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Using a Simple, Non Expensive *In Vitro* Model for Studying Chronic Myeloid Leukemia in Research Laboratories

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Abstract

Background: Chronic myeloid leukemia (CML) is the commonest neoplasm associated with a recurrent chromosomal aberration (fused bcr-abl gene on the Philadelphia chromosome), The current used methods for generating bcr-abl gene are very expensive and not feasible in labs with limited facilities. So this study aimed at using a simple, safe and non expensive method for generating bcr-abl gene to be used in CML researches.

Methods: Bcr-abl expression was evaluated in two groups; umbilical cord blood CD34$^+$HSCs (UCBCD34$^+$HSCs) nucleofected with the P210pcDNA3 plasmid (n=5) and UCBCD34$^+$HSCs nucleofected with the GFP-encoding plasmid (pMAX-GFP) (n=5). Then, neomycin (G418) antibiotic selection was done. Stable bcr-abl gene expression in G418 resistant P210pcDNA3 UCBCD34$^+$HSCs clones, was evaluated through quantitative PCR analysis of the expression of interleukin one receptor accessory protein (IL1RAP) gene. IL1RAP gene is newly discovered to be expressed on the surface of bcr-abl positive cells.

Results: G418 resistant P210pcDNA3 clones showed up to eleven-fold increase in IL1RAP gene expression compared to pMAX-GFP clones. Our results demonstrated how nucleofection of conventional plasmids is likely to induce gene expression in HSCs.

Conclusion: This simple method of generating bcr-abl gene expressing CD34$^+$ HSCs, shall open new revenues for chronic myeloid leukemia research especially in laboratories, not equipped for advanced and expensive cancer research. Furthermore IL1RAP over expression on the surface of bcr-abl +CD34$^+$HSCs, could be used as indicator for successful stable bcr-abl expression.

Keywords: Chronic myeloid leukemia; Nucleofection; IL1RAP Gene; P210pcDNA3

Abbreviations: HSCs: Hematopoietic Stem Cells; UCB CD34$^+$ HSCs: Umbilical Cord Blood Hematopoietic Stem Cells Positive for Cluster of Designation 34; CMV: Cytomegalovirus; Bcr-abl: Break Point Cluster Reigon-Abelson murine leukemia viral oncogene homolog 1; (P210): Protein210; CML: Chronic Myeloid Leukemia; IL1RAP: Interleukin One Receptor Accessory Protein; GFP: Green Fluorescence Protein; G418: Geneticin 418 Aminoglycoside Antibiotic; cDNA: complementary DNA; CMV: Cytomegalovirus; NeoR: Neomycin Resistance gene

Introduction

The biological properties of hematopoietic stem cells (HSCs) and their accessibility for ex vivo manipulation, render them one of the most promising targets of non viral gene modification approaches [1]. They represent the stem cell population where hallmark genetic aberrations of hematological disorders originate; the commonest of which is chronic myeloid leukemia (CML). Chronic myeloid leukaemia (CML) is a myeloproliferative disorder of pluripotent hematopoietic stem cells (CD34$^+$), affecting one or all cell lines (erythroid, platelet and myeloid). CML is characterized by a recurrent chromosomal aberration; the Philadelphia chromosome which carries the fused bcr-abl gene which has multiple isoforms with P210 being the commonest [2].

In the last few years, the advent of CML research has opened a new era in studying the disease pathogenesis and treatment modalities. However, resource limitations in many centers may hinder the establishment of valid study methods. Such centers' challenge is to tailor simple, non expensive, and accurate techniques that give high quality results. It was reported that simple *in vitro* CML models are preferred to the *in vivo* mouse models where CML stem cells showed poor or no engraftment in immune deficient mice [2]. Many researchers tried to generate CML stem cell models through retroviral transduction of umbilical cord blood CD34$^+$ hematopoietic stem cells (UCB CD34$^+$HSCs) to induce full length P210bcr-abl expression [2-4]. Although highly efficient gene transfer and long-term expression can be achieved with viral vectors, serious safety risks such as tumorigenicity have been identified due to insertional mutagenesis and uncontrolled gene expressions [5,6].

