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Expression of genes in normal fibroblast cells (WS1) in response to irradiation at 660 nm

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ABSTRACT

Photobiomodulation has been found to increase gene expression and release of various growth factors and cytokines involved in wound healing. Photobiomodulation has been used to treat a wide variety of disorders, and has been found to be beneficial in the treatment of chronic wounds, however the exact underlying mechanism is not well understood. This study aimed to profile 84 genes in response to irradiation at 660 nm. WS1 human skin fibroblasts were used in gene expression profiling studies in response to irradiation with a 660 nm diode laser at a fluence of 5 J/cm^2 and power density of 11 mW/cm^2. Forty-eight hours post-irradiation, 1 μg RNA was reverse transcribed and used in real-time qualitative polymerase chain reaction (qPCR). Genes involved in the extracellular matrix and cell adhesion, inflammatory cytokines and chemokines, growth factors and signal transduction were evaluated. A total of 76 genes were regulated by laser irradiation, 43 genes were up-regulated while 33 genes were down-regulated. Irradiation of WS1 cells at 660 nm modulates the expression of genes involved in collagen production, cellular adhesion, remodelling and spreading, the cytoskeleton, inflammatory cytokines and chemokines, growth factors and molecules involved in signal transduction.

1. Introduction

Photobiomodulation is the application of low powered (intensity) laser light to cells and tissue to accelerate cellular processes, including wound healing. It is non-thermal irradiation and often involves light in the visible red to near-infrared (NIR) range of the electromagnetic spectrum. It emits no heat, sound or vibration and there is no immediate increase in temperature post-irradiation [1–3]. *In vitro,* photoirradiation has shown to have variable effects on numerous cell types, including fibroblasts, keratinocytes and stem cells to name a few, and that these effects can be dependent on laser parameters. A multitude of cellular parameters, including cell morphology, migration, viability, proliferation, cytotoxicity, inflammatory cytokines and chemokines, growth factors, extracellular matrix (ECM) components, and growth factors have been studied, and all by various methodologies, of which the polymerase chain reaction (PCR) is one.

Reverse transcription of RNA into copy DNA (cDNA), followed by the quantitative polymerase chain reaction (qPCR) is an exceedingly sensitive method of quantifying gene transcripts from cells. The use of the fluorophor SYBR Green, which non-selectively binds to double-stranded DNA, and plate-format real-time PCR arrays, which measures the fluorescence at the end of each PCR cycle, enables researchers to quantify hundreds of gene transcripts in parallel [4]. The addition of running a melt curve, or dissociation curve, at the end of PCR cycling eliminates the need to perform gel electrophoresis to ensure the amplification of a single product.

During the highly controlled process of wound healing, various growth factors, cytokines and chemokines are released by a variety of cells to orchestrate this process. One of the many important aspects of wound healing is the formation of granulation tissue and the ECM, angiogenesis and inflammation. Growth factors, cytokines and chemokines not only control these events, but also influence gene expression involved in cellular migration, differentiation and proliferation. Recently, Peplow and Baxter [5] and Peplow et al. [6] published a review article on the expression of various growth factors in wounded diabetic and non-diabetic animal models, and cells in culture. A vast majority of the research in these reviews have analysed these growth factors and cytokines by PCR, and looked at growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), hypoxia-inducible factor (HIF), phosphatidylinositol-glycan biosynthesis class F protein (PIGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), various interleukins (IL), kerationocyte growth factor (KGF), stem cell factor (SCF), tumour necrosis factor (TNF), melanocyte stimulating factor (MSH), and endothelin-1 (ET-1).

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Irradiation of animal models and various cells have shown to stimulate gene expression of various growth factors, cytokines, chemokines and ECM components [5,6]. McDaniel and co-workers irradiated human skin fibroblasts with a 590/870 nm wavelength LED array (4.0 mW/cm^2) at various 590:870 nm ratios (100:0%; 75:25%; 50:50%; 25:75%; and 0:100% respectively) [7]. These ratios were derived by using pulsed duty cycle (250 ms on/100 ms off/100 pulses) and delivered 0.1 J/cm^2. Gene expression was then analysed 24 h post-irradiation using an ECM and adhesion molecule pathway focused microarray. They demonstrated that the gene expression profiles differed for each ratio combination used. When a ratio of 100:0% was used (590:870 nm), 77.5% of genes were altered, for 75:25% a total of 58.8% of genes were altered, for 50:50% a total of 21.3% of genes were altered, for 25:75% a total of 25% of genes were altered, and for 0:100% a total of 27.5% of genes were altered. The 75:25% ratio produced the best results with regards to an increase in ECM proteins (up-regulation of collagen and down-regulation in matrix metalloproteinases, MMPs). Gavish et al. [8] irradiated porcine primary aortic smooth muscle cells with a 780 nm diode laser at 1 and 2 J/cm^2 and incubated cells for 0, 0.5, 12, 24 or 36 h before RNA extraction and cDNA synthesis. Gene expression of MMP1, MMP2, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP2 and IL-1β was determined by semi-quantitative RT-PCR [8]. It was demonstrated that photobiomodulation up-regulated MMP1 (all time points) and TIMP2 (12 h) and down-regulated MMP2 (36 h) and IL-1β (0.5 h). Hakki and Bozkurt showed that irradiation of human gingival fibroblasts with a wavelength of 940 nm at different laser settings (power and fluence) significantly increased the expression of IGF, TGF-β, VEGF and collagen type I [9].

