

Enhanced Green Fluorescent Protein (EGFP) for Space Radiation Research using Mammalian Cells in the International Space Station

C. Baumstark-Khan, C.E. Hellweg, M. Palm, G. Horneck

DLR, Institute of Aerospace Medicine, Radiobiology Unit, D-51147 Köln (Germany)

Abstract

In the endeavour to assess radiation risks for humans in space the concerted action of all stimuli (e.g. radiation and microgravity) has to be known already at a cellular level. The introduction of reporter genes into mammalian cells which allows the visualisation of modified gene expression levels, signal transduction rates and cell metabolism activities will supply basic information on the cellular response to space radiation. The cloning of the gene for green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its subsequent expression in heterologous systems has established GFP as a unique genetic reporter system for use in a variety of organisms. Unlike other reporters, GFP fluorescence emerges in the absence of substrates or cofactors and allows for non-invasive monitoring in living and in paraformaldehyde-fixed cells. Enhancement of wild-type GFP by human codon optimisation and fluorophore mutation (EGFP) resulted in higher expression levels in mammalian cells and brighter fluorescence. The suitability of EGFP for gene expression studies to be performed on the ISS is shown for recombinant mammalian cells in response to UVC exposure.

KEYWORDS: Enhanced green fluorescent protein, mammalian cells, gene expression, space radiation.

1. Introduction

In space, man encounters with many strange and potentially harmful environmental factors. The impact of space radiation under microgravity conditions has to be understood for establishing guidelines to ensure health and fitness for work in the space habitat. Humans and whole animals' responses to microgravity have been studied in depth. There is increasing evidence that already basic cellular functions are sensitive to microgravity. For mammalian cells many microgravity dependent alterations have been reported, such as depression of cell proliferation [1], modifications in the cytoskeleton organisation of cultured cells [2] and in intracellular distribution of protein kinase C [3]. In addition to microgravity, cosmic radiation affects cells in space as revealed by cell inactivation, mutation induction and chromosomal aberrations in a variety of assay systems [4-6]. Experiments in space with irradiated bacteria and human fibroblasts suggest that a disturbance of cellular repair processes in the microgravity environment might not be the explanation for the so far reported synergism of radiation and microgravity [7]. Nevertheless, human beings exposed for extended time periods to space conditions display a amplified chance of displaying chromosomal aberrations [8]. Thus, extended space missions bear the risk of cancer induction for the astronauts.

It is hypothesised, that alterations in the expression of cell cycle regulatory proteins may represent important early events in the process of oncogenic transformation in vitro. Basic types of DNA damages lead to altered gene expression patterns of mammalian cells. Ionising and non-ionising radiation is known to induce certain cellular genes [9-11] and to decrease the expression of others [12]. Spe-

cific types of abnormal expression patterns are characteristic of particular genes. For example, members of the *myc* gene family are frequently overexpressed or amplified [13]. The introduction of heterologous genes into cultured mammalian cells represents an important means of investigating gene expression. In studies of this kind, the promoter or enhancer element of interest is inserted into a vector, so that the expression of a suitable reporter is a consequence of cellular response to radiation. Expression of the heterologous protein GFP, which was originally isolated from the bioluminescent jellyfish *Aequorea victoria*, represents a unique method for fluorescent labelling of viable cells [14]. The 27-kD monomer of GFP, which consists of 238 amino acids, retains its fluorescence proficiency under many severe conditions. Unlike other reporter system which require incubation of the cells with specific substrates or cofactors to produce a signal, GFP fluorescence only requires exposure to UV or blue light for visualisation. Wild type GFP was enhanced (EGFP) by modifications concerning the excitation peaks, the brightness and the expression level in mammalian cells by human codon optimisation [15-17].

