is released by cells, and then is picked up either by the cell itself, stimulating its own growth, or by neighbouring cells, stimulating their ability to divide. Receptors on the surface of the cell bind to EGF and relay the signal inside. Signaling proteins inside the cell then bind to these new phosphorylated tyrosines, initiating the signaling cascade that ultimately initiates DNA synthesis and cellular growth (Goodsell, 2003). It is also involved in tumour proliferation, metastasis, apoptosis, angiogenesis and wound healing (Bouis *et al*., 2006).

EGF induces phosphorylation of extracellular signal regulated kinase pathways. EGF participates in skin appendage development and differentiation, tissue repair and modeling and can activate ectodermal and mesodermal markers (Li *et al*., 2006 and Fu *et al*., 2002). It has also been found to increase cellular proliferation and viability in central nervous system precursor cells (Svendsen *et al*., 1995). Studies on EGF and stem cells have found that pre-treatment of human ADSCs with EGF increased neuronal differentiation (Safford *et al*., 2002 and Angenieux *et al*., 2006). EGF has been shown to stimulate differentiation of human mesenchymal stem cells into bone-forming cells (Kratchmarova *et al*., 2005). Furthermore, EGF completely blocks accumulation of lipids and this is associated with potent stimulation of cell proliferation (Hauner *et al*., 1995).

2.3.4 Fibroblast surface protein

Little is known about this surface protein found on human fibroblasts and fibroblast cell lines, as well as tissue macrophages and 95% of peripheral blood monocytes. This antigen has also been demonstrated on malignant fibrosarcoma tissue. Human synovial, foreskin and thymic fibroblasts also express this antigen but it is absent on human epithelial cells and lymphocytes. When fibroblast surface protein is detected by monoclonal antibodies, it has been described to be of molecular weight of 43 kDa and 72-80 kDa by Western blot analysis (Singer *et al*., 1989).

2.4 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). In 1967, Endre Mester in Semmeelweis 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 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 level lasers and high level lasers.

2.4.1 Low level laser therapy

Low Level Laser Therapy (LLLT), a form of phototherapy, involves the application of monochromatic light over biological tissue to elicit a biomodulative effect within that tissue. LLLT is now accepted in many countries and is used in medical and dental practices. LLLT 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. LLLT 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). Some of the therapeutic lasers are Helium-Neon (HeNe) and diode lasers. A diode laser was used in this study.

Diode lasers are solid state devices 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).

Low level laser light can accelerate wound and burn healing and improve conditions of patients after myocardial infarction and stroke. Low level 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). LLLT 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 LLLT 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). LLLT 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 LLLT on wound healing are attributed to increased cell proliferation. It is thought that when LLLT 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 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 level laser therapy 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).

LLLT 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). Low level laser irradiation 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 level lasers. For instance, He-Ne laser irradiation has produced degenerative effects on bovine oocytes (Ocana-Quero *et al*., 1997). However, the true effect of LLLT 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.

2.4.2 Lasers and stem cells

The combination of lasers and stem cells is quite novel in scientific research and therefore little data are currently available. Since lasers produce positive effects on cells such as cell proliferation, cell motility, activation of enzymes, the administration of lasers on stem cells would assist in tissue engineering and regenerative medicine. Studies on LLLT and stem cells have shown that low level lasers can alter the metabolism of stem cells, increase adenosine triphosphate (ATP) production and so increase migration (Gasparyan *et al*., 2004). Furthemore, low level laser irradiation has also been shown to promote the proliferation of rat mesenchymal bone marrow and cardiac stem cells *in vitro* (Tuby *et al*., 2007). However the effects of LLLT on human adipose derived stem cells have yet to be elucidated.

CHAPTER 3

METHODOLOGY

An overview of the study can be seen in the flow diagram in Appendix A. All items (chemicals/reagents) used are listed in Appendix C with the company names and catalogue numbers, and all solutions in Appendix D.

3.1 Isolation of Adipose Stem Cells

Dermal and adipose tissues excised from consenting adult donors undergoing abdominoplasty at Linksfield Park Clinic, Sandringham, Johannesburg and the University of the Witwatersrand were used for the isolation of ADSCs. All donors received a consent form and information regarding the donation of tissue (Appendix B). A signed copy of the consent form was returned by the donors before tissue collection. Donated tissue, surgically excised (dermal and adipose) or aspirated (lipo-aspirate), was collected and transported as biological specimens in a sealed polystyrene container at room temperature. The Academic Ethics Committee of the Faculty of Health Sciences, University of Johannesburg, approved this study in accordance with the Human Tissue Act 65, 1983 (Ethical Clearance Reference Number 09/06).

Adipose tissue was separated from the dermal layer (Figure 5) using a scalpel and placed in a sterile beaker with Hanks Balanced Salt Solution (HBSS) containing 10 000 units of Penicillin/Streptomycin and 250 µg/ml of Fungizone. HBSS was added in a ratio of 1:10. The covered beaker containing the tissue was then stored at room temperature overnight protected from light.

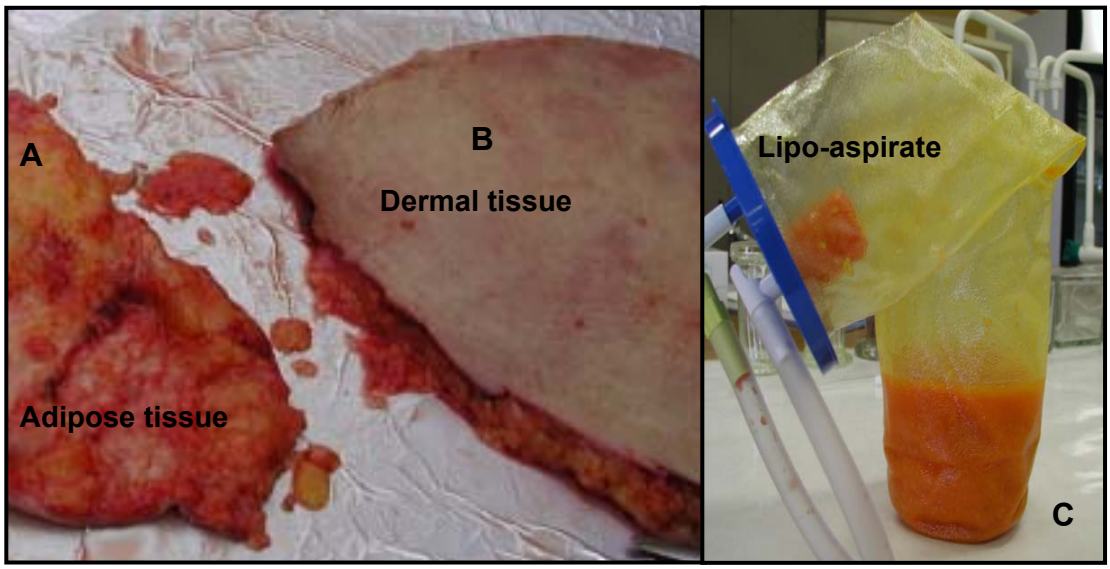


Figure 5. ADSCs were isolated from adipose tissue (A) separated from dermal tissue (B). Lipoaspirate (C) was used as an alternative source for isolating ADSCs.

The adipose tissue was removed from the beaker and minced into 3-5 mm pieces using 2 sterile scalpels. Typically 100 ml of minced tissue was placed in a collagenase type-1 solution containing 250 mg of collagenase type-1 powder (240 U/mg), 99.8 ml HBSS and 200 µl 5 M Calcium Chloride, sealed with parafilm and incubated in a shaking incubator (Labcon, Instrulab, SA) at 70 rpm for 80 min at 37 ^{0}C. The collagenase type-1 solution had a final concentration of 600 U/ml. Following incubation, equal volumes of complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal calf serum (FCS), 0.1% Penicillin/Streptomycin and 1 µg/ml Fungizone were added to all the tubes, and inverted to mix.

Following the centrifugation of the suspension at 1 000 rpm for 5 min at 20 ^{0}C (Orto Acresa, Mod. Digicen-R), the oil layer on the surface was removed and discarded using a plastic Pasteur pipette. The infranatant was then removed, resuspended and filtered through a 40 µm filter (BD Falcon cell strainer). The resultant suspension was centrifuged at 2 000 rpm for 5 min and the

supernantant removed. Cell pellets from the same sample were pooled in DMEM with 10% FCS and antibiotics, and re-pelleted at 2 000 rpm for 5 min. The supernatant was removed and the pellets were resuspended in erythrocyte lysis buffer (ELB) and incubated for 10 min at room temperature to lyse the red blood cells. The solution was centrifuged at 2 200 rpm for 5 min and the pellet resuspended in complete medium. Two and half millilitres of the cell suspension was added to seventeen and half millilitres of complete culture medium and seeded into 75 cm^2 culture flasks. The cells were incubated in a CO_2 incubator (Hera cell 150, Heraeus) at 37 ^0C in 85% humidity of 5% CO_2. Cells were observed in the flasks 24 h after incubation. The culture flasks are shown in the photograph below (Figure 6).

Figure 6. Adipose derived stem cells were cultured in complete media in 75 cm^2 culture treated flasks at 37 ^0C in 85% humidity and 5% CO_2.

3.2 Culturing of Adipose Derived Stem Cells

Cells were grown to semi-confluence (60-80%) before passage to avoid differentiation and the medium was changed once a week. Semi-confluent

cells were sub-cultured into 3.3 cm diameter culture plates for laser irradiation. In order to remove cells from the flask, complete media was discarded and cells were rinsed with 5 ml of HBSS three times to remove excess medium and then 1 ml of Trypsin/EDTA was added to each flask and incubated for 3-5 min at 37 ^{0}C. After incubation, 5 ml of 10% FCS in DMEM was added to each flask to deactivate trypsin and then the mixture was centrifuged at 2 000 rpm for 5 min at 20 ^{0}C (Orto Acresa centrifuge). The pellets were then suspended in 2 ml complete culture medium. The cell suspension, 200 µl, was then added to 1.8 ml of complete medium in 3.3 cm diameter culture plates. The plates were then incubated in a CO_2 incubator, allowing the cells to attach and grow for 4 days.

In order to increase proliferation and induce differentiation, some pellets were resuspended in complete medium containing 20 ng/ml of epidermal growth medium (EGF) and cultured in this medium as above.

3.3 Culturing of Human Skin Fibroblast (WS1) cells

Normal WS1 (ATCCRL 1502) were cultured in Eagle's Minimal Essential medium (EMEM) with Earle's BSS, 2 mM L-Glutamine, 1.0 mM Sodium Pyruvate, 0.1 mM Non-Essential Aminoacid, Penicillin and Fungizone.

Stock cells were removed from liquid nitrogen, thawed and immediately resuspended in 10 ml of plain medium (EMEM) and centrifuged at 2 200 rpm for 5 min. The supernatants were removed and the pellets resuspended in 1 ml complete medium and cultured in 75 cm^2 flasks containing 19 ml complete medium. Cells were grown to confluence, rinsed with 5 ml of HBSS three times to remove excess medium and then 1 ml of Trypsin/EDTA was added to each flask and incubated for 3-5 min at 37 ^{0}C. Trypsin was deactivated by adding an equal volume of 10% FCS in EMEM. The mixture was centrifuged at 2 200 rpm for 5 min and the pellets resuspended in 5 ml

complete medium. Cell viability analysis was performed using Trypan Blue exclusion test and 6 x 10^5 cells were seeded in 3.3 cm diameter culture plates. The plates were then incubated in a CO_2 incubator, allowing the cells to attach and grow overnight. The number of plates to be seeded and the volume of complete medium to be added to the cell suspension was determined by using the following formula.

Total viable cells/5 ml Required no. of cells /ml	=	No. of cells/5 ml 6 x 10^5 cells/plate	=	No. of plates
Each plate	=	5 ml No. of plates	=	ml/plate

Each small plate has a volume of 3 ml thus:

Volume of media to be added = 3 ml – ml/plate (cell suspension)

3.4 Laser Irradiation

The diode laser (LTIO00-PLT20) 636 nm (Figure 7) used in this study was supplied and set up by the National Laser Centre (NLC, South Africa). Both semi-confluent monolayers of ADSCs in complete medium and complete medium with EGF were irradiated with the lid off, in the dark, at room temperature with 5 J/cm^2 at 636 nm using a diode laser (Oriel, USA). Laser irradiation was delivered to the culture plate from above via an optical fibre. The beam was clipped to cover the entire area of the plate (3.3 cm^2). The correct eyewear was worn during irradiation. Duration of irradiation was calculated by measuring the power output of the laser using the formula as shown below. On average a power output of 110 mW was measured and this was calculated to take 6 min and 53 secs to deliver 5 J/cm^2. The power density was 12.1 mW/cm^2. Non-irradiated cells were used as controls and were kept under the same conditions. Both irradiated and non-irradiated samples were re-incubated at 37 ^{0}C in a humidified atmosphere of 5% CO_2.

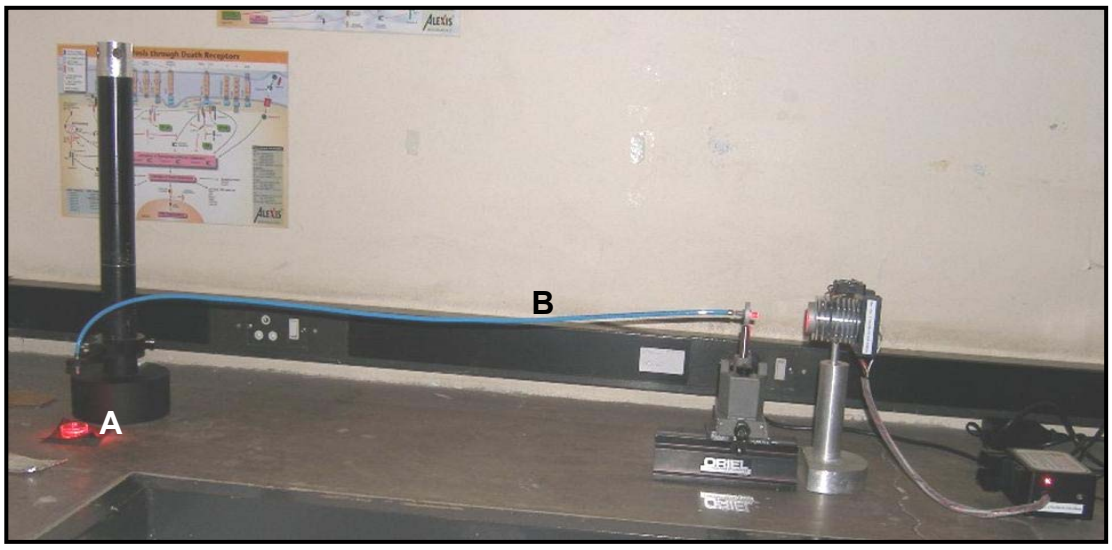


Figure 7. Diode laser with a wavelength of 636 nm and an average power output of 110 mW that was used to irradiate adipose derived stem cells with 5 J/cm^2. The beam was delivered to a 3.3 cm^2 petri dish (A) through the optical fibre (B).

Laser parameters are shown in Table 2 and the calculations for power density and duration of laser exposure are shown in Appendix E.

Table 2: Parameters of Diode laser that were used in this study.

Name and type	Semiconductor (diode)
Wavelength	636 nm
Spectrum	Red
Pulsed or continuous	Continuous wave (CW)
Output	110 mW
Power density	12.1 mW/cm^2
Energy density (fluence)	5 J/cm^2
Beam area	9.1 cm^2
Number of exposure	1 exposure on day 1
Duration of exposure	6 min 53 sec

3.5 Cell Morphology

Morphological observations were performed at 0, 24 and 48 h post irradiation on both irradiated and non-irradiated cells. An inverted light microscope (Olympus CKX41) was used to observe morphology (Figure 8). The microscope was coupled to a digital camera (Olympus C5060-ADUS) to document digital micrographs.

Figure 8. Inverted light microscope (Olympus CKX41) which was used to observe the morphology of the cells.

3.6 Cellular Responses

A cell suspension was used to measure cell viability through Trypan Blue staining and Adenosine Triphosphate (ATP) luminescence and cell proliferation through Optical density readings. Live cell monolayers were used to identify the presence of the markers, β1-integrin and Thy-1, through

immunocytochemical live cell labelling. Protein lysates of the cultured cells were used for Western Blot to determine the expression of β1-integrin and Thy-1. Cell medium was used to determine cellular proliferation and growth factor expression of basic fibroblast growth factor (bFGF).

3.7 Cell Viability

3.7.1 Trypan blue

Cellular viability was measured using Trypan Blue staining. Trypan Blue is a vital stain recommended for use in estimating the proportion of viable cells in a population (Phillips and Terrberry, 1957). The reactivity of this dye is based on the fact that the chromophore is negatively charged and does not react with the cell unless the membrane is damaged. Live (viable) cells do not take the dye and remain colourless while dead (non-viable) cells do and stain blue (Phillips and Terrberry, 1957).

A mixture of cell suspension in HBSS (20 µl) and Trypan Blue reagent (20 µl) was incubated at room temperature for 5 min. Viable and non-viable cells were counted using a Haemocytometer with Neubauer rulings using a light microscope (Olympus CKX41) and the percentage viability was determined. The percentage viability was calculated by dividing the number of viable cells (translucent) by the total number of cells and multiplied by 100.

3.7.2 Adenosine triphosphate (ATP) luminescence

The cell Titer-Glo luminescent Cell Viability assay provides a homogeneous method for determining the number of viable cells in culture based on the quantitation of ATP, which indicates the presence of metabolically active cells (Promega, 2005).

According to the protocol of the manufacturer, a mixture of cell suspension in complete medium (50 µl) was mixed with an equal volume of Glo reagent

(1 ml buffer and 0.007 g substrate) and mixed on a vortex (Snijders Scientific) for 2 min to induce lysis. The mixture (100 µl) was incubated for 10 min at room temperature and read on a luminometer (Hygiena International, Pi-102).

3.8 Cell Proliferation

3.8.1 Optical density

Optical density was used to measure cell proliferation. A hundred microlitre of cell suspension in complete medium (DMEM) was read at $A_{540\ nm}$ (Pinheiro *et al*., 2002) in a microplate reader (Benchmark Plus Microplate spectrophotometer, Bio-Rad).

3.8.2 Basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (bFGF) is a potent mitogenic agent for a wide variety of cells. It plays an important role in cellular proliferation and differentiation during tumour progression, wound healing, angiogenesis, tissue regeneration, embryogenesis and central nervous system development (Gospodarowicz *et al.,* 1987).

bFGF concentration was determined using the indirect ELISA (Enzyme linked immunosorbent assay). A serial dilution of 2.5 µg/µl bFGF was made in 100 µl carbonate-bicarbonate buffer ranging from 1.25 µg/µl to 0.0195 µg/µl (Figure 9).Human bFGF in PBS-T (10 mM phosphate buffer pH 7.4, 150 mm NaCl, 0.05% Tween 20) was used as a positive control and to establish a standard curve (Figure 10). The standards were used to determine the concentration of the unknown samples.

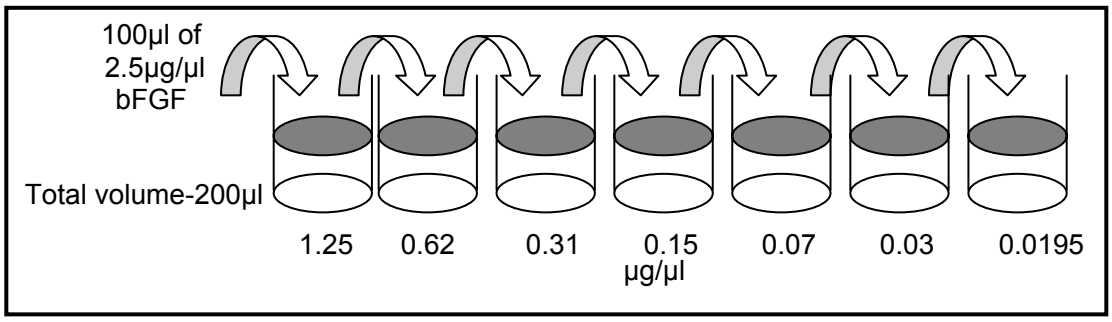


Figure 9. Serial dilution of bFGF standards. Concentrations ranging from 1.25µg/µl to 0.0195 µg/µl. Initially a 100 µl of 2.5 µg/µl bFGF was added to a 100 µl of carbonate bicarbonate making a concentration of 1.25 µg/µl .

Equal volumes (100 µl) of antigen solution (medium from the culture plate) and carbonate-bicarbonate buffer was mixed and incubated in a 96-well microplate overnight at 4 ^{0}C.

