

# The Effect of Low Level Laser Irradiation on Human Embryonic Stem Cells

Hossein Baharvand, Ph.D.<sup>☆‡</sup>, Masoud Soleimani, Ph.D.<sup>☆</sup>, Hamid Gourabi, Ph.D.<sup>☆☆</sup>

☆ Stem Cells Department, Royan institute

☆ Genetics Department, Royan institute

‡ Corresponding Address: P.O.Box:19395-4644, Stem Cells Department, Royan Institute, Tehran, Iran

Email: Baharvand50@yahoo.com

## Abstract

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**Introduction:** Different effects of low level laser irradiation (LLLI) on various cell types have already been demonstrated. However, its effects on embryonic stem cells have not yet been shown. The present study evaluates the morphological and immunocytochemical effects of LLLI on human embryonic stem cell (hESC) colonies.

**Material and Methods:** Equal-sized pieces of hESC line (Royan H1) were irradiated with a single dose of 830-nm Ga-Al-As diode laser (3, 5, and 8  $\text{jcm}^{-2}$ , 30mW) and cultured on mouse embryonic fibroblasts. The morphology of the colonies was evaluated qualitatively by observation under an inverted microscope (grades A, B, C, and D exhibited 0-30%, 30-50%, 50-80%, and 80-100% differentiation, respectively). The stemness area was assessed by expression of surface antigens using anti-Tra-1-60 and anti-Tra-1-81.

**Results:** Our data demonstrated a dose-dependent stimulatory effect of LLLI on hESC differentiation. Two doses of 5 and 8  $\text{jcm}^{-2}$  induced statistically significant differentiation (grades C and D).

**Conclusions:** These data showed that LLLI influenced hESC differentiation, which might be used for cell therapy after transplantation.

**Keywords:** differentiation, human embryonic stem cells, low level laser irradiation

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## Introduction

Over the last 30 years, low energy laser therapy has been used worldwide to treat musculoskeletal injury, pain, and inflammation (1). Various biostimulatory effects have been reported for low energy laser irradiation, such as the enhancement of DNA synthesis (2), collagen (3) and procollagen production (4, 5), as well as an increase in the proliferation rate of such different cells as fibroblasts (6, 7), endothelial cells (8), epithelial cells (9), osteoblasts (6), melanocytes (10), Schwann cells (11), keratinocytes, and lymphocytes (12).

On the other hand, some other published data indicate that low power laser irradiation can inhibit the proliferation of lymphocytes (13), haemopoietic cell lines (HL60 and U937) (14), and endothelial cells (15). Although these controversies

are attributed to problems such as different laser types, total doses applied, and target cells, the exact mechanism(s) of action is not known for both sides.

To date, several studies have addressed the direct effects of low intensity laser irradiation on stem cells, including the stimulation of satellite cell proliferation and the inhibition of their differentiation (16), and *in vitro* expansion of cord blood stem cells (17). Nevertheless, the effects of low power laser on embryonic stem cells have not yet been determined. Embryonic stem (ES) cells are most frequently derived from the inner cell mass (ICM) of blastocysts (18, 19). In fact, the ICM is used to give rise to an ES cell line that remains undifferentiated and is pluripotent, i.e. the cells having the potential to develop into any cell type from all three germ layers both *in vivo* and *in vitro*. In

this regard, an important development has been the recent establishment of human embryonic stem cells (hESCs) (20). The ability to direct the differentiation of hESC lines into a population of specific cell types, such as insulin-producing pancreatic cells, may provide a treatment measure for many diseases such as diabetes mellitus. Different treatments with physical and chemical agents which may enhance or inhibit the proliferation and differentiation of stem cells have their own impact in future researches in this field.

This study sought to illustrate the effects of low level laser irradiation on the RoyanH1 line of hESCs (21).

