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Evaluation of Four Tumor-Specific Promoters in Various Cancer Cell Lines

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ABSTRACT

Specific tumor cell targeting remains a challenge for cancer gene therapy. One strategy in overcoming this challenge is by using tumor-specific promoters to drive the expression of delivered genes selectively in cancer cells. In this study, we selected 5′-upstream promoters of survivin, high-mobility group protein B2 (HMGB2), cyclooxygenase-2 (cox-2) and alpha-lactalbumin (LALBA), which have been observed to be significantly upregulated in tumors but have a low level of expression in normal tissues. Plasmid DNA coding for the Discosoma sp. red fluorescent protein (DsRed) under control of the selected promoters were constructed. The activities of these promoters in cancer cell line models of breast cancer (MCF-7 and MDA-MB-231), glioblastoma (U-87 MG and C6), and medulloblastoma (DAOY) were assessed by flow cytometry and a microplate-reader assay. The promoter of cox-2 exhibited the highest transfection efficiency and activity among all the tested promoters, suggesting its broad applicability among multiple cancer types. Furthermore, the LALBA promoter showed the highest selectivity for breast cancer and medulloblastoma. These results should provide a framework for the further development of highly specific and active promoters for targeted cancer gene therapy.

INTRODUCTION

Gene therapy has been proposed to treat cancerous diseases when administered as a single agent or combined with standard cancer therapies [1-3]. The goal of cancer gene therapy is to replace a mutated or disregulated gene with its functional counterpart, or to hijack the cellular machinery to induce a therapeutic response in cancer cells with minimal deleterious side effects to the normal surrounding tissue. It is capable of overcoming the obstacle of conventional cancer therapy, such as low therapeutic ratio, dose limiting, normal tissue toxicity, and inefficiency in preventing tumor invasion [4,5].

Up to present, tumor targeting is the major challenge facing the routine use of gene therapy in clinic [5,6]. Thereby, much effort has been devoted to discover aberrant pathways in cancer that could be exploited in selective cancer cell killing [7-10]. However, this strategy is generally inefficient at killing cancer cells and yet amenable to formation of resistance, mainly resulted from the large amount of signaling pathways and processes that responsible for tumorigenesis as well as the ability of cancer cells in building redundant or alternative pathways when one or two are affected [6,11-13]. Therefore, toxic genes or genes expressing therapeutic proteins and pro-drug activated enzymes have been employed, among which the most widely used genes include Bcl-2-related protein, herpes simplex virus type-1 thymidine kinase, human tumor necrosis factor α-related apoptosis-inducing ligand and cytosine deaminase [14-17]. But, targeted delivery to cancer tissues is essential since the mechanism of action for most of such therapeutic gene-encoded proteins is not tumor-specific. In this regard, numerous studies have been focused on engineering both viral and non-viral delivery agents for the selective delivery of genes to target tumor cells [18,19]. For instance, molecular targeting of cancer cells through targeting ligands on the surface of viral or non-viral gene delivery vehicles has been shown to enhance the uptake and expression of genes in tumors to improve transfection efficiency [20-24]. However, molecular targeting alone is

insufficient to diminish the off target uptake and expression of the delivered genes to healthy cells [16,25]. In order to achieve truly targeted and effective gene therapy with highly toxic genes, the expression of the delivered gene must be limited to the target cancer cells.

One strategy to ensure tumor-selective expression is through transcriptional targeting of cancer cells with a tissue-specific promoter [20,26,27]. However, expression of transgenes could be toxic to both normal and tumor tissue derived from same cell type [5]. More specific cancer cell expression can be achieved using tumor-specific promoters that could drive the expression of toxic genes or genes encoding enzymes that turn prodrugs into toxic compounds or induce cellular apoptosis [28,29]. This way the off-target uptake associated with both viral and non-viral gene delivery vehicles would not cause harmful side effects since the delivered toxic gene would not be expressed.