The experiment Cellular Responses to Radiation in Space (CERASP), to be performed on the International Space Station (ISS) will supply basic information on the cellular response to radiation applied in microgravity. One of the biological end-points under investigation will be gene activation by space flight conditions in mammalian cells, based on fluorescent promoter reporter systems using green fluorescent protein. That is why the suitability of EGFP for gene expression studies is investigated using recombinant mammalian cells responding to DNA-damaging conditions.

2. Material and methods

Origin and purification of the plasmids used for the current study was already described in detail [18]. Only DNA preparations with an A_{260}/A_{280} ratio greater than 1.8 were used in transfections of Chinese Hamster Ovary (CHO) AA8 cells. The EGFP variants were transfected by liposome mediated DNA transfer in the presence of 5 % serum [18]. Stable transfectants were generated using the aminoglycoside antibiotic G418 for selection. The stably transfected clones (AA8-pCX-EGFP, AA8-pEGFP-N1 and AA8-pCMV-d2EGFP) were routinely cultured in medium containing 1.5 mg per ml of G418. UVC-irradiation was performed using a germicidal lamp (NN8/15, Heraeus). Cellular UV-sensitivity of cultured CHO cells was tested using the colony forming ability test [19]. EGFP expression was visualised using an inverted fluorescence microscope (Axiovert 135, Carl Zeiss, Germany), equipped with a filter set suitable for EGFP detection (Zeiss filter set 9, excitation BP 450-490 nm, emission LP 520 nm). Fluorescent excitation and emission spectra of EGFP-expressing cells were recorded using a F-2000 fluorescence spectrophotometer (Hitachi, Japan). Fluorescence intensities of EGFP expressing cells were read in the microplate reader Lambda Fluoro 320 (MWG Biotech, Germany) equipped with the high quality interference filters set (460/40 × 508/20) adjusted for measuring EGFP. Distribution patterns of EGFP expressing cells were analysed using a fluorescence activated cell scanner (FACScan, Becton Dickinson, San Jose, CA, USA) equipped with an argon laser (488 nm) as excitation source.

3. Results

For measurement of radiation effects on mammalian cells, the EGFP gene controlled by the CMV promoter, were stably transfected into Chinese Hamster Ovary (CHO) cells. The cells which stably integrated the foreign DNA into their genome survived the selection by the aminoglycoside antibiotic G418. EGFP expression, post-translational modification and folding works well in CHO cells, as has been already demonstrated for a number of cell lines and different applications. In a population of stably transfected AA8 cells after cultivation in G418 containing medium nearly all cells express EGFP (Fig. 1). After fixation with paraformaldehyde the EGFP-fluorescence yield is comparable to living cells.

An important question to be answered was whether EGFP expression modifies the cellular response to radiation. Possible reactions of EGFP to intense radiation could either be photoinstability leading to increased cellular radiosensitivity or the action of EGFP as a radical scavenger resulting in enhanced cellular survival after irradiation. Survival curves (Fig. 2) of the parental strain AA8 and the derived EGFP expressing strain AA8-pCX-EGFP obtained

