

RESEARCH ARTICLE

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Retracted: Luciferase-transfected colon adenocarcinoma cell line (DLD-1) for use in Orthotopic Xenotransplantation studies

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A retraction article was published for this article. It is available from the following link; <http://journal.chemistrycentral.com/content/6/1/121>.

Abstract

Background: Renilla Luciferase reporter gene (rLuc) GL4.82 and GL4.13 promoter are key player in transfection, but precise knowledge of its targets in colon cancer remains limited. The aim of this study was to characterize the best transfection technique to produce a stable transfected colon DLD1 (colorectal adenocarcinoma cell line), therefore imaging based approaches were employed.

Results: DLD1 cells were transfected with a Plasmid (SV40-RLuc) carrying Renilla luciferase under the control of the SV-40 promoter, by using two different transfection techniques. Cells expressing the required DNA were isolated after antibiotic (Puramycin) selection. Clones of DLD-1/SV40-RLuc were produced using two different techniques (96 well plates and Petri dish) and their florescence intensity was recorded using IMS machine (Calliper Life Sciences, Hopkinton, USA). Both techniques were characterized with the help of serial dilution technique. Results from this study substantiated that electroporation is the best. As expected, clones varied in their specific luciferase activity along with the dilutions. With the increase in cell concentration increase in intensity of florescence was recorded.

Conclusions: Based on the results we are confident that this transfected cell line DLD-1/SV40-RLuc (colorectal adenocarcinoma cell line) is the best for further Orthotopic Xenotransplantation Studies and *in-vivo* experiments as well. Investigation shows that DLD1/SV-rLuc cells have gained little bit resistance against both drugs therefore further study is suggested to know the reasons.

Keywords: DLD1, Reporter gene, Electroporation, ExGen transfection, MTT assay

Background

Human body is a complex of billions of cells, which grow, divide, and die in programmed fashion, and when any of these designed fashion undergoes uncontrolled or abnormal causes cancer. Cancer is a lethal disease; in 2007, 1554845 cancer related deaths were recorded in UK [1]. Good News is that despite increase in incidence, cancer mortality is decreasing [2] yet there is great need of new drug discovery and development. Drug discovery and development procedure involves; acquisition, preclinical screening, production and formulation,

toxicology, clinical trials, and general medical practice [3]. Screening drugs for their intracellular effects is a crucial part of the drug discovery and development process. To support any investigational new drug, there is an essential need of carefully designed preclinical drug *in vitro* and *in vivo* studies. Most of the researchers in drug development agree that the demonstration of anti-tumor efficacy in preclinical, typically animal based model of cancer is a key determinant in both, and compounds for testing should have shown target biomarker modulation *in vitro* and *in vivo* [4].

From 1950s to 1970s syngeneic murine models were used. Subcutaneous transplantation of human tumor xenograft in nude mice was used in next decades and now with the advancement of transgenic knock out models, interest

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in using genetically engineered mouse models (GEMMS) has sparked [5]. While syngeneic murine tumor models remain valuable for studying immunotherapeutic or targeting metastasis, the human tumor xenograft models established by inoculation of human cancer cell lines into the mice, which is immunodeficient, have been used for test of cytotoxic anticancer agents and new antineoplastic treatment modalities [6]. Since early 1990 new drug developments have moved from general cytotoxic agents to molecular target directed therapeutic, eventually, there was a need to identify tumor types and individual patient tumors that express the target and could benefit from therapies in clinical trials, therefore *in vivo* models used in preclinical development should be oriented, disease-to-disease or target directed [7]. This is still less clear and point of debate that which is the best model to use with respect to predict for clinical trails? Currently, nine out of ten experimental drugs fail in clinical studies because we cannot accurately predict how they will behave in people based on laboratory and animal studies. In Biomedical sciences use of animals as models to help understand and predict responses in humans, in toxicology and pharmacology in particular remains both the major tool for biomedical advances and a source of significant controversy [8].

During 19th century rise in use of animals rose up critically, which induced pressure on British government to amend Cruelty to Animal Act of 1876 in 1986 [9]. Recent developments of new techniques have permitted researchers to minimize the use of animals at a certain level. It has been very important element in the current success of biomedical research. Very first example of this was in the development of polio vaccine [10]. Since then human and animal cells culture techniques have improved greatly. Anyhow alternatives are nothing but new techniques that may lead to reduce the use of animal in research e.g. paper chromatography, radioimmunoassay, non-invasive imaging and use of Orthotopic models etc. has been main factor behind rapid development of biomedical research [11].

Currently research directions are towards specific cancer target, due to advanced development in cancer biology scientists are also focusing towards translational medicine and diagnosis. In use mouse tumor models have been improved in reliability of imitating human disease. Orthotopic implantation (Organ-specific) reproduces human tumor and metastasis, which has replaced ectopic subcutaneous implantation in experiments [12]. In Orthotopic models strong fluorescent are labeled with green fluorescent protein along with video detectors, allows the monitoring of details of growth, angiogenesis, and metastasis. External imaging is limited by light scattering in deep tissues, especially inside the skin. Signal reduction is markedly eradicated by opening a reversible skin-flap in

the light path, which increases the detection sensitivity at much higher extent [13]. The problem of this technique depth of tissue is thereby minimized and many tumors that were previously hidden are now clearly observable [14]. In recent years, a significant development has been made in the choice of imaging cancer non-invasively in murine tumor models. This includes new materials that are imaging probes, which selectively accumulate in tumors, or that become activated by tumor-specific molecules in animal experimentation [15]. Other tumor imaging's techniques that rely upon the detection of gene expression (reporter) in the body of the animals have been developed [16], which made a significant impact on both the versatility and the specificity of tumor imaging of animals.