Tumor-specific promoters provide another way to overcome the issues from conventional gene therapy by selectively allowing expression of the gene of interest in cancer cells [30,31]. Genes driven by tumor-specific promoters are usually highly upregulated in cancer cells, and the selective transgene expression only in tumor cells using tumor-specific promoters has significantly promoted their application in the development of novel cancer gene therapy strategies. Among these tumor-specific promoters, the promoters of survivin, high-mobility group protein B2 (HMGB2), cyclooxygenase-2 (cox-2) and alpha-lactalbumin (LALBA) have been shown as potential candidates for cancer gene therapy [5]. Survivin is a member of the inhibitor of apoptosis protein (IAP) family, which has been found to be expressed in a variety of human cancers, including brain, breast, pancreas, esophageal, and ovarian tumors [32-35]. It is completely absent in terminally differentiated cells making it a promising tumor-specific promoter candidate. Cox-2 is an inducible isoform of the cyclooxygenase family and is virtually undetectable in most tissues under physiological conditions. The overexpression of cox-2 is evidenced in breast cancer and closely linked to the progression of this type of cancer [36-38]. Cox-2 is also highly expressed in medulloblastoma and the cox-2 promoter is capable in enhancing transcriptional targeting in glioblastoma [39,40]. Similarly, HMGB2 has been shown to have high level expression in glioblastoma, medulloblastoma, and breast ductal carcinoma but a limited expression in adult organs [41,42]. The promoter of HMGB2 was recently reported to be effective in gene delivery in glioblastoma [29]. Human LALBA is a 15 kD protein involved in the modification of galactosyl transferase, an enzyme involved in lactose production. Its expression is completely absent in adult tissues except at the onset of parturition and then decrease a few weeks after birth [43,44]. It has been found highly upregulated in breast cancer tissue in more than half of clinical cases, and thus the promoter of LALBA has been reported as candidate for functional tumor-specific promoter in human breast cancer [45,46].

A better understanding of their behaviors across cancer types will be an important contribution to the improvement of the efficiency of cancer treatment and targeted gene therapies in the future. But to our knowledge, no comparative analysis has been carried out to identify the functions of these promoters in different types of cancer. Reporter gene assay has served as the most widely used approach in the evaluation of promoter activity in spite of its well-known disadvantages such as the necessity of the lysis of cells, the addition of the substrate and the time dependence of the luminescence signal. Flow cytometry has emerged as an efficient method mainly due to its capability of allowing direct quantification of reporter gene expression in individual cells of the transfected population without any processing steps. Also, concurrent measurement of promoter activity and transfection efficiency is enabled, which is often not feasible for reporter gene assays. In view of these facts, the selective expression of the fluorescence reporter protein, DsRed, provided by promoters of survivin, HMGB2, cox-2, and LALBA were tested in cell lines of breast cancer, glioblastoma, and medulloblastoma in the present study. Non-cancerous rat astrocytes and human umbilical vein endothelial cells (HUVECs) served as controls. Flow cytometry was used to identify the transfection efficiency and activity of each promoter in the cell lines. A comparison between flow cytometry and microplate-reader assay in assessing DsRed fluorescence intensity was also made.

MATERIALS AND METHODS

Cell Lines and Reagents

All cell lines were purchased from american type culture collection (ATCC) and all tissue culture reagents purchased from life technologies unless specified otherwise. Human breast cancer cell line MDA-MB-231, rat glioblastoma cell line C6 and human medulloblastoma cell line DAOY were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% antibiotic-antimycotic. Human breast cancer cell line MCF-7 and human glioblastoma cell line U-87 MG were cultured in eagle's minimum essential medium (ATCC) supplemented with 10% FBS and 1% antibiotic-antimycotic. Primary rat astrocytes (Lonza, catalog #: R-CXAS-520) were cultured in rat astrocyte growth medium (Cell Applications). Primary HUVECs (Lonza, catalog #: CC-2519) were cultured in endothelial cell growth medium (Cell Applications). All cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 2-3 days.

Construction of Plasmids

Genomic DNA was extracted from U-87 MG and MCF-7 cells using a DNA extraction kit following the manufacturer's protocol

(Qiagen). All the promoters were then PCR amplified using the following primers listed in **Table 1.** PCR products were cloned into PCR 2.1 vector using the TOPO cloning kit following the manufacture's protocol (Life Technologies). pDsRed-Max-N1 vector (Addgene plasmid 21718, Addgene Inc.) carrying the DsRed gene was chosen as the backbone vector. Both the promoter region and pDsRed-Max-N1 vector was excised with AseI and XhoI (New England Biolabs), and the digested fragments were subcloned into the vector upstream of DsRed [47] **(Figure 1a).**

Table 1. PCR primers.