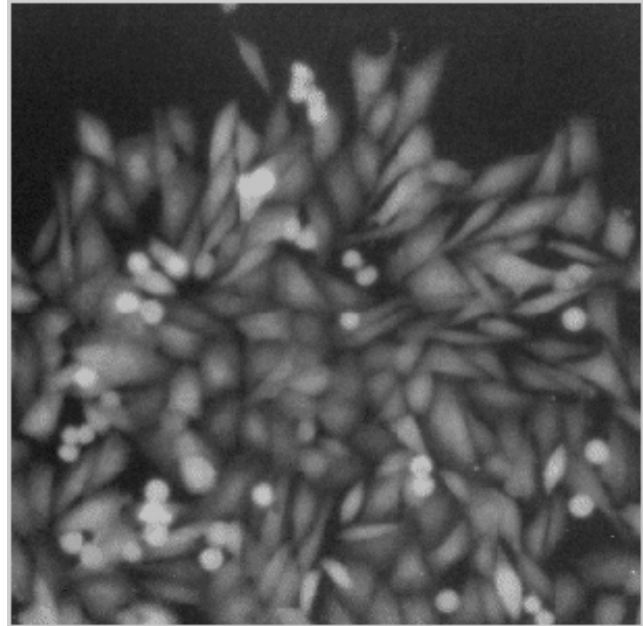


Fig. 1 – A single colony of AA8 cells stably transfected with pCX-EGFP. Life photographs were taken with an Olympus camera attached to an inverted fluorescence microscope (Zeiss Axiovert 135, magnification: 400-times) using a filter set suitable for fluorescein detection (Filter set 9, excitation filter bandpass 450-490, dichroic FT 510 and emission longpass filter 520).

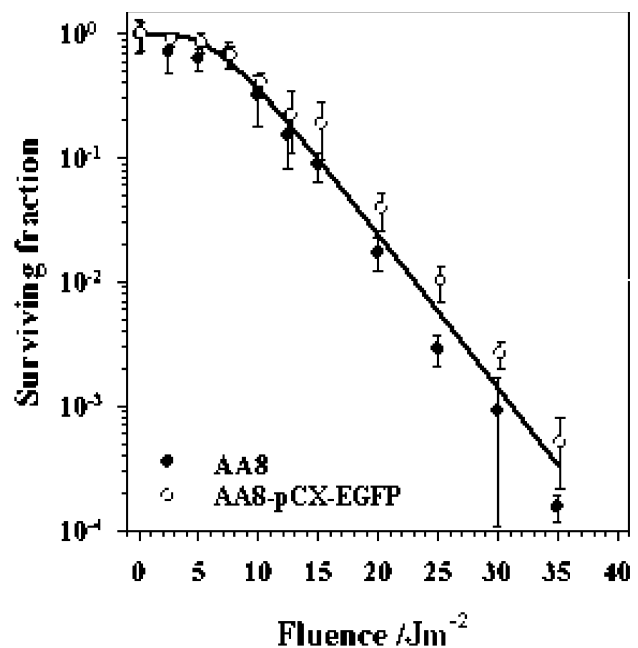


Fig. 2 – Survival curves of UVC-irradiated (254 nm) Chinese hamster ovary cells (AA8) stably transfected with the EGFP-expression vector pCX-EGFP (AA8-pCX-EGFP) compared to non-transfected AA8 cells. The dose-effect relationships were described by the formula $S = 1 - (1 - e^{-D/D_0})^n$ with D_0 -values of 3.5 ± 0.1 Gy and 3.8 ± 0.1 Gy and n -values of 7.3 ± 1.2 and 6.0 ± 0.7 for AA8 and AA8-pCX-EGFP, respectively.

after UVC-exposure show no major differences. Both cell strains display curvilinear survival curves with D_0 -values of 3.5 ± 0.1 Gy and 3.8 ± 0.1 Gy,

respectively. Surviving colonies of the stably transfected clone AA8-pCX-EGFP were additionally inspected for EGFP-expression by fluorescence microscopic surveillance. In any case, surviving cells produced green colonies (results not shown) and sectoring within single colonies could not be observed.

In order to evaluate the suitability of EGFP for gene expression studies using a microplate reader, the fluorescence output of several EGFP variants stably transfected into mammalian cells was previously measured and optimised measurement conditions were described [18]. Using these optimal conditions a linear dependence of fluorescence output from cell numbers could be shown for different EGFP-expressing cell clones. In order to detect possible growth differences between the parent cell line and the stably transfected cell line and to show cell growth by fluorescence determination, growth curves were established. For the stably transfected cells (AA8-pEGFP-N1) and the parent cell line AA8 growth was determined by counting cells (Fig. 3A) and measurement of fluorescence intensities in the microplate reader (Fig. 3B) dependent on incubation time. The growth curve determined by cell counts shows the typical three phases of cell growth, (i) the lag phase, (ii) the phase of exponential growth and (iii) the stationary phase when cell death compensates cell growth (Fig. 3A). Increase of fluorescence is delayed compared to the increase of cell numbers during the first two days of growth (Fig. 3B). This might be due to the fact that the fluorescence intensity is determined by the EGFP content per cell volume and the cell number per well, while the cell count is independent of cellular volume and protein, in this case especially EGFP, content. When the cell count reaches the stationary phase at 120 h, fluorescence still increases for 30 h (Fig. 3B), which means that the EGFP content per cell still increases due to ongoing protein synthesis. Taking this into consideration, the EGFP fluorescence allows a rapid estimation of cell growth without cell counting.