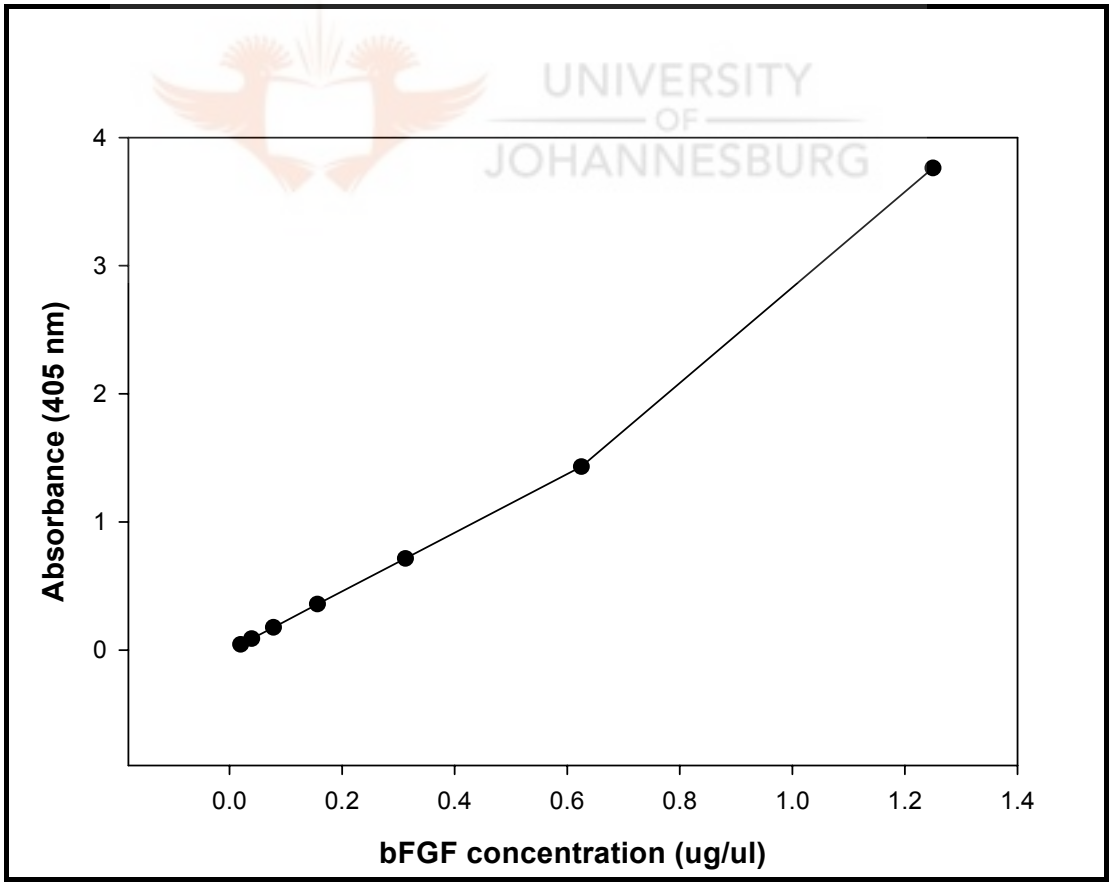


Figure 10. Absorbance and concentration of the bFGF standards. The concentrations are in µg/µl. The absorbances were read at A $_{405\ nm}$.

The coating solution was removed and washed four times with PBS-T using a microplate washer (Bio-Rad, SA, PW40). Primary antibody, monoclonal anti-fibroblast growth factor was diluted 1:6 500 (0.06 µg/ml) and 200 µl was added to each well and the plate was incubated for 2 h at room temperature. The solution was removed and the plate washed as before. Secondary antibody, goat anti-mouse IgG conjugated with horse radish peroxidase (HRP), diluted 1:4 000 (0.1 µg/ml) was made and 200 µl was added to all the wells. The plate was incubated for 2 h at room temperature. The solution was removed and the plate washed as before. Two hundred microlitres of freshly prepared TMB substrate was added and after 30 min the reaction was stopped by adding 50 µl of 1 mol/ml Sulphuric Acid (H_2SO_4) and read spectrophotometrically at $A_{405\ nm}$ (Benchmark Plus, Microplate spectrophotometer, Bio-Rad, SA).

3.9 Immunocytochemical live cell surface labelling

Cells were seeded onto heat sterilised glass coverslips, by slowly pipetting 200 µl of cell suspension into 1.8 ml of complete medium in 3.3 cm diameter culture plates containing coverslips (22 x 22 mm). The cells were allowed to attach to the coverslips and grow for 4 days to semi-confluence. The cultured cells were then irradiated.

Cells cultured on the sterile glass coverslips were removed from the incubator and rinsed twice with ice-cold PBS BSA/azide buffer (PBS; 0.1% w/v BSA-Bovine Serum Albumin; 0.01% w/v azide) and then incubated with anti-β1-integrin (0.8 µg/ml, 1:250) in PBS BSA/azide for 30 min on ice. Cells were then rinsed three times with PBS BSA/azide buffer and incubated with the secondary fluorescent antibody (0.4 µg/ml, 1:1 000; Goat anti-mouse IgG-Rhodamine, in PBS BSA/azide) for 30 min on ice, protected from light. Cells were rinsed three times as before and fixed in 3.7% formalin for 10 min.

Following fixation, cells were rinsed once briefly with PBS, and then once with tap water before being stained with 4'-6-diamidine-2-phenylindole (DAPI) and mounted on glass slides. The slides were viewed through a fluorescent microscope (Olympus BX41) to identify β1-integrin labelled cells (Red) with counter stained nuclei (Blue).

3.10 SDS PAGE and Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) separates proteins according to their molecular weights. Antibodies then bind the target proteins on the membrane. The method is a widely used immunoassay for detecting the presence of protein in a sample of cell lysates and tissue extracts. Two gels are made, the stacking gel and the running gel. The stacking gel is more porous and acidic (pH 6.8), while the running gel has small pores and is basic (pH 8.8). Proteins with low molecular weights will travel through the gels faster than those with high molecular weights. Enhanced Chemiluminescence process is used to visualise the protein and antibody complex (bands) on the membrane.

3.10.1 Protein isolation and determination of concentration

Protein lysates were prepared from irradiated and non-irradiated cells by scraping the cells from the culture dishes in HBSS using a cell scraper. The mixture was centrifuged at 13 000 rpm for 4 min in a microcentrifuge (Biofuge Pico-Heraeus), the supernatant removed and the pellet resuspended in an equal volume of HBSS and a two times sample buffer (2 M Tris(hydroxymethyl) aminomethane pH 6.8; 2% SDS; 100% glycerol and distilled H_2O). This mixture was sonicated (Bandelin Sonoplus-Bochem Laborbedarf) and then centrifuged as before and the supernatant (protein lysates) stored at -20 ^{0}C for protein analysis. All the above procedures were performed on ice to inhibit enzyme activity.

To determine the amount of protein in the lysates, the Bicinchoninic Acid (BCA) protein assay for the colorimetric detection and quantitation of total protein was used. In this method, cupric cations (Cu^{+2}) are reduced to cuprous cations (Cu^{+1}) by protein in an alkaline medium. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion (Smith *et al.*, 1985). A volume of 25 µl of the samples and standards were mixed with 200 µl of the working reagent (in U-bottom 96-well microplate) and incubated at 37 ^{0}C for 30 min. The standards, BSA, were diluted with two times sample buffer as shown in Table 3. The plate was allowed to cool at room temperature before being read at 562 nm in a microplate reader. The amount of protein in the lysates was determined using the standard curve (Figure 11).

Table 3. Table showing of nine BSA standards (A to I) with the concentrations from 2000 to 0 µg/ml. The diluent used was two times sample buffer.

Vial	Diluent volume(µl)	Volume and source of BSA	Final BSA Concentration (µg/ml)
A	0	300 µl of stock	2,000
B	125	375 µl of stock	1,500
C	325	325 µl of stock	1,000
D	175	175 µl of vial B dilution	750
E	325	325 µl of vial C dilution	500
F	325	325 µl of vial E dilution	250
G	325	325 µl of vial F dilution	125
H	400	100 µl of vial G dilution	25
I	400	0	0

3.10.2 SDS PAGE

SDS PAGE gels (Deionised water; 2.6% Acrylamide/bisacryl; 3 M Tris (stacking gel); 2 M Tris (running gel); 10% SDS; N,N,N^1,N^1, Tetramethylenediamine (TEMED); 10% Ammoniumperusulphate (APS)), were made. Mercaptoethanol (a reducing reagent) was added to each sample at a final concentration of 350 mM (0.25 µl of mercaptoethanol per 10 µl of sample). Samples were coloured with Bromophenol blue and boiled for 10 min and then allowed to cool down and 20 µg of protein per sample were loaded in the gels. A molecular weight marker (Precision Plus Dual Protein Colour Standard) was also loaded. Samples were run between 100 – 125 V at 60 mW in one times electrophoresis buffer (25 mM Tris, 192 mM Glycine and 0.1% SDS).

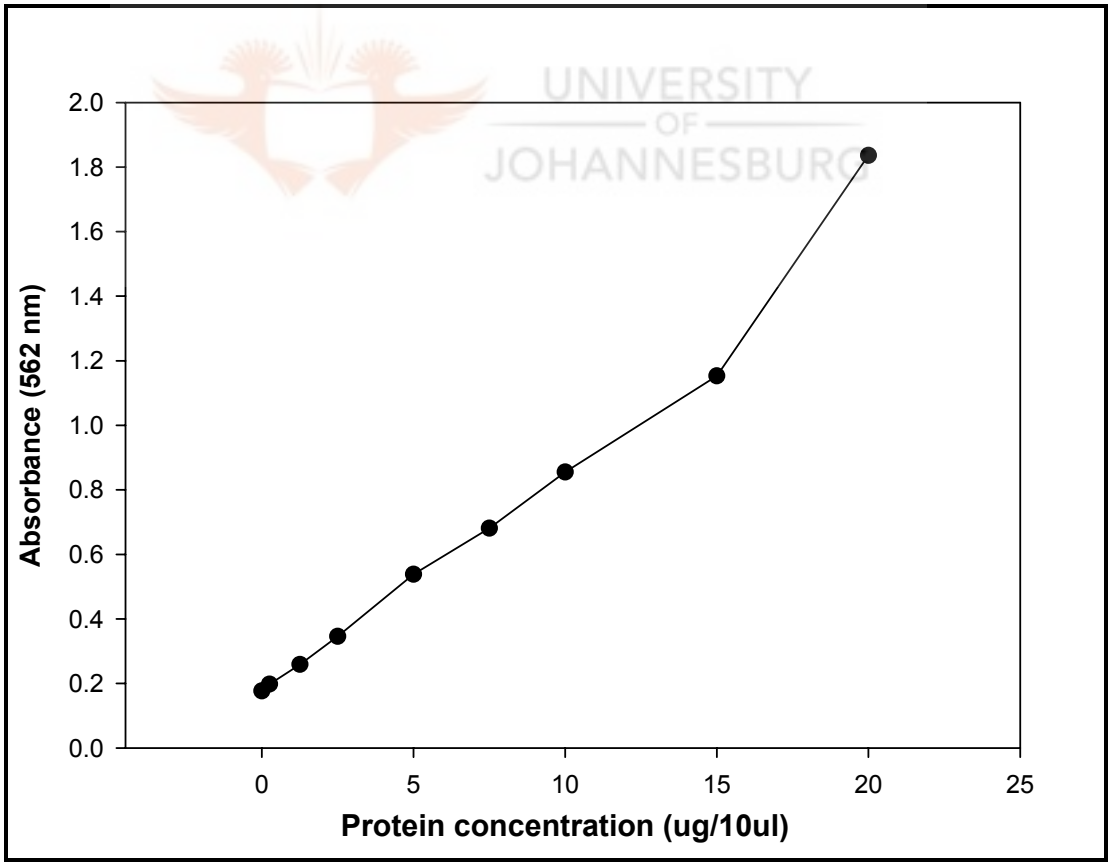


Figure 11. Absorbance and concentration of the BSA standards. The concentrations are in µg/10µl.

3.10.3 Immunoblotting and protein detection

Following electrophoresis, proteins from the gels were transferred to 0.2 µm immunoblot polyvinylidene diflouride (PVDF) membranes at 125 V for 1 h in one times transfer buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS and 20% Methanol). The blotting papers were then washed in Tween Tris Buffer Saline (TTBS; 50 mM Tris; 150 mM NaCl and 0.1% Tween-20) for 10 min at room temperature on a rotator (Heidolph Polymax 1040 – Labotec), ready for immunobloting. A schematic diagram of the whole western blot procedure is summarised in Figure 12.

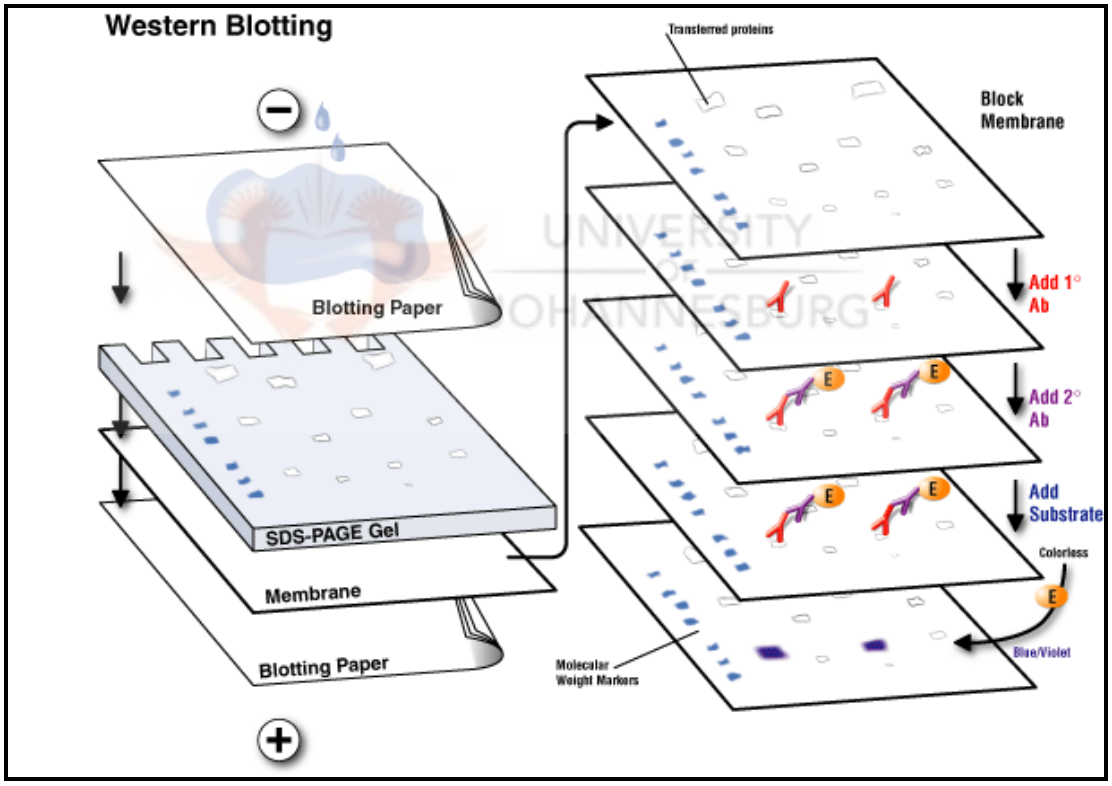


Figure 12. Schematic representation of the western blot procedure. Proteins are transferred from the gel to a PVDF membrane and detected using primary and secondary antibodies (Millpore Coorporation, 2007).

To detect the presence of β1-integrin, the blots were first blocked using 5% fat free milk in TTBS to block all remaining hydrophobic binding sites on the blots. This reduced background and prevented aspecific binding of the

primary antibody to the membrane (Wilson and Walker, 1995). The blots were incubated in primary antibody (2 µg/ml, 1:100, β1-integrin and Thy-1) diluted in blocking buffer (as above) for 1 h at room temperature. Blots were washed in TTBS. After incubation in secondary antibody (0.4 µg/ml, 1:1000, Goat anti-mouse horse-radish peroxidase for β1-integrin and 0.4 µg/ml, 1:1000, Goat anti-rabbit horse-radish peroxidase for Thy-1) diluted in the blocking buffer (as above) at room temperature for 2 h, blots were then washed again and incubated in chemiluminescent substrate for 10 min protected from light. The blots were then exposed to x-ray film for 2 and 4 min. The films were developed and then viewed.

3.11 Differention of ADSCs into Skin Fibroblast Cells

ADSCs were cultured in DMEM with 10% FBS and antibiotics. Semiconfluent cells were trypsinised, spun and resuspended in DMEM with 10% FBS, antibiotics. The cell suspension was adjusted to a cell concentration of 25 cells per µl and 2-Mercaptoethanol was added to a final concentration of $1x\ 10^{-6}$ Mol. Using a micropipette, 20 µl drops of the cell suspension were placed on the inside of the petri dish lids which were inverted so as to produce hanging drops. The lids were then put onto the petri dish bases into which 2 ml of HBSS were added (Figure 13). The petri dishes were incubated in CO_2 at 37^0C for 4 days.

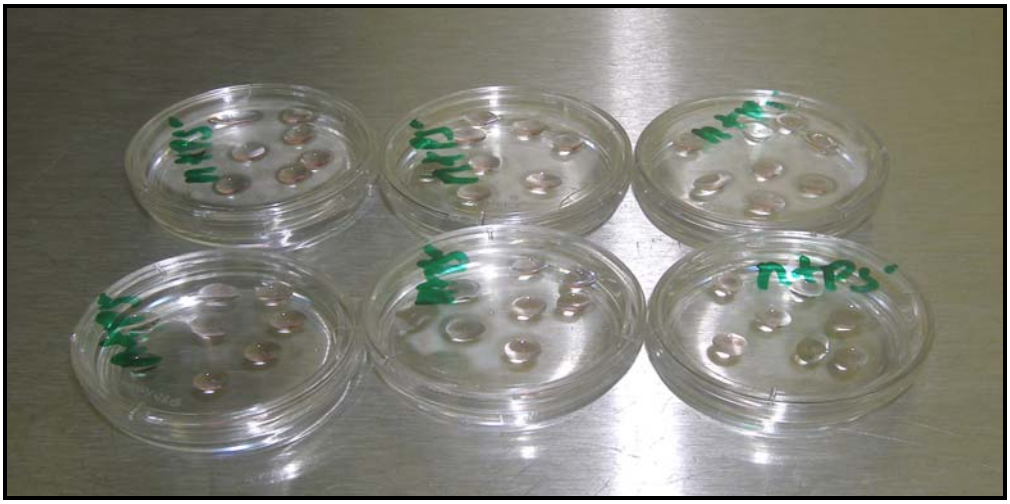


Figure 13. A photograph of hanging drops containing 25 cells/µl in DMEM medium with 1x10^{-6} Mol 2- Mercaptoethanol and antibiotics on the lids of petri dishes.

The small plates were coated with 0.1% gelatin and incubated in CO_2 at 37^{0}C overnight. Gelatin was removed the following day by aspirating just before the embryonic bodies were pipetted onto the plates. Embryonic bodies (ball/cluster of cells) that formed in the hanging drops were plated on 0.1% gelatin coated plates. The bodies were carefully pipetted onto the gelatin coated plates without dissociating them. Two millilitres of proliferation medium of 40 µl of B27, 20 ng/ml of EGF, 40 ng/ml of bFGF, antibiotics and DMEM was then carefully added to the plates (Schuldiner *et al*., 2000). The plates were then incubated in CO_2 at 37^{0}C and growth of the cells from the attached bodies were observed every day as observed in Figure 14 A, B, C and D shown by the arrows.

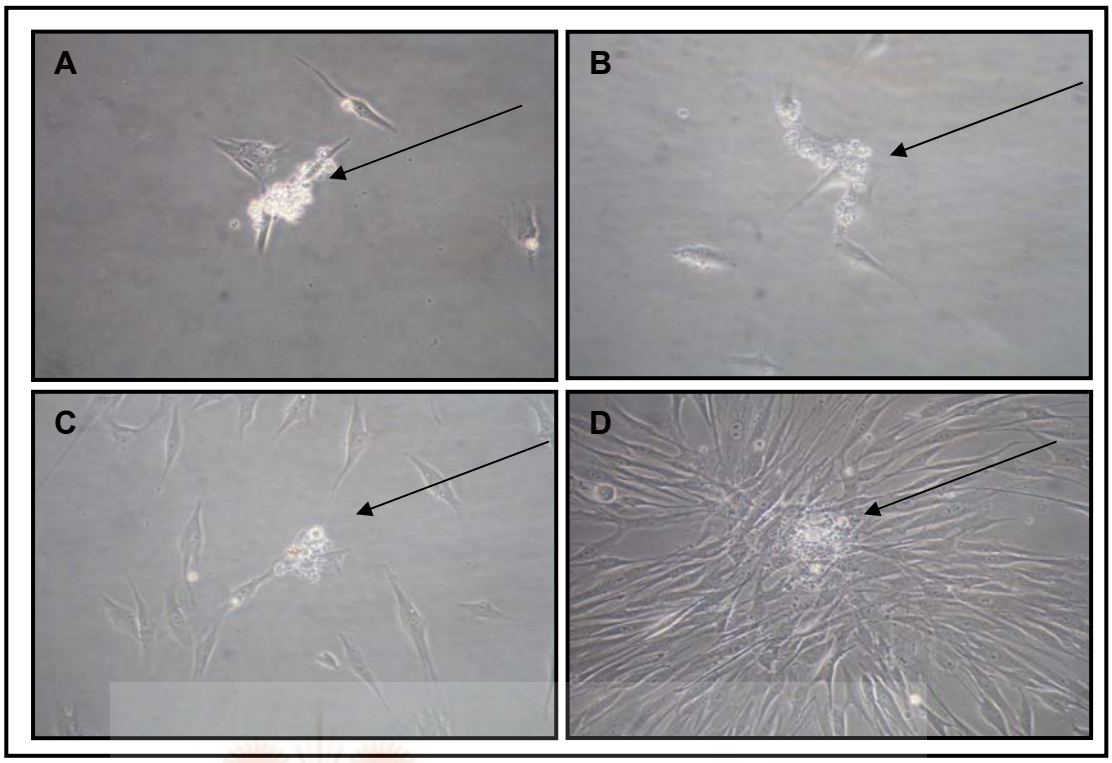


Figure 14. Differentiation of ADSCs in culture. A, B and C, cells growing from the attached bodies (cluster of cells) plated on gelatine coated plates indicated by arrows. D, a focus of cells extending from an embryoid body. 400x magnification.