Optical imaging is rapidly developing field of research aimed at non-invasively interrogates animals for disease progression, evaluating the effects of a drug, assessing the pharmacokinetics behavior of a drug, or identifying molecular biomarker of disease. Optical imaging is mainly based on quantifying the qualitative changes in the emission of light by bioluminescence and fluorescence, which requires incorporation of a vector reporter gene into the cells [17]. In fluorescence imaging, fluorescently tagged agent is injected in mice which carry cells or tissue expressing a florescent transgene, imaged using different light tight highly sensitive CCD camera [18]. Bioluminescence imaging is based upon the sensitive detection of visible light that is produced during enzyme mediated oxidation of a molecular substrate when that enzyme is expressed in animals as molecular reporter [19].

There are several other optical techniques being developed, as FTIR Spectroscopy, Raman Spectroscopy and Multiphoton Imaging but, very low cost and high throughput capability of Bioluminescence imaging and Fluorescence imaging make these important to the drug discovery and development process [20,21]. Key to non-invasive imaging is to incorporation of these reporter genes (talked above) in to the cells "Transfection". Transfection is the introduction of foreign DNA into eukaryotic or prokaryotic cells [22]. It is the delivery of DNA, mRNA, proteins, and macromolecules into the cells. Goal for the transfection is to study the regulation of gene as well as protein expression and function [23]. Frederick Griffith, 1928 first transformed nonpathogenic pneumococcus bacteria into virulent variety by mixing nonpathogenic pneumococcus with heat-killed pathogenic bacteria. Avery, et al., 1944 reported that the pure DNA is originally the transforming factor [24]. There are different methods of transfection commonly used such as DEAE-dextran for the delivery of nucleic acids into cells for transient transfection [25], calcium phosphate for transient and stable both type of transfection of a

variety of cell types [26], lipofection is much reproducible and efficient than other transfection modalities [27], micro-injection for the introduction of functional proteins, genes, inhibitors of enzyme activities and antibodies into living cells [28], protoplast fusion is very efficient with high level of transfection about 10% [29], electroporation is proven to be efficient method for transfection of many cell types [30] though it requires very precise and accurate setting for duration and strength of the current for each type of the cells used and ExGen 500 used both *in-vitro* and *in-vivo*, and wide range of cell types can be transfected with this reagent [31]. It has shown very higher transfection efficiencies as compared to other cationic lipids and polymers [32,33].

The objective of this study was to develop a stable transfected cell line DLD-1 colon adenocarcinoma and at the same time characterize two different techniques of transfection on the basis of their efficacy. Characterize these transfected cells on the basis of their growth and chemo sensitivity to two standard drugs (Doxorubicin, MB2) for further use in orthotopic xenotransplantation studies.

Results

Characterization of the techniques

DLD1 cells were transfected with a SV40-RLuc carrying Renilla luciferase under the control of the SV-40 promoter, using two different techniques. Cells expressing the required DNA vector (Figure 1) were isolated using antibiotic selection method [3]. To characterize the best technique among both techniques used for transfection (ExGen500 and Electroporation) in this study, substrate coelenterazine 50 µg/ml was added to each well and output was recorded, using IVIS machine with in 10 second of the

substrate addition. Electroporation and ExGen500 technique, Electroporation was found the best [3] shows, as ELec1's output was recorded 5.77×10^5 p/sec/cm²/sr while ExGen2's output was recorded 3.46×10^5 p/sec/cm²/sr. While from ExGen1, 2, and 3 ExGen 2 was the best and from ELec1 and 2, ELec1 was found the best. As expected, clones varied in their specific luciferase activity (measured by standard luminometer -data not shown-). Figure 2 represents light emission in live cells corresponding to five different clones after the treatment of cells with the substrate 50 µg/ml Coelenterazine.

Bioluminescence intensity is directly proportional to cell concentration

Following selection of the best clones serial dilutions were prepared to show correlation between cell number and photon output. Bioluminescence intensity of FLuc serial dilutions was recorded using IVIS machine. It was found that bioluminescence intensity of FLuc cells increases in a gradual manner, with the increase in cell concentration. At the concentration of 1×10^5 cells/well it shows an output of 1.52×10^4 (p/sec/cm²/sr). While a slight decrease has been noticed in the intensity of light at 5×10^5 cells/ml where it shows 1.02×10^4 (p/sec/cm²/sr) (Figure 3). Results were measured (mean ± s.d). All types of rLuc cells were serially diluted and treated with substrate. Results showed that bioluminescence intensity of the ELec1 increases in a gradual manner with the increase in cell concentrations, while ExGen1 and 2 did not show gradual increase in the light emission. On the other hand ExGen3 showed very higher output of 4.70×10^5 (p/sec/cm²/sr) (Figure 4) at the concentration of 5×10^5 cells/ml while at 1×10^6 cells/ml, it showed a decrease in the output 3.67×10^5 (p/sec/cm²/sr) (Figure 4). Anyhow ExGen2 showed consistent increase