Despite being the most studied prototypic hematpoietic neoplasm, no plasmid-based approaches were used to generate ex vivo models of CML cancer stem cells. For maintaining an ex vivo model of HSCs disorders, the ideal vector should reside into the nucleus of repopulating cells and should ensure expression of the trans gene for the entire lifespan of the cell and its progeny [1]. That is why many new research studies focused on developing episomal self-replicating plasmid-based systems for their efficiency in maintaining gene expression for multiple generations without integration in the host cell genome [7-9]. However,

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constructing such vectors could be laborious and needs a state-of-the art devices which couldn't be available in limited facilities.

Few reports are available about using conventional expression plasmids for stem cell reprogramming specially for the induction of the expression of full length bcr-abl gene through transfection $CD34^+$HSCs [10]. Even, these few reports used either a cell line (cos cell line) rather than primary culture [11] or a complicated promoter type (tet responsive) rather than a constitutional one [12].

In 2010, a group of scientists identified up regulation of a new surface biomarker on bcr-abl expressing cells; interleukin one receptor associated protein (IL1RAP). This discovery offers new options for CML stem cells identification [1]. IL1RAP was suggested to be regulated by the well- known signaling cascade, activated by bcr- abl fused gene [13]. To this end we tried to generate a simple *in vitro* model of CML like stem cells using a conventional expression plasmid; the P210pcDNA3 plasmid which carries the full length bcr-abl fused gene. Our aim was mainly to detect the efficiency of nucleofection of a simple constitutional expression vector in inducing stable gene expression and to assess this efficiency using the least number of advanced techniques.

Methods

We tailored a method to ensure the delivery of the bcr-abl gene into the nucleus using a simple conventional mammalian expression vector. Umbilical cord blood $CD34^+$ hematopoietic stem cells (UCBCD 34^+HSCs) were nucleofected with the P210pcDNA3 plasmid to induce bcr-abl expression in the $CD34^+$ HSCs. P210pcDNA3 plasmid, is a commercial conventional plasmid that encodes the full length bcr-abl gene (P210), the hallmark of the CML disorder. This plasmid is characterized by its Cytomegalovirus virus (CMV) promoter for high-level expression in mammalian cell lines in addition to the neomycin-resistance cassette (Neor) for antibiotic selection of stably transfected eukaryotic cells [14]. Bcr-abl expression in the UCBCD34^+HSCs was evaluated through real time pcr analysis of G418 resistant P210pcDNA3 clones for IL1RAP gene.

P210pcDNA3 plasmid harvesting and verification

P210 pcDNA3" **Addgene plasmid # 27481, USA** " was harvested according to the manufacturer's protocol after signing a material transfer agreement; MTA (Supplementary data). Then the plasmid was purified using the Plasmid Mini Kit **(Qiagen, USA)**, then verified by gel electrophoresis.

Nucelofection of cord blood-derived $CD34^+$ cells

UCB was obtained from Obstetrics and Gynaecology Department at Faculty of Medicine, Suez Canal University, Ismailia, Egypt and an informed consent obtained in accordance. After Ficoll-Hypaque **(Mediatech Cellgro, German)** gradient separation, UCB mononuclear cells were collected and enriched for $CD34^+$ cells using Miltenyi MACS separation techniques **(MiltenyiBiotec, USA)** $CD34^+$ cells were enriched to more than 93% purity, $CD34^+$ cells were washed with 1-phosphate buffered saline/0.5% bovine serum albumin **(Sigma-Aldrich, Germany)**. $CD34^+$ were analyzed morphologically using the phase contrast inverted microscope and were identified as small lymphocyte-like cells that lack cytoplasmic granules and have prominent nucleoli [15]. Then, cells were resuspended at 0.5 to 1-10^6 cells/0.1 mL human CD34 cell Nucleofector solution **(Amaxa® Human $CD34^+$ Cell Nucleofector® Kit, Amaxa Biosystems, USA)**. First group of cells were nucleofected with 1 – 5 µg of bcr- abl expressing P210pcDNA3 plasmid while the second group of cells was nucleofected with 2 µg of the empty pMAX-GFP plasmids as indicated using program U-08 on the Nucleofector **(Amaxa Biosystems, USA)** device. Nucleofected cells were immediately transferred to 12-well plates containing 37°C pre warmed medium (RPMI 1640 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 2 mM Ultra Glutamine, **(Lonza Bioproducts, USA)** and cells were incubated in humidified 37°C/5% CO_2 incubator for 48 hours. A third group was left un nucleofected.