Promoters	Forward primer	Reverse primer
Survivin	CC*ATTAAT*AGGAAACAGGCAAAACAT	CCG*CTCGAG*AGCGCACGCCCTCTTAG
HMGB2	CC*ATTAAT*GACAGACAGGACCTAAATGGTGGTC	CCG*CTCGAG*ATCCCCACTAATCTGATTGGTTCTG
cox-2	CC*ATTAAT*GAAGCGCTCGGGCAAAGAC	CCG*CTCGAG*TGCTGAGGAGTTCCTGGACG
LALBA	CC*ATTAAT*GGGCTCAAGTGATCCACCAG	CCG*CTCGAG*CGGGCAGGGAACAGGATGC

Cell Transfection

Transfection of cancer cell lines and rat astrocytes was conducted with Lipofectamine 2000 and Lipofectamine LTX (Life Technologies), respectively. One day before transfection, cells (1.5 × 105) were plated in 12-well plates with growth medium without antibiotics. After cells reached 85-90% confluence, they were transfected with a DsRed gene under the control of a testing promoter construct. The basic pDsRed vector containing the cytomegalovirus (CMV) promoter was used as a positive control for transfection efficiency. Lipofectamine 2000 at a ratio of DNA: Lipofectamine (ng:µl) of 2.5:1 was used throughout the study for cancer cell lines. Lipofectamine LTX was used in transfection of rat astrocytes due to its low toxicity. The ratio of DNA: Lipofectamine LTX: PLUS reagent (ng:µl:µl) is 1:1.5:1.

Fluorescence Microscopy

For each transfection agent, cells were seeded on to 22-mm glass coverslips 12-16 hr prior to transfection. Cells were transfected as described above, and then 48 hr after transfection were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and fixed with 4% formaldehyde (methanol free, Polysciences Inc.) in DPBS for 30 min. Fixative was then removed and cells were washed with DPBS to remove the formaldehyde. The slides were mounted using ProLong Gold antifade solution containing DAPI (Life Technologies) and imaged using a fluorescence microscope equipped with a 405 nm diode and 575 nm laser for collection of DAPI and DsRed emission signals, respectively.

Flow Cytometry Analysis

To determine the transfection efficiencies and activities of the promoters, cells were washed with DPBS, trypsinized, and suspended in DPBS containing 2% FBS 48 hr after transfection with plasmid vectors harboring different tumor-specific promoters. Analysis of at least 10,000 cells for each sample was performed on a BD FACS Canto flow cytometer (Beckton Dickinson). Forward and side scatter was used to distinguish viable cells and data was analyzed using the FlowJo software package (Tree Star).

Microplate-Reader Assay

To determine the fluorescence intensity of the entire cell population a microplate-reader assay was used. Cells were treated with each of the plasmids as described above. Forty-eight hours after transfection, cells were lysed in 0.05% Triton-X at 4°C for 20 min. After lysis, cell debris was removed by centrifuging at 12000 × g for 10 min and 600 µl of the cell lysate supernatant was used for microplate fluorescence assays. Protein concentrations were determined with Bradford protein assay kit (Biorad) according to the manufacturer's instructions. The fluorescence intensity was determined using a Spectrum microplate-reader with set of excitation and emission wavelength at 560 nm and 605 nm respectively for DsRed.

RESULTS

DsRed expression under the control of tumor-specific promoters.

Fluorescence reporter protein (DsRed) encoding plasmids were constructed under the control of survivin, cox-2, HMGB2, and LALBA promoters through subcloning into the pDs-Red-Max-N1 vector **(Figure 1a).** The constructs were confirmed by restriction digestion with AseI and XhoI, where the digested fragment had exactly the same size of the promoter inserted as indicated from the gel **(Figure 1b).**

To determine the activities of the different promoters, the plasmids were transfected into MCF-7 and MDA-MB-231 breast cancer cells, U-87 MG and C6 glioblastoma cells, and DAOY medulloblastoma cells. Rat astrocytes and HUVECs were used as a healthy cell control. Forty-eight hours after transfection, cells were examined using fluorescence microscopy. The CMV promoter provided the highest fluorescence signal through all cancer cell lines as expected for this high activity promoter. It also achieved higher transfections than the tumor-specific promoters in rat astrocytes and HUVECs **(Figure 2).** The cox-2 promoter showed substantially higher activity compared with other tumor-specific promoters in most tested cell lines, while the survivin and HMGB2

promoters demonstrated similar behavior within all cell lines. DsRed fluorescence signal with the LALBA promoter could be hardly observed except in MDA-MB-231 and DAOY **(Figure 3).**