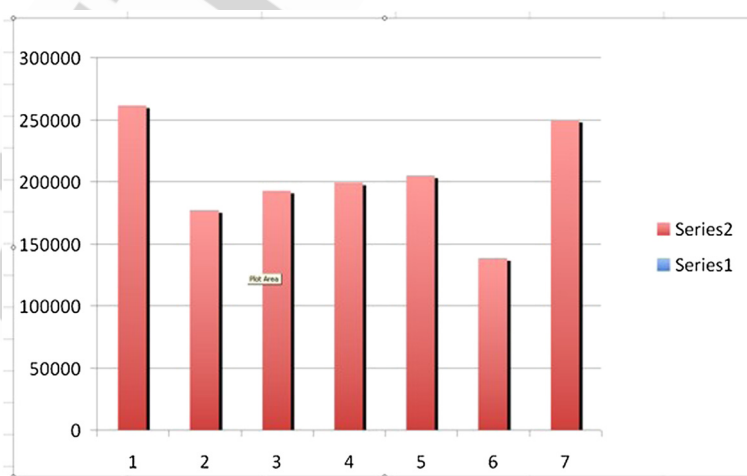


Figure 1 Cloning of SV40 region from GL4.13 into NheI & Hind III site of GL4.82 produces SV40-RLuc.

OUT PUT (P/SEC/CM2/SR)

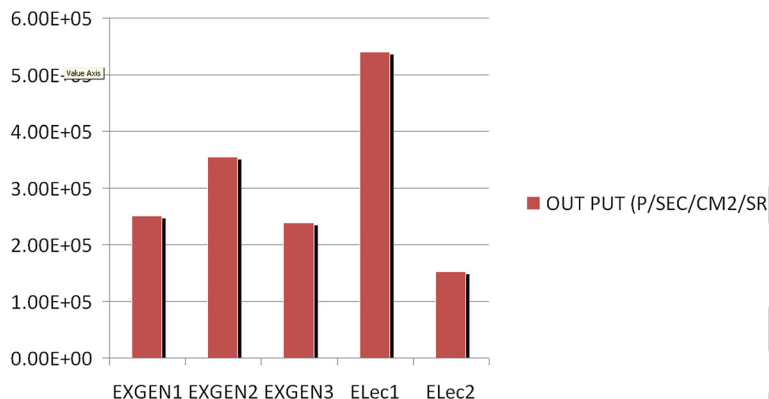


Figure 2 All transfected cells (ExGen1, 2,3 and ELEC1, 2) added substrate 5 μ l of coelenterazine (50 μ g/ml), Output (p/sec/cm2/sr) was recorded using IVIS machine with in 10 second of the substrate addition.

in output with higher concentrations of cells, 2.86×10^5 (p/sec/cm2/sr) at 1×10^6 cells/ml. ELec1 was found producing highest intensity than any other one, showing 6.12×10^5 (p/sec/cm2/sr) at 1×10^6 cells/ml.

ranging from 1.384×10^5 to 2.612×10^5 (p/sec/cm2/sr). In (Figure 5) it represents light emission in living cells corresponding to 7 clones after the treatment of cells with 25 μ g/ml Coelenterazine.

Characterization on the basis of cloning

96 well cloning

After the selection of best clones, we also evaluated the cloning techniques between 96 well plate cloning and ring cloning, single cell clones of ELec1 (Best transfected cells) from 96 well plates were imaged directly. As expected, clones varied in their specific luciferase activity. We selected 7 best clones (Figure 5) on the bases of their light emission

Ring cloning

In case of ring cloning we selected ELec1 and ExGen2 to image. Results shown in (Figure 6) light intensity of ELec1 ranging from 4×10^5 to 1.57×10^6 (p/sec/cm2/sr), and of ExGen2 it ranges from 1×10^5 to 1.275×10^6 (p/sec/cm2/sr). Three best resulting clones from ring cloning were selected for further process.

Serial Dilution FLuc

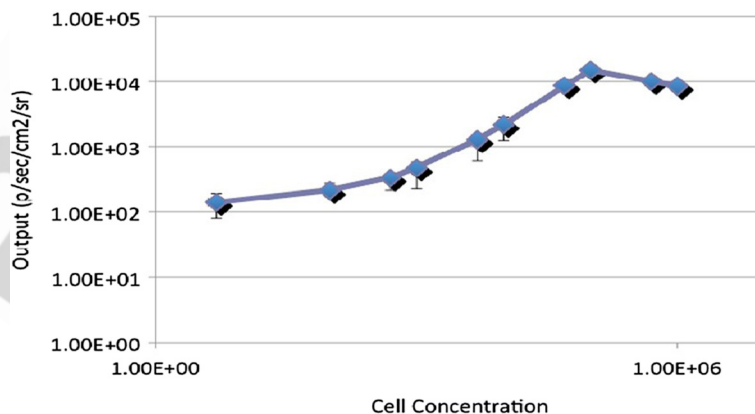


Figure 3 FLuc cells plated at range of concentration from 5×10 cells/well to from 1×10^6 cells/well, treated with 30 mg/ml D-Luciferin at 1:200 dilution and Imaged using IVIS machine. Shows highest intensity of light at the concentration of 1×10^5 cells/well. Results were measured (mean \pm sd).

