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ESTABLISHMENT OF A HIGHLY SENSITIVE CELL-BASED ASSAY FOR SCREENING OF HYPOXIA-INDUCIBLE FACTOR REGULATORS

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Abstract

Cell-based assay is a powerful tool in the fields of drug discovery. One of the main targets of drug development is transcription factors. Hypoxia-inducible factors (HIFs) are transcription factors involved in cellular oxygen homeostasis and associated with disease pathophysiology. Currently, a highly sensitive cell-based assay to measure HIF activity is still lacking. Using luciferase reporter constructs, we developed a highly sensitive, stable reporter cell line for monitoring the activity of HIF. Four copies of hypoxia response element (HRE) of the erythropoietin (EPO) gene, which is one of the targets of HIF, were used to drive the expression of the luciferase reporter. This stable reporter cell line gave a 500-fold increase in the luciferase signal in hypoxia compared to a normoxia condition. This robust increase in hypoxia response is crucial to ensure consistency and reproducibility of HIF assay results while reducing the overall assay cost. The establishment of this highly sensitive cell-based HIF assay may help expedite research in the discovery of HIF regulatory drugs.

INTRODUCTION

Hypoxia, a condition of lower oxygen concentration than normal level in the human body is reputed to be associated with many pathobiological diseases, including ischemic diseases [1, 2], infectious diseases [3, 4], heart diseases [5], chronic kidney diseases [6], vascular diseases [7], inflammatory diseases [8], neurodegenerative diseases [9] and advanced cancer progressions [10, 11]. The hypoxic microenvironment inside tumours is owing to the irregular growth of blood vessels and the vasculature system [12]. In addition to cell death, hypoxic tumours or cancer cells exhibit higher resistance to current cancer treatment modalities, including radiotherapy and chemotherapy, resulting in poor prognosis and increased mortality [13].

The master regulators that help cells to survive in hypoxia are hypoxia-inducible factors (HIFs) [14]. HIFs are transcription factors that consist of an oxygen-dependent α subunit and a constitutively-expressed β subunit. There are three isoforms of the α subunit, which are HIF-1α, HIF-2α and HIF-3α. These HIF transcription factors can orchestrate the downstream gene expression for cellular hypoxia acclimation, such as cell survival, growth and cell proliferation [15]. When cells are exposed to hypoxia conditions, HIF will be stabilized and specifically interact with the hypoxia response element (HRE) existing in the promoter regions of HIF target genes. This specific binding leads to the transcription and translation of genes responsible for cellular adaptation to lower oxygen concentration. In conjunction with cellular adaptation, HIF also serves as the crucial transcription factor to survive the cells in pathogenic hypoxic conditions, especially in ischemia and cancer progression. Hence, HIF is ideal to be therapeutically targeted for drug discovery and development in recent decades [16, 17].

Modern drug discovery has been accelerated by the use of cell-based assays. Currently, these assays are utilized for lead identification, drug dose optimization as well as target validation. The use of small-scale yet high-throughput techniques make these cell-based assays more efficient and cheaper for drug screening. These assays are preferred over other in vitro methods since they give direct cellular functional responses to the drug target of interest. This allows for a clearer understanding of physiological and pharmacological responses to the target. Demand for assays to quantitate HIF activities is tremendous. This is due to the fact that besides being involved in various cellular regulations from proliferation, apoptosis to cancer development, HIF has also been shown to be involved in stem cell renewal and differentiation [18]. The boost of stem cell research in the last decade has raised the need for a more robust cell-based assay system to measure HIF activities, particularly in HIF-related drug screening. Unfortunately, the systems which are currently available are costly, time-consuming and, most importantly, provide limited sensitivity. The choice of cell lines is also restrictive. To address these issues, more efficient, stringent, cost-effective and high-throughput methods are needed. New small-scale cell-based assays using well-studied cell lines will also allow for direct cellular functional responses to the drug target of interest.

The discovery of a new highly sensitive and robust cell-based assay system will fulfill this demand. The cell line used in this system will have to be extensively studied to correlate the assay findings to the *in vivo* situations. In the present study, we describe the development of a highly sensitive monoclonal cell-based assay for the screening of HIF activity through clonal selection.