## Material and Methods

### *Culture of human embryonic stem cells*

hESC line (Royan H1) (21) was grown on a mitomycin-C (M0503; Sigma, Germany) treated, mitotically inactivated mouse embryonic fibroblast (MEF) feeder layer (isolated from 13.5 day post-coital fetuses of NMRI out-bred mice used at 75,000 cell  $\text{cm}^{-2}$ ) in gelatin (G2500; Sigma)-coated tissue culture dish (3037; Falcon, USA). The hESCs were maintained in hESC medium consisted of 80% Knockout Dulbecco's modified Eagle's medium (10829-018; Gibco, UK) supplemented with 20% ES-qualified fetal calf serum (16141-079; Gibco), 2mM glutamine, (15039-027; Gibco), 0.1 mM  $\beta$ -mercaptoethanol (M7522; Sigma), 1% nonessential amino acid stock (11140-035, Gibco), insulin-transferrin-selenite (41400-045; Gibco), 100  $\text{Uml}^{-1}$  penicillin, and 100  $\mu\text{gml}^{-1}$  streptomycin (15070-063; Gibco). Colonies were further propagated in clumps of ~200-500 stem cell-like cells on MEF about every seven days.

### *Laser treatment of embryonic stem cells*

Seven days after having been plated, colonies (equal sized clumps of stem cells) were dissociated with a combined approach of mechanical slicing with a pipette, followed by exposure to 10  $\text{mgml}^{-1}$  dispase (17105-041; Gibco). The resulting slices were distributed randomly and irradiated with a low power Ga-As-Al laser apparatus (Endolaser 476;ENRAF

NONIUS, Asah Medico, Denmark) with a continuous wavelength of 830nm and power output of 30mW. Irradiation times were 100, 167, and 267 seconds, corresponding to energy densities of 3, 5, and 8  $\text{jcm}^{-2}$ , respectively. The cells were irradiated at a distance of 10 mm. Having been irradiated, the slices were cultured on new MEF in 5%  $\text{CO}_2$  and 95% humidity.

### *Grading criteria of human embryonic stem cells*

Colonies were evaluated on 7<sup>th</sup> day, when they were expected to be 1.5-2 mm in diameter. The colonies were assessed using 10X magnification of an inverted microscope (Olympus, CKX41, Japan). The quality of colonies was graded according to "ES Cell International" ([www.escellinternational.com](http://www.escellinternational.com)). The criteria for grading the colonies were as follows: (a) Grade A/ excellent: colonies with even morphology and well defined edges (Fig. 1A). The cells in these colonies were dense and hardly distinguished individually. They were thick, multi-layered and homogenous colonies and exhibited 0-30% differentiation. The differentiated cells were migrating and passing from the periphery of the colonies. (b) Grade B/ good: colonies with 30-50% differentiation in their peripheral areas (Fig. 1B). (c) Grade C/ fair: colonies exhibiting 50% to 80% differentiation (Fig. 1C). And finally, (d) Grade D/ poor: colonies of 80% to 100% differentiation and exhibiting well differentiated morphology at heterogeneous levels (Fig. 1D).

### *Cell surface markers*

The cells were rinsed twice with PBS, fixed with methanol: acetone (3:1) at -20°C, incubated with respective primary antibody [anti-tumor rejection antigens (1:20, TRA-1-60, 1:20, TRA-1-81; gifts from Peter Andrews, University of Sheffield, UK)], for 60 min at 37°C in a humid chamber. At the end of the incubation period, they were rinsed three times with PBS and incubated with the fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgG (1:100, Sigma, F9006) for 60 minutes at 37°C. After rinsing with PBS, the cells were analyzed

under fluorescent microscope (Nikon, Japan).

#### Statistical analysis

All experiments were repeated five times. Statistical evaluation was performed by means of Chi-square and Fisher's exact tests, and statistical significance was defined as  $p < 0.05$ .

#### Results

Table 1 and figure 3 show the effects of three different doses of low level laser irradiation on Royan H1 hESC line and different characteristics of hESCs.

The proportion of undifferentiated colonies (grade A+B) in those groups exposed to low level laser decreased compared to that in the control group ( $p < 0.05$  for  $5 \text{ j cm}^{-2}$  and  $8 \text{ j cm}^{-2}$  and  $p = 0.055$  for  $3 \text{ j cm}^{-2}$ ).