Serial dilutions of the nucleofected $CD34^+$ cells

Post nucleofection confluent $CD34^+$ cells, obtained 48 hours of culture were serially diluted in a 24 well plate till reaching a cell count of 4000-5000 cells in order to use it as a starting cell count for G418 antibiotic selection. 400 µl of media were added to each well of a 24 well plate. Next, 100 µl of a 5×10^6 cells/ml solution were added to the first well and pipetted to mix. This well had the maximum number of cells. Next, 100 µl from the first well were taken and added to the next well, and mixed. The process was repeated. Then 100 µl were taken from the last well, which contained the minimum number of cells, then discarded.

G418 selection of resistant clones

Serially diluted UCB $CD34^+$ HSCs were subjected to G418 selection using standard protocols [16]. Then each resistant clone was subcultured in a 24 well plate and was allowed to grow in a G418 selective medium, till suitable cell count was obtained (not more than 10^7 cells).

IL1RAP gene expression analysis

screening for successful bcr-abl gene stable expression was assessed. We didn't assess bcr-abl gene expression in the nucleofected cells, however, we assessed the expression of IL1RAP gene, a gene newly identified to be up regulated in bcr-abl expressing cells. Real-time PCR analysis was performed to cells nucleofected with the P210pcDNA3 plasmid and those nucleofected with the pMAX-GFP plasmid, both 48 hours and 14 days post nucleofection, using an ABI Prism 7500 analyzer **(Applied Bio systems, USA)** and Step One Plus **(Applied Bio systems, USA)** following manufacturer's protocols. All samples were analyzed in duplicates. Primers and probes for IL1RAP were obtained from **Invitro, USA** as assay-on demand primers. The relative quantity was calculated based on the ΔΔCt method [17] and normalized to GAPDH (Supplementary Figures S1-S9, Supplementary Tables S1and S6).

Statistical analysis

The mean, median and range of IL1RAP and GAPDH Ct values of test and control samples were tested for significance using the Mann-Whitney Test (Supplementary Tables S2). Expression levels are given as fold of expression considering the control sample equals one. The expression levels were tested for significance using Wilcoxon Signed-Ranks test [18]. The level of significance was set to $p<0.05$ (Supplementary Tables S3,S4,S5).

Results

P210pcdna3 Plasmid Harvesting and Verification Results

The presence of the desired bcr-abl gene fragment in the purified P210pcDNA3 plasmid (Figure 1A) was verified by gel electrophoresis after restriction enzyme digestion of the plasmid (Figure 1B). Two bands were visualized; the 7.2 Kb band referring to the bcr-abl gene fragment and the 5.4 Kb and referring to the pcDNA3 plasmid backbone.

Nucelofection of cord blood-derived $CD34^+$ cells results

Cell confluence of both nucleofected and non-nucleofected cells (cultured for 48 hours) reached up to 90% (Figure 2C).

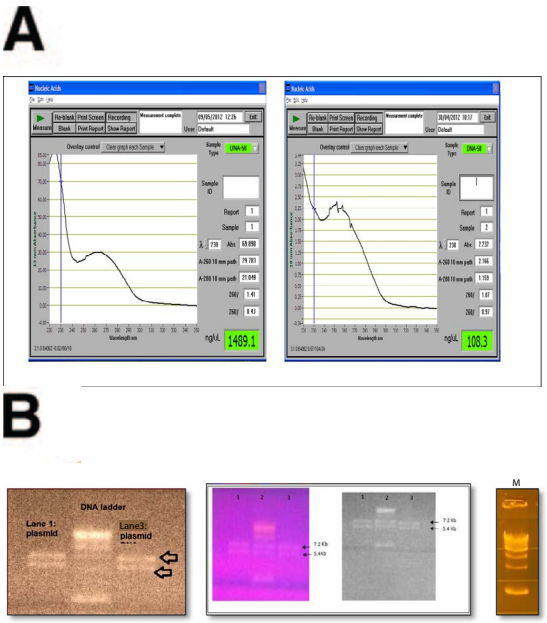

Figure 1: P210pcDNA3 plasmid purification and verification.(A) The P210pcDNA3 plasmid used for nucleofection, was recovered from the bacterial stab E-coli colonies, two E-coli colonies yielded plasmid DNA concentration ranging from 100 ng – 1.5 µg.(B) Agarose gel imaging showing the P210pcDNA3 fragments after digestion with the EcoRI restriction enzyme. Lane 1 and lane 3: Samples containing the two bands of the P210pcDNA3 plasmid. Lane 2: The DNA marker (M) is shown (2.5,5,7.5,10 and 12.5 kb shown).