UVC irradiation was evaluated as one kind of cytotoxic agent. The question was whether the expected cytotoxic effect of UVC, decreased growth, can be estimated by slower fluorescence increase after irradiation. In this experimental set-up, EGFP-expressing cells are seeded in low density per well. After UVC-irradiation in a microplate, AA8-pEGFP-N1 cells show a prolonged lag phase and lower maximal fluorescence (Fig. 4A), both effects being dependent on dose. Figure 4B shows the dose-effect relationships for growth inhibition for two analytic times from Figure 4A. For 72 hours after irradiation, the dose-effect curve is steeper than for 192 hours. This is relevant to the fact, that the analysis is performed when the non-irradiated cells are in exponential phase while the irradiated cells did not fully recover from the irradiation-induced growth delay. For the later analysis time, this growth delay is no longer evident, and the dose-response relationship describes the relative growth capacity of the cells.

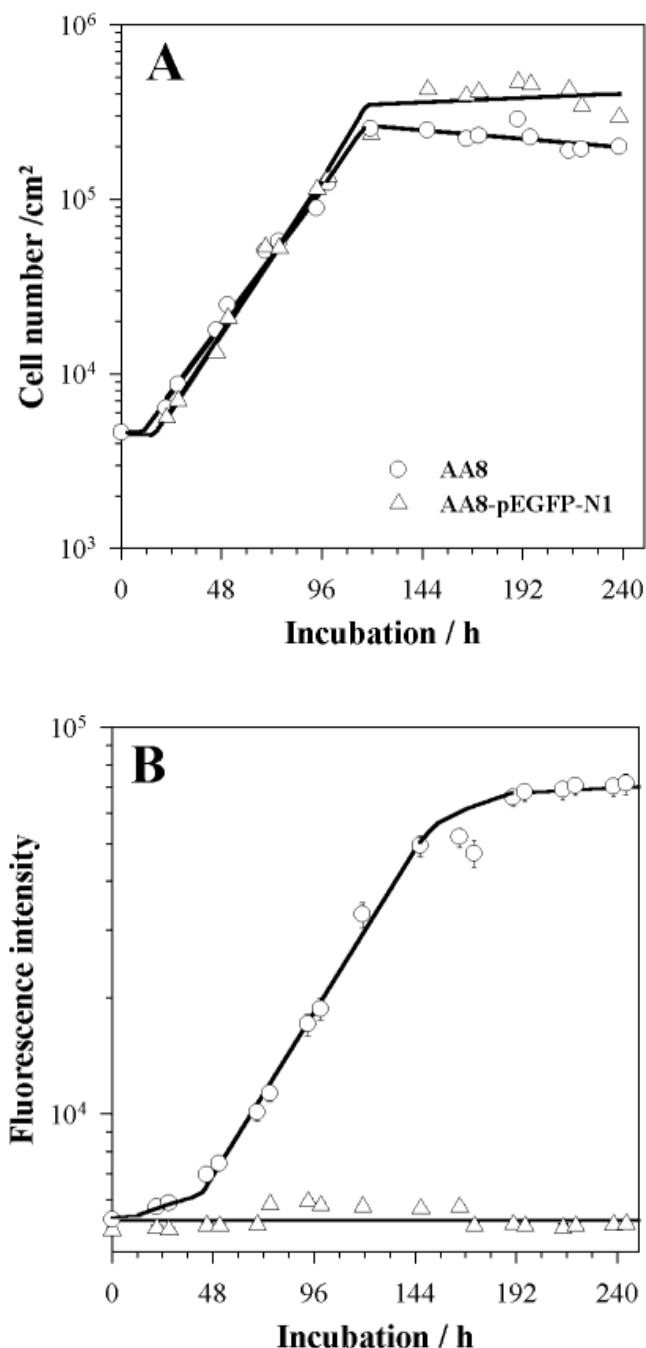


Fig. 3 – Growth curves of EGFP-expressing AA8 cells (AA8-pEGFP-N1) in comparison to non-transfected AA8. The cells were seeded into petri dishes and cells were counted at regular time intervals as described in Material and methods (A). In parallel, cells were seeded into a microplate and fluorescence was determined in the microplate reader twice a day. Bars show standard deviation of 48 wells (B).

4. Discussion

Green fluorescent protein from the jellyfish *Aequorea victoria* is a valuable tool for studying gene expression in mammalian cells. Green fluorescent protein (GFP) can be expressed in heterologous systems in the absence of substrates and for this reason, it can be used as reporter protein for gene expression in a variety of organisms [14, 18-21].