This study aimed to determine the effect of laser irradiation at a wavelength of 660 nm (output power of 100 mW) and a fluence of 5 J/cm^2 on the expression profiles of genes in WS1 fibroblast cells.

2. Materials and methods

All media and supplements were supplied by Sigma–Aldrich (Aston Manor, South Africa) and Whitehead Scientific (Brackenfell, Cape Town, South Africa), unless otherwise stated. Lasers were supplied and set up by the National Laser Centre (NLC, Pretoria, South Africa).

2.1. Experimental design

Experiments were repeated three times ($n = 3$) on randomised cell cultures. This was a controlled laboratory experiment. Cells were exposed to a low power laser (660 nm; 11 mW/cm^2; 5 J/cm^2) and incubated for 48 h. Total RNA was isolated and reverse transcribed into cDNA which was used in real-time qPCR. Non-irradiated cells were used as controls. qPCR results were normalised against 5 reference genes.

2.2. Cell culture

Human skin fibroblast cells (WS1, ATCC, CRL-1502) were grown in minimum essential medium (MEM, Sigma–Aldrich, M7278) supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma–Aldrich, F9665), 2 mM L-glutamate (Sigma–Aldrich, G7513), 1% (*v*/*v*) Penicillin–Streptomycin (Sigma–Aldrich, P4333), 1% (*v*/*v*) Amphotericine-B (Sigma–Aldrich, A2942), 1 mM sodium pyruvate (Sigma–Aldrich, S8636), and 0.1 mM non-essential amino acids (NEAA, Sigma–Aldrich, M7145). Cells were incubated at 37 °C in 5% carbon dioxide and 85% humidity.

2.3. Laser irradiation

Cells (6×10^5) were irradiated in 3.4 cm diameter culture dishes with a 660 nm diode laser at 5 J/cm^2. Cells were irradiated in the dark, with the culture dish lid off. Laser light was directed to cells via fibre optics at a distance such that the spot size was 9.1 cm^2. Non-irradiated controls (0 J/cm^2) were kept in a dark box on the bench. Power output was measured using a power meter (Fieldmate, 0398D05) and used to calculate the duration of exposure so as to deliver 5 J/cm^2. A summary of the laser parameters can be seen in Table 1. Cells were incubated for 48 h post-irradiation.

2.4. RNA isolation and cDNA synthesis

Post-incubation, cells were detached with TrypleExpress (Applied Biosystems, Johannesburg, South Africa, Invitrogen, 12604) rinsed with phosphate buffered saline (PBS) and lysed in 600 µl RLT Buffer which also inactivates RNase. RNA was isolated on the QIAcube (Qiagen) using the Qiagen RNeasy Mini Kit (Whitehead Scientific, 74104) with homogenizers (Whitehead Scientific, Qiagen, 79654). Isolated RNA was quantified on the Qubit™ fluorometer (Invitrogen) using the Quant-iT™ RNA Assay kit (Applied Biosystems, Johannesburg, South Africa, Invitrogen, Q32852). Purity was determined spectrophotometrically at A_{260} nm/A_{280} nm.

cDNA was synthesised from 1 µg total RNA using the QuantiTect Reverse Transcription Kit (Whitehead Scientific, Qiagen, 205311). Briefly samples were treated with DNase and incubated at 42 °C for 5 min. RT master mix was added and samples incubated at 42 °C for 15 min followed by 3 min incubation at 95 °C. cDNA was stored at -20 °C until ready for qPCR.