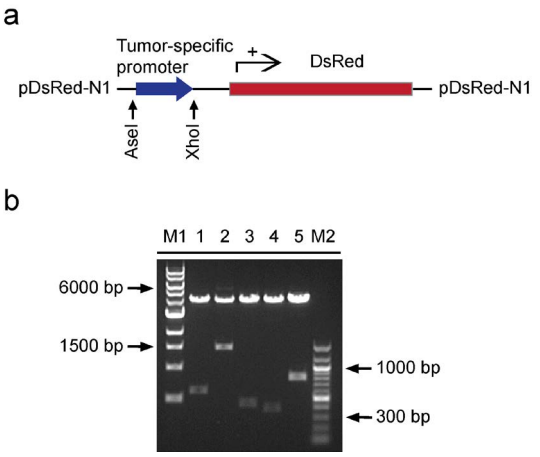

Figure 1. Construction of pDsRed plasmid containing tumor-specific promoters. (a) Schematic representation of plasmid construction. Arrows indicate the position of restriction sites used for cloning into pDsRed-N1 backbone. (b) Restriction digestion analysis of the plasmid containing promoters. M1, 1 kbp DNA marker; 1, pDsRed-MAX-N1; 2, pDsRed-Survivin; 3, pDsRed-HMGB2; 4, pDsRed-Cox-2; 5, pDsRed-LALBA; M2, 100 bp DNA Marker.

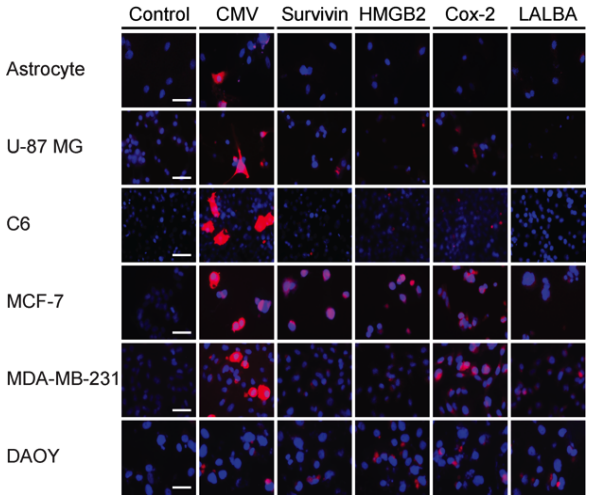

Figure 2. Overlaid fluorescent images of DsRed fluorescence (red) and nuclei-(blue) stained cells. The expression of DsRed under the control of CMV promoter acted as positive control. Images were taken with 20× lens. Scale bar = 100 µm.

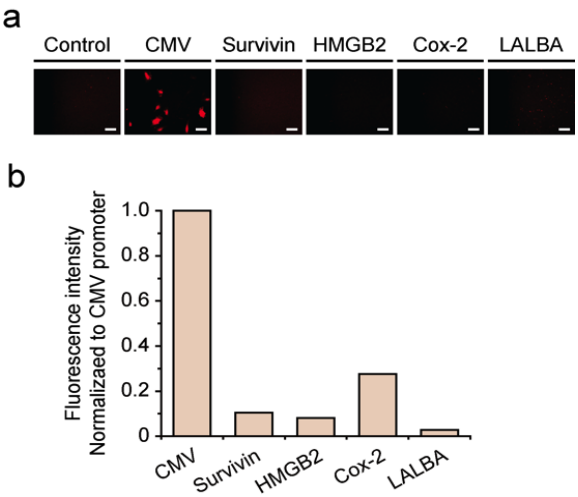

Figure 3. Assessment of promoter activities in HUVECs. (a) Fluorescent images of DsRed fluorescence. The expression of DsRed under the control of CMV promoter acted as positive control. Images were taken with 10× lens. Scale bar = 100 µm. (b) Mean fluorescence intensity of DsRed transfected cells normalized against the CMV promoter by flow cytometry.

Transfection Efficiency of DsRed under the Control of Tumor-Specific Promoters

The transfection efficiencies were quantified by flow cytometry. The percent of cells showing DsRed fluorescence signal

reflects the ability of promoters in driving transgene expression. **Figure 4** shows the typical flow cytometry plot, and the numbers indicate the percentage of transfected cell population in each of the cell lines. As expected, the CMV promoter exhibited the highest transfection efficiency through all testing cell lines, especially in U-87 MG where 42% of the entire cell population expressed DsRed. CMV had lower activity in rat astrocytes as compared to cancer cell lines, although optimal transfection reagent and conditions were adapted. Among the tumor-specific promoters examined, cox-2 promoter induced the highest percentage of transfected cells in all cell lines. Relative to the cox-2 promoter, the survivin promoter construct showed decreased transfection efficiency whereas the HMGB2 promoter indicated even a moderate lower value in the non medulloblastoma cell lines. In DAOY medulloblastoma cells, DsRed signal was hardly observed under the control of all tested promoters other than cox-2. In addition, no obvious expression of DsRed driven by LALBA promoter was detected, which corresponded well with the fluorescence images.