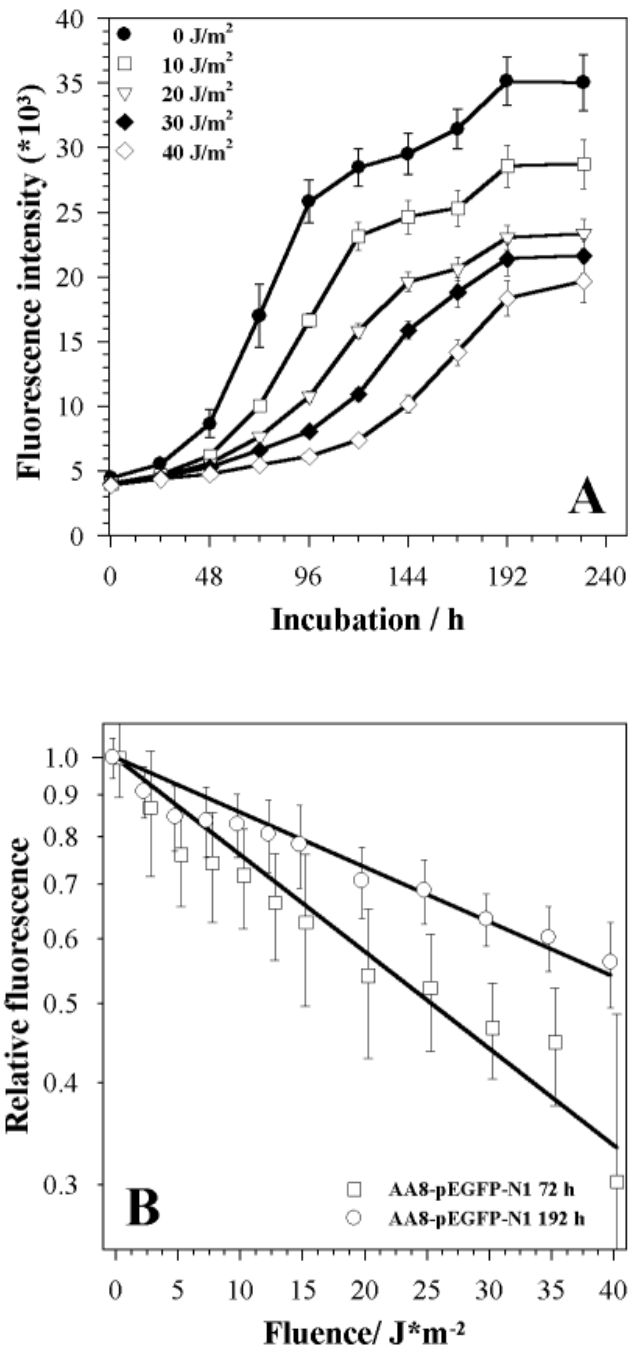


Fig. 4 – Cell growth of AA8-pEGFP-N1 after irradiation with several UVC doses, measured in a microplate reader (filter 460/508). The cells were seeded, irradiated 24 h later and fluorescence was measured every 24 hours. Representative example of one of three experiments, bars show standard deviation of 8 wells (A). For the 72 h and 192 h fluorescence data, growth inhibition was calculated according to the formula $GI = (F_{D>0} - F_B) / (F_{D=0} - F_B)$, where $F_{D=0}$ and $F_{D>0}$ are the fluorescence intensities of mock irradiated cells and of UVC irradiated cells, respectively. F_B is the fluorescence intensity of the empty microplate measured before cell inoculation. Data of three experiments were combined and linear regression analysis was performed, bars show standard deviation of 24 wells (B).

The wild-type GFP has been optimised for expression in mammalian cells. This Enhanced Green Fluorescent Protein (EGFP) carries a mutation in its chromophore which shifts the excitation peak to 488

nm and enhances its fluorescence intensity [22]. EGFP fluorescence has been reported to be stable, thus allowing its non-invasive monitoring in living cells and in paraformaldehyde-fixed cells [19]. It could also be shown that radiation sensitivity of transfected cells is not modified by high EGFP contents. Irradiation with moderate and lethal doses did neither impair the EGFP molecule itself, nor did the EGFP molecule impair cellular radiation responses.

The use of EGFP in space radiation research, as it is planned for the experiment CERASP, depends on quick and reliable data acquisition. The quantification of EGFP fluorescence in cells by different means was described previously [18]. The aim of the present study was to quantify cellular growth