Conversely, the proportion of differentiated colonies (grade C+D) in these groups increased ( $p < 0.05$  for  $5 \text{ j cm}^{-2}$  and  $8 \text{ j cm}^{-2}$  and  $p = 0.055$  for  $3 \text{ j cm}^{-2}$ ). All putative hESC areas expressed cell surface markers that characterized undifferentiated nonhuman primate and hESC, including Tra-1-60, and Tra-1-81 (Fig. 2 and 3).

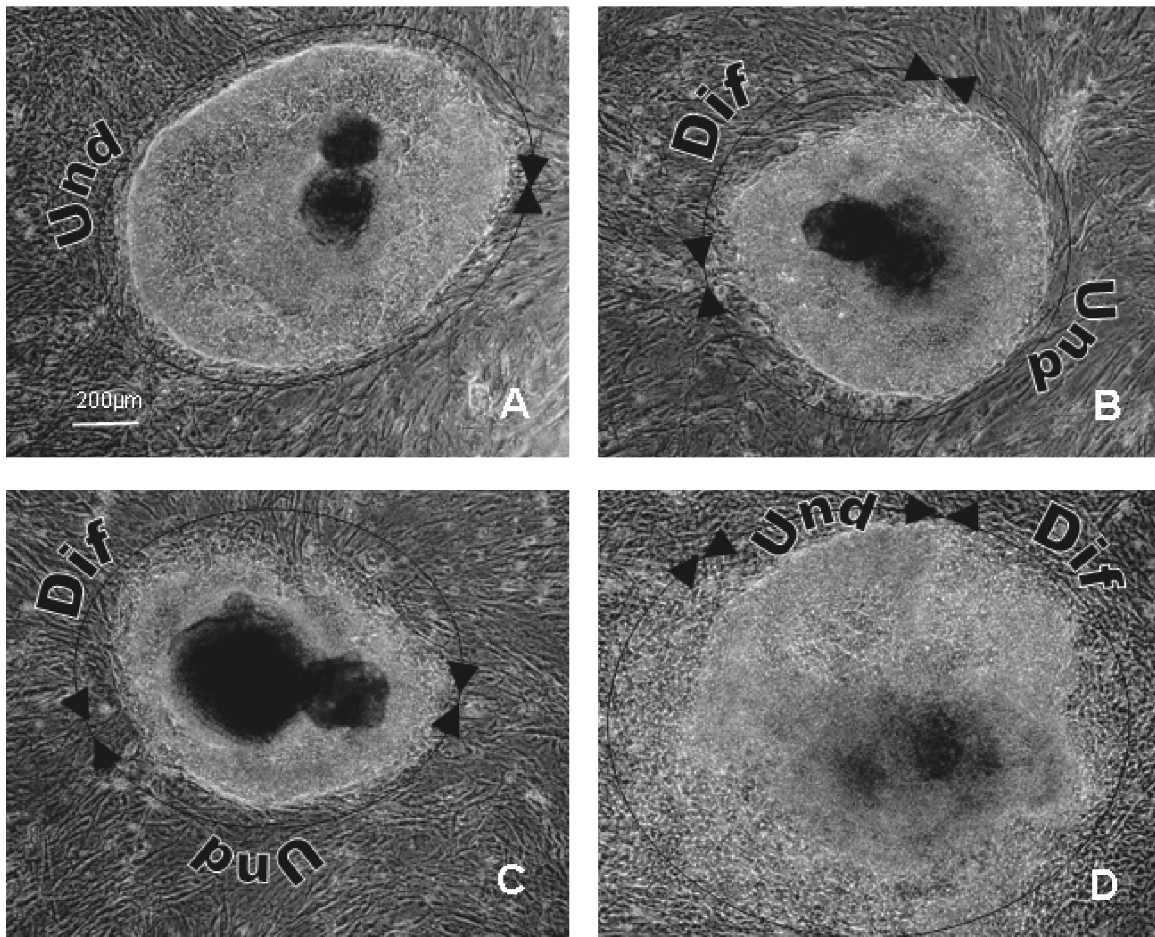


Fig. 1. Different grades of human embryonic stem cell colonies with phase contrast microscopy. (A), grade A; (B), grade B; (C), grade C; (D), grade D. Dif, differentiated area; Und, undifferentiated area.

