Femtosecond laser pulses for chemical-free embryonic and mesenchymal stem cell differentiation

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ABSTRACT

Owing to their self renewal and pluripotency properties, stem cells can efficiently advance current therapies in tissue regeneration and/or engineering. Under appropriate culture conditions *in vitro*, pluripotent stem cells can be primed to differentiate into any cell type some examples including neural, cardiac and blood cells. However, there still remains a pressing necessity to answer the biological questions concerning how stem cell renewal and how differentiation programs are operated and regulated at the genetic level. In stem cell research, an urgent requirement on experimental procedures allowing non-invasive, marker-free observation of growth, proliferation and stability of living stem cells under physiological conditions exists. Femtosecond (fs) laser pulses have been reported to non-invasively deliver exogenous materials, including foreign genetic species into both multipotent and pluripotent stem cells successfully. Through this multi-photon facilitated technique, directly administering fs laser pulses onto the cell plasma membrane induces transient submicrometer holes, thereby promoting cytosolic uptake of the surrounding extracellular matter. To display a chemical-free cell transfection procedure that utilises micro-litre scale volumes of reagents, we report for the first time on 70 % transfection efficiency in ES-E14TG2a cells using the enhanced green fluorescing protein (EGFP) DNA plasmid. We also show how varying the average power output during optical transfection influences cell viability, proliferation and cytotoxicity in embryonic stem cells. The impact of utilizing objective lenses of different numerical aperture (NA) on the optical transfection efficiency in ES-E14TG2a cells is presented. Finally, we report on embryonic and mesenchymal stem cell differentiation. The produced specialized cell types could thereafter be characterized and used for cell based therapies.

Keywords: Femtosecond laser pulses, multi-photon technique, optical transfection efficiency, optical stem cell differentiation, embryonic and mesenchymal stem cells, cell-based therapy, non-invasive gene delivery, chemical-free transfection, stem cell viability, proliferation and cytotoxicity.

1. INTRODUCTION

Several non-viral gene delivery methods for mouse embryonic stem cells including liposome, electroporation and nucleofection, have been developed to avoid potential safety issues of viral vector based gene transfer. Previous literature reports 20 – 70 % ES cell transfection efficiency using the effectene liposome based transfection system [1]. Further, Ward *et al*, 2002 [2] reported 50 – 80 % transfection efficiency expression of the EGFP plasmid in five undifferentiated mouse embryonic stem (mES) cells lines (BL/6III, D3, E14TG2a, MESC20 and 129) on using the lipofectamine transfection reagent in mES cells. Another study displayed that using EGFP as a reporter gene, nucleofection produced a ten fold transient transfection efficiency (i.e. 63.66 %) of mES cells compared to 6.41 % transfection efficiency achieved via electroporation [3]. Previously, electroporation and liposome-mediated methods were the most commonly used methods to transfect mES cells. However, electroporation has been reported to have typical transient mES transfection efficiency of less than 10 % [3].

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Although offering improvement to both the stable and transient mES transfection efficiencies, chemical transfection via reagents can lead to the change in metabolism of these cells and limit their use as a cell-based therapy. For example, liposome-based methods are reported to promote embryonic stem (ES) cell differentiation since the protocol of this cell transfection method requires that the cells be exposed to serum-free conditions [3]. The risk of spontaneous differentiation of stem cells under serum-free conditions could lead to heterogeneity within the ES cell population, hence, complicating and altering *in vitro* stem cell investigations.

Optical transfection via femtosecond laser pulses is a non-invasive, chemical-free transfection procedure that utilizes minimum reagent volumes and can be applied under sterile tissue culture protocols. It offers the targeted treatment of a large number of individual stem cell colonies, different cell types and their subcellular organelles [4, 5]. Targeted transfection of both multipotent (single cells) and pluripotent (colonies) stem cells via this technique has been previously reported [4, 6]. Therefore, it is becoming an attractive methodology for both the transfection and differentiation of stem cells as these cells are important for cell-based therapies. In this paper we investigated for the first time, the transfection of mES cells (ES-E14TG2a) via fs pulses emitted by a portable 1064 nm Fianium ultra-fast fiber laser. 70 % transfection efficiency in ES-E14TG2a cells through EGFP DNA plasmid was successfully achieved. Secondly, post altering the average power output at the sample plane, we present data on the cellular changes in adenosine triphosphates (ATP), alkaline phosphate (ALP) and lactate dehydrogenase (LDH) in mES cells. Our results on transfection efficiency versus objective lens used, confirmed the requirement of a high NA lens during optical transfection. Lastly, we comment on embryonic and mesenchymal stem cell differentiation. Overall these investigations will promote the production of specialized cell types that could be essentially applied in tissue regeneration or engineering as cell based therapies.