by fluorescence measurement of EGFP expressing cells, and to determine the amount of growth inhibition after treatment of cells with UV-light. Constitutive expressed EGFP accumulates at a high level in the cells. In such a case, EGFP fluorescence yields can be used for measuring cellular viability and cytotoxic effects using a microplate reader. EGFP content is a parameter comparable to cellular protein content due to its expression characteristics, but it offers the advantage that once stably expressed in cells, it can be detected by simple fluorescence measurement. A fast estimation of cytotoxic effects can be done by measuring the fluorescence of a cell layer in a microplate reader repeatedly for a certain time. This offers the possibility to monitor rapidly the effects of cytotoxic agents in a mammalian cell system.

Furthermore, FACS analysis results in more accurate descriptions of radiation modified EGFP expression patterns than a simple determination of fluorescence yields obtained by fluorescence readings using a microplate reader. Thus, after UVC-exposure of exponentially growing cells the FACS analysis showed the appearance of two sub-populations of different responding cells with time [19]. In one certain sub-population a total loss of EGFP was observed with increasing UVC-doses, probably due to a leak-out of the soluble EGFP molecules through impaired cellular membranes as it is the case in apoptotic cells. A second sub-population was found to enrich EGFP in a dose-dependent way. These irradiated cells probably continue protein synthesis (as well as synthesis of EGFP) while cell division breaks down leading to accumulation of cell mass. In experiments inhibiting DNA synthesis by hydroxyurea, an inhibitor of ribonucleotide reductase, a similar effect was achieved [23].