Resistant clones to G418 appear after two weeks of nucleofection

The resistant clone appeared at the well with the G418 concentration of 0.8mg/ml and cell concentration of 10^3 cells after two weeks from nucleofection. The resistant clone was sub cultured in another 96 well plate containing 0.8mg/ml in all wells and a single clone was observed in nearly every well.

IL1RAP over expression in cells nucleofected with P210pcDNA3 relative to those nucleofected with the empty vector Pmax-GFP

IL1RAP proved to be over expressed in cells nucleofected with P210pcdna3 relative to cells not nucleofected with P210 pcDNA3 or those nucleofected with pMAX-GFP plasmid (an empty control vector). The expression levels after 48 hours ranged from two to nine folds change (Figure 2D and supplementary Table S4) while the expression levels in the subcultured G418 selected nucleofected cells ranged from four to eleven folds (Figure 2E).

Discussion

In this study, The P210 pcDNA3 plasmid was used for inserting the bcr-abl gene into the $CD34^+$ hematopoietic stem cells. The PmaxGFP plasmid wasn't used directly as a source of gene but used as a reporter plasmid because it has been previously tested by Hammer, who inserted the bcr-abl gene into two different cell types ;the BAF3 and the Hela cells. The gene was successfully inserted into the Hela cells but failed in the BAF3 cell line [19].

Thus, Pmax GFP plasmid was used as a reporter plasmid only to avoid the risk of failure of the gene insertion into the $CD34^+$ hematopoietic stem cells. However, the use of pcDNA3 plasmid as an expression vector, proved to be successful, in inducing the bcr-abl gene expression in our study. This was consistent with the results of another study [20] in which they used the same vector as an expression vector for encoding M2-tagged JNK1 wild-type and led to successful expression of the inserted gene. They used the G418 selection system at a concentration of 0.4–0.8 mg/ml that was similar to the concentration used in our study (0.75 mg/ml).

In the current study, nucleofection, the modified electroporation technique, was used for gene transfer in order to avoid the disadvantages of electroporation; cell damage and non specific transport of the intracellular components across cell membrane through the time of membrane electro premeability. Nucleofection uses a combination of electrical parameters, generated by a device called Nucleofector, with cell-type specific reagents to transfer a substrate directly into the cell nucleus (mainly extra-chromosomally, in case of circular DNA or intra- chromosomally, in case of linearized DNA), thus optimizing the electrical current intensity and length and increasing the specificity and proper targeting to the nucleus. Nucleofection preserves cell viability and intracellular systems are conserved [21]. In order to avoid the risk of improper gene integration and meticuolous linearization [22], we didn't use lineraized DNA molecule (despite inducing stable gene expression) [23], we used circular DNA instead.

In this work IL1RAP gene over expression, in P210pcDNA3 nucleofected cells, was considered an indicator for stable bcr-abl gene expression according to Ren [13].

The difference between the fold change expression of IL1RAP gene in the nucleofected cells with bcr- abl gene after 48 h (up to 5 fold) and after 10 days (up to 11fold) in comparison with non nucleofected cells could be attributed to the timing of gene expression analysis. In this study, after forty eight hours, the DNA introduced in the transfection process didn't integrate into the nuclear most probably due to DNA dilution through mitosis or degradation leading to weaker expression of the desired gene [24]. However, after selection by the G418 antibiotic, the IL1RAP gene expression was reassessed in the sub-cultured resistant clones containing the P210pcDNA3 plasmid. We found that the fold change of IL1RAP gene expression in the G418 selected cells,