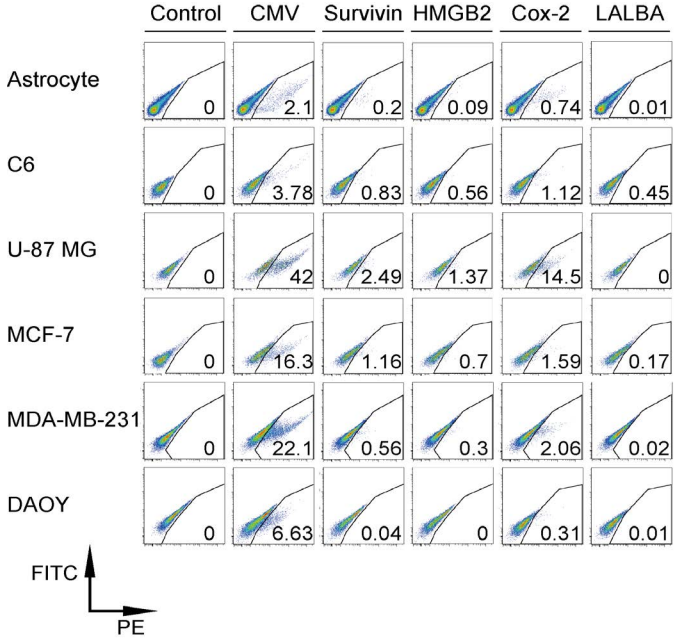

Figure 4. Flow cytometriy analysis of DsRed expression under the control of tumor-specific promoters. Representative dot plots illustrate DsRed-expressing cells and numbers indicate percent of DsRed positive cells.

Transfection efficiency of tumor-specific promoters was also normalized against CMV promoter so that promoter activities could be directly compared between cell lines. As seen from **Figure 5a**, cox-2 promoter had the highest transfection efficiency among the four tumor-specific promoters in both glioblastoma cell lines, while no transfection efficiency was observed with LALBA promoter. Promoters of survivin and HMGB2 displayed higher transfection efficiency in C6, whereas cox-2 promoter appeared to have nearly 2.3-fold higher transfection efficiency in U-87 MG. Unlike the glioblastoma cells, higher transfection efficiency of DsRed under the control of all promoters was detected in MCF-7 as compared to MDA-MB-231 **(Figure 5a)**, although cox-2 promoter also indicated the highest transfection. Transfection profiles of the four tested promoters in DAOY were fairly similar with the other tested cancer cells with cox-2 and LALBA promoters exhibiting the highest and lowest transfection, respectively **(Figure 6a).** Regarding cancer type selectivity, promoter of survivin indicated the highest and lowest transfection efficiency in C6 and DAOY, respectively, and the behaviors of this promoter in U-87 MG and MCF-7 was close. The HMGB2 promoter displayed similar selectivity with survivin in all cancer cell lines examined, while cox-2 promoter had the highest transfection efficiency in U-87 MG. Additionally, the LALAB promoter was more effective in driving DsRed expression in MCF-7 as compared to MDA-MB-231.

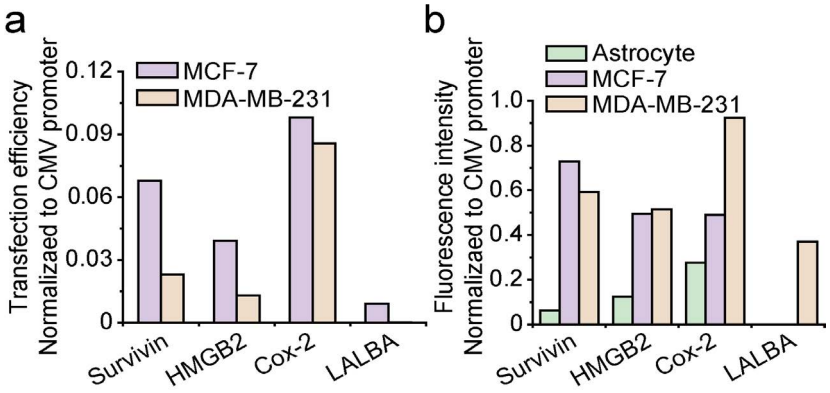

Figure 5. Quantitative assessment of promoter activities in glioblastoma cells by flow cytometry. (a) Transfection efficiency with DsRed expression. The percentage of DsRed positive cells with tumor-specific promoters was normalized against the CMV promoter. (b) Median fluorescence intensity of DsRed transfected cells normalized against the CMV promoter.