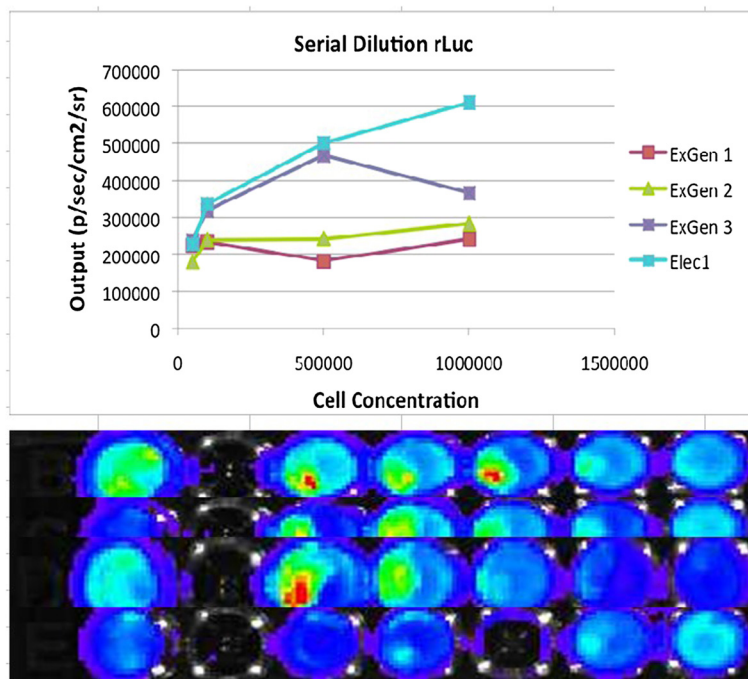


Figure 4 Three independent DLD1/SV-rLuc/ExGen and Two independent DLD1/SV-rLuc/Elec clones were seeded in 96-well plates at different cell densities, ranging from 5×10^3 cells/well to 1×10^6 cells/well and treated with the Renilla luciferase substrate Coelenterazine. 50 $\mu\text{g/ml}$, output (p/sec/cm²/sr) was recorded using IVIS machine with in 10 second of the substrate addition.

Growth curve results

To ensure that transfected cell shows no difference in their proliferation activity after transfection, we determined growth curve for Normal DLD1, FLuc, rLuc cells, results shown in (Figure 7). Following subculture, the control cells

progressed through a characteristic growth cycle, nearing the peak phase at Day4. Normal DLD1 cells along with the other transfected cells, FLuc and rLuc, were cultivated at the concentration of 5×10^4 cell/ml. Growth of all cells appears to be very similar with FLuc having the highest rate of

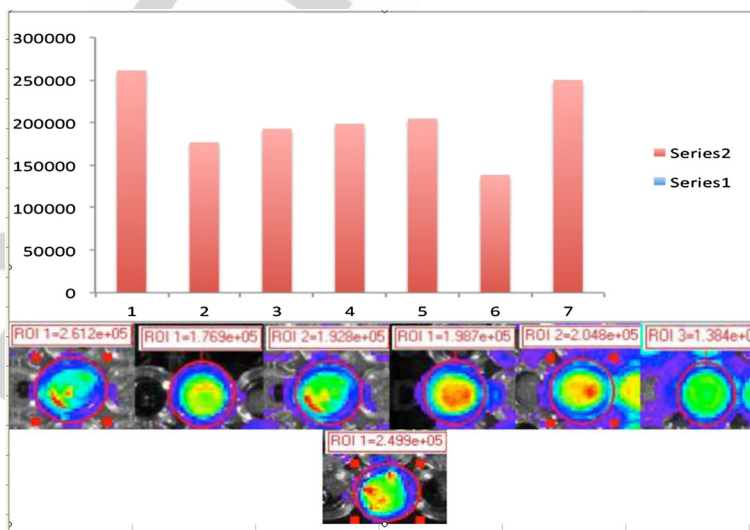


Figure 5 Seven independent DLD1/SV-rLuc/Elec clones were selected after seeding of 10 cells/ml in 96-well plates and treated with coelenterazine 25 $\mu\text{g/ml}$ after overnight incubation. Output (p/sec/cm²/sr) was immediately quantified using IVIS machine.

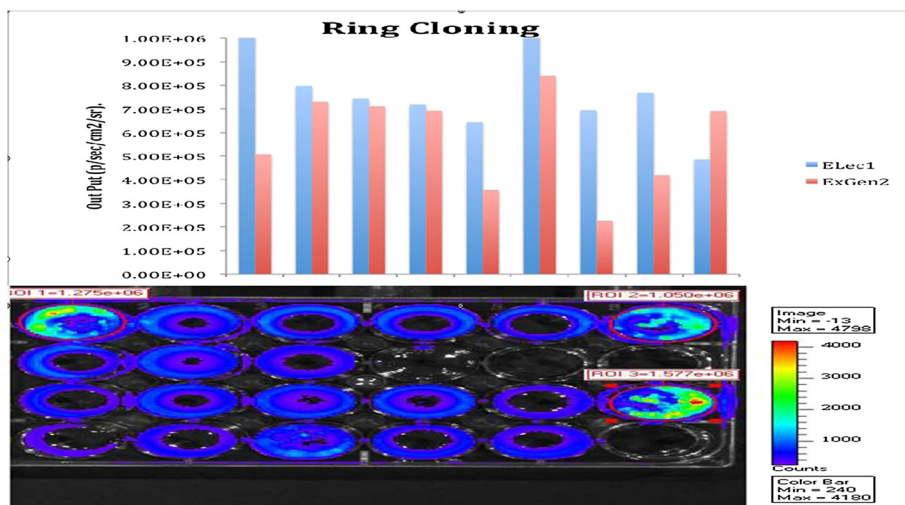


Figure 6 Nine independent clones of DLD1/SV-rLuc/ExGen2 and of DLD1/SV-rLuc/ELEC1 were transferred from patri dishes to the 24-well plates at and treated with coelenterazine 25 µg/ml after overnight incubation. Output (p/sec/cm2/sr) was immediately quantified using IVIS machine.

growth as compare to others. All the cells have shown nearly same trend of growth.