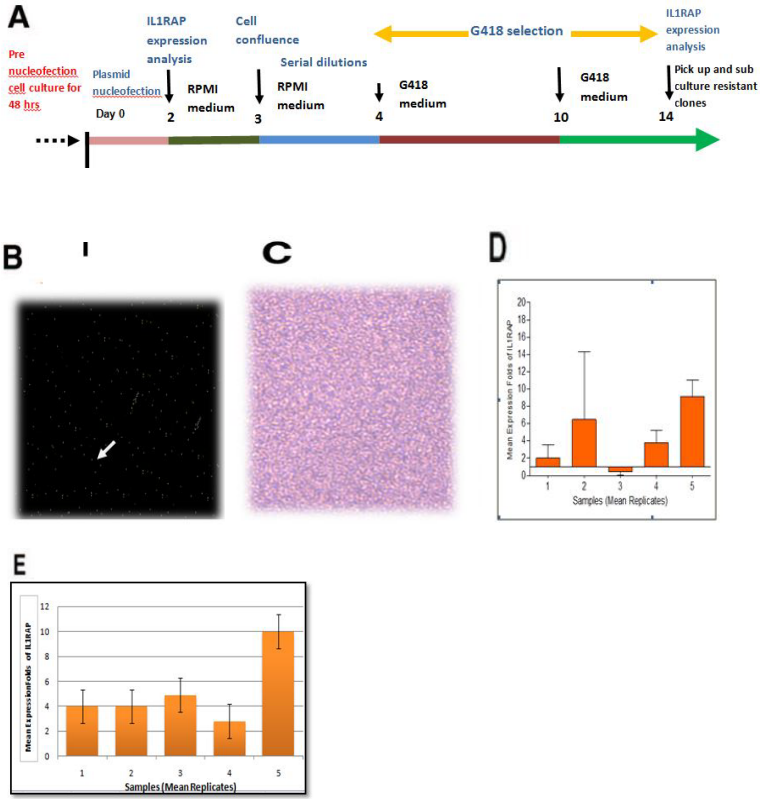

Figure 2: Umbilical cord blood CD34⁺HSC, nucleofection with P210pcDNA3. (A) A diagram illustrating the process of nucleofection and the fate of nucleofected cells. The nucleofection was done in two settings (12 and 12). Each nucleofection setting included 6 test samples of 10^6 cells (of purified CD34+ hematopoietic stem cells nucleofected with the P210pcDNA3), 5 negative control (purified CD34+ hematopoietic stem cells nucleofected with no plasmids) and 1 positive control sample (purified CD34+ hematopoietic stem cells nucleofected with pmaxGFP plasmid). The six successfully nucleofected test sample were cultured for 48 hours till cell confluence (up to 90%) was achieved. Five test samples were harvested for real time PCR analysis of IL1RAP gene expression after 48hrs while the sixth sample was serially diluted in a 24 well-plate in a 1:2 ratio. A cell count of 5000 cells was achieved at well number B5 on day 4. The cell suspension in well B5 was seeded in a 96 well plate for G418 selection of resistant clones. By the 14th day, the resistant clones were picked up, sub-cultured cells were then harvested for IL1RAP expression analysis. (B)The success of the nucleofection process was evaluated by the successful nucleofection of pmax-GFP plasmid into the purified CD34+ hematopoietic stem cells (white arrows showing green fluorescent signals from nucleofected CD34+ with pmax-GFP plasmid). (C) Post nucleofection confluent cultured CD34⁺ hematopoeitic stem cells, cell count 2 × 10^6 cells (Brightfield X 20). (D) The expression levels of nucleofected cells, 48 hours post nucleofection, ranged from 2 to 9 folds with *p* value 0.015. (E) The IL1RAP gene expression levels in subcultured G418 selected nucleofected cells, ranged from 3 to 11 folds change with a *p* value < 0.05

was doubled (fold change: 11) relative to that of the non-G418 selected cells (fold change: 5). This selection guaranteed the assessment of only the cells expressing the IL1RAP gene and thus yielded high expression fold change.

Our results of over expression were close to but not identical to those reported by Jaras et al. where the retroviral P210 bcr-abl UCB CD 34⁺ expressing cells (n:3) in their study showed higher expression level (up to 12 folds) compared to empty vector MIG control-expressing cells and normal bone marrow CD34⁺ stem cells (n: 3) which were not retrovirally transduced. This discrepancy could be attributed to the use of a different expression system in our study (expression plasmids; P210 pcDNA3) to induce the bcr-abl gene expression instead of the retroviruses used in their study. Stable gene expression characterized for retroviruses in comparison to transient expression achieved by plasmid vectors, is suggested to be responsible for such variation [25].

Conclusion

On overall, we conclude that, conventional plasmids could be used for studying Chronic Myeloid Leukemia especially in labs or research centers with limited resources. The nucleofection of a conventional plasmid is likely to induce stable gene expression in HSCs. Furthermore, bcr-abl mediated IL1RAP over expression on CML CD34⁺HSCs, offers major target for CML anticancer drug development

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