3.12 Buffy Coat Cell lysate Preparation

Cell lysate from the buffy coat of human peripheral blood was used as a positive control in immunodetection of fibroblast surface protein. Whole blood was collected in 4 anticoagulated tubes. The tubes were spun down and plasma was removed. The buffy coat (creamy layer just above the red cells) was aspirated and washed in 500 µl HBSS for 3 times, 5 min each wash at 4^0C at 13,300 rpm in a microcentrifuge (Haraeus Fresco 17). Equal amounts of HBSS and 2 times sample buffer were added to the washed buffy coat and the mixture was sonicated and spun down at 4^0C for 5 min at 13,300 rpm. The sample was then stored at -20^0C for Western blotting and dot blotting.

3.13 Dot Blot Method

Cell lysate samples were diluted in denaturing buffer to a final concentration of 100 ng/µl. The absorbances of the diluted lysates were measured at A_{280} and concentrations of the lysates were determined. PVDF membrane was moistened in TBS buffer until evenly wet. The membrane was then placed on dry filter to drain for 5 min. The membrane was marked with a pencil as a guide for spotting and 1 µl of diluted lysate was applied directly onto the membrane and dried at room temperature. For higher concentration, an additional 1 µl was applied to the spot and then allowed to dry prior to immunodetection.

3.14 Statistical Analysis

All laser irradiation experiments and biochemical assays associated with measuring changes as a result of laser irradiation were performed six times (n=6). Statistical analysis was performed using Sigma Plot 8.0 software. Differences between groups were determined using the student T-test for each independent variable.

CHAPTER 4

RESULTS

ADSCs were cultured in DMEM with 10% FBS and antibiotics (Penicillin/Streptomycin and Fungizone) irradiated at 636 nm with 5 J/cm^2 and 12.1 mW/cm^2 power density. The cells were also cultured in DMEM supplemented with EGF and irradiated at 636 nm with 5 J/cm^2 and 12.1 mW/cm^2 power density. Cells were irradiated once. Cell morphology, viability, proliferation and the expression of proteins were evaluated at 0, 24 and 48 h. WS1 cells, cultured in EMEM were used as controls in monitoring the differentiation of ADSCs into skin fibroblast cells. The protein expression of Fibroblast Surface Antigen was used to monitor differentiation of ADSCs into skin fibroblast cells.

4.1 Cell Morphology

Morphologically, the ADSC's present as a monolayer of smooth elongated cells as viewed using an inverted microscope (Figure 15). No noticeable difference in morphology was noted between the irradiated and control cells at 0, 24 or 48 h. However compared to the non-irradiated cells (Figure 15 A), irradiated cells at 24 and 48 h (Figure 15 B) showed a greater density, indicative of higher proliferation. Addition of EGF to the cultures did also not noticeable alter the morphology of ADSCs. However an increase in proliferation was observed in irradiated cultures with EGF compared to cultures with EGF only. The proliferation rate was higher at 48 than at 24 and 0 h (Figure 15 C and D).

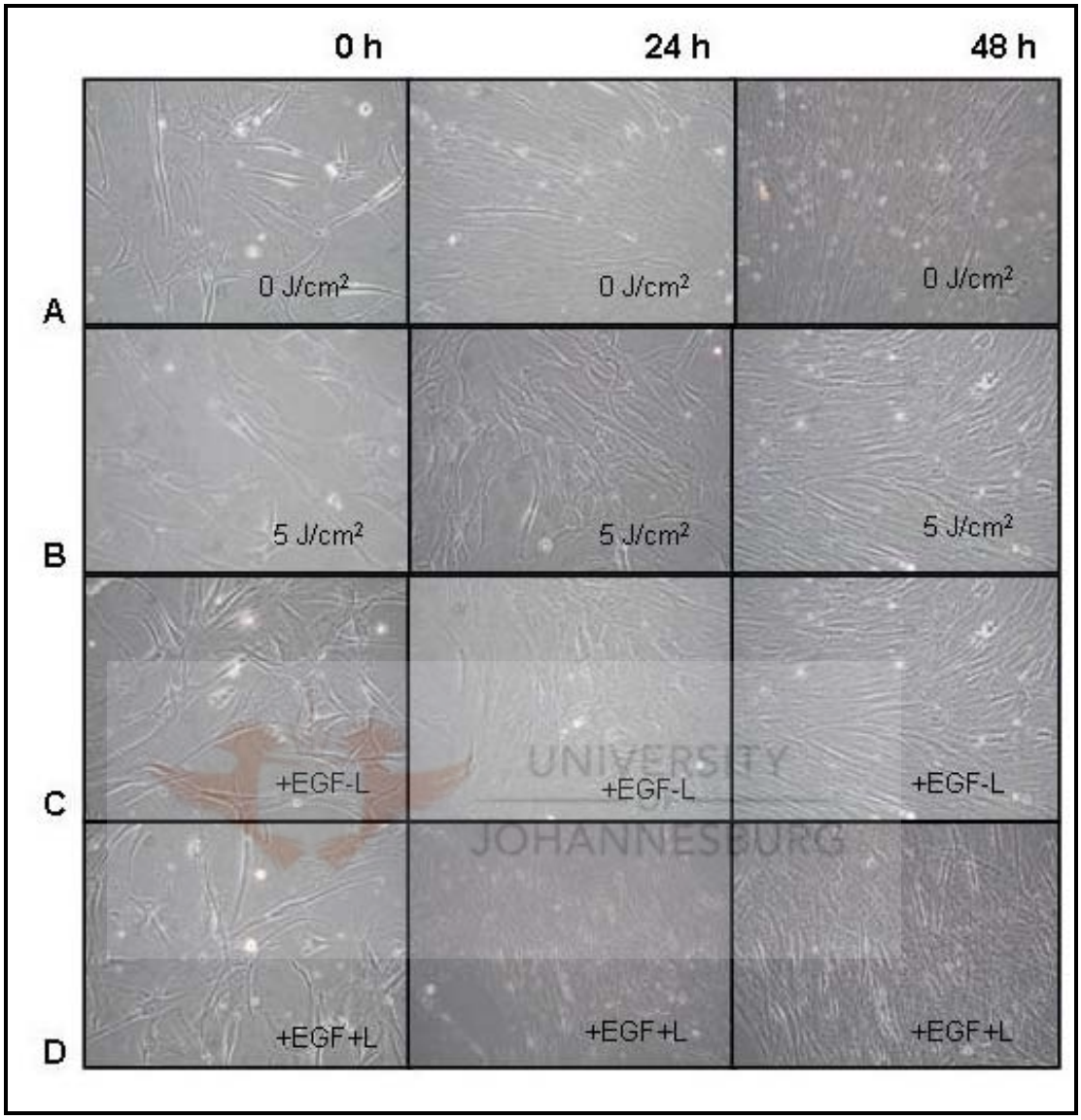


Figure 15. Morphology of ADSC's at 0, 24 and 48 h. Morphologically, there was no difference between irradiated and non-irradiated cells. However, compared to the non-irradiated control (A), irradiated cells (B) proliferated more at 24 and 48 h. Addition of EGF to the cultures did not appear to alter the morphology. However, compared to the control and irradiated cells on diagrams A and B respectively, proliferation increased in both the EGF only (C) and EGF and irradiated (D) groups. The proliferation was more in EGF and irradiated as compared to EGF only. This was also observed more at 48 than at 24 and 0 h. 400x magnification.

4.2 Cell Viability

Trypan Blue exclusion test indicated an increase in percentage cell viability in cells that were irradiated compared to control cells (non-irradiated) at 0, 24

and 48 h. The increase was statistically significant at 48 h (P<0.05) while at 0 and 24 h the increase was not significant (P=0.248 and P=0.126 respectively) (Figure 16).

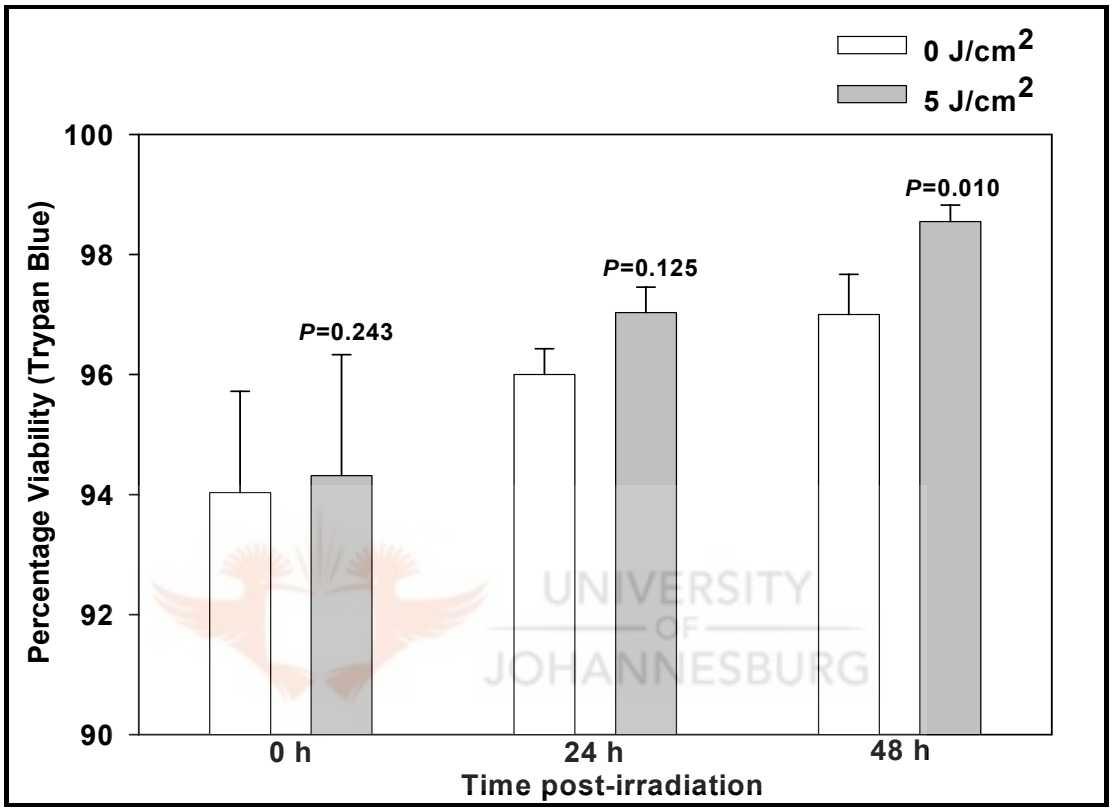


Figure 16. Trypan Blue exclusion test was used to measure percentage cell viability in ADSCs irradiated with 5 J/cm^2. Non-irradiated cells were used as controls. Percentage cell viability showed an increase in irradiated cells evaluated at 24 and 48 h compared to their respective control cells but at 0 h, there was no significant difference in percentage cell viability.

The increase in percentage cell viability was not significant in control cells at 48 h compared to control cells at 24 and 0 h (P=0.237 and P=0.188 respectively). The increase was also not significant in control cells at 24 h compared to control cells at 0 h (P=0.357). In irradiated cells, percentage cell viability showed a significant increase at 48 h compared to cells at 24 and at 0 h (P=0.05 and P<0.05 respectively) but the increase was not significant at 24h compared to cells at 0 h (P=0.216).

Trypan Blue exclusion test showed an increase in percentage viability in cells that were incubated with EGF and irradiated compared to their respective control cells at 0, 24 and 48 h (P<0.001, P<0.001 and P<0.0001 respectively) (Figure17).

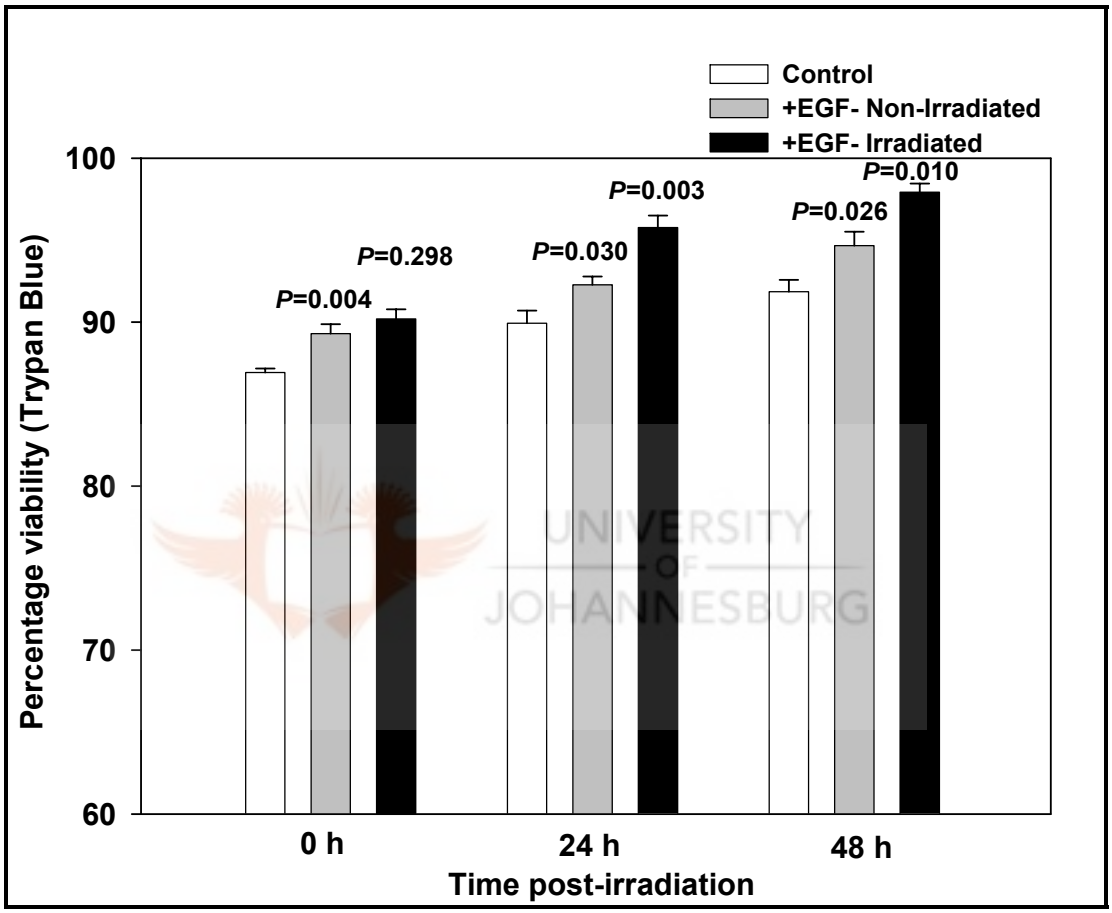


Figure 17. Trypan Blue exclusion was used to measure cell viability in ADSCs. Cells incubated with EGF were either irradiated with 5 J/cm^2 or non-irradiated. Cells without EGF and non-irradiated were used as controls. Percentage viability showed an increase in cells incubated with EGF and irradiated compared to their respective controls at 0, 24 and 48 h. There was an increase in percentage viability in cells incubated with EGF and non- irradiated compared to their respective controls at 0, 24 and 48 h. The results for percentage viability showed an increase in cells incubated with EGF and irradiated compared to cells incubated with EGF and non-irradiated at 24 and 48 h but at 0 h, there was no significant difference.

The percentage viability results showed significant increases in cells incubated with EGF and irradiated compared to cells incubated with EGF alone at 24 and 48 h (P<0.001and P<0.05 respectively), however the increase was not significant at 0 h (P=0.298). The increase in percentage viability was significant at 0, 24 and 48 h in cells that were incubated with EGF and non-irradiated compared to their respective controls (P<0.01, P<0.05 and P<0.05 respectively).

In cultures incubated with EGF and irradiated, the increase in percentage viability was significant at 48 h compared to cells at 0 h (P<0.001), at 48 h compared to cells at 24 h (P<0.01) and at 24 h compared to cells at 0 h (P<0.05). Cells incubated with EGF and non-irradiated showed significant increases in percentage viability at 48 h compared to cells at 0 h (P<0.001), at 48 h compared to cells at 24 h (P<0.01) and at 24 h compared to cells at 0 h (P<0.05). In control cells, the increase in percentage viability was significant at 48 h compared to cells at 0 h (P<0.01). The increase was not significant at 48 h compared to cells at 24 h (P=0.102) and at 24 h compared to cells at 0 h (P=0.085).

ATP luminescence showed that there was a statistically significant increase in ATP concentration in irradiated cells when compared to non-irradiated cells (control cells) at 24 and 48 h (P<0.05 and P<0.05 respectively) but at 0 h the increase was not significant (P=0.520) as shown in figure 18. In non-irradiated cells, the increase in ATP concentration was not significant at 48 h compared to cells at 0 h (P=0.137) and 24 h (P=0.496). The results also showed no significant increase at 24 h compared to cells at 0 h (P=0.371). In irradiated cells, the increase was significant at 48 h compared to cells at 0 h (P<0.005) and at 24 h compared to cells at 0 h (P<0.05) but the increase at 48 h was not significant compared to cells at 24 h (P=0.396).

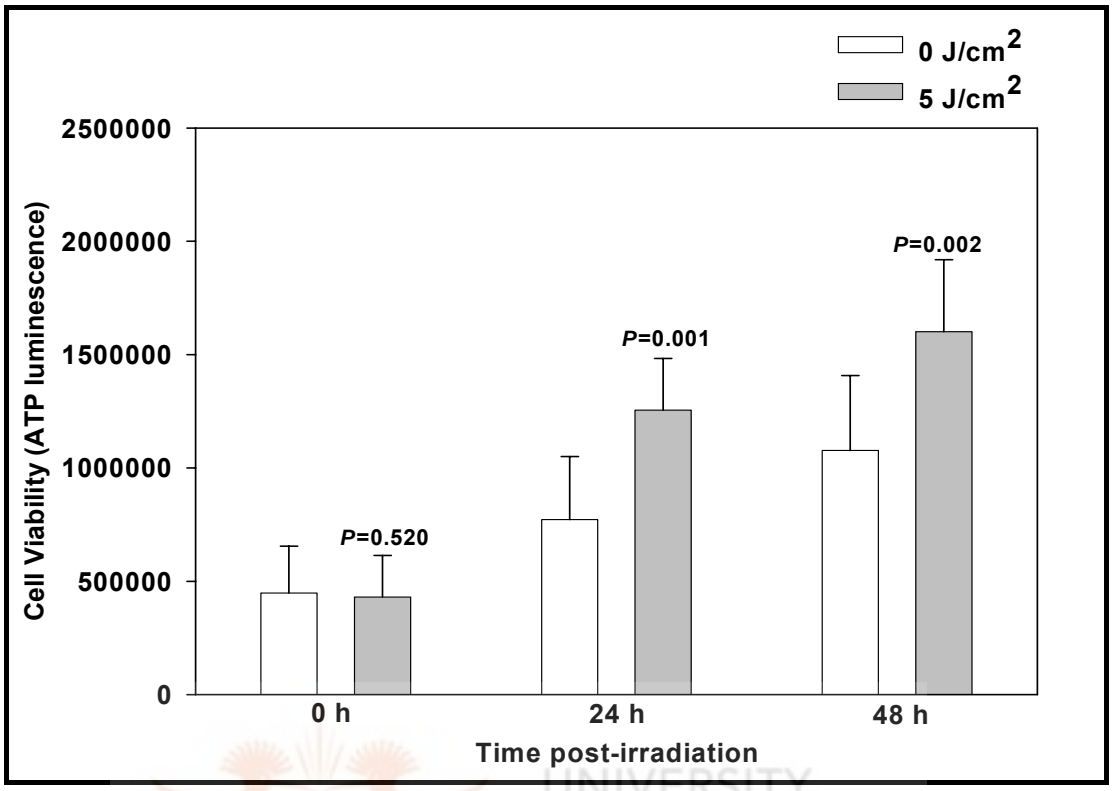


Figure 18. ATP luminescence assay was used to determine cell viability of ADSCs irradiated with 5 J/cm^2. Non-irradiated cells were used as controls. ATP luminescence showed an increase in cell viability in irradiated cells compared to their respective control cells at 24 and 48 h, however there was no difference in ATP luminescence at 0 h.

ATP luminescence showed a significant increase in cells with EGF and irradiated compared to their respective controls at 0, 24 and 48 h (P<0.001, P<0.05 and P<0.001 respectively) (Figure 19). The increase was also significant in cells with EGF and non-irradiated compared to their respective controls at all time points (P<0.001, P=0.05 and P<0.001 respectively). The increase in ATP luminescence in cells with EGF and irradiated compared to cells with EGF and non-irradiated was significant at 24 and 48 h (P<0.05 and P<0.01 respectively), however at 0 h there was no difference in ATP luminescence (P=0.626). In cells with EGF and irradiated, the increase in ATP luminescence was significant at 48 h compared to cells at 0 h (P<0.001).