MATERIALS AND METHODS

Plasmids, Cells and Transfection

Four copies of the HRE of the EPO gene (4xEPO) [19] were cloned into the pLUC-MCS plasmid vector (Stratagene, USA). Cells, seeded in triplicates, were co-transfected with an HRE-driven firefly luciferase reporter construct and pRL-CMV (Promega), expressing Renilla luciferase (internal control for transfection efficiency), using the Effectene reagent (QIAGENE) [20-21]. The resulting plasmid construct designated as 4XEPO-HRE-FLuc was co-transfection with the pcDNA3 plasmid (Invitrogen, Massachusetts, USA) carrying a neomycin-resistant gene into the human osteosarcoma cells (Saos-2, ATCC# HTB-85). The transfection was performed using a 10:1 ratio of 4XEPO-HRE-FLuc:pcDNA3 with the Lipofectamine 2000 (Invitrogen, Massachusetts, USA) following protocols suggested by the manufacturer. Transfected cells were cultured in antibiotic-free DMEM (PAA Laboratories, Pasching, Austria) supplemented with 5% FBS (PAA Laboratories, Pasching, Austria) for 48 hours in a humidified incubator supplied with 5% CO_2 at 37ºC.

Selection of Stably-transfected Cells

Spent media in the transfected cells were replaced with fresh DMEM containing 1 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS. The cells were incubated for another three days before the media was replaced again with the G418-containing DMEM. This media replacement was repeated every three days for three weeks. After three weeks, the surviving cells were trypsinized and propagated further. A single luciferase assay was performed to confirm the HIF reporter activity of this neomycin-resistant mass population of cells.

Luciferase Reporter Assay

Initially, 2.5 X 10^4 of the cells were plated in wells of a 48-well plate in antibiotic-free DMEM with 10% FBS for 16 hours. One of the plates was then incubated for 24 hours in normoxia (21% O_2) in a humidified CO_2 incubator while the other was incubated in hypoxia (0.5% O_2) in a hypoxic chamber (BioSpherix, USA) controlled by an oxygen regulator (ProOx model 110, BioSpherix, USA). After 24 hours of exposure to different oxygen concentrations, Bright-Glo™ Luciferase Assay (Promega, USA) was used to analyze the HIF reporter activity. To do this, the spent media in each well was discarded and the cells were rinsed once with 300 µl of 1XPBS. Room temperature-equilibrated Glo Lysis Buffer (GLB; 60 µl) was then added to the wells. Plates were then gently rocked on a shaker for 5 min at room temperature. Cell lysis was confirmed visually by observing the wells of the 48-well plate under a light microscope. Thirty microliters of the resulting cell lysates were then transferred into individual glass vials. Bright-Glo™ Assay Reagent was then added to each tube at a 1:1 (v/v) ratio. Luminescence intensity was immediately read and recorded by a Luminometer (Sirius-2, Titertek- Berthold, Germany).

Isolation of Single Cells and Clonal Expansion

After confirmation of the HIF reporter activity in the mass population of the stable parental G418-resistant cells, single-cell cloning was performed using a limiting serial dilution cloning strategy. The parental cells were seeded in the first-row wells (A1-A8) of a 96-well plate at 1.5 X 10^4 cells/well. After overnight incubation, spent media in the wells was pipetted out carefully, and the cells were washed with 50 µl 1X PBS. Cells were trypsinized and resuspended in 100 µl of DMEM supplemented with G418 and 10% FBS. One hundred microliters of the same media were pipetted into the remaining nine rows of empty wells. Cell suspension from the first well (100 µl) was then transferred into the adjacent well, and the mixture was pipetted up and down. The same volume of cell suspension was then transferred to the next

well. This process was repeated to obtain a two-fold serial dilution of the cell suspension. All the cell suspension from the last well (200 µl) was diluted in 10 ml of conditioned DMEM supplemented with G418 and 10% FBS. The mixture was then inverted up and down to ensure the even distribution of cells. This cell suspension (100 µl) was subsequently aliquoted into wells of a 96-well plate. The plate was then incubated in a 45º slanted position for 5 hours at 37ºC. After the incubation, each well was examined for the presence of single attached cells. Wells containing single cells were properly labelled. The plate was subjected to further incubation at 37 ºC for 7 to 14 days. Individual cells in the wells were visualized, and images were captured every four days. Single cells which grew into a colony were trypsinized and transferred into wells of a 48-well plate. As the number of cells gradually increased, the subculturing was performed into bigger tissue-culture containers. The resulting cell clones were then cryopreserved for long-term storage.

HIF Activity Verification using HIF Regulatory Drugs

To verify the property of the stable single-cell population as an assay system to measure HIF activity, a proof of concept study was performed using commercially available HIF inhibitors. They were Bortezomib (Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) and Cisplatin (Sigma-Aldrich, St. Louis, MO, USA). Cells were seeded at 1.5 X 10^4 cells per well in 96-well plates for 24 h, followed by treatment with selected concentrations of Bortezomib [20] and cisplatin. The cells were then incubated under either normoxic or hypoxic conditions for 24 hours. HIF inhibitory effects of the drugs were examined using the single luciferase assay system as described above. To ensure that the cells were still viable in the presence of drug treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide MTT assay was simultaneously performed on replicate plates.