2. METHODOLOGY

The pluripotent mES cells utilized during these studies are called ES-E14TG2a cells. These cells were purchased from the European Collection of Cell Cultures (ECACC) via Sigma-Aldrich, South Africa (SA). These divided frequently when cultured in the presence of the leukemia inhibitory growth factor (LIF) (Millipore, SA). LIF is an interleukin 6 class cytokine, which is a chemical that affects cell growth, development and influences embryogenesis. Since embryonic stem cells arise from the inner cell mass (ICM) at the blastocyst stage, their removal from this location results in their removal from their natural supply and source of LIF. Consequently, removal of LIF imposes stem cell differentiation. Therefore, during *in vitro* culturing of mES cells LIF supplementation is crucial to maintain the stem cells in an undifferentiated state.

2.1 Cell culturing

The ES-E14TG2a cells were always cultured in a 37^{o}C, 5 % carbon dioxide (CO_2) and 85 % humid incubator (optimum growth condition). These adherent cells were grown in 0.2 % gelatin (Sigma, SA) coated on the surface of a T25 vented top culture flasks (*Nunc*^{TM}). They were sub-cultured twice weekly at a concentration of 1 X 10^6 cells/ml in complete growth medium constituting of Knockout DMEM (KDMEM) (*Invitrogen, UK*) modified with 1 % non-essential amino acids (*Invitrogen, UK*), 1 % L-glutamine (*Invitrogen, UK*), 1 % sodium pyruvate (*Invitrogen, UK*), 0.1 % β-mercaptoethanol (Si*gma, SA*), supplemented with LIF (*Millipore, SA*) and 10 % FBS (*Biosera, UK*).

2.2 DNA preparation

The enhanced green fluorescent protein plasmid (pEGFP from *Clontech Laboratories, Inc*) carries a red-shifted variant of wild-type GFP which facilitates brighter fluorescence and higher expression in mammalian cells. Additionally, this pEGFP (3.4 kb) encodes the GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. pEGFP was prepared from transformed *Escherichia coli* (*E. coli)* cells by utilizing the mini-prep DNA purification system according to the manufacturer's instructions (*Qiagen, UK*).

2.3 Optical setup

The optical transfection setup consisted of a dual objective optical system as depicted in Figure 1. Briefly, the femtosecond laser beam pulses emitted by a Fianium FemtoPower1060 laser (1064 nm, 80 MHz, 320 fs, average power = 60 mW at the focus) were magnified by a simple two lens telescope to match the back aperture of the 60 X air objective (*Nikon*) lens with numerical aperture (NA) 0.8. This created a diffraction limited spot of ($1/e^2$) diameter = 1.1 µm. The pulse energy at the objective front focal plane was calculated to be ~ 0.75 nJ with an associated pulse peak power = 3.75 kW. An electronic shutter (*Newport, SA,* model: M-76992) on the beam path was used to regulate the time of beam exposure at the cell sample plane on an XYZ translation stage (*Newport*). The sample chamber was illuminated via a Koehler arrangement, and imaged by a Watec color camera (WAT-250D) situated below the sample stage.