MTT ASSAY

In vitro growth inhibition by MB2 (ICT in-house drug) and Doxorubicin was tested against all the cell types wild type DLD1 and transfected DLD1/SV-FLuc, DLD1/SV-rLuc cells, to ensure that transfected cells have no change in their response to drug as compared to normal DLD-1 cells. Cells were tested after 96 hours of drug exposure. Data was recorded by using Teacan reader set at the 540 nm filter. Results (Figures 8 and 9) described

that DLD1/SV-rLuc transfected cells have gained little bit resistant against both of the drugs as ic50 for normal DLD-1 cells for MB2 is (30 nM +/- 10 nM), for DLD1/SV-FLuc = (30 nM +/- 10 nM) and DLD1/SV-rLuc = (100 nM +/- 30 nM) (Figure 4). Ic50 of DLD1 cells for doxorubicin is = (100 nM +/- 30 nM) (Figure 6) and of DLD1/SV-FLuc = (100 nM +/- 30 nM) and DLD1/SV-rLuc = (300 nM +/- 100 nM) was observed. Anyhow all cell types have shown more or less same behavior to the drug exposure. With increase in drug concentration, all cells have shown gradual decay in percentage viability. Results were measured as (mean ± s.d).

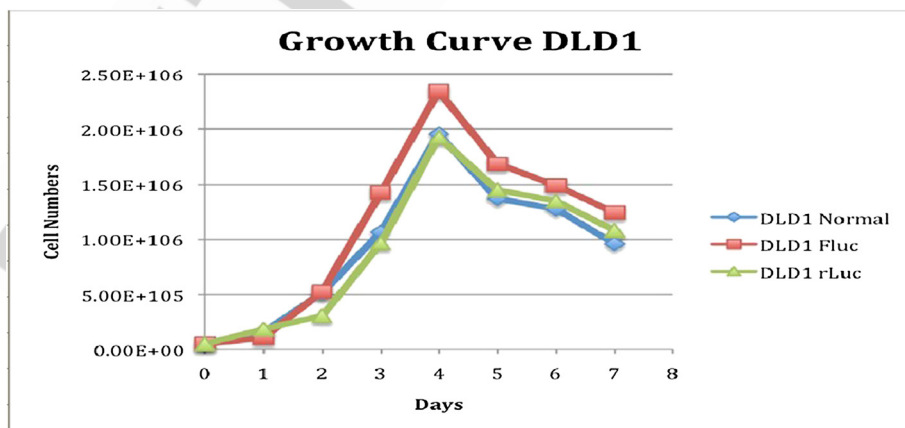


Figure 7 Above showing growth curve of Normal DLD1, DLD1/SV-FLuc, rLuc cells over the period of seven days. Cells were maintained in 10 ml RPMI supplemented with 1% L-glutamine, 1% Sodium Pyruvate, and 5% Fetal bovine syrup (FBS), incubated at 37°C 5% CO2. Counted manually using haemocytometer. Results were measured (mean ± s.d).

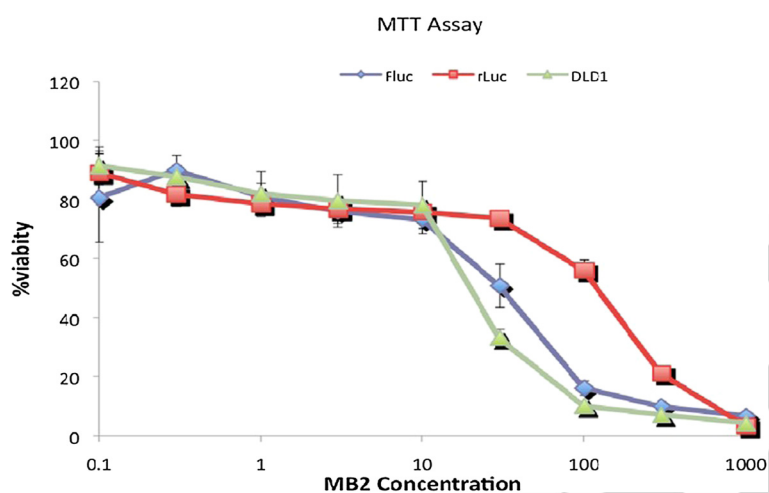


Figure 8 MTT assay result; The effects of MB2 on DLD-1, DLD1/SV-rLuc, and DLD1/SV-FLuc cells exposed to serial dilution of MB2 ranging from .1 nM to 1 μM for 96 hours showing gradual decrease in % viability with the increasing dose of MB2. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (i.e. no drug added) was taken as 100% cell viability.