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REFERENCES

- [1] Cogoli-Greuter M, Meloni MA, Sciola L, Spano A, Pippia P, Monaco G, Cogoli A. Movements and interactions of leukocytes in microgravity. *J Biotechnol* 1996; 47: 279-287.
- [2] Hughes-Fulford M, Nelson K, Blaug S, Summer CG, Lukefahr BD, Lewis ML. MC3T3-E1 osteoblasts grown in microgravity on STS-56 have reduced cell growth, glucose utilization with altered actin cytoskeleton and increased prostoglandin synthesis. *ASGSB Bull* 1993; 7: 31
- [3] Schmitt DA, Hatton JP, Emond C, Chaput D, Paris H, Levade T, Cazenave J-P, Schaffar L. The distribution of protein kinase C in huamn leukocytes is altered in mcirogravity. *FASEB* 1996; 10: 1627-1634.
- [4] Kiefer J, Schenk-Meuser K, Kost M. Radiation biology. In: D Moore, P Bie and H Oser Eds. *Biology and Medical Research in Space*. Berlin. Springer 1996: 300-367.
- [5] Horneck G. Impact of spaceflight environment on radiation response. In: *Terrestrial Space Radiation and Its Biological Effects*. PC McCormack, CE Swenberg and H Bückler Eds. New York. Plenum Press 1988: pp.707-714.
- [6] Bender MA, Gooch PC, Kondo S. The Gemini 3 S4 spaceflight radiation interaction experiment. *Radiat Res* 1967: 31; 91-111.
- [7] Horneck G, Rettberg P, Kozubek S, Baumstark-Khan C, Rink H, Schäfer M, Schmitz C. The influence of microgravity on repair of radiation-induced DNA damage in bacteria and human fibroblasts. *Radiat Res* 1997: 147; 376-384.
- [8] Obe G, Johannes I, Johannes C, Hallmann K, Reitz G, Facius R. Chromosomal aberrations in blood lymphocytes of astronauts after long-term space flights. *Int J Radiat Biol* 1997; 6; 727-734.
- [9] Filatov D, Bjorklund S, Johansson E, Thelander L. Induction of the mouse ribonucleotide reductase R1 and R2 genes in response to DNA damage by UV light. *J Biol Chem* 1996: 271; 23698-23704.
- [10] Narayan S, He F, Wilson SH. Activation of the human DNA polymerase beta promoter by a DNA-alkylating agent through induced phosphorylation of cAMP response element-binding protein-1. *J Biol Chem* 1996: 271; 18508-18513.
- [11] Trautinger F, Kindas-Mugge I, Knobler RM and Honigsmann H. Stress proteins in the cellular response to ultraviolet radiation. *J Photochem Photobiol B* 1996: 35; 141-148.
- [12] Assefa Z, Garmyn M, Bouillon R, Merlevede W, Vandenneede RJ, Agostinis P. Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. *J Invest Dermatol* 1997: 108; 886-891.
- [13] Amundson SA, Zhan Q, Penn LZ, Fornace AJ Jr. Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents. *Oncogene* 1998: 17; 2149-2154.
- [14] Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 1994: 263; 802-805.
- [15] Yang TT, Cheng L, Kain SR. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucl Acids Res* 1996: 24; 4592-4593.
- [16] Zhang G, Gurtu V, Kain SR. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem Biophys Res Commun* 1996: 227; 707-711.
- [17] Tsien R. The green fluorescent protein. *Ann Rev Biochem* 1998: 67; 509-544.
- [18] Hellweg CE, Baumstark-Khan C, Rettberg P, Horneck G. The Suitability of Enhanced Green Fluorescent Protein (EGFP) as a Reporter Component for Bioassays. *Anal Chimica Acta* 2001: 246; 175-184.
- [19] Baumstark-Khan C, Palm M, Wehner J, Okabe M, Ikawa M, Horneck G. Green Fluorescent Protein (GFP) as a Marker for Cell Viability After UV-Irradiation. *J Fluoresc* 1999; 9; 37-43.
- [20] Kain SR, Adams M, Kondepudi A, Yang TT, Ward WW, Kitts P. Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* 1995: 19; 650-655.
- [21] Pines J. GFP in mammalian cells. *Trends Genet* 1995: 11; 326-327.
- [22] Zhang G, Gurtu V, Kain SR. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem Biophys Res Commun* 1996: 227; 707-711.