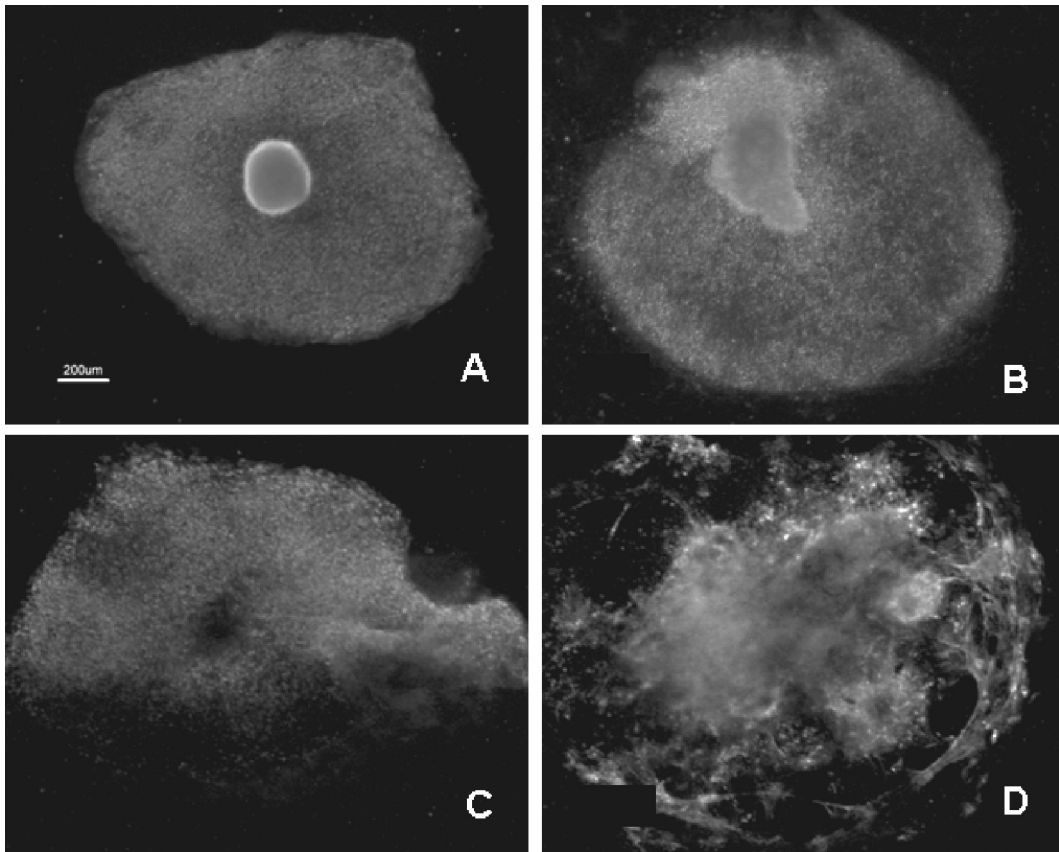


Fig. 2. Expression of Tra-1-60 as a surface antigen marker on hESCs. (A), grade A; (B), grade B; (C), grade C; (D), grade D.

(This figure has also been printed in full-color at the end of the issue.)