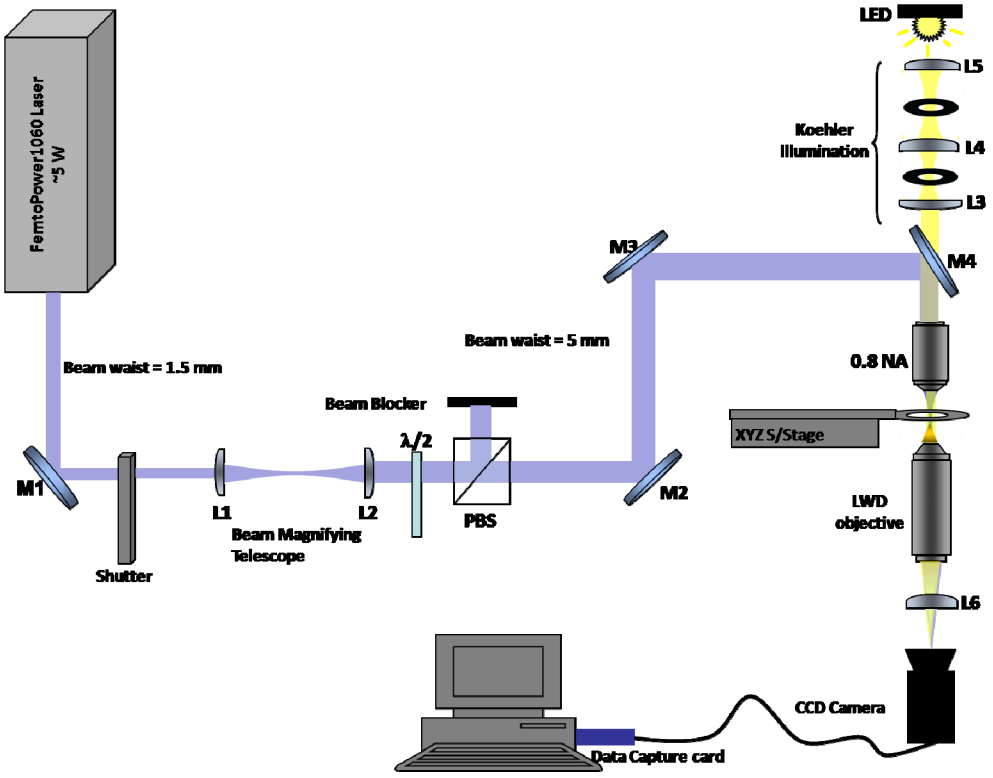

Figure 1: Optical transfection setup for my studies. An infrared Gaussian beam (beam diameter ($2w_o$) = 1.5 mm) is emitted by a portable Fianium FemtoPower1060 fs fiber laser bounced off mirror (M1) and expanded via lenses (L1 (f = 50 mm) and L2 (f = 175 mm). A half wave plate (λ/2) and polarizing beam splitter cube (PBS) were used to attenuate the power output from 5 W down to between 20 – 130 mW at the sample plane depending on the experiment, while mirrors (M2 and M3) served as a periscope. Mirror M4 reflected the beam onto the back aperture of a 60 X objective lens (NA 0.8). A light emitting diode (LED) provided sample lighting when arranged into Koehler illumination via passing through lenses (L5, L4 & L3) and two apertures. The sample imaging system consisted of: a long working distance (LWD), f = 200 mm, 50 X Mitutoyo objective lens (NA = 0.55), a tube lens (L6, f = 200 mm) and a charge coupled device (CCD) camera. These were connected to the output computer by a data capture card.

2.4 Optical transfection parameters

During our experiments, the optimum fs pulse transfection parameters for efficient transfection of ES-E14TG2a cells were determined to be 60 mW, 40 ms using a 60 X air objective lens (NA = 0.8) from Nikon. These parameters were calculated following conducting a series of experiments at different average power output values and as well as using objective lenses of varying NA. Details summarised in both Tables 1 and 2 below.

2.4.1 Average power output

Table 1: Using a half wave plate and polarizing beam splitter aligned in the optical path of our fs laser transfecting setup, a range of average power outputs (column 1) was used for chemical-free transfection of ES-E14TG2a pluripotent stem cells.

Average Power Output (mW)	Peak power (kW)	Pulse Energy (nJ)
20	2.5	0.25
40	5	0.5
60	7.5	0.75
80	10	1
100	12.5	1.25
120	15	1.5
140	17.5	1.75

2.5 Cell Sample preparation for fs laser transfection

For fs pulse transfection experiments, the ES-E14TG2a cells were seeded at approximately 10^6 cells/ml and plated in gelatin coated 35 mm diameter type zero glass bottomed petri dishes (23 mm diameter = glass working area, *World Precision Instruments, Stevenage, UK*). These cells were suspended in 2 ml of complete growth medium when incubated to sub-confluence over 24 hrs under optimum growth condition. Then the cell colonies were carefully washed twice with 2 ml of serum-free KDMEM each time, to remove the serum. Thereafter they were submerged in 60 µl of serum-free medium containing 10 µg/ml of EGFP plasmid DNA (pDNA). The sample chamber was then covered with a 22 mm diameter type-1 coverslip (*BDH, Poole UK*). Targeted optical transfection of individual cell colonies was then performed via laser irradiation through administering three shots of ultra-short duration while avoiding a visual cellular response. Irradiation of the adherent stem cell colonies facilitated by targeted delivery of the infrared fs laser beam pulses (60 mW and 40 ms) permitted diffusion of surrounding plasmid DNA into individual groups of stem cells per colony. Following laser irradiation the DNA containing medium was aspirated, the stem cell colonies washed once with serum-free KDMEM, covered in 2 ml complete medium and incubated under optimum growth conditions for 48 hrs before live cell colony fluorescence analysis and imaging.