RESULTS AND DISCUSSION

The Saos-2 human osteosarcoma cell line was co-transfected with the recombinant 4XEPO-HRE-FLuc and the pCDNA3 plasmids harbouring the Neomycin-resistant gene. The resulting transfectants were then treated with G418 antibiotics. Throughout the course of treatment, changes in the cell culture were monitored under a light microscope. Representative images of cellular morphological changes at selected times are shown in Figure 1A. On day three post drug treatment, most of the cells in the culture became rounded up and detached from the cell culture flasks. These cells were removed together with the spent media during the 3-day interval media replacement steps. On day nine post-treatment, cells with typical epithelial Saos-2 morphology were visible. By day 14, the number of these cells appeared to be increased. Despite a slight reduction in the number of cells with normal morphology on day 16, these cells continued to multiply and reached a high degree of confluency from day 20 onwards. In order to determine whether this G418-resistant cell population retained a functional recombinant 4XEPO-HRE-FLuc gene construct, they were subjected to a luciferase reporter assay. When the cells were cultured under a normoxia condition, there was a negligible level of luciferase expression (Fig. 1B). However, when the cells were cultured under a hypoxic condition, the luciferase expression increased by 88-fold compared to the normoxia level. These data confirmed that the stable transfectant population was capable of expressing luciferase in response to hypoxia conditions. To establish single-cell clones from this mass cell population, they were then subjected to serial limiting dilutions and subsequent cultures.

In order to obtain the single-cell clones, which exhibit higher sensitivity in HIF responsiveness in the study, single-cell cloning by limiting serial dilutions was performed on mass cell populations. Following a serial limiting dilution cloning strategy, a number of single cells were observed in individual wells of a 96-well plate. A representative image of such a cell is shown in Figure 2A. Four of these individual cells proliferated to form cell colonies. These colonies were visible when they were observed on day ten following the initial culture (Fig. 2A). They continued to multiply, and by day 21, the wells of the 96-well plate became confluent. To test the level of hypoxia-responsive luciferase signal of each of the four cell clones, they were subjected to the luciferase reporter assay. Fold difference of luciferase signals was made against their corresponding cultures in the normoxic condition. Data obtained showed that all four clones showed an increased fold difference (Fig. 2B) compared to the parental mass population (Fig. 1B). Out of the four clones, clone 3 (C3) gave the highest luciferase signal which was 517-fold higher than its normoxia control (Fig. 2B). To the best of our knowledge, this is the highest fold-difference of a hypoxia-responsive signal shown in a stable cell clone.