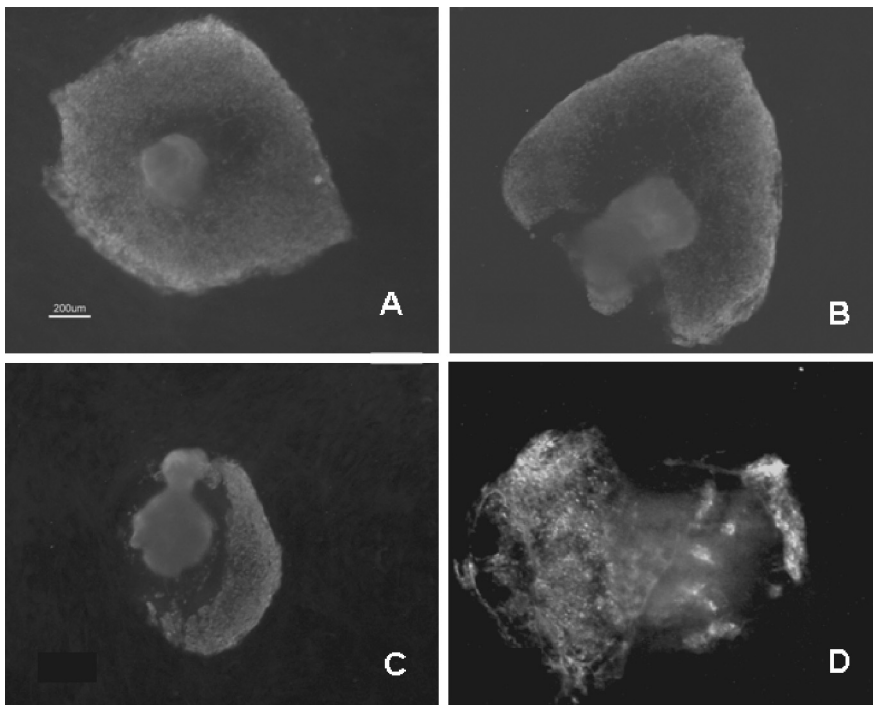


Fig. 3. Expression of Tra-1-81 as a surface antigen marker on hESCs. (A), grade A; (B), grade B; (C), grade C; (D), grade D.

(This figure has also been printed in full-color at the end of the issue.)

## Discussion

ES cells are routinely expanded to give rise to relatively homogenous, undifferentiated populations as judged by the morphology and expression of a range of markers. ES cells undergo symmetrical self-renewal, and their expansion can be continued indefinitely. Nonetheless, the maintenance of the undifferentiated human embryonic stem cell phenotype is not cell-autonomous, and co-culture with a feeder layer is essential. Our findings suggest that low-energy laser irradiation (5 and 8 jcm<sup>-2</sup>) significantly stimulates human embryonic stem cell differentiation even if they have been co-cultured with a feeder layer. This is more or less different from reports by other authors, who conducted their experiments with other cell lines and different laser types and levels. Some similarities, however, do exist. Soleimani et al. (22) have shown increased differentiation of human mesenchymal stem cells into osteoblastic cells after low power laser irradiation. Rood et al. (12) have reported no keratinocyte differentiation by this way. On the other hand, Ben-Dov et al. (16) have shown that He-Ne low energy laser irradiation, in addition to its positive effect on the activation and proliferation of an established line of mouse satellite cells, significantly inhibits muscle cell differentiation. They suggested that low energy laser irradiation induced early cell-cycle proteins, such as cyclin D1, upregulated in the G1 phase, and these factors in turn activated quiescent satellite cells and propelled them more in cell cycle. They concluded that laser irradiation could affect satellite cell proliferation and prolong the cell cycle, prompting a delay in cell differentiation. Ozwaet et al. (6) have reported two stimulating effects of low energy laser irradiation: one on the cellular proliferation, especially the proliferation of nodule forming cells of osteoblast lineage, and the

second on cellular differentiation, in particular to committed precursors, resulting in an increase in both the number of more differentiated osteoblastic cells and bone formation.

In addition to expressing the transcription factors oct-4, Stat3, and Nanog (23, 24), the cell cycle of the ES cells also seems to play a role in preventing differentiation. ES cells, like the epiblastic cells in the preimplantation embryos, have an unusual cell cycle; the G1 checkpoint, specifically,

does not appear to operate in these cells (25, 26). This may explain why it has not been possible to induce a quiescent withdrawal from the cell cycle to a G1 or G0 state in undifferentiated ES cells (27). However, if ES cells begin to differentiate by forming embryoid bodies, cyclin D expression increases, the G1 phase of the cell cycle becomes longer, and the rate of cell division slows (25). This can occur if the feeder layer and LIF are withdrawn from mouse ES cell cultures, or the former from human ES cell cultures. Constant cell proliferation may somehow inhibit cell differentiation, and once the signals for cell division are removed, differentiation can occur (28).

Moreover, laser irradiation could affect the cell

cycle and lengthen it, ultimately converting the quality of human embryonic stem cells from an undifferentiated state to a differentiating one.

## Conclusion

Our data demonstrate that low power laser can interact with human embryonic stem cells to stimulate colony differentiation.

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