Discussion

Imaging techniques are emerging as important research tools in the scientific industry, very useful especially in diagnostics which are the main problem of the time. Even though our understanding of functional roles of these reporter genes in cancers is steadily increasing, knowledge about Renilla Luciferase reporter gene GL4.82 and GL4.13 promoter used in colon adenocarcinoma is still largely missing. Hence focused on the characterization of the best transfection technique to get the stable cell line after transfection, which could result in a fine biomarker in targeting colon adenocarcinoma cells. Supporting previous findings that imaging techniques are largely used in

tumor studies, cell growth profile of DLD1, DLD1/SV-rLuc Cells and DLD1/SV-FLuc cells were established. Cell lines demonstrated that proliferation levels of DLD1, DLD1/SV-rLuc Cells and DLD1/SV-FLuc cells were significantly same relative to non-tumorigenic cell lines [34,35]. Growth reduction of DLD-1 cells upon transient miR-145 transfection, which implies that miR-145 possesses a tumor-suppressor function *in vitro* has already been reported [36].

Here, immunocytochemistry has been used as a method of identification of potential targets using two different drugs. It has the advantage over strictly computational target prediction that it is not only based on the presence of a

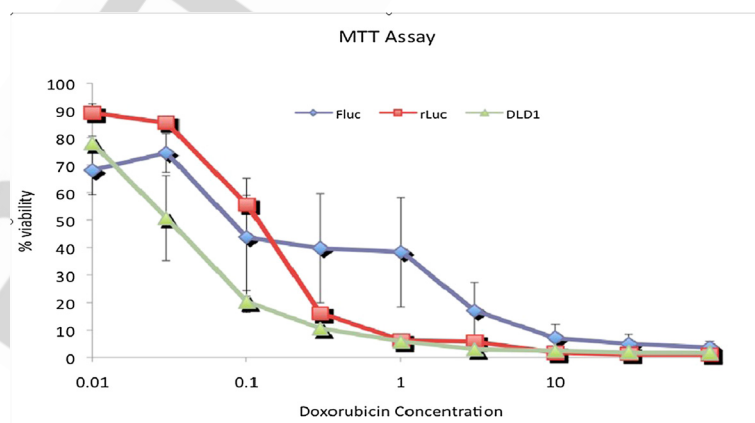


Figure 9 MTT assay result; The effects of Doxorubicin on DLD-1, DLD1/SV-rLuc, and DLD1/SV-FLuc cells exposed to serial dilution of Doxorubicin ranging from 10 nM to 100 μM for 96 hours showing gradual decrease in % viability with the increasing dose of Doxorubicin. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (i.e. no Drug added) was taken as 100% cell viability.

seed site and sequence features of the potential target, but takes into account whether the target is expressed in the considered cell line and whether the target is regulated on the transcript level [37-39]. Since this approach is solely based on changes observed on transcript level, targets exclusively regulated by translational repression will not be identified. Many of the targets identification techniques used here have previously been implicated in cancer [40,41]. A number of fluorescence genes with fluorescence function, many of which have also been associated with colon cancer, were identified as potential biomarkers based on the microarray analysis [42-44]. The cell membrane associated fraction of VANGL1 increases with differentiation and was demonstrated to co-localize with E-cadherin in human colon cells [45,46]. Previously reported miR-145 targets including OCT4, SOX2 and KLF4 involved in the promotion of stem cell proliferation were not expressed in DLD-1 cells [47,48].

Experimental validation of these biomarkers demonstrated a significant success in *in-vitro* studies as well as in *in-vivo* [49]. The regulation in luciferase assays further validates that this is the result of direct interactions [50]. The reason of the different effects of luminescence in the different assays is likely due to a lack of direct comparability between these assays [51]. This difference could also be due to other binding factors involved in regulation of the endogenous transcript, as these binding sites are not present in the 3'UTR or cDNA fragments used in the cloned luciferase reporter constructs [52-54].

A number of studies have linked increased use of these biomarkers in cancer studies with increased cell motility and tumor invasion [55]. In conclusion, using a microarray based approach we have identified additional targets for the cancer-associated miRNA miR-145 in colon cancer cells [56]. These transfection techniques could be a milestone in identifying different targets in colon cancers as well as other cancer types [57-59]. As the problem for the early diagnose stays the same in this era we need to have more studies following these lines of the studies which could save time and get very early diagnose of the cancer at the same time.

On the same time we strongly suggest the further use of the same cell lines been transfected in this studies in Orthotopic Xenotransplantation Studies, as we best stable cells were frozen down already. These studies can elaborate the use of such biomarkers in *In-Vivo* studies.

Conclusions

Renilla Luciferase reporter gene GL4.82 and GL4.13 promoter Renilla luciferases (rLuc) GL4.82 have emerged as important gene regulators and are recognized as key players in transfection (rLuc) GL4.82 is reported to be

down regulated in several cancers, but knowledge of its targets in colon cancer remains limited. To investigate the role of (rLuc) GL4.82 in colon cancer, we have employed imaging based approach to characterize the best transfection technique to get a stable transfected colon DLD1 cell line. Based on the best of its imaging results *in-vitro* we hereby urge the use of this cell line in Orthotopic Xenotransplantation Studies and *in-vivo* experiments. DLD1/SV-rLuc cells gained little bit resistance against both drugs; more studies designed for this particular plasmid need to be carrying out to know the reason why they gained that resistance while other transfected line did not.