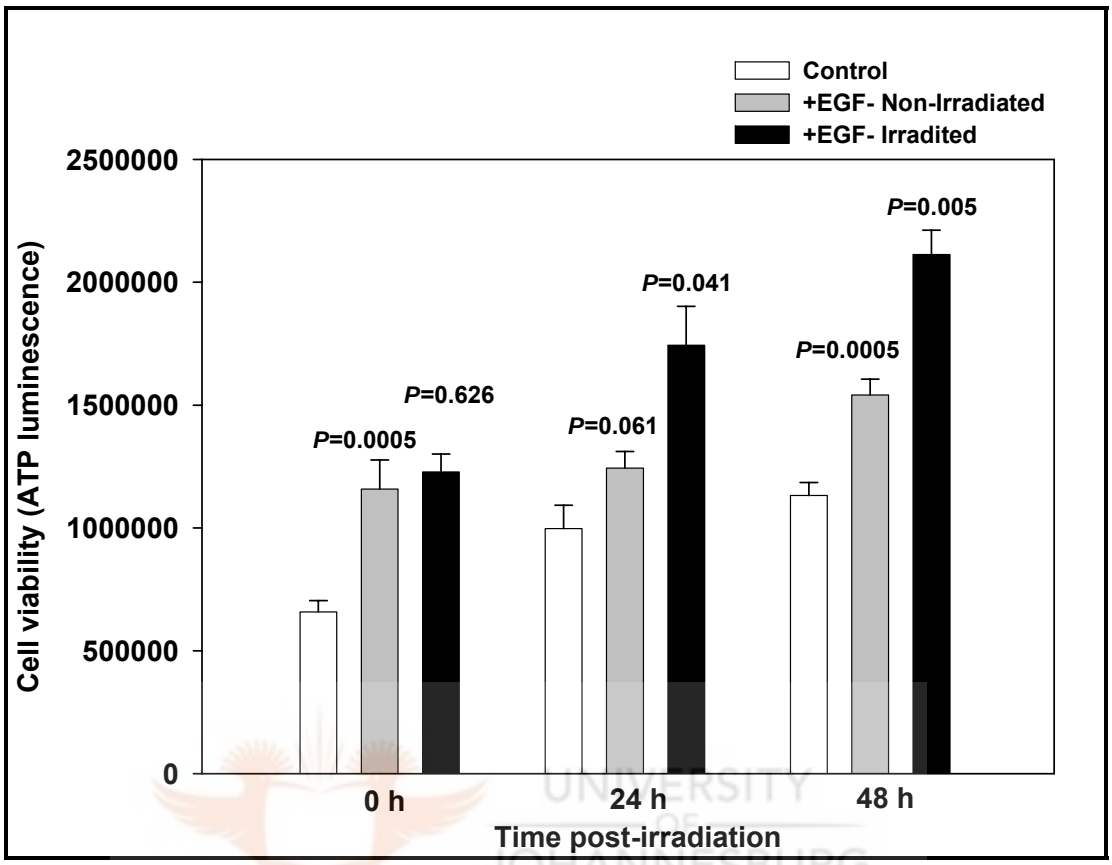


Figure 19. **ATP luminescence was used to determine cell viability of ADSCs. Cells with EGF were either irradiated with 5 J/cm^2 or non-irradiated. Cells without EGF and non-irradiated were used as controls. ATP luminescence showed an increase in cells with EGF and irradiated compared to their respective control cells at 0, 24 and 48 h. The results showed an increase in cell viability in cells with EGF and non-irradiated compared to their respective controls at 0, 24 and 48 h. At 24 and 48 h ATP luminescence showed an increase in cells cultured with EGF and irradiated compared to non-irradiated cells cultured with EGF, however there was no significant difference in ATP luminescence at 0 h.**

The significant increase was also observed at 48 h compared to cells at 24 h (P=0.05), and at 24 h compared to cells at 0 h (P<0.05). Cells with EGF and non-irradiated showed a significant increase in ATP luminescence at 48 h compared to cells at 0 h (P<0.01). This observation was also seen at 48 h compared to cells at 24 h (P<0.05), however the increase was not significant at 24 h compared to cells at 0 h (P=0.145). In control cells, the increase in ATP luminescence was significant at 48 h compared to cells at 0 h (P<0.001).

The increase was not significant at 48 h compared to cells at 24 h (P=0.20) and this was also observed at 24 h compared to cells at 0 h (P=0.12).

4.3 Cell Proliferation

Irradiated cells at 24 and 48 h showed a statistically significant increase in OD compared to their respective controls (P=0.050 and P<0.05 respectively), however at 0 h there was no significant increase (P=0.986) (Figure 20).

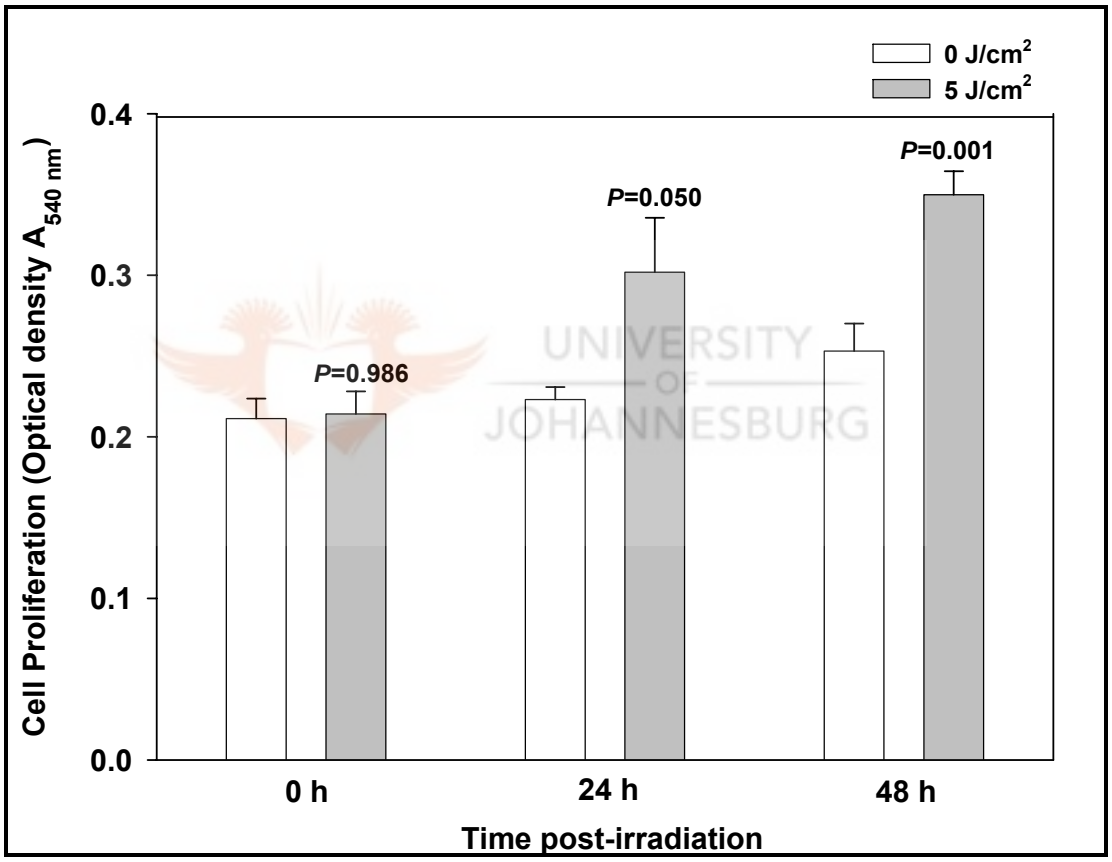


Figure 20. Optical density assay was used to determine cell proliferation in ADSCs irradiated with 5 J/cm^2. Non-irradiated cells were used as controls. The results showed an increase in OD in irradiated cells compared to their respective control cells at 24 and 48 h post irradiation, but at 0 h there was no difference in OD.

In non-irradiated cells, the OD results showed no significant increase at 48 h compared to cells at 0 h (P=0.072) and this was also observed at 48 h

compared to cells at 24 h (P=0.138). The increase was not also significant at 24 h compared to cells at 0 h (P=0.436). In irradiated cells, the increase in OD was significant at 48 h compared to cells at 0 h (P<0.01) and this observation was seen at 24 h compared to cells at 0 h (P<0.05), however, the increase at 48 h compared to cells at 24 h was not significant (P=0.138).

Optical density results showed an increase in cell proliferation in cells incubated with EGF and irradiated compared to their respective controls (Figure 21). The increase was significant at 24 and 48 h (P<0.01 and P=0.001 respectively), however the increase was not significant at 0 h (P=0.126). Cells incubated with EGF and non-irradiated also showed an increase in cell proliferation compared to their respective controls. The increase was significant at 24 and 48 h *(*P<0.05) not at 0 h *(*P=0.161). Cells incubated with EGF and irradiated showed a significant increase in cell proliferation compared to cells with EGF and non-irradiated at 48 h (P=0.050), however at 0 and 24 h the increase was insignificant (P=0.792 and P=0.116 respectively).

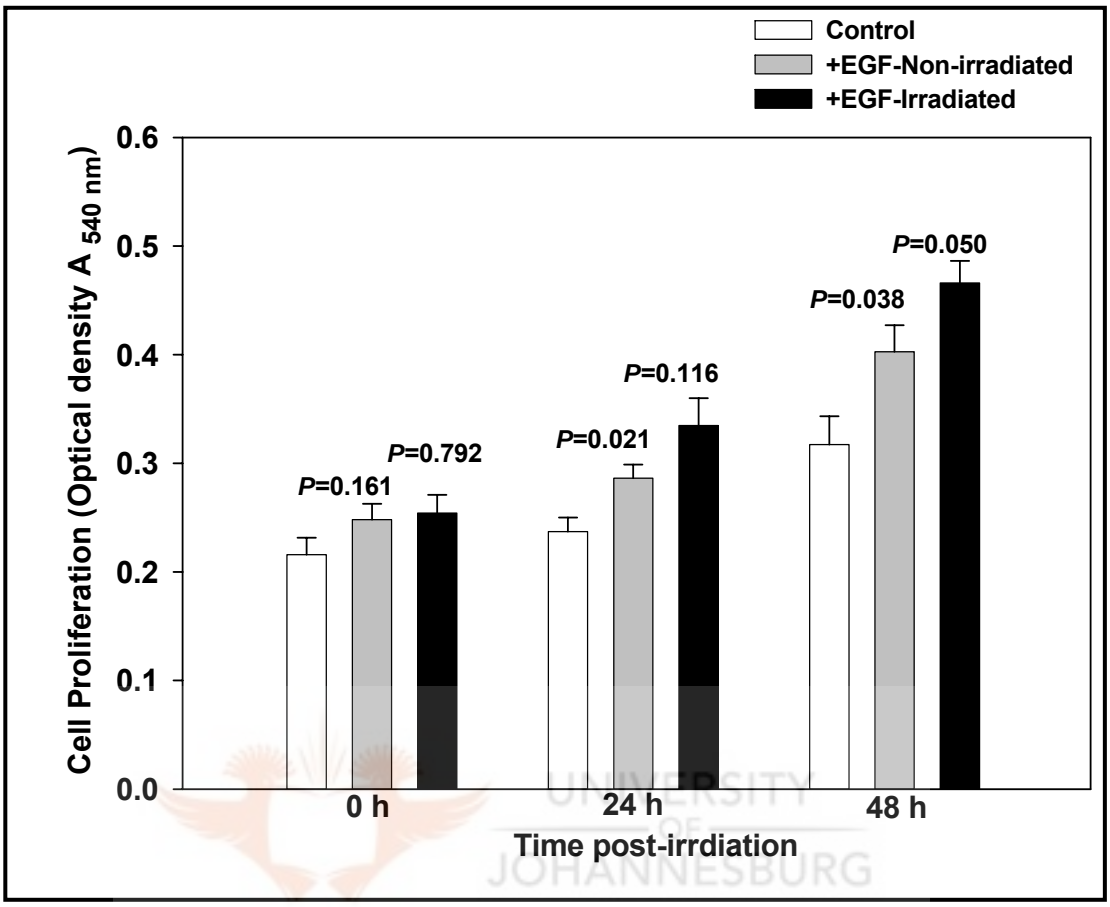


Figure 21. Optical density assay was used to determine cell proliferation in ADSCs. Cells incubated with EGF were either irradiated with 5 J/cm^2 or non-irradiated. Cells without EGF and not irradiated were used as controls. The results showed an increase in cell proliferation in cells with EGF and irradiated compared to their respective control cells at 0, 24 and 48 h. The increase in OD was also observed in cells with EGF and non-irradiated compared to their respective control cells at 0, 24 and 48 h. Cells incubated with EGF and irradiated also showed an increase in OD compared to cells incubated with EGF and non-irradiated at 48 and 24 h. At 0 h, there was no significant difference.

In cells incubated with EGF and irradiated, the increase in cell proliferation was significant at 48 h compared to cells at 0 h (P<0.001). This was also observed at 48 h compared to cells at 24 h (P<0.001), and at 24 h compared to cells at 0 h (P<0.05). Cells incubated with EGF and non-irradiated showed a significant increase in cell proliferation at 48 h compared to cells at 0 h (P<0.001). The observation was also seen at 48 h compared to cells at 24 h (P<0.001), however the increase at 24 h compared to cells at 0 h was not

significant (*P*=0.076). In control cells, the increase in cell proliferation through OD was significant at 48 h compared to cells at 0 h (*P*<0.01) and at 48 h compared to cells at 24 h (*P*<0.05) but the increase was not significant at 24 h compared to cells at 0 h (*P*=0.323).

bFGF results (Figure 22) showed a significant increase in irradiated cells compared to control cells at 48 h (*P*=0.050). The increase was not significant at 0 and 24 h (*P*=0.990 and *P*=0.347 respectively).

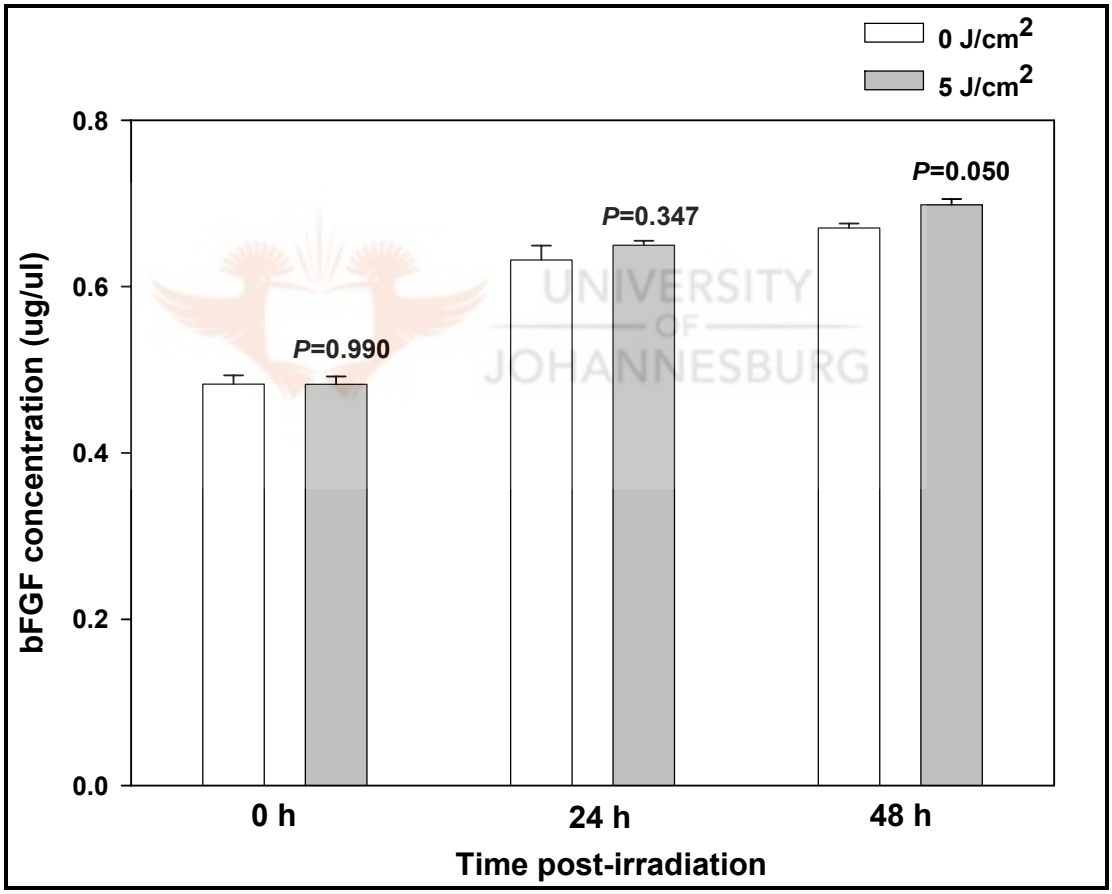


Figure 22. bFGF concentration was used to determine cell proliferation in ADSCs irradiated with 5 J/cm^2. Non-irradiated cells were used as controls. bFGF concentration showed an increase in irradiated cells compared to their respective control cells at both 24 and 48 h, however there was no difference at 0 h.

The increase in bFGF in non-irradiated cells was significant at 48 h compared to cells at 0 h (P<0.01) and this was also observed at 24 h compared to cells at 0 h (P<0.05), however, the increase at 48 h compared to cells at 24 h was not significant (P=0.062). In irradiated cells, the increase in bFGF was significant at 48 h compared to cells at 0 h (P<0.01). The observation was also seen at 48 h compared to cells at 24 h (P<0.01) and at 24 h compared to cells at 0 h (P<0.05).

bFGF results showed a significant increase in cell proliferation in cells with EGF and irradiated compared to their respective controls at 0, 24 and 48 h (P<0.05, P<0.001 and P<0.0001 respectively) (Figure 23). The increase was also significant in cells with EGF and non-irradiated compared to their respective control cells at 0 and 24 h (P<0.05 and P<0.01 respectively), but at 48 h the increase was not (P=0.444). The increase in bFGF concentration in cells with EGF and irradiated compared to cells with EGF and non-irradiated was not significant at all time points (P=0.978, P=0.060 and P=0.329 respectively).

In cells with EGF and irradiated, the increase in bFGF concentration was significant in cells at 48 h compared to cells at 0 h (P<0.001). The observation was also seen at 48 h compared to cells at 24 h (P<0.05) and at 24 h compared to cells at 0 h (P<0.05). Cells with EGF and non-irradiated showed a significant increase in bFGF concentration at 48 h compared to cells at 0 h (P<0.001), at 24 h compared to cells at 0 h (P<0.05), however the increase in bFGF concentration at 24 h compared to cells at 0 h was not significant (P<0.14). In control cells, the increase in bFGF concentration was significant at 48 h compared to cells at 0 h (P<0.01) and this was also observed at 48 h compared to cells at 24 h (P<0.05). The increase was not significant at 24 h compared to cells at 0 h (P=0.072).

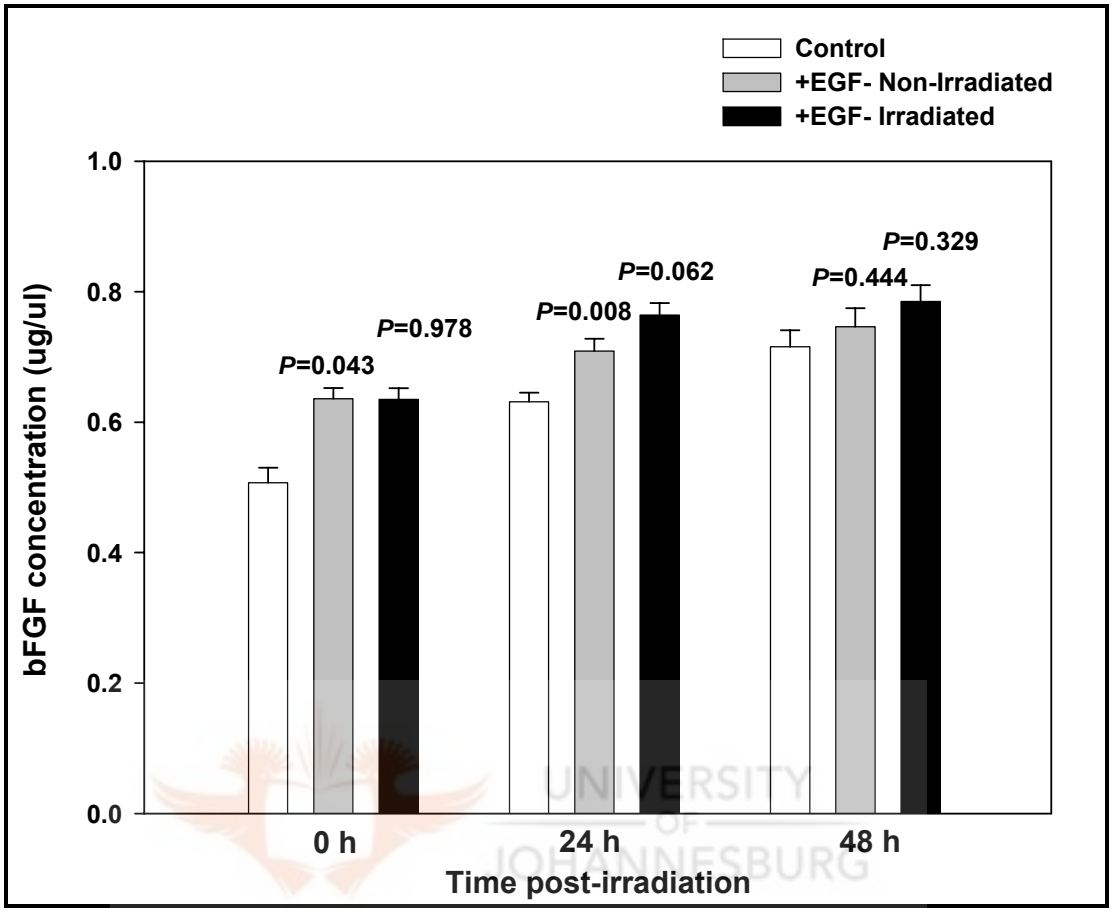


Figure 23. **bFGF concentration in µg/µl (n=6) was used to determine cell proliferation in ADSCs. Cells with EGF were either irradiated with 5 J/cm^2 or non-irradiated. Cells without EGF and not irradiated were used as controls. The results showed an increase in cells that had EGF and were irradiated compared to their respective control cells at 0, 24 and 48 h. bFGF results showed an increase in cells with EGF and irradiated compared to cells with EGF and non-irradiated at 24 and 48 h but at 0 h, there was no significant difference. Cells with EGF and non-irradiated showed significant increases in bFGF concentration compared to their respective controls at 0, 24 and 48 h.**

4.4 Protein Expression

4.4.1 β1-Integrin expression

Immunocytochemical live cell labelling showed the expression of β1-integrin in irradiated cells (Figure 24). Western blot analysis showed an increase in β1-integrin expression in cells irradiated at 24 and 48 h compared to

non-irradiated cells (Figure 25). This expression was increased at 24 h compared to cells incubated at 37 ^{0}C for 48 h.