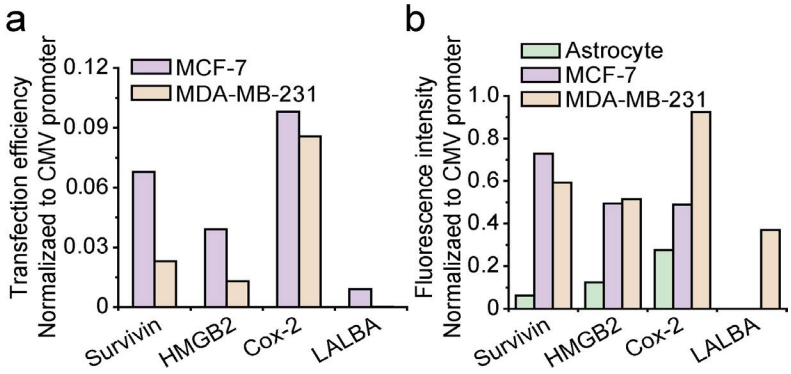

Figure 6. Quantitative assessment of promoter activities in breast cancer cells by flow cytometry. (a) Transfection efficiency with DsRed expression. The percentage of DsRed positive cells with tumor-specific promoters was normalized against the CMV promoter. (b) Median fluorescence intensity of DsRed transfected cells normalized against the CMV promoter.

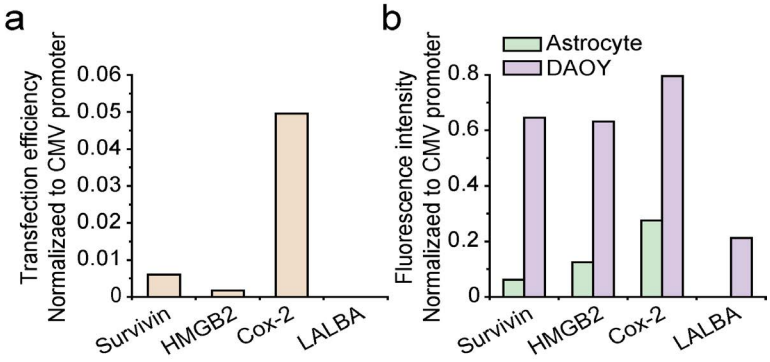

Figure 7. Quantitative assessment of promoter activities in medulloblastoma cells by flow cytometry. (a) Transfection efficiency with DsRed expression. The percentage of DsRed positive cells with tumor-specific promoters was normalized against the CMV promoter. (b) Median fluorescence intensity of DsRed transfected cells normalized against the CMV promoter.

Analysis of Fluorescence Intensity of DsRed under the Control of Tumor-Specific Promoters

Along with transfection efficiency, the fluorescence intensity of DsRed in transfected cells provides quantitative information on the activity of the promoters. The successfully transfected cells expressing DsRed were analyzed using flow cytometry to determine the amount of gene product expressed in each transfected cell. To accurately compare promoter activities among cell lines, the value for all promoters were normalized to the CMV promoter.

Normalized fluorescence intensities of all tumor-specific promoters were lower in rat astrocytes than in cancer cell lines, and similar results were observed in HUVECs **(Figure 2).** Promoters of survivin, HMGB2 and cox-2 showed significantly higher DsRed fluorescence intensity in C6 as compared to that in U-87 MG, while LALBA promoter did not indicate any fluorescence signal in either of the glioblastoma cell lines **(Figure 5b).** This is in line with the transfection efficiency analysis that survivin and HMGB2 promoters exhibited more pronounced transfection efficiency in C6, whereas no transfection was observed with LALBA promoter **(Figure 5a).** Similar to the results acquired from glioblastoma cell lines, all promoters had considerably higher activities in breast cancer cells than astrocytes and HUVECs **(Figure 6b and Figure 2).** Survivin promoter displayed higher DsRed fluorescence intensity in MCF-7 when compared with MDA-MB-231, while promoters of HMGB2 and cox-2 showed totally opposite transfection profile as shown in **Figure 6b.** Additionally, the normalized DsRed expression was decently high under the control of LALBA promoter in MDA-MB-231 cells although transfection efficiency was low. The completely opposite phenomenon was observed in MCF-7 cells, where relatively higher transfection efficiency was accompanied with neglectable DsRed fluorescence intensity **(Figures 6a and 6b).** Furthermore, we assessed the behaviors of tumor-specific promoters in human DAOY medulloblastoma cells, which were noticed fairly close to their performances in MDA-MB-231 **(Figure 7b).**