2.6 Cellular transformation assays

No plasmid DNA was utilized during cellular transformation experiments. Each assay was performed to test change in cell viability (ATP), proliferation (ALP) and cytotoxicity (LDH) following irradiating mES cells using fs laser pulses at different pulse energies. Briefly, subsequent to constructing and aligning the fs transfection setup, a sample of ES-E14GT2a cells was plated and prepared as mentioned in section 2.5. However, the cell monolayer was submerged in 150 µl of complete culture medium. Then for each experiment, targeted optical transfection of individual mES cell colonies was performed as explained in 2.5 at various average power output values (see Table 1). Following laser irradiation the cells in culture supernatant were incubated at optimum growing conditions. 24 hours later culture supernatant was harvested and cells trypsinised cells for sample preparation. Then changes in cellular parameters were measured as shown in Figures 3 – 5 below. A negative control was prepared for each experiment, where cells were exposed to on laser irradiation, i.e. cells allowed incubating at room temperature in 150 µl complete culture medium for ± 20 min.

2.6.1 Cell viability assay

The CellTiter-Glo luminescent cell viability assay, which is a homogeneous method of determining the number of viable cells in culture based on the quantifying of ATP present, was employed in this study. ATP signals the presence of metabolically active cells in culture. 100 µl of reconstituted CellTiter-Glo reagent was added to an equal volume of cell suspension ($1X10^5$ cells/ 100 µl) after irradiation in a luminometer tube. The tube contents were mixed on an orbital shaker for 2 minutes to induce cell lyses. These samples were thereafter incubated for a further 10 minutes at room temperature to stabilize the luminescent signal. Luminescence was recorded using a Junior EG & G Berthold Luminometer [7].

2.6.2 Cell proliferation assay

ALP is a membrane bound enzyme released during inflammation, remodeling and cell proliferation. This enzyme has been used as a marker for wound healing and its activity was measured by the colorimetric assay using ρ-nitrophenyl phosphate (ρ-NPP) as a substrate. Following irradiation, 100 µl of culture supernatant was pre-incubated with an equal volume of 0.5 M N-methyl-D-glucamine buffer, pH 10.5, 0.5 mM magnesium acetate, 110 mM sodium chloride and 0.22 % Triton X-100 for 30 minutes at 37^{o}C in a 96 well plate. 20 mM ρ-NPP was added and the reaction was incubated at 37^{o}C for 30 minutes. The amount of ρ-nitrophenol liberated was measured at 405 nm [8].

2.6.3 Cell cytotoxicity assay

The CytoTox 96®, a non-radioactive cytotoxicity assay, was utilized to measure LDH, a stable cytosolic enzyme that is released upon cell lyses. After laser treatment 100 µl of culture supernatant was mixed with an equal volume of reconstituted substrate mix in a 96 well plate. The plate was covered with tin foil and incubated at room temperature for 30 minutes, protected from light. 100 µl of the stop solution was added and the absorbance read at 490 nm [9].

3. RESULTS

3.1 Laser Parameters

For statistical purposes all experiments were performed in triplicate, and each experiment was repeated five times. In all cases, the transfection efficiency measured in percent was calculated according to Tsukakoshi *et al.* (1984) [10] and Mthunzi *et al.*(2010) [4] using the expression: $N\text{cor} = [(E/D).100]/X_D$, where *N*cor is the population corrected transfection efficiency, *E* is number of cells transiently expressing the pDNA after a suitable amount of time has passed, *D* number of cells dosed on a given experiment, and X_D is the ratio of proliferation that has occurred by the dosed cells between dosing and the measurement of expression. During all the photo-transfection experiments undertaken in my studies three different types of negative controls were run.