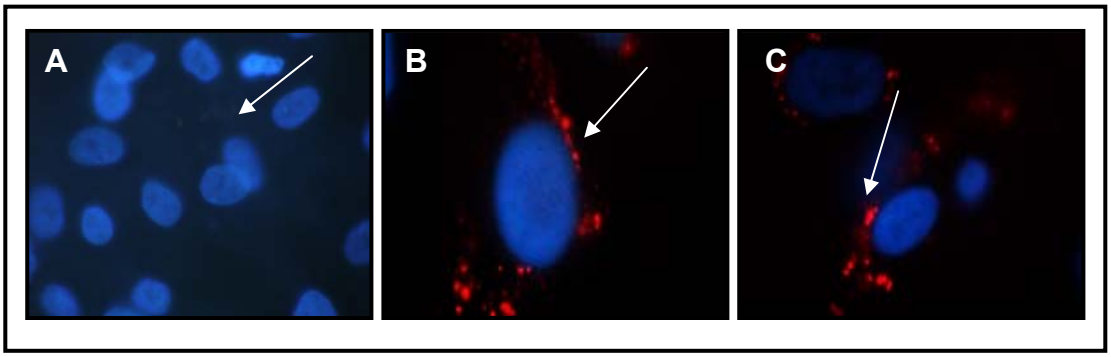


Figure 24. **β1-integrin expression. Expression of β1-integrin (red) in ADSCs was determined by immunocytochemical staining. Weak expression of β1-integrin in non-irradiated ADSC's. The arrow shows faintly β1-integrin (A). Post irradiation surface expression of β1-integrin is shown by the arrows on diagrams (B) and (C). Nuclei are counter stained with DAPI (blue).**

Western blot analysis revealed an increased expression of β1-integrin in irradiated cells compared to non-irradiated cells. The expression was higher at 24 than 48 h as seen in Figure 25 below.

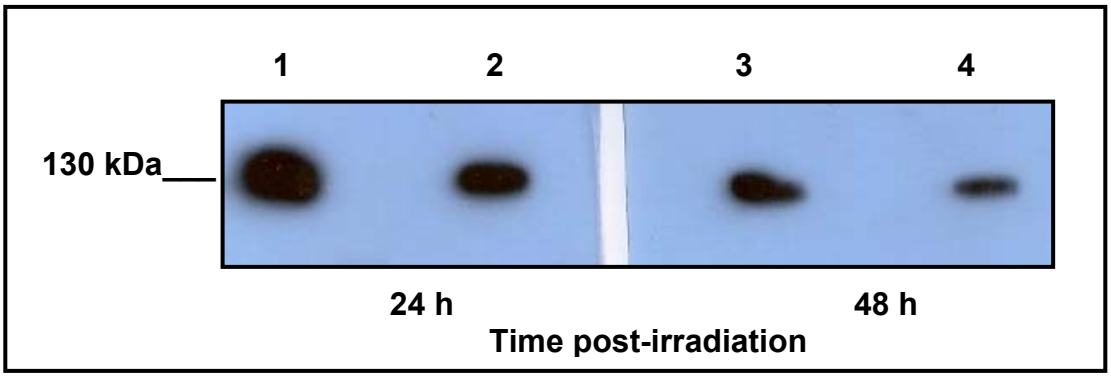


Figure 25. **Western blot analysis of the expression β1-integrin in irradiated and control cells (non-irradiated cells) at 24 and 48 h post irradiation. Expression of β1- integrin is higher in irradiated cells than in non-irradiated cells. Lane (1) irradiated 24 h, (2) non-irradiated 24 h, (3) irradiated 48 h and (4) non-irradiated 48 h.**

Western blot analysis of ADSCs cultured with EGF and irradiated revealed an increase in expression of β1-integrin at 24 and 48 h compared to 0 h however the expression of β1-integrin at 24 h was higher than at 48 h (Figure 26).

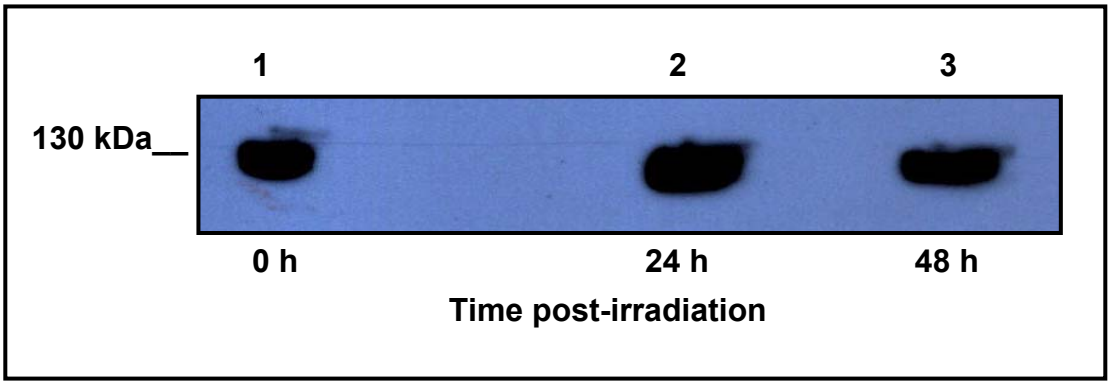


Figure 26. Western blot analysis of the expression of β1-integrin in ADSC's with EGF and irradiated. There was higher expression of β1-integrin at 24 h than at 48 and 0 h.

4.4.2 Thy-1 expression

Western blot analysis of ADSCs showed an increase in expression of the stem cell marker Thy-1, in irradiated cells at 24 and 48 h. However the expression was higher at 24 h than at 48 h as shown in Figure 27.

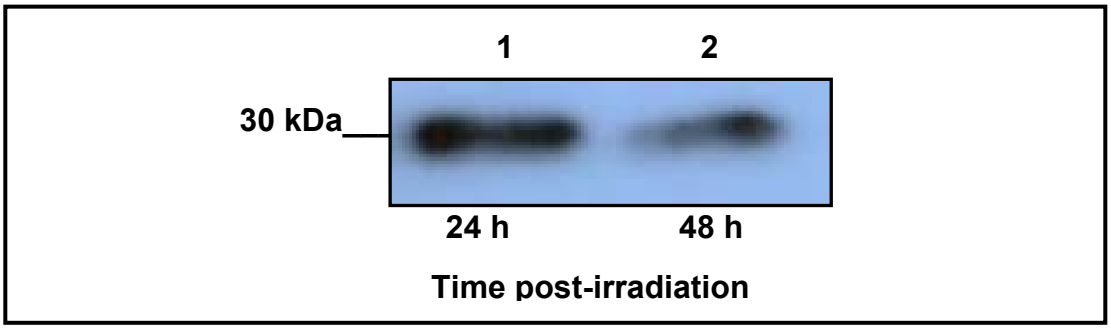


Figure 27. Western blot analysis of the expression of Thy-1 in irradiated cells at (Lane 1) and 48 h (Lane 2) post irradiation. There is more expression of Thy-1 at 24 than at 48 h.

Similar to β1-Integrin expression, there was an increase in expression of Thy-1 in cells that were irradiated and incubated with EGF at 24 h than at 48 h (Figure 28).

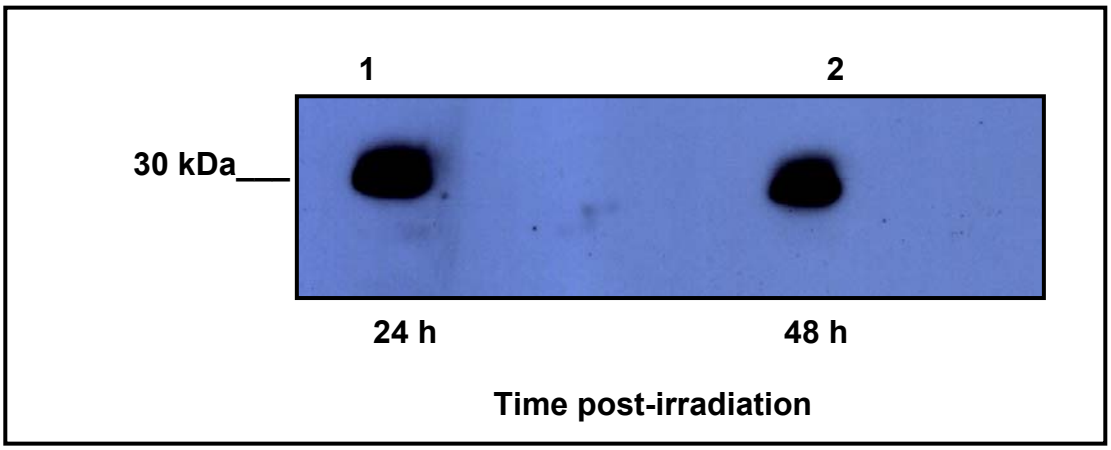


Figure 28. **Western blot analysis of the expression of Thy-1 in irradiated cells incubated with EGF at 24 and 48 h post irradiation. There was higher expression of Thy-1 at 24 than at 48 h.**

4.4.3 Fibroblast surface protein expression

Fibroblast surface protein was observed in buffy coat cell lysate samples through a dot blot method (lane A-4, B-1 and B-2). The marker was not observed in ADSCs and differentiated ADSCs lysate samples (lane A-1, A-2 and A-3) or in WS1 cell lysate samples (lane B-3 and B-4) (Figure 29).

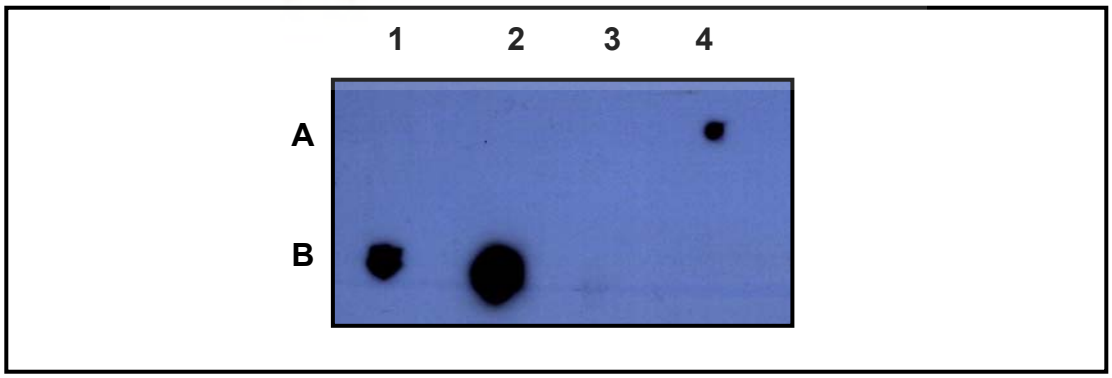


Figure 29 **Dot blot results showing the expression of fibroblast surface protein in buffy coat cell lysates prepared from human peripheral blood(lane A-4, B-1 and B-2). The presence of fibroblast surface protein was not observed in ADSCs and differentiated ADSCs samples or in WS1 samples (lane A-1, A-2, A-3, B-3 and B-4).**

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Adipose tissue contains an abundant, accessible source of adult stem cells, and the stem cells provenient from this tissue are termed adult adipose derived stem cells (ADSCs) (Gimble and Guilak, 2003; Safford and Rice, 2005). ADSCs have been shown to be able to differentiate into smooth muscle, bone, fat, cartilage and neuronal tissue when treated with specialised induction media *in vitro* (Rodriguez *et al*., 2006; Serakinci and Keith, 2006). Once differentiated, these cells could potentially be used in stem cell therapy to replace or repair damaged tissues and organs. A number of disorders that are amenable to this approach include neurological and cardiovascular diseases, as well as bone defects and diabetes (Keith, 2006). In this study, ADSCs were derived from human adipose tissue and the effects of low level laser irradiation alone as well as in combination with EGF were evaluated *in vitro* at 0, 24 and 48 h post irradiation. To date, no research conducted on the effect of laser irradiation on ADSCs alone as well as in combination with EGF other than the work presented in this dissertation have been published.

LLLT has been shown to have a variety of biostimulatory effects such as wound healing (Houreld and Abrahamse, 2005; Hawkins *et al.*, 2005), fibroblast proliferation (Kana *et al.*, 1981; Boulton and Marshall, 1986; Van Breugel, and Bar, 1992), nerve regeneration (Anders *et al*., 1993), and collagen synthesis (Lams *et al.*, 1986). Studies on LLLT and stem cells have shown that LLLT increases migration of stem cells *in vitro* (Gasparyan *et al.*, 2004). Furthermore, LLLT has also been found to promote proliferation and differentiation of human osteoblast cells *in vitro* at 632nm with power output of 10 mW (Stein *et al*., 2005) as well as promote proliferation of mesenchymal and cardiac stem cells in culture at 1 and 3 J/cm^2 (Tuby *et al*., 2007). To our

knowledge, the work presented in this dissertation is the first study investigating the effect of low level laser irradiation on human ADSCs.

This study found out that LLLT had no effect on cellular morphology at all time points, however there was more confluency at 24 and 48 h than at 0 h. This may be due to fact that the stimulatory effect of LLLT is responded by the cells when more time is given to react (Houreld and Abrahamse, 2006b). There was no damage to the cell morphology. This corresponds to the findings of Hawkins and Abrahamse (2005; 2006) who found out that lower fluences of 2.5 and 5 J/cm^2 had more positive effects on cells than at higher fluence (10 J/cm^2).

In this study LLLT was found to increase cell viability, cell proliferation and the expression of β1-integrin and Thy-1 in ADSCs. Cell viability increased at 24 and 48 h in irradiated cells compared to non-irradiated cells. Both Trypan blue and ATP luminescence showed an increase at 24 and 48 h post irradiation agreeing with literature (Hawkins and Abrahamse, 2005). The effect of LLLT on cells is based on an increase in mitochondrial oxidative metabolism caused by excitation of components of the respiratory chain leading to an increase in ATP (Karu, 2003). Therefore ATP luminescence is a significant test in cell viability. Optical density measurements at 24 and 48 h indicated an increase in cell proliferation in the irradiated cells, however the proliferation was greater at 48 than at 24h. Although an increase in optical density can be used to measure cellular proliferation (Pinheiro *et al*., 2002), optical density may also increase due to cellular lysis and fragmentation (Hawkins and Abrahamse, 2005) therefore this test should be done in combination with other tests which are more sensitive like bFGF. Cell proliferation studies monitoring the expression of bFGF revealed an increase in cell proliferation in irradiated cells compared to non-irradiated ones.

However, at 0 h there was no difference in cellular responses as the cells needed more time to respond to LLLT.

In 2005, Hawkins and Abrahamse found that helium-neon laser irradiation of 5 J/cm^2 resulted in an increase in cellular proliferation of human skin fibroblast cells (WS1). At the same dosage and wavelength, this study obtained similar results using ADSCs, proving that LLLT can produce positive effects on different cell types. These results demonstrate that LLLT significantly promotes the increase in cell number or proliferation of ADSCs. The results agree with those found by Mvula *et al*., (2007) who found that LLLT increased cell viability, proliferation and expression of β1-integrin at 24 and 48 h at 635 nm and 5 J/cm^2.

The findings of this study concur with those of Aust *et al* 2004 who reported that ADSCs were positive for the stem cell surface marker, β1-integrin. This study, showed an increase in β1-integrin expression in irradiated cells with a higher expression at 24 h. Further investigation into the higher expression at 24 h and not at 48 h is required. However, one hypothesis for this phenomenon could be that the effect of the irradiation is only transient, and in order to maintain the high level integrin expression, the cells would require further doses of irradiation - this requires further investigation. Immunocytochemical live cell surface labelling also confirmed the expression of β1-integrin at 24 h post irradiation. The study also observed an increase in another stem cell marker, Thy-1, in irradiated cells compared to non-irradiated through immunocytochemical live cell surface labelling and Western blot analysis. The increased expression of both markers was also observed in cells that were incubated with the growth factor, EGF.

It has been found that EGF as a growth factor plays important roles in the regulation of cell growth, proliferation and differentiation (Carpenter and

Cohen, 1990). It is also involved in metastasis, apoptosis, angiogenesis and wound healing, as well as in tumour proliferation (Bouis *et al*., 2006). EGF is a powerful mitogen which elicits DNA synthesis and proliferation in a variety of cell types. The mitogenic effect of EGF has been shown to be related to the activity of receptor tyrosine kinase, which induces other protein phosphorylation associated with signal transduction from the plasma membrane to the nucleus (Pouyssegur and Seuwen, 1992). EGF has been found to increase [^{3}H] Thymidine and BrdU incorporation in a time and dose dependent manner. EGF increased calcium ions influx and hydrogen peroxide in mouse embryonic stem cells (Heo *et al*., 2005). It has been reported that EGF-induced hydrogen peroxide increased intracellular calcium ions in Rat-2 fibroblasts (Lee *et al*., 2000). Calcium is an essential intracellular signal involved in many biological processes including proliferation, differentiation, fertilization, secretion, contraction and apoptosis (Heo *et al*., 2005). When EGF attaches to receptors on the stem cell surface, it activates many pathways that can influence stem cell proliferation, migration and differentiation (Trfton, 2007).

EGF activates the ERK pathway. This pathway results in cell proliferation and in the increased transcription of Bcl-2 family members and inhibitor of apoptosis proteins, thereby promoting cell survival. EGF also promotes cell survival through the activation of P13 kinase/AKT signaling. EGF triggers the recruitment of P13 to activated ErbB receptors, which is mediated by the binding of SH2 domains in P13 kinase to phosphorylated tyrosine residues. AKT promotes cell survival through the transcription of anti-apoptotic proteins. Intermediate factors involved in this process are NFkB and CREB (Henson and Gibson, 2006). Another pathway initiated by EGF is the JAK/STAT pathway. JAK posphorylates STAT proteins localised at the plasma membrane. This leads to the translocation of STAT proteins to the nucleus

where they activate the transcription of genes associated with cell survival (Kisseleva *et al*., 2002; Henson and Gibson, 2006).

The addition of EGF to the cultures brought about an increase in cell viability and proliferation however this addition had no effect on cellular morphology. The basic morphology (fibroblastic-like) of the cells was retained, however the cultures were more confluent 24 and 48 h than at 0 h. At 0 h, the confluence was higher in cultures that with EGF and irradiated than in cultures that did not contain EGF and not irradiated.

Trypan blue, ATP luminescence, optical density and bFGF results showed an increase at 0, 24 and 48 h post irradiation following addition of EGF to the cultures. However at 0 h there was no difference in cellular responses in cells with EGF only and in cells where EGF was added and irradiated. This could be due to irradiated cells requiring more time to respond to LLLT. The increase in cellular responses in cells incubated with EGF agrees with the experiment carried out by Svendsen *et al*., (1995) with EGF- supplemented cultures that showed a higher increase in cell number of central nervous system precursor cells and remained viable for a longer time compared to cultures lacking EGF. This is also in agreement with the results by Hauner *et al*., (1995), who found that incubating stromal cells from human adipose tissue with EGF completely blocked accumulation of lipids and this was associated with a potent stimulation of cell proliferation. EGF in concentrations of 10, 100, 500 ng/ml have been found to stimulate the proliferation of human glioma cells or prostate cell through the direct induction of cyclin D1 (D'Onofrio *et al*., 2003 and Perry *et al*., 1998). Moreover, EGF (0.6 - 80 ng/ml) has been supplemented in experiments to maintain neural stem cell self renewing and multilineage potential (Pitman *et al*., 2004 and Tropepe *et al*., 1999). Heo *et al*., (2005) found that EGF stimulated the proliferation of mouse embryonic stem cells at doses of 10, 50 and 100 ng/ml

in agreement with the results of this study which used a dosage of 20 ng/ml. Western blot results also revealed an increase in the markers on cells that were irradiated and incubated with EGF.

The increase in cell viability, proliferation as well expression of the markers presented in this dissertation in this study was more pronounced in cells that were also irradiated.

Fibroblast surface protein is found on human fibroblasts and fibroblast cell line as well as tissue mcrophages and peripheral blood monocytes (Singer *et al*., 1989). This protein was used as a marker to confirm the differentiation of ADSCs into skin fibroblast cells. WS1 cells are human skin fibrolast cell lines and was used in this study as a positive control. The buffy coat cells were prepared from human peripheral blood and since they contain monocytes which express fibroblast surface protein were used as a positive controls as well. The normal ADSCs were used as negative controls. Dot blot results showed the presence of fibroblast surface protein in buffy coat cell lysates. This shows the protein is expressed in these cells. Neither the Western blot nor the dot blot experiment did show the presence of fibroblast surface protein in the normal ADSCs, the differentiated ADSCs and the WS1 cell lysates. This could mean that either differentiated ADSCs and WS1 cells do not express the protein or the protein was not expressed by these cells at that time of the experiment due to a number of reasons. One reason would be due to the culturing conditions that the cells were exposed to. *In vitro* culture, high serum concentrations and extended culture periods are some of the environmental stresses that have been reported to affect the genetic stability of the cultured cells (Thompson *et al*., 2001). However this study used ADSCs of passage 2 and were differentiated in a non-FCS medium. WS1 cells were of passage 27.

Recent studies have determined that ADSCs reduce their expression of surface histocompatibility antigens opposed to freshly isolated ADSCs (McIntosh *et al*., 2006). Serially passaged ADSCs have also displayed karyotypic abnormalities at a frequency of more than 30% and formed tumors at a frequency of 50% (Rubio *et al*., 2005). There have been reports that telomerase activities have been maintained, decreased or absent with progressive passage in ADSCs (Gimble *et al*., 2007). ADSCs have undergone malignant transformation after more than four months of passaging (Rubio *et al*., 2005). The above discoveries indicate that it is possible to get negative results even if the ADSCs were differentiated because above changes could manipulate the genetic make up of the cells thereby not expressing the required protein. The other reason would be that the protein might be expressed in the differentiated cells at a later passage after differentiation. Deng *et al*., 2001 also differentiated human marrow stromal cells into early progenitors of neural cells and could not observe the presence of a marker for mature neurons.