2.5. Gene expression profiling

The SABiosciences human wound healing RT^2 Profiler PCR Array System (Whitehead Scientific, SABiosciences, PAHS-121) was used to evaluate 84 genes (Table 2). These included genes involved in the ECM, remodelling enzymes, cellular adhesion, cytoskeleton, inflammatory cytokines and chemokines, growth factors and signal transduction.

cDNA was thawed, made up to a final volume of 111 µl, added to the master mix (containing ROX as a reference dye and SYBR green) and 25 µl was added to each well. Real-time qPCR was performed on the Stratagene MX3000p (Anatech, Olivedale, South Africa) using the following cycles: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 1 min at 60 °C. A dissociation (melting) curve was also performed at 95 °C for 1 min and 65 °C for 2 min; readings were taken at 65 to 95 °C at 2 °C per minute intervals. A single peak at temperatures greater than 80 °C had to be obtained.

Threshold cycle (C_t) values greater than 35 were considered negative. C_t values were exported to an Excel-based Data Analysis Template (available from the SABiosciences website) with the appropriate pathway-focused genes. Results were normalised

Table 1
Laser parameters used in this study.

Parameter	
Manufacturer	RGBlase, Fremont, California, USA
Wavelength (nm)	660
Wave emission	Continuous wave
Power output (mW)	100
Power density (mW/cm^2)	11
Spot size (cm^2)	9.1
Fluence (J/cm^2)	5
Duration of irradiation	7 min 35 s

Table 2
Genes evaluated using the human wound healing RT^2 Profiler PCR Array System. Wells A1 through G12 contained genes involved in wound healing. Wells H1 through H5 contained housekeeping genes, H6 contained a human genomic DNA control (HGDC), H7 through H9 contained replicate reverse transcription controls (RTC), and H10 through H12 contained replicate positive PCR controls (PPC).

Pathway	Genes
Extracellular matrix & cell adhesion	
ECM components	COL14A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A3, COL5A1, COL5A2, COL5A3, VTN
Remodelling enzymes	CTSG, CTSK, CTSL2, F13A1, F3 (Tissue Factor), FGA (Fibrinogen), MMP1, MMP2, MMP7, MMP9, PLAT (tPA), PLAU (uPA), PLAUR (uPAR), PLG, SERPINE1 (PAI-1), TIMP1
Cellular adhesion	CDH1 (E-cadherin), ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6
Cytoskeleton	ACTA2 (a-SMA), ACTC1, RAC1, RHOA, TAGLN
Inflammatory cytokines & chemokines	
	CCL2 (MCP-1), CCL7 (MCP-3), CD40LG (TNFSF5), CXCL1, CXCL11 (ITAC/IP-9), CXCL2, CXCL5 (ENA-78/LIX), IFNG, IL10, IL1B, IL2, IL4, IL6
Growth factors	
	ANGPT1, CSF2 (GM-CSF), CSF3 (GCSF), CTGF, EGF, FGF10, FGF2, FGF7, HBEGF (DTR), HGF, IGF1, MIF, PDGFA, TGFA, TGFB1, TNF, VEGFA
Signal transduction	
TGFßx	TGFB1, TGFBR3, STAT3
WNT	CTNNB1, WISP1, WNT5A
Phosphorylation	MAPK1 (ERK2), MAPK3 (ERK1), PTEN
Receptors	EGFR, IL6ST (GP130)
Other	PTGS2
Housekeeping genes	
	B2M, HPRT1, RPL13A, GAPDH, ACTB

against an average of all five housekeeping/reference genes. All the necessary calculations ($\Delta\Delta C_t$ method) and interpretation of the control wells was carried out by the program. Fold-change ($2^{(-\Delta\Delta C_t)}$) was also calculated and reported as fold up-regulation if greater than 1, and fold down-regulation if less than 1. Non-irradiated normal cells (N 0 J/cm^2) were used as the control.

2.6. Statistical analysis

Experiments were repeated three times ($n = 3$). Results were normalised against an average of all five housekeeping/reference genes. The student T-test was performed by the SABiosciences Excel-based Data Analysis Template and reported as significant if $P < 0.05$.

3. Results

Forty-eight hours post-irradiation at a wavelength of 660 nm with 5 J/cm^2 the profile of 84 genes involved was determined by real-time RT-qPCR in WS1 fibroblast cells. A total of 43 genes were found to be significantly up-regulated, while 33 genes were significantly down regulated (Table 3).