Methods

Materials

DLD1 (colon adenocarcinoma cells) obtained from European Collection of Cell Cultures Salisbury UK (ECACC™). DLD1/Sv-Fluc transfected cells obtained from IN HOUSE CELL CULTURE COLLECTION of ICT. Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with, 1% L-glutamine, 1% Sodium Pyruvate, 10% fetal bovine serum (FBS), all purchased from Sigma Aldrich, St. Louis, USA. Hanks Balanced Salt Solution (HBSS), T/E .25% Trypsin EDTA Solution, Serum free media (SFM), Doxorubicin Hydrochloride, MTT (Thiazolyl Blue Tetrazolium Bromide, Approx 98% TLC), DMSO (Dimethyl Sulfoxide, ≥ 99.9% ACS Spectrophotometric grade) and Puramycin dihydrochloride solution 10 mg/ml also obtained from Sigma Aldrich, St. Louis, USA. Gene Pulser Xcell Machine, Renilla Luciferase reporter gene GL4.82, and GL4.13 promoter obtained from Promega, Madison, USA. Fermentas, Maryland, USA provided 150 mM NaCl, and ExGen 500. Coelenterazine (native coelenterazine), a substrate for Renilla Luciferase from Biotium Hayward, CA (the compound (1 mg/ml) was dissolved in methanol). D-luciferin Firefly potassium salt, a substrate for Fire Fly Luciferase from Xenogen Alameda, CA (A 30-mg/ml stock in PBS was filtered through 0.22 μm filters before use). IVIS 50 imaging machine from Calliper Life Sciences, Hopkinton, USA. MB2 (Vascular Disrupting Agent) was taken from in house production of ICT.

Cell lines, culture conditions

DLD1 cells, DLD1/Sv-Flu previously cultured and DLD1/SV40-rLuc transfected with Renilla luciferase (rLuc) GL4.82 reporter gene under the control of Simian Virus 40 GL4.18 promoter, cells were maintained in complete RPMI 1640 medium and incubated in humidified incubator at 37°C and 5% CO₂ level. Passage of all types of cells was always done by seeding in complete RPMI 1640, incubating at 37°C and 5% CO₂ level, washing twice by 10 ml/wash

Table 1 ExGen500's Volume used for transfection

Sample	Amount of DNA	Volume Of ExGen500 μ l at equivalents
DLD1/SV-rLuc/ExGen1	1 μ g	8.23 μ l
DLD1/SV-rLuc/ExGen2	1 μ g	9.87 μ l
DLD1/SV-rLuc/ExGen3	1 μ g	11.52 μ l

HBSS and counting manually with the help of haemocytometer after trypsinization.

Transfection of the cells

Renilla Luciferase reporter gene GL4.82 (Figure 2) and SV40 GL4.13 (Figure 1) promoter been used to make the reporter gene by cloning SV40 region from GL4.13 into the NheI & Hind III site of GL4.82 made SV40-RLuc as a vector reporter to transfect the DLD1 cells [60].

Transfection methods

Two different techniques of transfection;

- I. Electroporation
- II. ExGen500

Electroporation

1×10^7 cells/ml were used for Electroporation Transfection, so resuspended the required concentration of the cells in 400 μ l of serum free media (SFM) after trypsinization of the cells. Transferred the cell suspension to the 2 mm vial. 0.88 μ g/ml SV40-RLuc were added to each sample (needed 20 μ g of Plasmid in 400 μ l of cell suspension). Gene Pulser Xcell Machine was used for electroporation. Two methods of electroporation were used;

Exponential electroporation; the machine was set at voltage 250volts, Capacitance 1000volts, and resistance ∞ . Sample 1 was electroporated and these cells were named DLD1/SV- rLuc/ELec1.

Square Wave Method; the machine was set at voltage 250volts, Pulse Length 10 nm, and Number of Pulse 1. Sample 2 was electroporated and these cells were named DLD1/SV-rLuc/ELec2. Following the transfection, Cells were transferred in to T25 flasks and incubated in humidified incubator at 37°C 5% CO₂.

ExGen500 Transfection

Initially, we recommend the use of 1ug of DNA and 3.3 ul (6 equivalents) of ExGen 500 per well of 6-well plate. Cells were plated in 6 well plates at 5×10^4 cells/well and incubated overnight at 37°C 5% CO₂ level. 150 mM NaCl solution was made up. The ExGen 500 and DNA complex was made by adding 1 μ g of SV40-RLuc in to 100 μ l of 150 mM NaCl, vortaxing gently and spinning down and then adding ExGen 500 at the concentrations shown in (Table 1). Following this, the solution was mixed immediately for 10 second then incubated for 10 minutes at room temperature. Then 100 μ l of each sample was added to each well containing 3 ml complete RPMI 1640 respectively. Then plate was incubated at 37°C and 5% CO₂ level for 48 hours. Colonies were visible after 2 to 3 weeks. Colonies were picked using sterile cloning rings and expanded to form sub lines.