ADSCs are considered to be of great source of stem cells for tissue engineering and regenerative medicine. The results presented here suggest that EGF in combination with LLLT increase cell number and viability, which in turn could play an important role in the differentiation of ADSCs into other cell types that would be beneficial in tissue engineering and regenerative medicine.

5.2 Conclusion

This study concludes that low level laser irradiation at 5 J/cm^2 using diode laser at 636 nm with power density of 12.1 mW/cm^2 in the dark at room temperature, can positively affect ADSCs *in vitro* by increasing cell viability, cell proliferation and the expression of β1-integrin and Thy-1. Low level laser

irradiation of 5 J/cm^2 alone as well as in combination with addition of EGF to the cells does not change the morphology of ADSCs.

The study therefore suggests that low level laser irradiation alone as well as in combination with the addition of EGF in cultures could enhance the viability, proliferation and the maintenance of the stem cell properties of ADSCs in culture, therefore not only expanding the number of the stem cells, but also avert premature differentiation of these cells into other tissue types. However, this study does not conclude that the addition of EGF to the cultures could differentiate the cells.

ADSCs are multipotent because of their ability to differentiate into a variety of different cells or tissues. These cells could be differentiated into skin fibroblast cells. At present there is lack of information on specific markers that define the skin fibroblast cells differentiated from ADSCs. One of the aims of this study was to differentiate ADSCs into skin fibroblast cells. This study therefore does not conclude that it had differentiated ADSCs into skin fibroblast cells but it suggests that more experiments on many proteins expressed in human skin fibroblast cells, should be investigated on the ADSCs that undergo differentiation procedures.

By preventing spontaneous differentiation *in vitro*, the ADSCs could possibly be cultured for longer periods without the loss of their stem cell characteristics. This in turn would also allow for further studies to be conducted into the stem cell dynamics of ADSCs, as well as their differentiation potentials further aiding the development of future stem cell treatments and therapies. However, further exploration into the stability and biological safety of these cells would be required before such treatments could be implemented.

PUBLICATION

B. Mvula, T. Mathope, T. Moore and H. Abrahamse (2007) The effect of low level laser irradiation on adult human adipose derived stem cells, *Lasers in Medical Science* (Published online: 23 August 2007).

Lasers in Medical Science

10.1007/s10103-007-0479-1

Original Article

The effect of low level laser irradiation on adult human adipose derived stem cells

B. Mvula[1], T. Mathope[1], T. Moore[1] and H. Abrahamse[1]

(1) Laser Research Group, Faculty of Health Sciences, University of Johannesburg, P. O. Box 17011, Doornfontein, Johannesburg, South Africa, 2028

H. Abrahamse
Email: habrahamse@uj.ac.za

Received: 3 April 2007 **Accepted:** 18 June 2007 **Published online:** 23 August 2007

Abstract This study investigated the effect of low level laser irradiation on primary cultures of adult human adipose derived stem cells (ADSC) using a 635-nm diode laser, at 5 J/cm^2 with a power output of 50.2 mW and a power density of 5.5 mW/cm^2. Cellular morphology did not appear to change after irradiation. Using the trypan blue exclusion test, the cellular viability of irradiated cells increased by 1% at 24 h and 1.6% at 48 h but was not statistically significant. However, the increase of cellular viability as measured by ATP luminescence was statistically significant at 48 h ($p<0.05$). Proliferation of irradiated cells, measured by optical density, resulted in statistically significant increases in values compared to non-irradiated cells

($p < 0.05$) at both time points. Western blot analysis and immunocytochemical labeling indicated an increase in the expression of stem cell marker β1-integrin after irradiation. These results indicate that 5 J/cm^2 of laser irradiation can positively affect human adipose stem cells by increasing cellular viability, proliferation, and expression of β1-integrin.

Keywords Primary human adipocyte stem cells - 635 nm diode laser - Trypan blue test viability - ATP luminescence - β1-integrin differentiation expression

Introduction

Stem cells have the ability to self-renew, an extensive proliferative potential and an ability to give rise to one or more differentiated cell types [1, 2]. Due to their broad differentiation potential, stem cells are of particular interest from a therapeutic point of view. One may envisage a scheme in which a patient's own somatic stem cells from a particular tissue might be used in autologous cell therapy to replace tissue(s) of the same or different tissue [3]. However, before such therapies are able to be applied, further research is necessary to determine the differentiation potential of different stem cell types, the means by which to stimulate controlled differentiation and the stability and safety of the differentiated tissue(s).

Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is relatively easily isolated. ADSCs have shown a rather impressive differentiation potential *in vitro*, from adipocytes, to osteogenic, myogenic, chondrogenic, and neurogenic lineages when treated with established lineage-specific factors [4]. Signals through cell–cell contact, extracellular matrix (ECM) proteins and other factors secreted from surrounding tissue are instrumental in triggering specific pathways that

subsequently lead to differentiation. Many of these inducing factors have yet to be elucidated; however, known growth factors and signals are currently being researched in great detail to further define and understand their roles in tissue growth and differentiation. Stem cells can be identified by their expression of certain genes and proteins. One such protein expressed on surface membranes of stem cells is β1-integrin. β1-integrin is a known stem cell marker and has previously been shown to be expressed in adipose-derived stem cells [5,6].

Laser irradiation at different intensities has been shown to inhibit and stimulate cellular processes. Recent findings suggest that at the cellular level, laser energy of a particular wavelength can initiate signaling cascades, such as those that promote cellular proliferation [7]. Studies on low level laser therapy (LLLT) and stem cells have shown that low level laser irradiation increased migration of stem cells and suggests that LLLT could affect the metabolism of stem cells [8], which in turn, could also be indicative of increased cell proliferation.

Materials and Methods

Isolation of adipose cells

Adipose tissue from consenting donors undergoing abdominoplasty was used for the isolation of the adipose stem cells. Ethical approval in accordance with the Human Tissue Act 65, 1983, was obtained from the Academic Ethics Committee of the Faculty of Health Sciences, University of Johannesburg.

Adipose tissue was separated from the dermal layer using a scalpel and placed in a sterile beaker with Hanks Balanced Salt Solution (HBSS; Adcock-Scientific SA., P04-34500) containing 10,000 U/ml penicillin/streptomycin (Pan Biotec-GmBH, SA, PO6-07100) and 250 µg/ml fungizone (GIBCO, SA, 15290-026). The covered beaker containing the tissue was then stored at

room temperature overnight protected from light. The adipose tissue was removed from the beaker and cut into 3 to 5 mm pieces using two scalpels. Equal volumes (12.5 ml) of the minced tissue and a collagenase solution containing 600 U/ml collagenase type-1 (240 U/mg, Pan Biotec-GmBH, SA, LS0004196), HBSS, and 5 M CaCl were then placed in 50-ml Falcon tubes, sealed with parafilm and incubated in a shaking incubator (labcon, Instrulab, SA) at 20×*g* for 80 min at 37°C. After incubation, equal volumes of complete medium, consisting of Dulbecco's Modified Eagle Medium (DMEM- (GIBCO, SA, 21331-020) supplemented with 10% fetal calf serum (FCS; deltabioproducts, SA, 14-501BI), 0.1% penicillin/streptomycin, and 1 µg/ml fungizone were added to the tubes, and inverted to mix. After centrifugation of the suspension at 300 ×g for 5 min at 20°C, the oil layer on the surface of the suspension was removed and discarded using a plastic Pasteur pipette. The infranatant was then removed, resuspended, and filtered through a 40-µm filter (BD Biosciences, SA, 352340). The resultant suspension was spun at 650 ×g for 5 min, and the supernatant was removed. Cell pellets from the same sample were pooled and resuspended in complete medium, and spun at 650 ×g for 5 min.

The supernatant was removed, and the pellets were resuspended in Erythrocyte Lysis Buffer [(ELB; NH_4Cl, $KHCO_3$, ethylenediaminetetraacetic acid (EDTA)] and incubated for 10 min at room temperature. The solution was spun at 650 ×g for 5 min and the pellet resuspended in complete medium. Using 75 cm^2 tissue culture flasks, in 75 cm^2 2.5 ml of the cell suspension was added to 17.5 ml of complete culture medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. ADSCs were observed in the flasks 24 h after incubation. The cells were grown to semi-confluence before passage, and the medium was changed once a week. Semi-confluent cells were subcultured in 3.3 cm diameter culture plates for laser irradiation.

Laser irradiation

Semi-confluent mono-layers of ADSC were irradiated in the dark at room temperature with 5 J/cm^2 at 635 nm using a diode laser (Oriel, USA). Laser irradiation was delivered to the culture plate of 3.3 cm diameter via an optical fiber with a spot size of 3.3 cm diameter covering the entire surface of the culture dish uniformly. On average, 50 mW of power output was measured, and this was calculated to take 15 min to deliver 5 J/cm^2 irradiation at a power density of 5.5 mW/cm^2. Non-irradiated control cells were kept under the same conditions. Both irradiated and non-irradiated samples were re-incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cell morphology

Morphological observations using an inverted light microscope (Olympus CKX41) were performed at 24 and 48 h post irradiation on both irradiated and non-irradiated cells. A digital camera (Olympus C5060-ADUS) coupled to the microscope was used to document digital micrographs.

Cell Viability

Trypan blue

Cellular viability was measured by using trypan blue [9]. Trypsin/EDTA (0.02–0.05 μg) cell suspensions of the culture ADSCs were used to measure cell viability through trypan blue and adenosine triphosphate (ATP) luminescence. A mixture of cell suspension (500 cells/μl) in complete medium (20 μl) and (20 μl) trypan blue reagent (Sigma, SA, 200-786-7) was incubated at room temperature for 5 min. Viable and nonviable cells were counted using a hemocytometer with Neubauer rulings using a light microscope (Olympus CKX41), and the percentage viability was determined. The percentage viability was calculated by dividing the number of viable cells (translucent) by the total number of cells and multiplied by 100.

Metabolically active cell detection: adenosine triphosphate (ATP) luminescence

The cell Titer-Glo luminescent cell viability assay (Promega, SA, G7571) provides a homogeneous method for determining the number of viable cells in culture based on quantitation of ATP, which indicates the presence of metabolically active cells (Product information, Fact sheet # G757rev02). According to the manufacturer's protocol, a mixture of cell suspension (500 cells/µl) in complete medium (50 µl) was mixed with equal volume of Glo reagent (1 ml buffer and 0.007 g substrate) and mixed on a vortex for 2 min to induce lysis. The mixture was incubated for 10 min at room temperature and read on a luminometer (Hygiena International, Pi-102, Germany).

Cell proliferation

Optical density (OD) was used to measure cell proliferation. A hundred microliters of cell suspension in complete medium (DMEM) was read at $A_{540\ nm}$ in a microplate reader (BioRad, Benchmark Plus Microplate spectrophotometer).

Protein Expression

Expression of β1-integrin

Cells were seeded onto coverslips, by slowly pippetting 200 µl of cell suspension (500 cells/µl) into 1.8 ml of complete medium in 3.3 cm diameter culture plates containing heat sterilized glass coverslips (22 × 22 mm) (Deckglaser, Lasec, SA). The cells were allowed to attach to the coverslips and grow for 4 days to semi-confluence. The cultured cells were then irradiated. Cells cultured on the sterile glass coverslips were rinsed twice with ice-cold phosphate-buffered saline (PBS) bovine serum albumin (BSA)/azide buffer (PBS, 0.1% w/v BSA, Sigma Aldrich, SA, 9048-46-8; 0.01% w/v azide, BDH Lab supplies, UK, 103692K) and then incubated with β1-integrin

(0.8 µg/ml, 1:250; Whitehead Scientific Group, SA, Sc-9970) in PBS/azide for 30 min on ice. Cells were then rinsed three times with PBS BSA/azide buffer and incubated with the secondary fluorescent antibody (0.4 µg/ml, 1:1,000, Goat anti-mouse IgG-Rhodamine; Whitehead Scientific Group, SA, Sc-2092) in PBS/azide for 30 min on ice, protected from light. Cells were rinsed three times as before and fixed in 3.7% formalin for 10 min. After fixation, cells were rinsed once briefly with PBS, and then once, with tap water before being stained with 4′-6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, SA, D9564) and mounted on glass slides. The slides were viewed through a fluorescent microscope (Olympus BX41).

Western blotting

Cultured cells were lysed in lysis buffer (equal volumes of HBSS and sample buffer-2 M Tris (hydroxymethyl) aminomethane pH 6.8, BioRad, SA, 161-0719; 2% sodium dodecyl sulfate (SDS), BioRad, SA, 161-0302; 100% glycerol, Separation Scientific, SA, 56-40-6 and H_2O) on ice. Cell extracts were sonicated, protein was determined using Bicinchoninic Acid Protein Kit Assay (BCA™, Pierce, USA, 23228) [10].

Ten microgram of protein was loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene diflouride membranes, 0.2 µm, (Immunoblot PVDF membrane, BioRad, SA, 162-0177). Membranes were blocked overnight in blocking buffer containing Tris buffer saline (TTBS-50 mM Tris; 150 mM NaCl, Separation Scientific, SA, 7647-14-5) containing 0.1% Tween 20 and 5% non-fat milk. This reduced background and prevented binding of the primary antibody to the membrane [11]. The membranes were then incubated in primary antibody (2 µg/ml, 1:100, β1-integrin, Whitehead Scientific Group, SA, Sc-9970) diluted in blocking buffer (as above) at room temperature for 1 h. The membranes were washed in TTBS and then incubated in secondary antibody (0.2 µg/ml, 1:1,000, goat

anti-mouse horseradish peroxidase, Whitehead Scientific, SA, sc-2005) diluted in blocking buffer (as above) at room temperature for 2 h.

The membranes were washed as before and incubated in chemiluminescent substrate (SuperSignal West Pico, Pierce, USA, 34080) for 10 min protected from light. The blots were then exposed to X-ray film (Kodak MXG, Rockester, USA, 326052) for 2 and 4 min. The films were developed and then viewed.

Statistical analysis

All laser irradiation experiments were performed at least six times ($n=6$). Statistical analysis was performed using Sigma plot 8.0 software. Differences between groups were determined using the Student T test for each independent variable (viability, proliferation, protein expression).

Results

Cell morphology

A single layer of smooth elongated fibroblast-like cells were observed as shown in Fig. 1. The observation revealed no difference between the irradiated and the control cells at both 24 and 48 h.

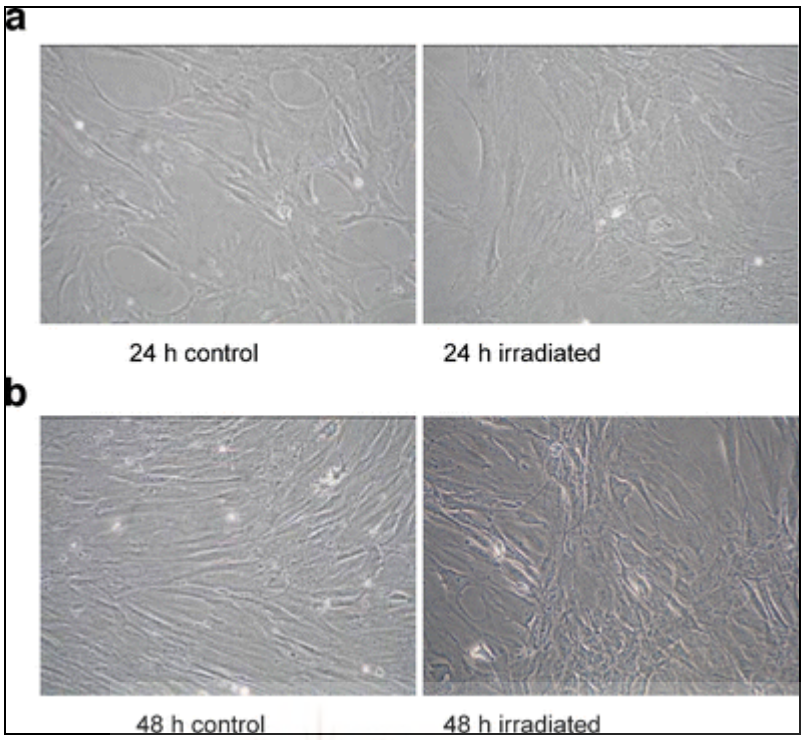


Fig.1 Morphology of ADSCs. Monolayer of ADSCs 24 h (a) and 48 h (b) post irradiation showing typical smooth elongated cell shape. No discernable change was observed although was an increase in cell number

Cell viability

Trypan blue indicated an increase in percentage viability in cells that were irradiated compared to non-irradiated cells at 24 and 48 h; however, the difference did not prove to be statistically significant (Table 1). The increase in viability may indicate a stimulatory effect of laser irradiation. ATP luminescence showed that there was a statistically significant increase in ATP concentration at 48 h ($p < 0.05$) in irradiated cells when compared to non-irradiated cells at 24 and 48 h as shown in Fig. 2.

Table 1. Cell viability

Time post irradiation (h)	Control (non-irradiated; %)	Irradiated (%)
24	96	97
48	97	98.6

Percentage cell viability using trypan blue ($n=6$) showed an increase in irradiated cells evaluated at 24 and 48 h compared to the non-irradiated. The increase was however not statistically significant.

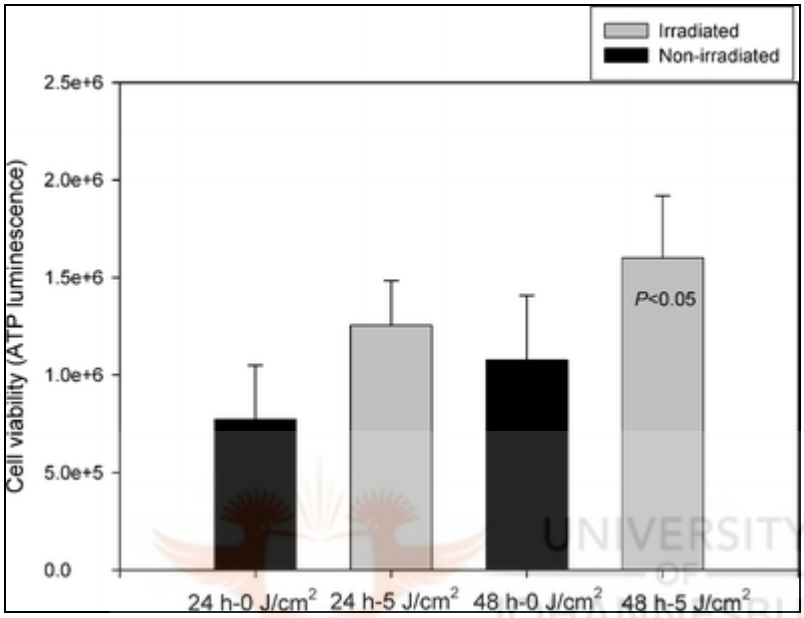


Fig. 2 Cell viability. ATP luminescence showed an increase in cell viability in irradiated cells compared to non-irradiated cells both at 24 and 48 h. The increase was statistically significant at 48 h ($P<0.05$)

Cell proliferation

Irradiated cells both at 24 and 48 h showed a statistically significant increase ($p<0.05$) in OD compared to their respective controls (Fig. 3)

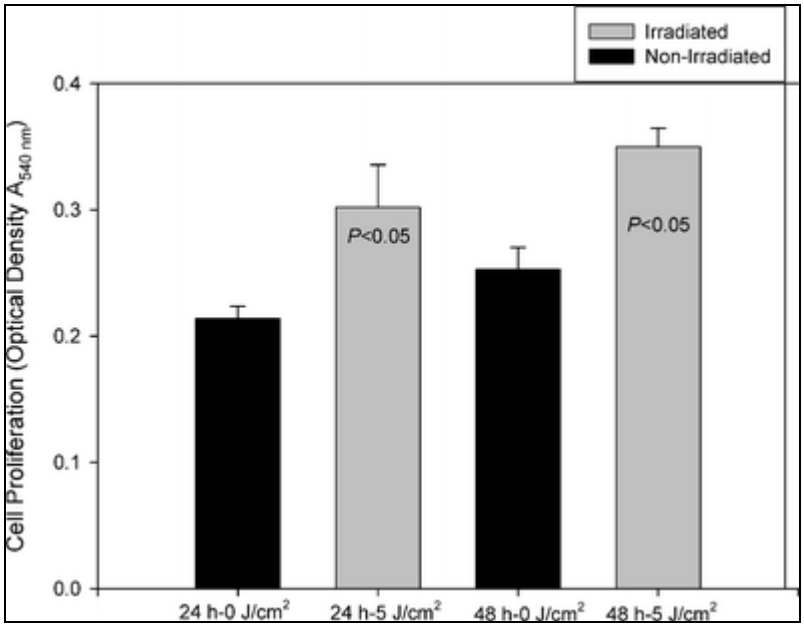


Fig. 3 Cell proliferation. Optical density assay ($n=6$) showed a statistically significant increase in cell proliferation in irradiated cells at 5 J/cm^2 compared to the non-irradiated cells ($P<0.05$) at 24 and 48 h post irradiation

Protein expression

Immunocytochemical live cell labeling showed the expression of β1-integrin in irradiated cells (Fig. 4). Western blot analysis showed an increase in β1-integrin expression in cells irradiated at 24 and 48 h (Fig. 5).