Of these 43 genes which were up-regulated, five were involved in the ECM components (COL14A1, COL4A1, COL4A3, COL5A3 and VTN), nine were remodelling enzymes (CTSG, CTSL2, F13A1, F3, FGA, MMP7, MMP9, PLAT, and PLG), six were involved in cellular adhesion (CDH1, ITGA3, ITGA4, ITGA6, ITGAV, and ITGB3), two were involved in the cytoskeleton (ACTC1 and RAC1), seven formed part of the inflammatory cytokines and chemokines (CD40LG, CXCL11, CXCL2, IFNG, IL10, IL2, and IL4), nine were growth factors (CSF2, CSF3, EGF, FGF10, HGF, IGF1, PDGFA, TGFA, and TNF) and five were involved in signal transduction (TGFBR3, STAT3, CTNNB1, WISP1, and EGFR). Of the 33 genes down-regulated, five were involved in the ECM components (COL1A1, COL1A2, COL3A1, COL5A1, and COL5A2), seven were remodelling enzymes (CTSK, MMP1, MMP2, PLAU, PLAUR, SERPINE1, and TIMP1), four were concerned with cellular adhesion (ITGA2, ITGA5, ITGB1, and ITGB5), three were connected to the cytoskeleton (ACTA2, RHOA, and TAGLN), three were inflammatory cytokines and chemokines (CXCL1, CXCL5, and IL1B), five were growth factors (ANGPT1, CTGF, FGF2, MIF, and TGFB1), and six were connected to signal transduction (WNT5A, MAPK1, MAPK3, PTEN, IL6ST, and PTGS2).

4. Discussion and conclusion

Photobiomodulation has been used in a wide number of disciplines, such as somatology, dentistry, physiotherapy, dermatology, neurology, tissue engineering and regeneration and veterinary science to stimulate biological processes. The proposed mechanism of action is the absorption of the photon energy by cellular chromophores or photoacceptors which assume an electronically excited state [10]. These chromophores convert the light energy to chemical energy which stimulates cellular mechanisms such as DNA synthesis, transcription, cellular migration, differentiation and proliferation. Irradiation of isolated mitochondria at 660 nm with 5 J/cm^2 altered mitochondrial enzyme activity [11]. There was an increase in cytochrome c oxidase activity (terminal electron acceptor in the mitochondrial electron transport chain, ETC) which ultimately lead to an increase in adenosine triphosphate (ATP). This increase in ATP leads to further downstream processes, such as the release of growth factors and gene transcription. This study aimed to determine the expression of 84 genes related to ECM components, remodelling enzymes, cellular adhesion, cytoskeleton, inflammatory cytokines and chemokines, growth factors and signal transduction in normal, unstressed human skin fibroblast cells (WS1) in response to irradiation at 660 nm with a fluence of 5 J/cm^2. This paper looked at the response of normal, unstressed cells *in vitro*, and a completely different genetic profile will more than likely be obtained using stressed cells. It should also be kept in mind that this is an *in vitro* repose of normal fibroblast cells to a wavelength of 660 nm, and a different response to the same wavelength might be seen in cells irradiated *in vivo* due to the interaction with a number of other cells and factors.

The up- and down-regulation of genes in response to photobiomodulation has been shown in a variety of cells both *in vitro* and *in vivo*. There has been an up-regulation in MMP1 [8], TIMP1 [12], TIMP2 [8], IL-6 [12], Col-I [9,12–15], Col-II [16], Col-III [12], IGF [9], TGF-β [9,12,17,18], VEGF [9], endothelial nitric oxide synthase (eNOS) [19], PDGF [18], cytochrome c oxidase [20], pyrophosphatase (complex V) [20] and STAT3 [21]. Some of the genes which have been found to be down-regulated include MMP1 [12], MMP2 [8,12,22], MMP9 [22], IL-1β [8,18,23], TGF-β [24], TNF-α [24,25], COX-2 [25] and interferon-gamma (IFN-γ) [19]. In a similar study, stressed fibroblast cells irradiated at 660 nm resulted in the up-regulation of genes involved in the ETC [20].

Table 3
Gene profile for normal fibroblast cells irradiated at 660 nm with 5 J/cm^2 (n = 3). This table shows only genes which were significantly up- (fold change greater than 1) or down-regulated (fold change less than 1), and provides a summary of their functions. Results are reported as significant if reported as significant if $P < 0.05$.