These were labeled: 1. laser /no DNA, i.e. cells irradiated using the laser conditions mentioned above (60 mW, 40 ms) in the absence of DNA, 2. DNA /no laser, i.e. cells allowed incubating at room temperature in 60 µl of transfection mixture (10 µg/ml DNA made up in neat KDMEM) for ± 20 min and 3. No DNA /no laser, i.e. cells allowed incubating at room temperature in 60 µl neat KDMEM for ± 20 min. For all three negative controls no fluorescence was detected, meaning no intracellular transportation of any exogenous material was possible. This observation led to a confirmation that merely bathing cells in the presence of whatever foreign naked genetic or non-genetic matter does not result to its intracellular inclusion in the absence of perforation.

3.1.1 Objective lens and transfection efficiency

During fs laser transfection and due to the non-linear nature of this membrane perforation process, there are rigorous requirements not only in the accurate positioning of the laser focus on the cell plasma membrane but very importantly in creation of a tightly focused diffraction limited laser beam spot. The latter can be achieved through slightly overfilling the back aperture of a high NA objective lens with a laser light beam. Table 2 indicates typical transfection efficiency data obtained during our experiments upon employing lenses of different NA for mES cell optical transfection.

Table 2: Experimental data of ES-E14GT2a cells optically transfected with pEGFP at 60 mW, 40 ms using a 40 X, 60 X and 100 X objective lenses per experiment (n = 5) . The photo-transfection efficiency obtained under these conditions was highest (73 %) when the beam was tightly focussed compared to the case of a weakly focused beam spot (12 %).

Magnification (X)	NA	WD	Immersion Media	Photo-Transfection Efficiency (%)
40	0.65	0.65	-	12
60	0.80	0.3	-	70
100	1.25	0.23	Oil	73

3.2 Transfection efficiency of ES-E14GT2a cells using EGFP

In our studies we obtained for the first time 70 % transfection efficiency of ES-E14TG2a mES cell colonies using pEGFP. Figure 2 below depicts successful photo-transfection of non-differentiated pluripotent stem cells, also indicating the possibility of the selection and targeted treatment of specific cells within a mass of cells.

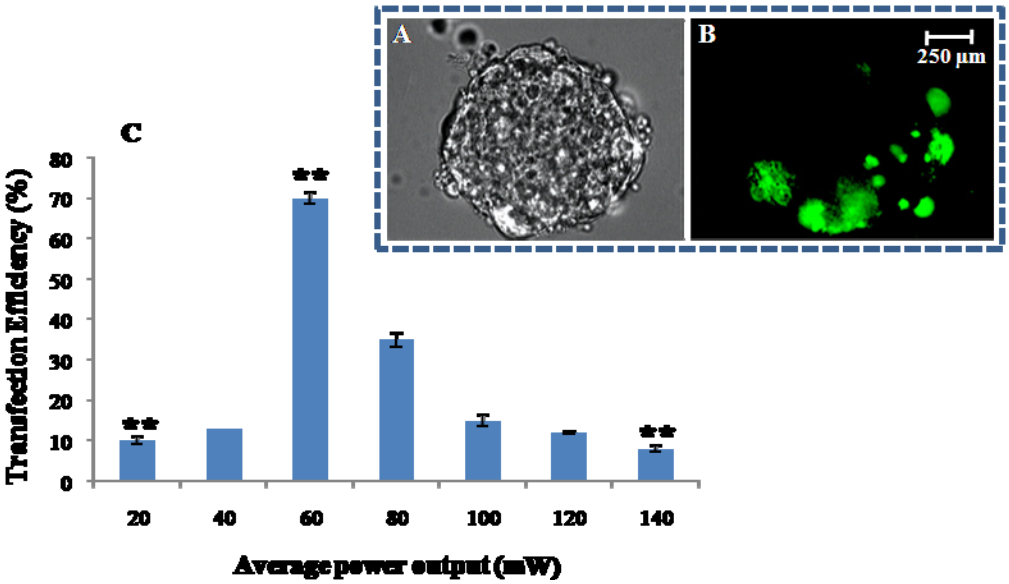

Figure 2: Pictures A and B respectively illustrate the brightfield and fluorescent images of ES-E14TG2a cells photo-transfected with pEGFP at 60 mW and 40 ms. C is the transfection efficiency plot measured at different average power output values. When compared to those irradiated at 140 mW and fs pulses with peak powers around 17.5 kW, cells optically transfected at 60 mW had the highest transfection efficiency. The data herein presents the corrected transfection efficiency calculated as mentioned before. Error bars represent the SEM (n = 5 experiments of 50 dosed cell colonies). Using ANOVA followed by Dunnett's and Fisher's tests, ** means the data sets are significantly different from each other.