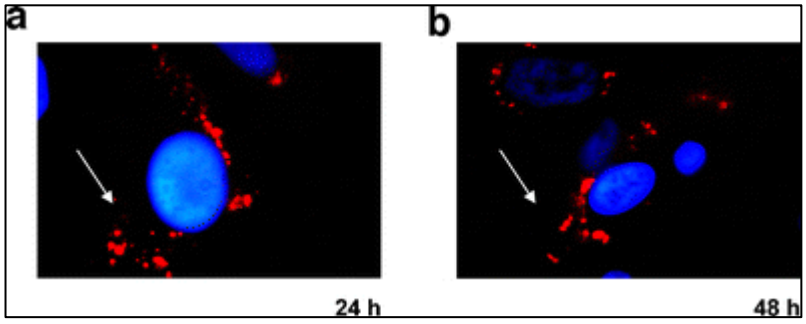


Fig. 4 β1-integrin expression. Post irradiation surface expression of β1-integrin (*red*) in ADSCs. Localization of β1-integrin on the cell surface is shown by the arrows on both diagrams (a) and (b). Nuclei are counter stained with DAPI (*blue*)

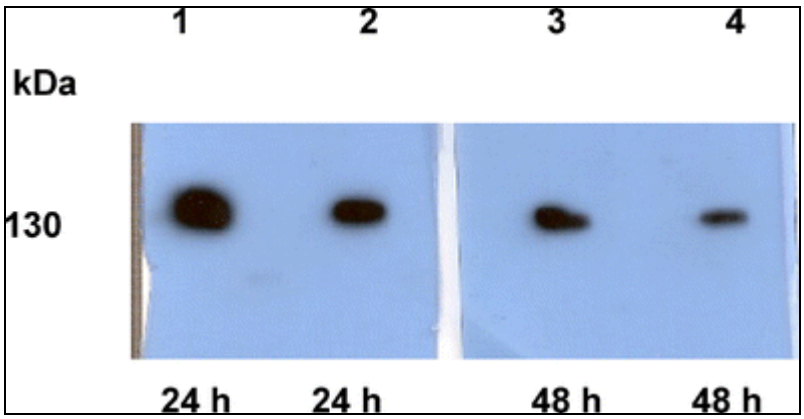


Fig. 5 Western blot analysis of the expression of β1-integrin in irradiated and non-irradiated cells at 24 and 48 h post irradiation. Expression of β1-integrin appears to be higher in irradiated cells compared to non-irradiated cells. Lanes 1 irradiated 24 h; 2 non-irradiated 24 h, 3 irradiated 48 h, and 4 non-irradiated 48 h

Discussion

To our knowledge, this is the first study investigating the effect of low level laser irradiation on human ADSCs *in vitro*. Adipose tissue contains an abundant, accessible source of adult stem cells, and the stem cells prevenient from this tissue are termed adult adipose derived stem cells [12,13]. ADSCs have been shown to be able to differentiate into bone, fat, cartilage, neuron, and smooth muscle when treated with specialized induction media in vitro [14,15]. Once differentiated, these cells could potentially be used in stem cell therapy to replace or repair damaged tissues and organs. A number of disorders that are amenable to this approach include neurological, cardiovascular diseases, and bone defects and diabetes [15]. In this study, ADSCs were derived from adipose tissue, and the effect of low level laser irradiation was evaluated *in vitro* at 24 and 48 h post irradiation.

LLLT has been shown to have a variety of biostimulatory effects such as wound healing [16,17], fibroblast proliferation [18-20], nerve regeneration [21], and collagen synthesis [*22*]. Studies on LLLT and stem cells have shown that LLLT increases migration of stem cells [8], but as yet, there have been no studies on the effect of LLLT on ADSCs.

In this study, we found that LLLT increased cell viability, cell proliferation, and the expression of β1-integrin in ADSCs. Cell viability increased at both 24 and 48 h in irradiated cells compared to non-irradiated cells. Although the trypan blue exclusion assay did not show a statistically significant increase in viability, the more sensitive and reliable ATP luminescence assay showed a significant increase 48 h after irradiation.

Optical density measurements at both 24 and 48 h indicated an increase in cell proliferation in the irradiated cells. Proliferation was greater after 48 than at 24 h. This is in agreement with the results of Hawkins and Abrahamse [23], who found that there was an increase in cellular proliferation of human skin fibroblast cells (WS1) when treated with a Helium Neon laser at the same dosage used in this study and wavelength of 632.8 nm.

In addition, this study found that β1-integrin showed an increased expression in irradiated cells, and the expression was greater after 24 h. β1-integrin is a cell surface marker for ADSCs [24]. Immunocytochemical live cell surface labeling also confirmed the expression of β1-integrin at 24 h post irradiation.

We conclude that low level laser irradiation at 5 J/cm^2, a power density of 5.5 mW/cm^2 and a wavelength of 635 nm can positively affect ADSCs in vitro by increasing cell viability, cell proliferation, and the expression of β1-integrin. We therefore suggest that low level laser irradiation could enhance the viability, proliferation, and the maintenance of the stem cell properties of ADSCs in culture, therefore, not only expanding the number of the stem cells, but also avert premature differentiation of these cells into other tissue types. By preventing spontaneous differentiation *in vitro*, the ADSCs could possibly be cultured for longer periods without the loss of their stem cell characteristics. This, in turn, would also allow for further studies to be conducted into the stem cell dynamics of ADSCs, as well as their

differentiation potentials, further aiding the development of future stem cell treatments and therapies.

Acknowledgements This project was supported by the National Laser Centre of South Africa, National Research Foundation of South Africa, Council for Scientific and Industrial Research of South Africa and University of Johannesburg.

References

1. Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98-104

2. Reya T, Morrison S, Clarke MF, Weissman I (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105-111

3. Clarke D, Frisen J (2001) Differentiation potential of adult stem cells. Curr Opin Genet Dev 11:575-580

4. Zuk PA, Zhu M, Ashijian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279-4295

5. Hudson DL (2004) Epithelial stem cells in human prostate growth and disease. Nature 7:188-194

6. Gimble JM (2003) Adipose tissue derived therapeutics. Opin Biol Ther 3(5):705-713


7. Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD (2005) Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation *in vitro*. Lasers Surg Med 36:8-12

8. Gasparyan L, Brill G, Makela A (2004) Influence of low level laser radiation on migration of stem cells. Laser Florence 1-7

9. Philips HL, Terrberry JE (1957) Counting actively metabolizing tissue cells. Exp Cell Res 13:341-347

10. Smith PK, Krohn RJ, Hemanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76-85

11. Wilson K, Walker J (1995) Practical biochemistry, principles and techniques, UK, Cambridge University Press 4:434-438

12. Gimble JM, Guilak F (2003) Adipose derived adult stem cells: isolation, characterisation, and differentiation potential. Cytotherapy 5(5):362-369

13. Safford KM, Rice HE (2005) Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells. Curr Drug Targets 6(1):57-62

14. Rodriguez LV, AlfonsoZ, Zhang R, Leung J, Wu B, Ignarro LJ (2006) Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cell. Proc Natl Acad Sci 103(32):12167-12172

15. Serakinci N, Keith NW (2006) Therapeutic potential of adult stem cells.

Eur J Cancer 42(9):1243-1246

16. Houreld N, Abrahamse H (2005) Low level laser for diabetic foot wound healing. Diabet Foot 8(5):182-193
17. Hawkins D, Houreld N, Abrahamse H (2005) Low level laser therapy (LLLT) as an effective therapeutic modality for delayed wound healing. Ann NY Acad Sci 1056:486-493
18. Kana JS, Hutschenreiter G, Haina D, Waidelich W (1981) Effect of low power density laser radiation on healing on open skin wound in rats. Arch Surgery 116:293-296
19. Boulton M, Marshall J (1986) He-Ne laser stimulation of human fibroblast proliferation and attachment *in vitro*. Lasers Life Sci 1:123-34
20. Van Breugel H, Bar PRD (1992) Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photobiomodulation of human fibroblasts *in vitro*. Lasers Surg Med 12:528-537
21. Anders JJ, Borke RC, Woolery SK, Van der Merwe WP (1993) Low power laser irradiation alters the rate of regeneration of rat facial nerve. Lasers Surg Med 13:72-82
22. Lam TS, Abergel RP, Meeker CA, Castel JC, Ewyer RM, Uitto J (1986) Laser stimulation of collagen synthesis in human skin fibroblasts culture. Lasers Life Sci 1:61-77
23. Hawkins D, Abrahamse H (2005) Biological effects of helium-neon laser

irradiation on normal and wounded fibroblasts. Photomed Laser Surg 23(3):251-259

24. Aust L, Devlin B, Foste SJ, Halvorsen YDC, Hicok K, Laney TDU, Sen A, Willingmyre GD, Gimble JM (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. Cytotherapy 6(1):714(8)

Another publication emanating from this study is: **Mvula B., Moore T. and Abrahamse H. (2008) The effect of low level laser irradiation and epidermal growth factor (EGF) on human adult adipose derived stem cells**, (In progress).

REFERENCES FOR THE DISSERTATION

- 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.
- Ades, E.W., Zwerner, R.K., Acton, R.T. and Balch, C.M. (1979) Isolation and partial characterization of the human homologue of Thy-1, *Journal of Experimental Medicine*, **151**: 400-406.
- Alster, T.S. and BettenCourt, M.S. (1998) Review of cutaneous lasers and their applications, *Current Concepts*, **91(9)**: 806-812.
- Alonso, L. and Fuchs, E. (2003) Stem cells of the skin epithelium, *Proceedings of the National Academy of Sciences*, **100(1)**: 11830-11835.
- Anders, J.J., Borke, R.C., Woolery, S.K. and Van der Merwe, W.P. (1993) Low power laser irradiation alters the rate of regeneration of rat facial nerve, *Lasers in Surgery and Medicine*, **13**: 72-82.
- Angenieux, B., Schrorderet, D.F. and Arsenijevic, Y. (2006) Epidermal growth factor is a neuronal differentiation for retinal stem cells *in vitro*, *Stem Cells*, **24**: 696-706.
- 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.
- Aust, L., Devlin, B., Foster, S.J., Halvorsen, Y.D.C., Hicok, K., Laney, T.D.U., Sen, A., Willingmyre, G.D. and Gimble, J.M. (2004) Yield of Human Adipose-Derived Adult Stem cells from Liposuction Aspirates, *Cytotherapy*, **6(1)**: 7-14(8).

- Basford, J.R. (1995) Low intensity laser therapy: still not an established clinical tool, *Lasers in Surgery and Medicine*, **16**: 331-342.
- 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.
- Becker, A.J., McCulloch, E.A. and Till, J.E. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells, *Nature,* **197**: 452-454.
- Bianco, P. and Robey, P.G. (2001) Stem cells in tissue engineering, *Nature*, **414**: 118-121.
- Bishop, A.E., Buttery, L.D.K. and 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.
- Bouis, D., Kusumanto, Y.K., Meijer, C., Mulder, N.H. and Hospers, G.A.P. (2006) A review on pro- and anti-angiogenic factors as targets of clinical intervention, *Pharmacological Research*, **53**: 89-103.
- Boulton, M. and Marshall, J. (1986) He-Ne laser stimulation of human fibroblast proliferation and attachment *in vitro*, *Lasers Life Science*, **1**:123-134.
- Brakebusch, C. and Fassler, R. (2005) β1-Integrin function *in vivo*: Adhesion, migration and more, *Cancer and Metastasis Reviews*, **24**: 403-411.
- Butler, D.L., Goldstein, S.A. and Guilak, F. (2000) Functional tissue engineering: The role of biomechanics, *Journal of Biomechanics Engineering*, **122**: 570-575.

- Campos, L.S., Decker, L., Taylor, V. and Skarnes, W. (2006) Notch, EGFR and β1-Integrin pathways are coordinated in neural stem cells, *Journal of Biology and Chemistry*, **281(8)**: 5300-5309.
- Carpenter, G. and Cohen, S. (1990) Epidermal growth factor, *Journal of Biology Chemistry*, **265(14)**: 7709-7712.
- Chambers, I., Colby, D. and Robertson, M. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, *Cell*, **113(5**): 643-655.
- 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, D., Zhao, M. and Mundy, G.R. (2004) Bone morphogenetic proteins, *Growth Factors*, **22**: 233-241.
- Choi, Y.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 and Biophysical Research Communications*, **345(2)**: 631-637.
- Cullen, B., Watt, P.W., Lundqvist, C., Silcock, D., Schmidt, R.J., Bogan, D. and Light, N.D. (2002) The role of oxidised regenerated cellulose/collagen in chronic wound repair and its potential mechanism of action, *The International Journal of Biochemistry and Cell Biology*, **34(12)**: 1544-1556.
- Darby, I.A. and Hewitson, T.D. (2007) Fibroblast differentiation in wound healing and fibrosis, *International Review of Cytology*, **257**: 143-179.

- Deng, W., Obrocka, M., Fischer, I. and Prockop, D.J. (2001) *In vitro* differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular Cyclic AMP, *Biochemical and Biophysical Research Communications*, **282**: 148-152.
- D'Onofrio, M., Arcella, A., Bruno, V., Ngomba, R.T., Battaglia, G., Lombari, V., Ragona, G., Calogero, A. and Nicoletti, F. (2003) Pharmacological blockade of mGlu2/3 metabotropic glutamate receptors reduces cell proliferation in cultured human glioma cells, *Journal Neurochemistry*, **84(6)**: 1288-1295.
- 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.
- Fu, X.B., Sun, X.Q., Sun, T.Z., Dong, Y.H., Gu, X.M., Chen, W. and Sheng, Z.Y. (2002) Epidermal growth factor stimulates tissue repair in skin through skin stem cell activation, *Zhongguo Xiu Fu Chong Jian Wai ke Za Zhi*, **6(1)**: 31-35.
- Gardner, R.L. (2002) Stem cells: potency, plasticity and perception, *Journal of Anatomy*, **200(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. (2003) Adipose tissue derived therapeutics, *Expert Opinion Biology Therapy*, **3(5)**: 705-713.
- Gimble, J.M. and Guilak, F. (2003) Adipose derived adult stem cells: isolation, characterisation, and differentiation potential, *Cytotherapy*, **5(5)**: 362-369.
- Gimble, J.M., Kartz, A.J. and Bunnell, B.A. (2007) Adipose derived stem cells for regenerative medicine, *Circulation Research*, **100**: 1249.

- Goldwasser, S.M. (1997) Lasers: safety, diode lasers, helium neon lasers, drive, information, parts, *A Practical Giude for Experimenters and Hobbyists*, *Sci.Electronics.FAQ*, Version 2.62.
- Goodsell, D.S. (2003) The molecular perspective: Epidermal growth factor, *The Oncologist*, **8(5)**: 496-497.
- Gospodarowicz, D., Schweigerer, L., Neufeld, G., Friedman, J., Abraham, A. J. and Fiddes, J. (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their growth, *Nature*, **325**: 257-259.
- Grinnemo, K.H., Mansson-Broberg, A., Leblanc, K., Corbascio, M., Wardell, E., Siddiqui, A.J., Hao, X., Sylven, C. and Dellgren, G. (2006) Human mesenchymal stem cells do not differentiate into cardiomyocytes in a cardiac ischemic xenomodel, *Annals of Medicine*, **38(2)**: 144–153.
- 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.
- Hauner, H., Rohrig, K. and Petruschke, T. (1995) Effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) on human adipocyte development and function, *European Journal of Clinical Investigation*, **25(2)**: 90-96.
- Hawkins, D. and Abrahamse, H. (2005) Biological effects of helium-neon laser irradiation on normal and wounded human skin fibroblasts, *Photomedicine and Laser Surgery*, **23(3)**: 251-259.
- Hawkins, D. and Abrahamse, H. (2006) The role of laser fluence in cell viability, proliferation and membrane integrity of wounded human skin

fibroblasts following helium-neon laser irradiation, *Lasers in Medical Surgery*, **38**: 74-83.

- 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., Houreld, N. and Abrahamse, H. (2005) Low level laser therapy (LLLT) as an effective therapeutic modality for delayed wound healing, *Annals New York Academy of Sciences*, **1056**: 486-493.
- Henson, E.S. and Gibson, S.B. (2006) Surviving cell death through epidermal growth factor signal transduction pathways: Implications for cancer therapy, *Cell Signal*, **18**: 2089-2097.
- Heo, J.S., Lee, J.L. and Han, J.H. (2005) EGF stimulates proliferation of mouse embryonic stem cells: Involvement of calcium ions influx and p44/42 MAPKS, *American Journal of Physiology-Cell Physiology*, **290(1)**: 123-133.
- Houreld, N. and Abrahamse, H. (2005) Low level laser for diabetic foot wound healing, *The Diabetic Foot*, **8(5)**: 182-193.
- Houreld, N. and Abrahamse, H. (2006b) Frequency of helium-neon laser irradiation on viability and cytotoxicity of diabetic wounded fibroblast cells, *Photomedicine Laser Surgery*, In press.
- 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. (2007) 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.
- Hynes, R.O. (2002) Intergrin bidirectional, allosteric signalling machines, *Cell*, **110(6)**: 673-687.

- Hudson, D.L. (2004) Epithelial stem cells in human prostate growth and disease, *Nature,* **7**: 188-194.
- 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).
- Jones, P.H. and Watt, F.M. (1993) Separation of human dermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression, *Cell*, **73**: 713-724.
- Jones, P.H., Harper, S. and Watt, F.M. (1995) Stem-cell patterning and fate in human epidermis, *Cell*, **80**: 83-93.
- Kana, J.S., Hutschenreiter, G., Haina, D. and Waidelich, W. (1981) Effect of low power density laser radiation on healing on open skin wound in rats, *Arch Surgery*, **116**: 293-296.
- Karu,T. (2003) Low-power laser therapy, Vo-Dind T (Ed), *Biomedical Photonics Handbook*, CRC Press Boca Raton, USA, **pp**: 48-1 – 48-25.
- Keith, R.S. (2006) Fate of embryonic stem cells, *Cell Transplantation*, **15**: 369-380.
- Kemshead, J.T., Ritter, M.A., Cotmore, S.F. and Greaves, M.F. (1982) Human Thy-1: expression on the cell surface of neuronal and glial cells, *Brain Research*, **236**: 451-461.
- Kesseleva, T., Bhattacharya, S., Braunstein, J. and Schinder, C.W. (2002) Signalling through the JAK/STAT pathway, recent advances and future challenges, *Genetics*, **285**: 1-24.
- 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.
- Kratchmarova, I., Blagoev, B., HHaack-Sorensen, M., Kassem, M. and Mann, M. (2005) Mechanisms of divergents of growth factor effects in mesenchymal stem cells differentiation, *Science*, **308(5727)**: 1472-1477.
- 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=PIIS00928674060097 67& highlight (Accessed 24/02/07).
- Lam, T.S., Abergel, R.P., Meeker, C.A., Castel, J.C., Ewyer, R.M. and Uitto, J. (1986) Laser stimulation of collagen synthesis in human skin fibroblasts culture, *Lasers in Life Science*, **1**: 61-77.
- Lavker, R.M. and Sun, T. (2000) Epidermal stem cells: Properties, markers and location*, Proceedings of the National Academy of Sciences*, **97(25)**: 13473-13475.
- Lee, Z.W., Kweon, S.M., Kim, S.J., Kim, J.H., Cheong, C., Park, Y.M. and Ha, K.S. (2002) The essential role of hydrogen peroxide in the regulation of intracellular calcium ions by epidermal growth factor in rat-2 fibroblasts, *Cell Signal*, **12(2)**: 91-98.
- Levy, L., Broad, S., Diekmann, D., Evans, R.D. and Watt, F.M. (2000) β1-Integrins regulate keratinocyte adhesion and differentiation by distinct mechanisms, *Molecular Biology of Cell*, **11**: 453-466.
- Li, H., Fu, X., Ouyang, Y., Cai, C.L., Wang, J. and Sun, T. (2006) Adult bone-marrow derived mesenchymal stem cells contribute to wound healing of skin appendages, *Cell Tissue Research*, **326**: 725-736.
- Lindvall, O. and Kokaia, Z. (2006) Stem cells for the treatment of neurological disorders, *Nature*, **441**: 1094-1096.

- Masson, N.M., Currie, I.S., Terrace, J.D., Garden, O.J., Parks, R.W. and Ross, J.A. (2006) Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1, *American Journal of Physiology and Gastrointestinal Liver Physiology*, **291**: G45-G54.
- 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.
- McKenzie, J.L. and Fabre, J.W. (1981) Human Thy-1: Unusual localisation and possible functional significance in lymphoid tissues, *Journal of Immunology*, **126**: 843.
- McIntosh, K., Zvonic, S., Garrett, S., Mitchell, J.B., Floyd, Z.E., Hammill, L., Kloster, A., Halvorsen, Y.D., Ting, J.P., Storms, R.W., Goh, B., Kilroy, G., Wu, X. and Gimble, J.M. (2006) The immunogenicity of human adipose derived cells: Temporal changes *in vitro*, *Stem Cells*, **24**: 1245-1253.
- Mester, E., Szende, B. and Tota, J.G. (1967) Effect of laser on hair growth of mice, *Kiserl Orvostud*, **19**: 628-631.
- Miller, R.H. (2006) The promise of stem cells for neural repair, *Brain Research*, **1091**: 258-264.
- Millipore Coorporation (2007) Introduction to antibodies-Protein (western) blotting, http://www.cheicon.com/resouerce/ANT101a2B.asp (Accessed 22/03/07).
- 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

- Moore, T.J. (2007) Stem cell Q and A- An introduction to stem cells and their role in scientific and medical research, *Medical Technology SA*, **21(1)**: 3-6.
- Morrison, S.J. and Kimble, J. (2006) Asymmetric and symmetric stem cell divisions in development and cancer, *Nature*, **441**: 1068-1074.
- Mvula, B., Mathope, T., Moore, T. and Abrahamse, H. (2007) The effect of low level laser therapy on adult human adipose derived stem cells, *Lasers in Medical Science*, in press.
- 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.
- 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.
- Orlic, D., Hill, J. and Arai, A. (2002) Stem cells for myocardial regeneration circulation, *Stem Cell*, **91**: 1092.
- 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, *Vetenary Clinical Small Animal*, **32**: 517-534.
- Perry, J.E., Grossmann, M.E. and Tindall, D.J. (1998) Epidermal growth factor induces cyclin D1 in a human prostate cancer cell line, *Prostate*, **35(2)**: 117-124.
- 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.
- Pitman, M., Emery, B., Binder, M., Wang, S., Butzkueven, H. and Kilpatrick, T.J. (2004) LIF receptor signaling modulates neural stem cell renewal, *Molecular Cell Neuroscience*, **27(3)**: 255-266.
- Pouyssegur, J. and Seuwen, K. (1992) Transmembrane receptors and intracellular pathways that control cell proliferation. *Annual Reveiw Physiology*, **54**: 195-210.
- Promega, 2005, Cell Titer-Glo^{TM} Luminescent, Cell Viabilty Assay, Product information, Fact sheet # G757rev02.
- Reya, T., Morrison, S., Clarke, M.F., Weissman, I. (2001) Stem cells, cancer, and cancer stem cells, *Nature*, **414**: 105-111.
- 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.