Gene symbol	Gene name	Fold difference	P value	Gene ID	Gene location	Summary [28]
ACTA2	Actin, alpha 2, smooth muscle, aorta	0.02	<0.001	59	10q23.3	Part of the actin family which play a role in cell motility, structure and integrity
ACTC1	Actin, alpha, cardiac muscle 1	382.15	<0.001	70	15q14	Part of the actin family
ANGPT1	Angiopoietin 1	0.25	<0.01	284	8q23.1	Secreted glycoprotein and plays a role in angiogenesis
CD40LG	CD40 ligand	762.54	<0.001	959	Xq26	Regulates B cell function by engaging CD40 on the B cell surface
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	2.42	<0.05	999	16q22.1	Part of the cadherin superfamily. Calcium dependent cell–cell adhesion glycoprotein
COL14A1	Collagen, type XIV, alpha 1	42.56	<0.001	7373	8q23	Encodes the collagen alpha 1(XIV) chain. Interacts with fibril surface and regulates fibrillogenesis
COL1A1	Collagen, type I, alpha 1	0.00	<0.001	1277	17q21.33	Encodes the pro-alpha 1 chains of type I collagen. Fibril-forming collagen
COL1A2	Collagen, type I, alpha 2	0.00	<0.001	1278	7q22.1	Encodes the pro-alpha 2 chain of type I collagen
COL3A1	Collagen, type III, alpha 1	0.00	<0.001	1281	2q31	Encodes the pro-alpha 1 chains of type III collagen, a fibrillar collagen that is found in connective tissue frequently in association with type I collagen
COL4A1	Collagen, type IV, alpha 1	19.85	<0.001	1282	13q34	Encodes the major type IV alpha collagen chain of basement membranes
COL4A3	Collagen, type IV, alpha 3	4570.07	<0.001	1285	2q36-q37	Encodes for the alpha subunit 3 in type IV collagen
COL5A1	Collagen, type V, alpha 1	0.25	<0.001	1289	9q34.2-q34.3	Type V collagen appears to regulate the assembly of heterotypic fibres composed of both type I and type V collagen. Encodes for alpha chain 1
COL5A2	Collagen, type V, alpha 2	0.24	<0.001	1290	2q14-q32	Encodes for alpha chain 2 for type V collagen
COL5A3	Collagen, type V, alpha 3	176.64	<0.001	50509	19p13.2	Encodes for alpha chain 3 for type V collagen
CFS2	Colony stimulating factor 2 (granulocyte–macrophage)	133.87	<0.001	1437	5q31.1	Controls the production, differentiation and function of granulocytes and macrophages
CSF3	Colony stimulating factor 3 (granulocyte)	6.28	<0.01	1440	17q11.2-q12	Controls the production, differentiation and function of granulocytes
CTGF	Connective tissue growth factor	0.05	<0.001	1490	6q23.1	Plays a role in chondrocyte proliferation and differentiation and cell adhesion
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	14.98	<0.001	1499	3p21	Part adherens junctions proteins. Creates and maintains epithelial cell layers by regulating cell growth and adhesion between cells
CTSG	Cathepsin G	859.89	<0.001	1511	14q11.2	Part of peptidase S1 protein family and have specificity similar to that of chymotrypsin C. May participate in connective tissue remodelling at sites of inflammation
CTSK	Cathepsin K	0.13	<0.001	1513	1q21	Encodes for a lysosomal cysteine proteinase involved in bone remodelling and resorption
CTSL2	Cathepsin L2	1.88	<0.05	1515	9q22.2	Encodes for a lysosomal cysteine proteinase
CXCL1	Chemokine (C-X-C motif) ligand 1	0.03	<0.001	2919	4q21	Part of the CXC subfamily of chemokines. Plays a role in inflammation and as a chemoattractant for neutrophils
CXCL11	Chemokine (C-X-C motif) ligand 11	3463.46	<0.001	6373	4q21.2	Part of the CXC subfamily of chemokines. Chemoattractant for activated T-cells and is the dominant ligand for CXC receptor-3
CXCL2	Chemokine (C-X-C motif) ligand 2	188.88	<0.001	2920	4q21	Part of the CXC subfamily of chemokines. Mobilizes cells by interacting with a cell surface chemokine receptor, CXCLR2
CXCL5	Chemokine (C-X-C motif) ligand 5	0.30	<0.01	6374	4q13.3	Part of the CXC subfamily of chemokines. This chemokine is a potent chemotaxin involved in neutrophil activation
EGF	Epidermal growth factor	30.51	<0.001	1950	4q25	Potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types
EGFR	Epidermal growth factor receptor	1.70	<0.05	1956	7p12	Cell surface glycoprotein and part of protein kinase superfamily. Receptor that binds to EGF leading to cell proliferation
F13A1	Coagulation factor XIII, A1 polypeptide	3703.48	<0.001	2162	6p25.3–p24.3	Encodes coagulation factor XIII A subunit. Coagulation factor XIII is the last zymogen to become activated in the blood coagulation cascade and acts as a transglutaminase stabilizing the fibrin clot
F3	Coagulation factor III (thromboplastin, tissue factor)	1.85	<0.05	2152	1p22–p21	Encodes for coagulation factor III, an initiator of coagulation and receptor for coagulation factor VII
FGA	Fibrinogen alpha chain	335	<0.001	2243	4q28	Encodes for the alpha component of fibrinogen, which plays a role in coagulation. Fibrinogen is cleaved by thrombin to form fibrin
FGF10	Fibroblast growth factor 10	4195.62	<0.001	2255	5p13–p12	Part of the FGF family. This protein exhibits mitogenic activity for keratinizing epidermal cells
FGF2	Fibroblast growth factor 2 (basic)	0.39	<0.05	2247	4q26	Part of the FGF family. Implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumour growth
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	26.62	<0.001	3082	7q21.1	Part of the plasminogen subfamily of S1 peptidases. Regulates cell growth, motility, and morphogenesis
IFNG	Interferon, gamma	131.72	<0.001	3458	12q14	Part of the type II interferon family. IFNG is a soluble cytokine with anti-viral, immunoregulatory and anti-tumour properties and is a potent activator of macrophages
IGF1	Insulin-like growth factor 1 (somatomedin C)	523.24	<0.001	3479	12q23.2	Involved in mediating growth and development
IL10	Interleukin 10	17.24	<0.001	3586	1q31–q32	Has a pleiotropic effect in immunoregulation and inflammation