Puramycin selection/cloning

To select the cells expressing the required DNA vector (Figure 1), all types of cell were treated with 50 μ g/ml of Puramycin because SV40-RLuc is Puramycin resistant that works as selection marker. Optimal concentration for puramycin was tested earlier at different concentrations with normal DLD1 cells data not included here. So, purely transfected cells expressing the required DNA vector (Figure 1), sustained within the puramycin were harvested and plated in Petri dishes and 96 well plates at 10 cells/ml following the procedure described earlier. Plates were incubated at 37°C 5% CO₂ level for 2–3 days. Single cell clones were selected in both, Petri dishes and 96 well plates for imaging. Single cell best observed clones in 96 well plates were imaged directly. While best clones from Petri dishes were passaged to the 24 well plates using method descried earlier.

Serial dilutions

To check the ratio of bioluminescence and cell numbers both DLD1/SV-FLuc and DLD1/SV-rLuc cells were serially diluted. Each type of cells were serially diluted at the concentrations ranging from 1×10^4 cells/well to 1×10^6 cells/well and DLD1/SV-FLuc at the concentrations ranging from 5×10 cells/well to 1×10^6 cells/well was plated in 96 well plates. Incubated overnight at 37°C 5% CO₂ level for imaging.

Table 2 Drug concentrations of MB2 and Doxorubicin used in MTT assay

Blank	Control	MB2 drug concentration								
Blank	Control	.1 nM	.3 nM	1 nM	3 nM	10 nM	30 nM	100 nM	300 nM	1 μ M
Blank	Control	Doxorubicin Drug Concentration								
Blank	Control	10 nM	30 nM	100 nM	300 nM	1 μ M	3 μ M	10 μ M	30 μ M	100 μ M

Imaging and quantification of bioluminescence data

The *in vivo* Imaging System (IVIS), consists of a cooled camera (CCD) mounted on a light-tight chamber (dark box), a camera controller, a camera cooling system, and a Windows based computer system, were used for data acquisition and analysis. Each 96/24-well plate sample was placed in the specimen chamber mounted with the CCD camera cooled to -90°C , with a field of view set at 25 cm above the sample shelf. The photon emission, transmitted from cell samples was measured in p/sec/cm²/sr. The bioluminescence color images were viewed using the living image software overlay (Xenogen). Bioluminescence was measured for the plates set up for imaging using substrate 50 $\mu\text{g/ml}$ Coelenterazine for RLuc cells and 30 mg/ml stock solution of D-Luciferin at 1: 200 dilution for FLuc cells. To check the effect of lower concentration of Coelenterazine, 25 $\mu\text{g/ml}$ of Coelenterazine was used to image ring cloning and 96 well plate cloning. Images were taken within the 10 sec of the Coelenterazine addition to the cells, due to the quick nature of the reaction. Coelenterazine was tested alone 1st in blank 2nd in only media.

Growth curve

Growth curves were determined to ensure that transfected cells show no difference to normal cells in their proliferation activity. Normal DLD1 cells, DLD1/SV-rLuc Cells and DLD1/SV-FLuc cells were seeded at 5×10^4 cells/ml. Fourteen flasks of each line were seeded. To count, cells were washed with Hanks, trypsinized, centrifuged, and resuspended in 5 ml of media and then counted manually using Haemocytometer every day for 7 days. Two flask of each type of cells were used every day to have precise average count of the cell.

MTT assay

The MTT assay was done to check that transfected and normal cells had same Chemosensitivity (transfection has not changed their nature to react with the chemicals). In brief, normal DLD1, DLD1/SV-rLuc Cells and DLD1/SV-FLuc cells were seeded at 1×10^5 cells/ml into 96 well plates. Column 1 containing culture medium 200 $\mu\text{l/well}$, only to blank the Spectrophotometer. Some drugs affect the optical density (OD) of the medium and require a separate blank. Column 2 containing 200 $\mu\text{l/well}$ only cell suspension but no drug exposure serves as control wells to calculate the control cell survival percentage rest of the plate cultivated with 180 $\mu\text{l/well}$ of cell suspension. The cells then left to adhere for 24 h in incubator at 37°C 5% CO₂ level. Next day, serial dilutions of both drugs were prepared (Table 2).

MB2 ranges from 1 μM to .1 ηM and Doxorubicin ranging from 100 μM to 10 ηM , and added 20 μl of each dilution to the plates respectively leaving Blank and

control with no drug. The plates were incubated at 37°C with 5% CO₂ level for 96 hours. MTT stock solution 5 mg/ml was prepared in distilled water. Stock solution of MTT was then diluted in complete RPMI 1640 at 1/10 ratio. All the media from the plates were removed and MTT solution 200 $\mu\text{l/well}$ was added in each well leaving blank. The plates were incubated for 4 hours at 37°C with 5% CO₂. MTT was removed from the plates very carefully and 150 μl of DMSO was added. Finally the absorbance at 540 nm of the plates was read with the Tecan plate reader. MTT assay were done in triplicate. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only i.e. no Drug added was taken as 100% cell viability i.e. the control.

Freezing down the cells

The best clones resulting high intensity of bioluminescence were washed with hanks, trypsinized, centrifuged and resuspended in 1 ml of complete RPMI 1640. Cells suspension was transferred to the 1.5 ml vials with 1:10 of DMSO and stored in freezer at -80°C for future use.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MRS and SS carried out the reporter gene cloning SV40 to make SV40-RLuc as a vector reporter to transfect the DLD1 cells and biochemical characterizations, MAA performed transfection studies. IY and AW carried out imaging and quantification of bioluminescence data. All authors have read and approved the final manuscript.

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