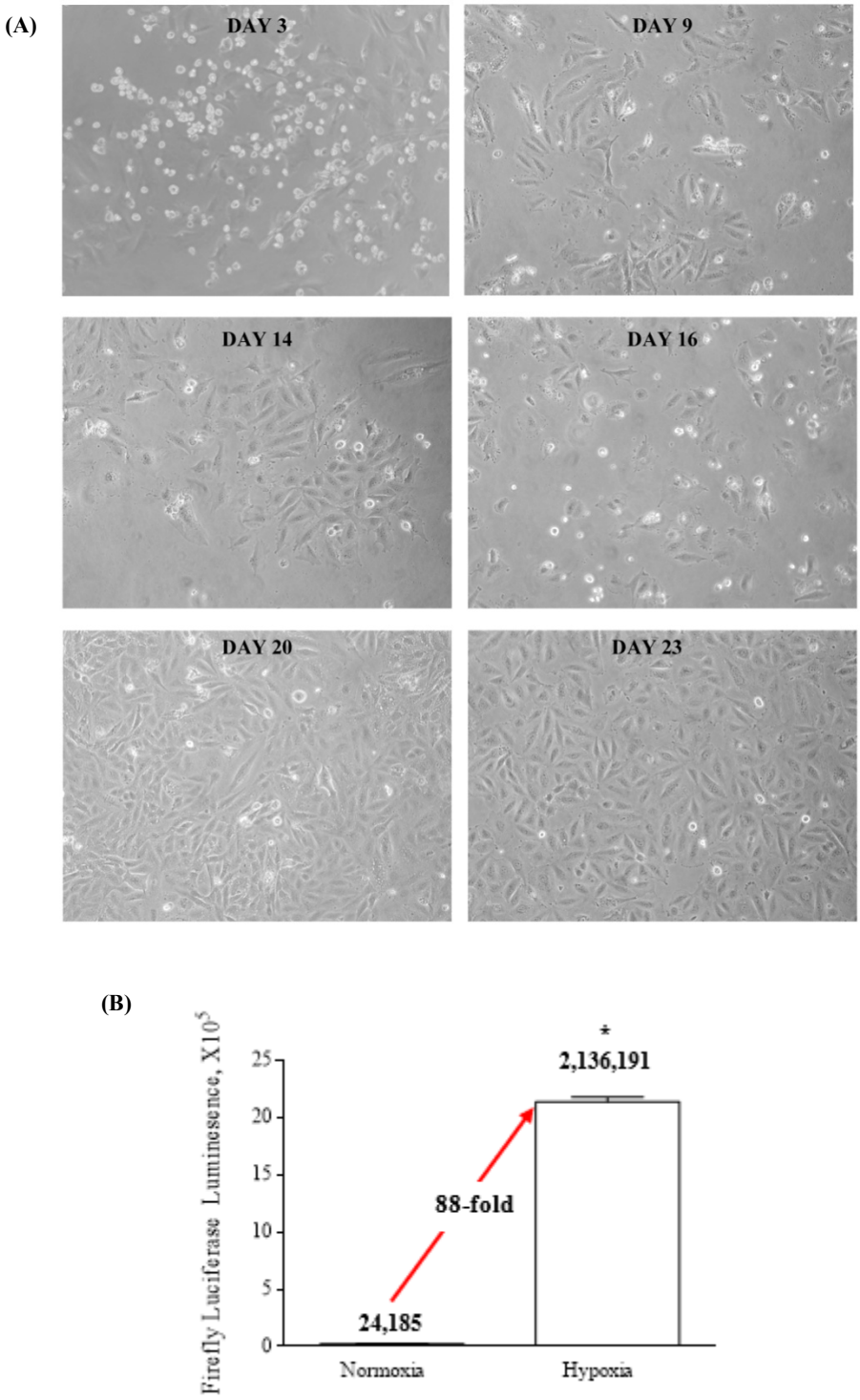
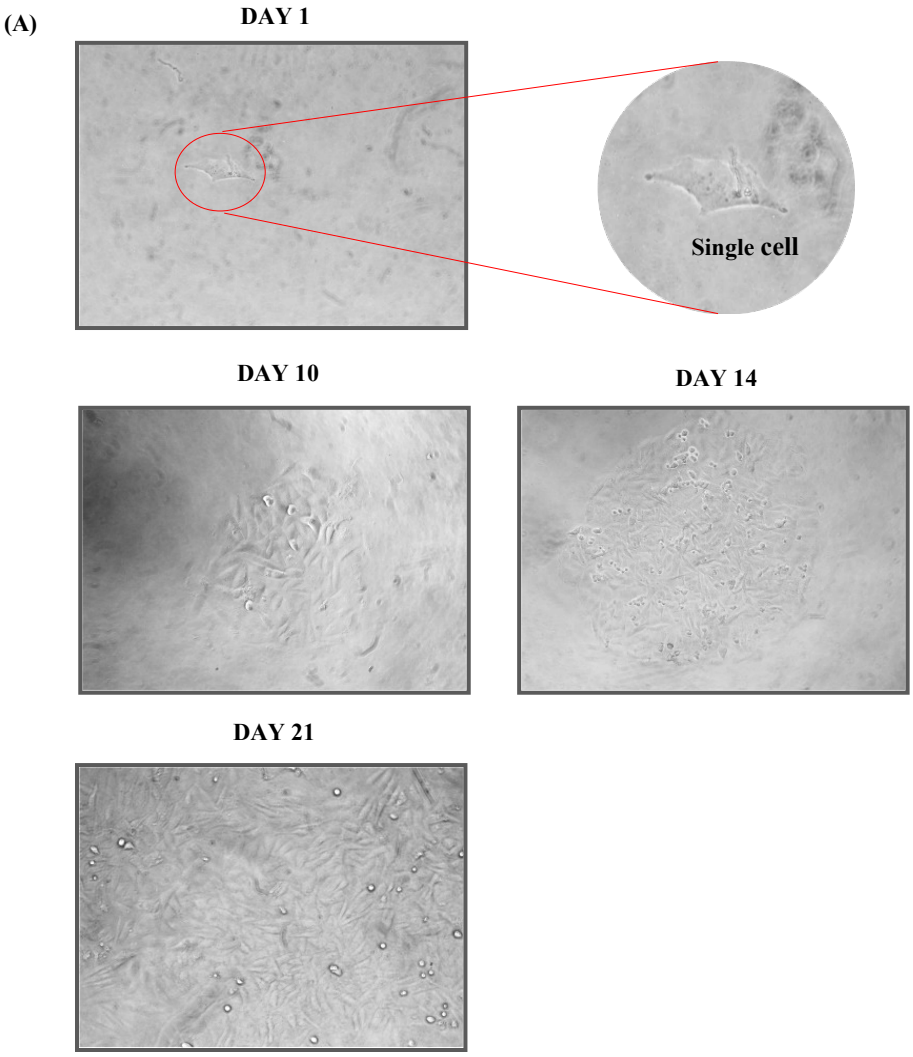
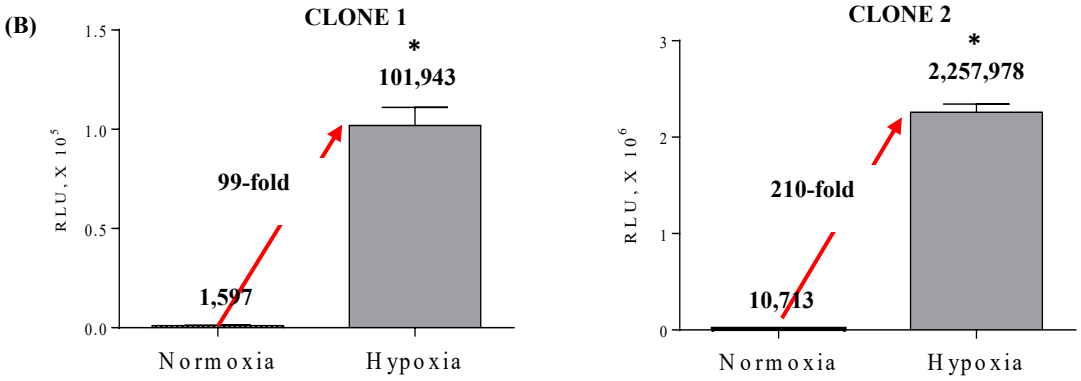