(*continued on next page*)

Table 3 (*continued*)

Gene symbol	Gene name	Fold difference	*P* value	Gene ID	Gene location	Summary [28]
IL1B	Interleukin 1, beta	0.01	<0.001	3553	2q14	An important mediator of the inflammatory response
IL2	Interleukin 2	1998.45	<0.001	3558	4q26–q27	Proliferation of T and B lymphocytes
IL4	Interleukin 4	62.74	<0.001	3565	5q31.1	Pleiotropic cytokine which activates macrophages
IL6ST	Interleukin 6 signal transducer	0.08	<0.01	3572	5q11.2	Signal transducer for many cytokines, including IL-6
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.39	<0.01	3673	5q11.2	Part of the integrin family; group of membrane receptors involved in cell adhesion and signal transduction. Encodes the alpha subunit of a transmembrane receptor for collagens and related proteins
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	4.99	<0.01	3675	17q21.33	Part of the integrin family. Encodes alpha 3 subunit and interacts with many ECM proteins
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	3.11	<0.01	3676	2q31.3	Part of the integrin family. Encodes an alpha 4 chain. Associates with either beta 1 chain or beta 7 chain
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	0.01	<0.001	3678	12q11–q13	Part of the integrin family. Encodes the integrin alpha 5 chain. Associates with beta 1 to form a fibronectin receptor
ITGA6	Integrin, alpha 6	23.50	<0.001	3655	2q31.1	Part of the integrin family. Encodes the integrin alpha 6 chain. Combines with multiple partners resulting in different integrins
ITGAV	Integrin, alpha V	12.98	<0.001	3685	2q31–q32	Part of the integrin family. Encodes integrin alpha chain V. Combines with multiple integrin beta chains to form different integrins
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.01	<0.001	3688	10p11.2	Part of the integrin family. Encodes integrin beta 1 chain. Multiple alternatively spliced transcript variants have been found for this gene
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	1.47	<0.05	3690	17q21.32	Part of the integrin family. Encodes integrin beta 3 chain
ITGB5	Integrin, beta 5	0.00	<0.001	3693	3q21.2	Part of the integrin family. Encodes integrin beta 5 chain
MAPK1	Mitogen-activated protein kinase 1	0.49	<0.05	5594	22q11.21	Part of the MAP kinase family. Involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development
MAPK3	Mitogen-activated protein kinase 3	0.03	<0.001	5595	16p11.2	Part of the MAP kinase family
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.02	<0.001	4282	22q11.23	Involved in cell-mediated immunity, immunoregulation, and inflammation. Regulation of macrophage function
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	0.12	<0.001	4312	11q22.3	Part of the MMP family, which breaks down the ECM. MMP1 Breaks down interstitial collagens, types I, II, and III
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	0.10	<0.01	4313	16q13–q21	Degrades type IV collagen
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	11.75	<0.001	4316	11q21–q22	Degrades proteoglycans, fibronectin, elastin and casein
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	2.51	<0.01	4318	20q11.2–q13.1	Degrades type IV and V collagens
PDGFA	Platelet-derived growth factor alpha polypeptide	3.46	<0.001	5154	7p22	Part of the platelet-derived growth factor family. Regulator of cell growth and division
PLAT	Plasminogen activator, tissue	33.24	<0.001	5327	8p12	Encodes for a serine protease which converts the pro-enzyme plasminogen to plasmin, a fibrinolytic enzyme
PLAU	Plasminogen activator, urokinase	0.08	<0.001	5328	10q24	Encodes for a serine protease involved in degradation of the ECM. Converts the plasminogen to plasmin
PLAUR	Plasminogen activator, urokinase receptor	0.04	<0.001	5329	19q13	Encodes the receptor for PLAU and influences many cell-surface plasminogen activation processes and ECM degradation
PLG	Plasminogen	45.40	<0.001	5340	6q26	Encodes for a zymogen that is activated by proteolysis and converted to plasmin and angiostatin
PTEN	Phosphatase and tensin homolog	0.00	<0.001	5728	10q23.3	Encodes for a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. Tumour suppressor gene involved in cell cycle regulation
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase, COX-2)	0.54	<0.05	5743	1q25.2–q25.3	Key enzyme in prostaglandin biosynthesis. Elevated levels are found during inflammation
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	2.00	<0.01	5879	7p22	Part of the RAS superfamily which are small GTP-binding proteins, which regulate diverse cellular events, including cell growth, cytoskeletal reorganization, cell–cell adhesion, motility and activation of protein kinases
RHOA	Ras homolog family member A	0.01	<0.001	387	3p21.3	Part of the RAS superfamily
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.00	<0.001	5054	7q22.1	Encodes for a member of the serine proteinase inhibitor (serpin) superfamily, and is an inhibitor of PLAT and PLAU
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	94.88	<0.001	6774	17q21.31	Part of the STAT protein family, which are phosphorylated in response to cytokines and growth factors. Mediates the expression of a variety of genes in many cellular processes such as cell growth and apoptosis
TAGLN	Transgelin	0.41	<0.001	6876	11q23.2	Encodes for a transformation and shape-change sensitive actin cross-linking/gelling protein. A functional role of this protein is unclear
TGFA	Transforming growth factor, alpha	504.25	<0.001	7039	2p13	Growth factor which activates signalling pathways involved in cell proliferation, differentiation and development