3.3 Cellular transformation assays

There are a number of successfully and widely employed transfection techniques, each of these come with pros and cons. Nonetheless, the suitability of each technique for a specific application will heavily depend on the cell type under investigation. Stem cell research conducted *in vitro* requires employment of biomedical techniques with the least amount of cytotoxicity, which also avoids altering of normal biochemical, morphological and physiological characteristics of cells. As a result of low irradiation threshold, localized energy deposition, non-heating effects and minimum collateral damage fs laser assisted transfection has proved to be very effective for transfection studies *in vitro*. Figures 3 – 5 below determines experimental data on changes in ATP, ALP and LDH levels in ES-E14TG2a cells following optical treatment at different pulse energy levels.

3.3.1 ATP change in ES-E14TG2a cells irradiated at different average power output levels

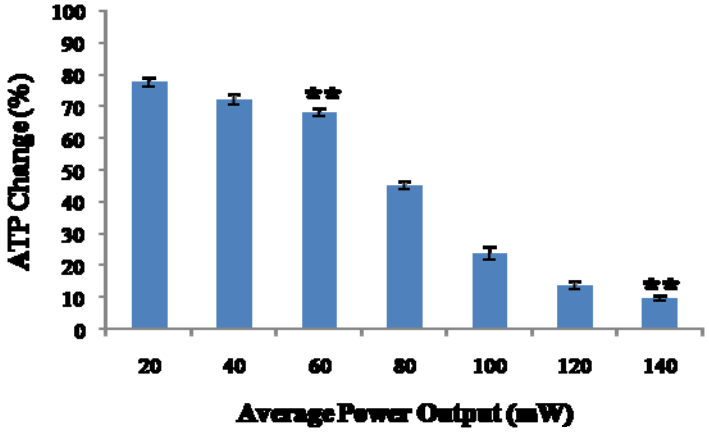

Figure 3: Irradiating mES cells with low power output levels (20 – 60 mW) had limited compromise to cell viability. However, exposure of ES-E14Tg2a cells to fs peak powers of roughly 17.5 kW had a ~7 fold decrease in cell viability. In negative controls which received no laser irradiation, an ATP change of 88 % was obtained (data not shown). Error bars represent the SEM (n = 5 experiments of 50 dosed cell colonies). Using ANOVA followed by Dunnett's and Fisher's tests: ** means data sets are significantly different from each other.

3.3.2 Cell proliferation changes when ES-E14TG2a cells were irradiated at different pulse energies

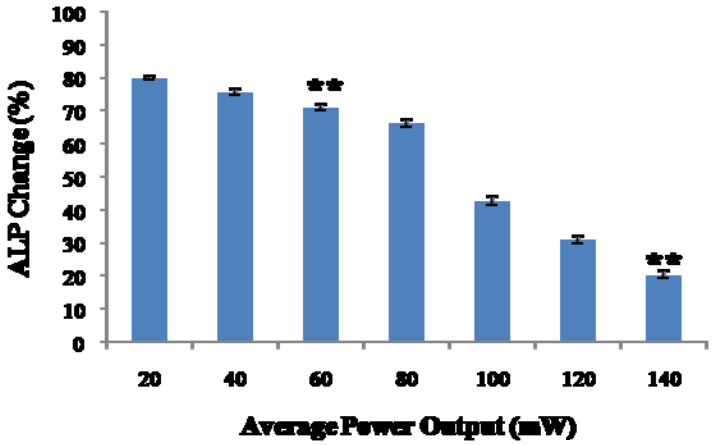

Figure 4: Upon optical treatment at low doses of 0.25 – 1 nJ pulse energies, ES-E14TG2a cells continued to proliferate producing ALP values closely comparable to that of the negative control (ALP change = 92 %, data not shown). Irradiating mES cells using the average power output of 140 mW decreased the amount of ALP produced by the cells and hence, their proliferation. Error bars represent the SEM (n = 5 experiments of 50 dosed cell colonies). Using ANOVA followed by Dunnett's and Fisher's tests: ** means data sets are significantly different from each other.