- Rubinstein, P. (2006) Why cord blood? *Human Immunology*, **67**: 398-404.
- Rubio, D., Garcia-Castro, J., Martin, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C. and Bernad, A. (2005) Spontaneous human adult stem cell transformation, *Cancer* Research, **65**: 3035-3039.
- 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.
- Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.D., Wilkison, W.O., Gimble, J.M. and Rice, H.E. (2002) Neurogenic differentiation of murine and human adipose derived stromal cells, *Biochemical Biophysics Research Communication*, **294(2)**: 371-379.
- Safford, K.M., and Rice, H.E. (2005) Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells, *Current Drug Targets*, **6(1)**: 57-62.
- Scadden, D.T. (2006) The stem cell niche as an entity of action, *Nature*, **441**: 1075-1079.
- 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 Benveninisty, 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(21)**: 11307-11321.
- Serakinci, N. and Keith, W.N. (2006) Therapeutic potential of adult stem cells, *European Journal of cancer*, **42(9)**: 1243-1246.

- Singer, K.H., Scearce, R.M., Ronnov-Jessen, L., Celis, J.E., Van deurs, B. and Petersen, O.W. (1989) Removal of fibroblast from human epithelial cell cultures, *Journal of Investigative Dermatology*, **92**:166.
- Siminovitch, L., McCulloch, E.A. and Till J.E. (1963) The distribution of colony-forming cells among spleen colonies, *Journal of Cellular and Comparative Physiology,* **62**: 327-336.
- Singer, K.H., Scearce, R.M., Ronnov-Jessen, L., Celis, J.E., Van deurs, B. and Petersen, O.W. (1989) Removal of fibroblast from human epithelial cell cultures, *Journal of Investigative Dermatology*, **92**:166.
- Smith, P.K., Krohn, R.J., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid, *Analytical Biochemistry*, **150**: 76-85.
- Spees, J.L., Olson, S.D., Whitney, M.J. and Prockop, D.J. (2006) Mitochondrial transfer between cells can rescue aerobic respiration, *Proceedings of National Academy of Science, USA*, **103**: 1283-1288.
- Spradling, A., Drummond-Barbosa, D. and Kai, T. (2001) Stem cells find their niche, *Nature*, **414**: 98-104.
- Stadler, L., Lanzafame, R., Oskoui, P., Zhang, R., Coleman, J. and Whittaker, M. (2004) Alteration of skin temperature during low level laser irradiation at 830 nm in a mouse model, *Photomedicine and Laser Surgery,* **22(3)**: 227-231.
- 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.
- Strem, B.M., Hicok, K.C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R.E., Frazer, J.K. and Hedrick, M.H. (2005) Multipotential

differentiation of adipose tissue-derived stem cells, *Keo Journal of Medicine*, **54**: 134-141.

- Srivastava, D. and Ivey, K.N. (2006) Potential of stem-cell-based therapies for heart disease, *Nature*, **441**: 1097-1099.
- Svendsen, C.N., Fawcett, J.W., Bentlage, C. and Dunnett, S.B. (1995) Increased survival of rat EGF-generated CNS precursor cells using B27 supplemented medium, *Experimental Brain Research*, **102**: 407-414.
- Thompson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A. and Hearn, J.P. (1995) Isolation of a primate embryonic stem cell line, *Proceedings of the National Academy of Sciences,* **86**: 7844-7848.
- Thompson, J.A. Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshal, V.S. and Jones, J.M. (1998) Embryonic stem cell lines derived from human blastocysts, *Science*, **282**: 1145-1147.
- Thompson, S.L., Kanfortova, G., Gregory, R.I., Reik, W., Dean, W. and Feil, R. (2001) Environmental effects on genomic imprinting in mammals, *Toxicology Letters*, **120**: 143-150.
- Trafton, A. (2007) Adult stem cell survival, promise advanced, *MIT Tech Talk*, **51(19)**: 4-6.
- Tropepe, V., Sibilia, M., Ciruna, B.G., Rossant, J., Wagner, E.F. and van der Kooy, D. (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon, *Development Biology*, **208(1)**: 166-188.
- 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

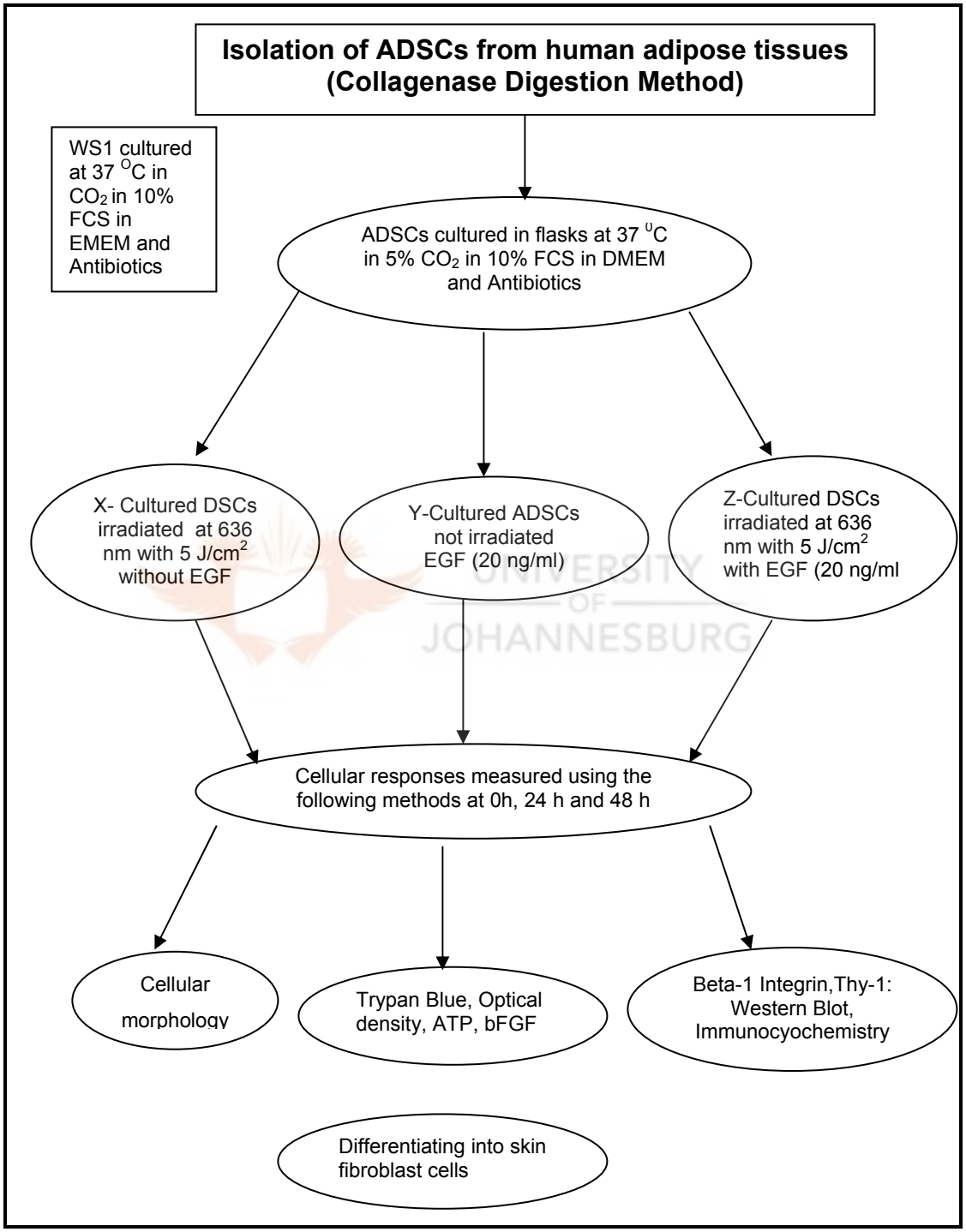
cardioprotection and enhanced angiogenesis, *Laser in Surgery and Medicine*, **38**: 682-688.

- Tuby, H., Maltz, L. and Oron, U. (2007) Low level laser irradiation (LLLI) promotes proliferation of mesenchymal and cardiac stem cells in culture, *Lasers in Surgery and Medicine*, **39(4)**: 373-378.
- 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).
- 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.
- Van Breugel, H. and Bar, P.R.D. (1992) Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photobiomodulation of human fibroblasts *in vitro*, *Lasers in Surgery and Medicine,* **12**: 528-537.
- Wang,T., Xu, Z., Jiang, W. and Ma, A. (2006) Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell, *Introductory Journal of Cardiology*, **109**: 74-81.
- 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.
- Wu, D.C., Boyd, A.S. and Wood, K.J. (2007) Embryonic stem cell transplantation: potential applicability in cell replacement therapy and regenerative medicine, *Front. Biosciences*, **12**: 4525-4535.
- Wilson, K. and Walker, J, *Practical Biochemistry, principles and techniques*, 4th ed, UK, Cambridge University Press, 1995, **pp.** 434-438.

- 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 Biophysical Research Communications*, **342**: 662-670.
- 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.
- Zhou, J. Chen, S., Liu, W., Cao, Y. and Duan, E. (2004) Enrichment and identification of human foetal epidermal stem cells, *Human Reproduction*, **9(4)**: 968-974.
- Zuk, P.A., Zhu, M., Ashjian, P., Daniel A., Ugarte, D., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. (2002) Human adipose tissue is a source of multipotent stem cells, *Molecular Biology of the Cell*, **13**: 4279-4295.
- Zuk, P.A., Zhu, M., Mizuno, H., Huang, J.I., Chaudhari, S., Lorenz, H.P., Benhaim, P. and Hedrick, M.H. (2001) Mutilineage cells derived from human adipose tissue, a putative source of stem cells for tissue engineering, *Tissue Engineering*, **7(2)**: 211-216.

APPENDIX A

Methodology Flow Diagram



APPENDIX B

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: *Monitoring the Effects of Laser Radiation on Dermal and Adipose Stem Cells - A study to evaluate the effects of Low Level Laser Therapy (LLLT) on dermal and adipose stem cells*.

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,

Dr. T.J. Moore
Laser Research Group
University of Johannesburg

AUTHORISATION FOR DONATION OF EXCESS TISSUE

LASER RESEARCH GROUP

UNIVERSITY OF JOHANNESBURG

TITLE: Monitoring the Effects of Low Level Laser Irradiation on Dermal and Adipose Stem Cells: A study to evaluate the effects of Low Level Laser Therapy (LLLT) on dermal and adipose stem cells.

Principal Investigator: Dr. T. J. Moore

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. Prof A. Widgerow 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

Group, 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 C

MATERIALS, COMPANIES, COUNTRIES AND CATALOGUE NUMBERS

Item	Company/Country	Catalogue number
DMEM	GIBCO, UK	21331-020
HBSS	Pan Biotech-GmBH	P04-34500
Penicillin/Streptomycin	Pan Biotech-GmBH	P06-07100
Fungizone	GIBCO, UK	15290-026
Collagenase Type-1	Pan Biotech-GmBH	LS0004196
40 µM cell strainer	BD Biosciences, USA	352340
WS1 cells	Scientific Group, SA	ATCCRL 1502
EMEM	GIBCO, USA	32360
L- Glutamine	Cambrex BioScience, USA	25030-024
Sodium Pyruvate	Cambrex BioScience, USA	13-115E
Non-Essential Aminoacid	Cambrex BioScience, USA	13-114E
EDTA	Merck, SA	223 60 20 EM
FCS	Deltabioproducts, SA	14-501 BI
75 cm^2 culture flasks	Corning, USA	CR-430720
Cell culture dishes (3.3 cm)	Becton Dickinson, USA	BD 353001
Trypsin/EDTA	Pan Biotech-GmBH	P10-023100SP
EGF	Pan Biotech-GmBH	CB-1101003
Trypan Blue	Sigma, USA	200-786-7
ATP Glo-reagent	Promega, SA	G7571
Human bFGF	Sigma, USA	F0291
NaCl	USB,USA	7647-14-5
Tween-20	USB,USA	9005-64-5
Carbonate bicarbonate buffer	Sigma, USA	C-3041
Mouse IgG Kappa	Sigma, USA	M90935
F96 Maxisorp Nunc	Nunc, Apogent, Denmark	439454
Goat anti-mouse IgG HRP conjugate	Santa Cruz, USA	Sc-2005

TMB	BD Biosciences, USA	51-2607KC
Sulphuric Acid	BDH Laboratory Supplies, UK	191687E
Azide	BDH Laboratory Supplies, UK	103692K
Anti β1-integrin	Santa Cruz, USA	Sc-9970
Goat anti-mouse IgG Rhodamine	Santa Cruz, USA	Sc-2092
DAPI	Sigma, USA	D9564
Mounting Medium	Santa Cruz, USA	Sc-24941
Tris	Bio-Rad, USA	161-0719
SDS	Bio-Rad, USA	161-0302
Glycerol	USB,USA	56-81-5
BSA	Sigma, USA	9048-46-8
BSA Standards	Pierce, USA	23209
BCA Reagent	Pierce,USA	23225
Acrylamide/Bisacryl	Bio-Rad, USA	161-0158
TEMED	Bio-Rad, USA	203-744-6
APS	Pan Biotech-GmBH	7727-54-0
Mercaptoethanol	Sigma, USA	60-24-2
Bromophenol Blue	Sigma, USA	263-653-2
Marker-Precision Plus Dual Protein Color Standard	Bio-Rad, USA	161-0374
Glycine	USB,USA	56-40-6
Polvinylidene Diflouride membrane	Bio-Rad, USA	162-0177
Methanol	Merck chemicals, SA	416 40 80 LC
Milk powder	Spar brand, SA	648
Chemiluminescent reagent (SuperSignal West Pico)	Pierce, USA	34080
MXG X-ray film	Kodak, U SA	326052
Gelatin Powder Porcine	US Biological, USA	G2024
bFGF	Pan Biotech-GmBH	CB-1102021
EGF	Pan Biotech-GmBH	CB-1101003
B27	GIBCO, USA	17504-044

Anti-Fibroblast Surface Protein	Affinity BioReagents, USA	MA1-25071
Secondary antibody, anti-mouse, IgM	Affinity BioReagents, USA	SA1-25256
Urea	Holpro Lovasz, RSA	79702 M
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	Merck Chemicals, RSA	BB102494C

APPENDIX D

SOLUTIONS, MEDIUM AND CHEMICALS

Cell Isolation and Culture				
D1	Collagenase type-1 solution (600 U/ml-100 ml)	Collagenase type-1	240 U/mg	250mg
		HBSS		99.8 ml
		Calcium Chloride		200 µl
D2	DMEM 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
D3	Complete medium with EGF (100 ml)	DMEM F12		88.580 ml
		FBS	10%	10 ml
		Penicillin/Streptomycin	0.1%	1 ml
		Fungizone	1 µg/ml	400 µl
D4	Complete medium - EMEM (100 ml)	EMEM		84 ml
		FBS	10%	10 ml
		L-Glutamine	2 mM	2 ml
		Sodium Pyruvate	1.0 mM	1 ml
		Non-Essential Amino Acid	0.1 mM	1 ml
		Penicillin/Streptomycin	0.1%	1 ml
		Fungizone		1 ml
D5	Erythrocyte lysis buffer (500 ml)	NHCl		4.1 g
		$KHCO_3$		0.5 g
		EDTA		0.019 g
		Distilled H_2O		500 ml

D6	Trypan Blue solution 0.4% (100 ml)	Trypan Blue powder		0.4 g
		HBSS		100 ml
Basic Fibroblast Growth Factor				
D7	Phosphate buffer saline-Tween (PBS-T)	Phosphate buffer pH 7.4	10 mM	
		NaCl	150 mM	
		Tween-20	0.05%	
D8	Monoclonal anti-fibroblast growth factor 0.06 µg/ml (1:6500)	Antibody		1 µl
		PBS-T		6 499 µl
D9	Goat anti-mouse IgG HRP 0.1 µg/ml (1:4 000)	Antibody		1 µl
		PBS-T		3 999 µl
D10	Sulphuric Acid 1 mol/ml (500 ml)	Sulphuric Acid	l mol/l	5 ml
		Distilled H_2O		495 ml
D11	PBS BSA/azide buffer (100 ml)	PBS		100 ml
		BSA	0.1%	0.1 g
		Azide	0.01%	0.01 g
D12	β1-Integrin 0.8 µg/ml (1:250)	Antibody		1 µl
		PBS BSA/azide		249 µl
D13	Formalin 3.7% (100 ml)	Formaldehyde	37%	3.7 ml
		PBS BSA/azide buffer		96.3 ml
Western Blotting				
D14	Two times sample buffer (50 ml)	Tris pH	2 M	3.12 ml
		SDS	2%	2 g
		Gycerol	100%	10 ml
		Distilled H_2O up to 50 ml		

D15	Stacking gel 4 gels	Acrylamide/bisacryl	37.5:1	1 000 µl
		Tris	2 M	500 µl
		SDS	10%	80 µl
		TEMED		16 µl
		APS	10%	80 µl
		Distilled H_2O		6.8 ml
D16	Running gel 12.5% 4 gels	Acrylamide/bisacryl	2.6%	10 ml
		Tris	3 M	3 ml
		SDS	10%	240 µl
		TEMED		24 µl
		APS	10%	80 µl
		Distilled H_2O		10.8 ml
D17	One times electrophoresis buffer	Tris	5 mM	3.03 g
		Glycine	192 mM	14.4 g
		SDS	0.1%	1 g
		Distilled H_2O up to 1000 ml		
D18	One times transfer buffer	Tris	5 mM	3.03 g
		Glycine	192 mM	14.4 g
		SDS	0.1%	1 g
		Methanol	20%	200 µl
		Distilled H_2O up to 1000 ml		
D19	Tris buffer saline (TBS)	Tris/HCl	50 mM	6.05 g
		NaCl	150 mM	8.77 g
		Distilled H_2O up to 1000 ml		
D20	Tween tris buffer saline (TTBS)	Tris/HCl	50 mM	6.05 g
		NaCl	150 mM	8.77 g
		Tween-20	0.1%	1 ml
		Distilled H_2O up to 1 000 ml		

D21	Blocking buffer (5% fat	Fat free powder		2.5 g
		TTBS		50 ml
D22	β1-Integrin 2 µg/ml (1:100)	β1-Integrin		10 µl
		Blocking buffer		990 µl
D23	Secondary antibody, Goat anti-mouse IgG HRP 0.4 µg/ml (1:1 000)	Antibody		1 µl
		Blocking buffer		999 µl
D24	Secondary antibody, Goat anti-mouse IgG-Rhodamine 0.4 µg/ml (1:1 000)	Antibody		1 µl
		Blocking buffer		999 µl
D25	Tris 3 M pH 8.8 (100 ml)	Tris		36.33 g
		pH with HCl		
		Distilled H_2O up to 100 ml		
D26	Tris 2 M pH 6.8 (100 ml)	Tris		24.2 g
		pH with HCl		
		Distilled H_2O up to 100 ml		
D27	APS 10%	APS		100 mg
		Distilled H_2O		1 ml
D28	SDS 10%	SDS		1 g
		Distilled H_2O		10 ml
D29	Gelatin 0.1%	Gelatin		250 mg
		Distilled H_2O		250 ml
		Dissolve and sterilise at 121 ^{0}C for 1 h store at 4 ^{0}C		
D30	bFGF (growth factor), 10 µg/ml	bFGF powder		50 µg
		Distilled H_2O		5 ml
D31	EGF 0.1 mg/ml	EGF powder		1 mg
		Distilled H_2O		10 ml

D32	2-Mercaptoethanol ($1x10^{-6}$ Mol in cell suspension)	2-Mercaptoethanol		1 µl
		Cell suspension		1 ml
D33	Fibroblast surface protein (1:500)	Antibody		2 µl
		Blocking buffer		998 µl
D34	Secondary antibody anti-mouse IgM (1:1000)	Antibody		1 µl
		Blocking buffer		999 µl
D35	Proliferation medium (50 ml)	DMEM		49.250 ml
		B27		20 µl
		EGF		10 µl
		bFGF		20 µl
		Penicillin/Streptomycin		500 µl
		Fungizone		200 µl
Dot Blotting				
D36	Denaturing buffer (100 ml)	di-Sodium hydrogen orthophosphate (Na_2HPO_4)	10 mM	0.121 g
		Urea	8 M	48.05 g
		Tris	10 mM	0.141 g
		Distilled H_2O up to 100 ml		
		pH to 8.0		

APPENDIX E

LASER PARAMETER CALCULATIONS

E1. Power Density

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

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

E2. 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:

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

$$\frac{413.22}{6\ 0} = 6 \text{ min}$$

6 X 60 = 360 sec

413-360 = 53 sec

Total duration of exposure = 6 min 53 sec