Table 3 (*continued*)

Gene symbol	Gene name	Fold difference	*P* value	Gene ID	Gene location	Summary [28]
TGFB1	Transforming growth factor, beta 1	0.16	<0.01	7040	19q13.1	Part of TGF-β family of cytokines, which regulate proliferation, differentiation, adhesion and migration amongst others. Plays an important role in regulating the immune system
TGFBR3	Transforming growth factor, beta receptor III	79.05	<0.001	7049	1p33–p32	Membrane receptor that often functions as a co-receptor with other TGF-β receptor superfamily members
TIMP1	TIMP metallopeptidase inhibitor 1	0.08	<0.001	7076	Xp11.3–p11.23	Encodes for protein which forms part of the TIMP family which are inhibitors of MMPs
TNF	Tumour necrosis factor	2443.39	<0.001	7124	6p21.3	Pro-inflammatory cytokine. Regulation of wide spectrum of processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
VTN	Vitronectin	65.25	<0.001	7448	17q11	Member of the pexin family. Promotes cell adhesion and spreading, and binds to several serpin serine protease inhibitors
WISP1	WNT1 inducible signalling pathway protein 1	171.41	<0.001	8840	8q24.22	Part of the WNT1 inducible signalling pathway (WISP) protein subfamily. Mediate diverse developmental processes
WNT5A	Wingless-type MMTV integration site family, member 5A	0.02	<0.001	7474	3p21–p14	Part of the WNT family. Ligand for transmembrane receptor frizzled-5 and the tyrosine kinase orphan receptor 2. Regulates developmental pathways