3.3.3 Change in LDH in ES-E14TG2a cells optically treated with various average power output levels

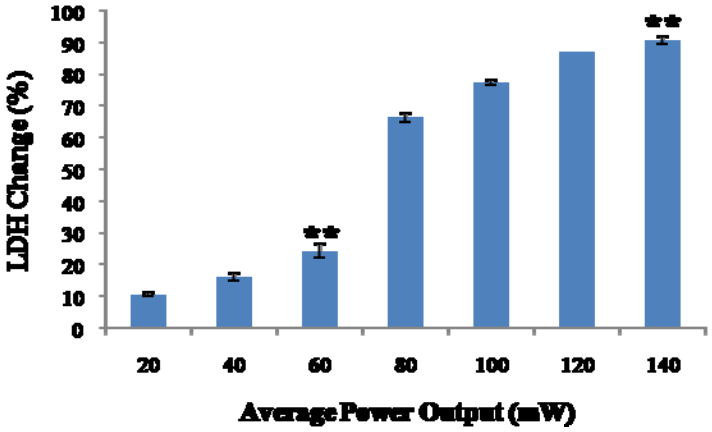

Figure 5: A dose of 140 mW during fs laser pulse treatment of mES cells caused cell cytotoxicity leading to a detectable LHD change of 90 % compared to 8 % change obtained in the negative control samples (data not shown) as well as, 30 % LDH change on treatment with the optimum dose of 60 mW. Error bars represent the SEM (n = 5 experiments of 50 dosed cell colonies). Using ANOVA followed by Dunnett's and Fisher's tests: ** means data sets are significantly different from each other.

4. DISCUSSION

Fs laser pulses allow a chemical-free delivery of both molecular and non-molecular particles of various sizes into mammalian cells. Key characteristics to this all optical methodology include its ability to keep cell viability intact, retain the quality of genetic materials being introduced, producing a minimum immunogenic response and its use under sterile tissue culture protocols. The ability to specifically tailor the optical transfection conditions and parameters during photo-transfection renders this technique versatile. As a result of their high peak powers, focused fs pulses, possess sufficient density of photons to initiate nonlinear processes such as multiphoton absorption of photons precisely at the location of the focus. Therefore employment of lenses of high NA during optical transfection studies is essential. Our results in Table 2 depicted that upon using a 60 X and a 100 X lens, transient transfection efficiency data ranged between 70 and 73 % compared to 12 % efficiency obtained using a 40 X lens. Although use of the 100 X oil immersion lens resulted in the highest transfection efficiency data, this outcome was not significantly different to that attained using a 60 X lens with a lower NA (0.8) compared to that of the 100 X (1.2). Nonetheless, since the geometry of our setup was upright and also considering our sample chambers, using a dry objective for optical transfection was uncomplicated compared to using an oil immersion lens which resulted in oil contaminations of our mES cell samples.

The possibility of using fs laser pulses for targeted transient transfection of ES-E14GT2a using pEGFP was explored for the first time. Thus, the problems of cytotoxicity and stem cell population heterogeneity resulting from using other cell transfection methodologies can be avoided. The experimental data presented in this paper shows how an all optical system as photo-transfection (naked pDNA introduction) can be used for genetic introduction into pluripotent cells. During our investigations 70 % transfection efficiency in pluripotent stem cells was successfully achieved. This result is very interesting as ES cell lines can assist in the understanding of pathological diseases including the origin of cancers, testing the efficacy of drugs and in monitoring the development of genetic disorders.

Because of the intensity-dependent nature of the multiphoton process, utilization of fs pulses for optical transfection offers a high degree of spatial confinement of the deposited energy, causing fine, highly localized disruptive effects at the target area, therefore preventing any collateral damage.

Our cell transformation data confirmed how irradiating mES cells at low power output typically conserved cell viability and proliferation. Conversely, fs laser treatment of mES cells at average power output levels as high as 140 mW (~ 17.5 kW, peak powers) led to an increase in cytotoxicity.

5. CONCLUSION

Notably Mthunzi *et al*. (2010) [4], reported on the capability to photo-transfect successfully mES (E14g2a cells) both in a targeted manner but also with the ability of transforming these cells into a new cell type. The controlled ability of ES cells and adult stem cells to differentiate (non-spontaneous differentiation) into specific cell types holds immense potential for therapeutic use in cell–based and gene therapy. The next step of our work therefore entails optical differentiation of both embryonic and mesenchymal stem cells. These investigations will promote the production of specialized cell types that could be essentially applied in tissue regeneration or engineering as cell-based therapies.

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