Among the tested cancer cell lines, all of the promoters except for LALBA showed the highest expression of DsRed in C6 and lowest in U-87 MG. Fluorescence intensity of DsRed dominated by promoter of cox-2 in MDA-MB-231 was almost the same as that in C6, while nearly two times and 20% higher than that in MCF-7 and DAOY, respectively. The promoters of survivin and HMGB2 had similar performance cross tested cancer cell lines, except that the survivin promoter showed higher activity in MCF-7 than in MDA-MB-231 while it was the opposite for HMGB2. The relative fluorescence intensity of LALBA promoter was only acquirable in MDA-MB-231 and DAOY, and it was roughly 76% higher in MDA-MB-231 than DAOY. The amount of gene product expressed in astrocyte cells was the lowest among all the promoters.

Comparison of Microplate-Reader Assay with Flow Cytometry Analysis

To determine the fluorescence intensity of the entire cell population (expressing and non-expressing cells), microplate-reader

assay was conducted. Although still measuring fluorescence intensity with this technique, different information could be acquired since off-target cells are also considered, which is more representative of the gene expression in tumor. Forty-eight hours after transfections, cells were lysed with Triton X-100 and the fluorescence intensity of the cell population was measured. DsRed fluorescence intensity acquired from tumor-specific promoters was also normalized to CMV promoter for the purpose of comparing promoter activity among cells lines.

Overall, DsRed fluorescence intensity measured by microplate-reader assay coordinates with that from flow cytometry in indicating promoter activities. No detectible DsRed fluorescence was observed in astrocyte cells, except a low expression level with survivin promoter (data not shown). However, DsRed expression manipulated by promoters was higher from flow cytometry analysis as compared to the microplate-reader assay. As shown in **Figure 8a**, all tumor-specific promoters indicated higher activities in C6 as compared to U-87 MG. Unlike flow cytometry analysis, DsRed expression under LALBA promoter was revealed by microplate-reader assay in both glioblastoma cell lines **(Figure 8a).** In MCF-7, the activity of survivin promoter was slightly lower than that of cox-2 promoter, which was somehow opposite to the result from flow cytometry assay. All promoters displayed lower activities in MDA-MB-231 **(Figure 8b)**, which is also different from flow cytometry analysis indicating higher surviving promoter only in MCF-7 **(Figure 6b)**. In DAOY, LALBA and survivin promoters exhibited similar activity, which was about 57% of that indicated by cox-2 promoter **(Figure 8c)**. This correlates with the flow cytometry quantification well, except for the higher LALBA promoter activity than promoters of surviving and HMGB2.

It was also noticed that the promoter of survivin showed the highest specificity in MCF-7, although it displayed very little activity in another breast cancer cell line, MDA-MB-231. Promoters of survivin, HMGB2 and LALBA all indicated relatively high activities in C6 followed successively by DAOY and U-87 MG. Cox-2 promoter exhibited equally high activities in C6 and MCF-7 and similar relative DsRed fluorescence intensity in U-87 MG (0.08 ± 0.005), MDA-MB-231 (0.07 ± 0.003) and DAOY (0.07 ± 0.02).

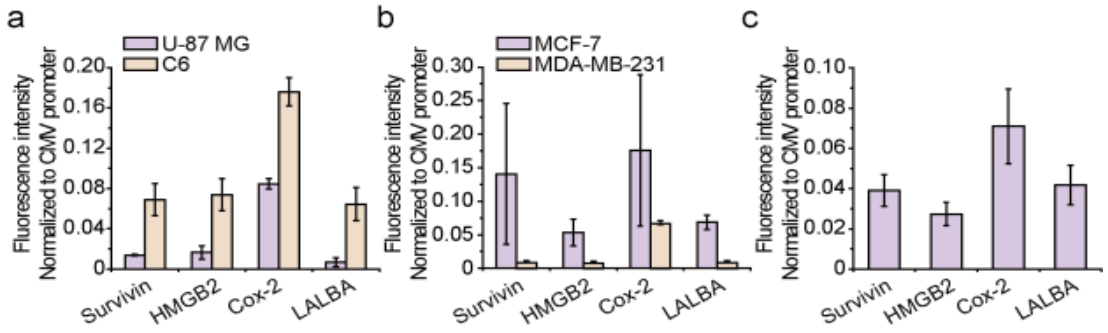

Figure 8. Activities of tumor-specific promoters assessed by microplate-reader assay. Bar charts show Fluorescence intensity of DsRed manipulated by the tumor-specific promoters in (a) glioblastoma cells, (b) breast cancer cells, and (c) medulloblastoma cells after normalization to the CMV promoter. Data are expressed as the mean ± SD of three independent experiments.