In this study, the expression of 76 genes, out of the 84 genes tested (90.5%), was altered in normal human skin fibroblast cells (WS1). A total of 43 genes were up-regulated (51.2%) and 33 genes were down-regulated (39.3%). One of fibroblast cells main functions is to produce collagen, a vital structural protein in the ECM. During tissue remodelling, new collagen and other ECM constituents are lay down, and old ECM proteins are removed by proteinases. Thus both reparative and destructive process take place at the same time, and this is a carefully controlled process. Tissue remodelling is regulated by a number of proteinases (e.g. MMPs and cathepsins) and their inhibitors (TIMPs) at the level of expression, deposition, activation, and inhibition [26,27]. A number of ECM components (COL14A1, COL4A1, COL4A3, COL5A3, and VTN) and remodelling enzymes (CTSG, CTSL2, F13A1, F3, FGA, MMP7, MMP9, PLAT, and PLG) were found to be up-regulated in response to irradiation at 660 nm with 5 J/cm^2. A number of genes were also down-regulated: ECM components (COL1A1, COL1A2, COL3A1, COL5A1 and COL5A2) and remodelling enzymes (CTSK, MMP1, MMP2, PLAU, PLAUR, SERPINE1, and TIMP1).

Cell adhesion is important in maintaining tissue architecture, and involves the binding of cells to each other and to the ECM. Adhesion is through cell-surface proteins or adhesion molecules such as selectins, cadherins and integrins. Cadherin is a calcium dependent glycoprotein which ensures that cells are bound together. It is made up of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail [25]. They act as both receptors and ligands. Irradiation resulted in the up-regulation of cadherin 1 (CDH1). Integrins are membrane bound proteins involved in cell-surface adhesion as well as signal transduction. They act as collagen receptors. They are heterodimeric proteins composed of an alpha and beta chain. There are a number of integrin family members and cells can have multiple types on their cell surface. Irradiation at 660 nm resulted in the up-regulation of five integrin genes (ITGA3, ITGB3, ITGA4, ITGA6 and ITGAV) and the down-regulation of four integrin genes (ITGB1, ITGA2, ITGA5 and ITGB5).

The cytoskeleton is a cellular skeleton, or scaffolding, contained within the cytoplasm and provides the cell with shape and support. The cytoskeleton is also involved in cell motility, intracellular movement of organelles and muscle fibre contraction. There are three primary cytoskeletal filaments, namely microfilaments (actin filaments), microtubules (α- and β-tubulin) and intermediate filaments (e.g. keratin). When genes related to the cytoskeleton were evaluated in irradiated WS1 cells, only two genes were significantly up-regulated, (ACTC1 and RAC1), while three genes were down-regulated (ACTA2, RHOA and TAGLN). Cytokines are signalling molecules used in intercellular communication, and are classified as proteins, peptides or glycoproteins. The term cytokine has also been used to describe immunomodulating molecules; largely interleukins and interferons. Chemokines are chemotactic cytokines and are divided up into two main subfamilies, CXC and CC. Seven out of the 13 evaluated genes involved with inflammatory cytokines and chemokines were up-regulated in irradiated normal WS1 cells: CD40LG, CXCL11, CXCL2, IFNG, IL2, IL4 and IL10. Three genes were down-regulated: CXCL1, CXCL5 and IL1B.

Growth factors stimulate a variety of cellular processes involved in cell growth, proliferation and differentiation. Evaluation of genes in irradiated normal WS1 fibroblast cells concerned with growth factors showed that nine genes were up-regulated (CSF2, CSF3, EGF, FGF10, HGF, IGF1, PDGFA, TGFA and TNF), and five genes were down-regulated (ANGPT1, CTGF, FGF2, MIF and TGFB1) in response to photobiomodulation at 660 nm. Signal transduction is an important function in any cell as it is the process by which external signals, such as a hormones or growth factors, stimulate a cell to carry out a specific function, e.g. enzyme activity or gene expression. The external stimulus interacts with a membrane receptor, which transfers the information into the cell where second messengers may relay the information. A common method of relaying such information is by protein phosphorylation, and many second messengers stimulate responses by activating protein kinases. When genes involved with signal transduction were evaluated in irradiated normal WS1 cells, five genes were up-regulated (TGFBR3, STAT3, CTNNB1, WISP1 and EGFR), while six were down-regulated (WNT5A, MAPK1, MAPK3, PTEN, IL6ST, and PTGS2).

This study has demonstrated the effects of irradiation with a 660 nm diode laser at 5 J/cm^2 on the expression of genes in unstressed WS1 human skin fibroblast cells. There was a significant change in the expression of a number of genes involved in collagen production, cellular adhesion and spreading, remodelling enzymes, the cytoskeleton, inflammatory cytokines and chemokines, various growth factors and signal transduction. These genes are involved in the normal function of a normal fibroblast cell in culture subjected to light at 660 nm. This study shows clear evidence that photobiomodulation results in a change in a large number of genes post-irradiation in normal fibroblast cells.

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