DISCUSSION

The rapid development of cancer gene therapeutics and limitations on accuracy and efficiency of gene delivery have prompted the need for effective gene therapy vectors, which can be achieved by the use of promoters to drive the expression of gene of interest in specific cancer types. Several groups of proteins have been found overexpressed in tumor while undetectable in normal tissues and thereby the upstream regulatory sequences of the genes coding those proteins are being increasingly used for direct expression of heterologous genes specifically in cancer cells. In this study, we aimed at evaluating the preferential activities of tumor-specific promoters in various cancer cell lines as there is insufficient information on the crossing behavior of tumor-specific promoters.

The commonly used reporter genes include firefly luciferase, β-galactosidase, and chloramphenicol acetyl transferase [48,49]. However, these methods demand the lysis of cells and the addition of substrate followed by detection of the generated luminescence signal, while the sensitivity of the assay cannot be assured. By contrast, it is more convenient and straightforward to use fluorescence reporter genes such as DsRed. Stable expression of this protein provides living cells with a bright internal fluorescence signal detectable through microscopy. In addition, cellular auto-fluorescence is much lower in the wavelengths used for DsRed detection as compared to green fluorescent protein (GFP), another common used florescent protein [50]. We also noticed that DsRed has greater fluorescence signal than GFP (data not shown), suggesting the high sensitivity of our assay.

In this study, we selected certain 5' upstream promoter regions of the four genes based on previous reports and investigated their activities in different cancer cell lines by testing promoter-driven DsRed expression. Rat astrocytes, HUVECs, and CMV promoter served as negative control cell lines and positive control promoter, respectively. In fact, transfection could vary among plasmids harboring different promoters due to their different quality and quantity since each of these plasmid vectors was separately prepared. Additionally, transfection varied among different cell lines owing to their respective response to the transfection conditions as indicated by the flow cytometric analysis **(Figure 4)**. In this regard, distinct transfection reagents were used for non-cancerous cells and cancer cells, and transfection conditions were also optimized. Nevertheless, such variations

should affect the expression of DsRed gene in a similar manner in the same cell lines. In this regard, we assumed that promoter activity could be reflected from the normalized fluorescence intensity.

Among the four tumor-specific promoters, the promoter of cox-2 exhibited the highest transfection efficiency and activity across cancer cell lines with even a little activity in astrocytes as observed from flow cytometry. It coordinates with other studies that the expression of cox-2 is upgraded in several cancer tissues [51,52]. It was also noticed that these promoters indicated specificity in only certain cancer cell lines instead of cancer types. For instance, the promoter of survivin indicated lower activity in MDA-MB-231 as compared to MCF-7, while the rest of tested promoters were the opposite. It suggests that promoter activity is closely associated with the genetic complexity and heterogeneity of individual cell types, such as different gene profiles and cellular contexts. This finding could actually complicate cancer therapy because of the difficulties in efficient targeting of entire tumor cell populations considering the cell heterogeneity related differentiation states and tumor progression. In this regard, utility of a ubiquitous promoter, or a promoter with balanced activity and specificity would be appropriate in cancer gene therapy in order to ensure the expression of transgenes only in the majority of cell populations.

From the comparison between microplate-reader assay and flow cytometry assay we noticed that results obtained from these two methods were not identical. It was speculated that the fluorescence intensity measured by flow cytometry is more accurate as it permits the analysis of individual cells manifesting DsRed expression, particularly when taking into consideration the low transfection efficiency due to resistant to gene transfection of many cell lines used in promoter studies. In contrast, the entire lysed cell population is included in microplate-reader assay even with protein concentration normalization afterwards. The results thereby are always subject to some reservations. But it is surprising that results from these two assays were not dramatically distinct in our studies. We suspected that the high efficiency of DsRed fluorescence contributed to increasing the sensitivity of microplate-reader assay.

CONCLUSION

In this study, we demonstrated the availability of DsRed in evaluating promoter activities, and revealed the selectivity of tumor-specific promoters in cancer cell lines. Both flow cytometry and microplate-reader assays displayed the universal therapeutic potential of cox-2 promoter in glioblastoma, breast cancer, and medulloblastoma. Moreover, our studies can give valuable suggestion on the selective application of these promoters in individual cell lines.

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