Figure 1. Development of stable cell population with hypoxia-driven luciferase expression. (A) Representative images of morphological changes in Saos-2 on different days following co-transfection with pLUC-MCS carrying the 4XEPO-HRE and pCDNA3 harbouring the Neomycin resistant gene and subsequent antibiotic treatment. (B) The intensity of luciferase produced by the mass population of the stable transfectants upon hypoxia induction.


(A)
DAY 1
Single cell
DAY 10
DAY 14
DAY 21


(B)
CLONE 1
*
101,943
99-fold
1,597
RLU, X 10^5
1.5
1.0
0.5
0.0
Normoxia
Hypoxia
CLONE 2
*
2,257,978
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RLU, X 10^6
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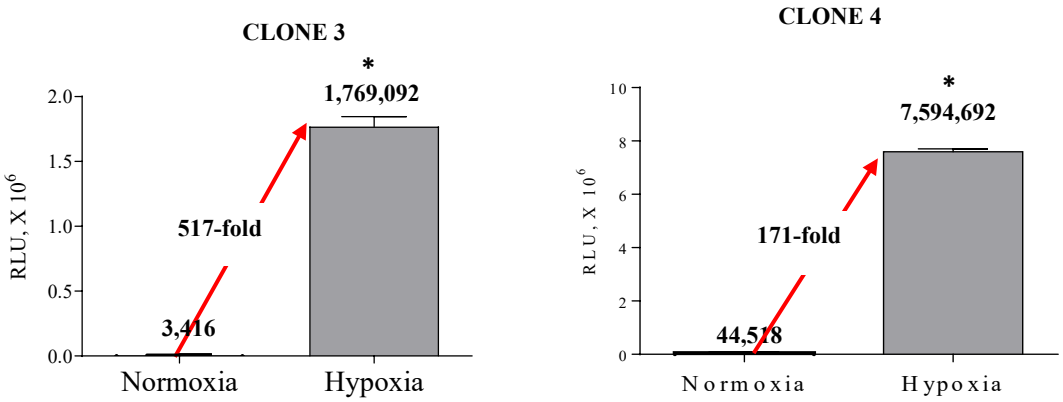

Figure 2. Expansion of clonal cells with increased hypoxia-responsive luciferase expression. (A) Representative images for the development of a cell population from a single isolated cell. A single cell was obtained by performing single-cell cloning using the limiting dilution method in a 96-well plate. Over a period of three weeks, a single cell was propagating, growing into a group of colonies, and then reaching 100% of cell confluence. (B) Fold difference in bioluminescence intensity in each of the four clones upon hypoxia induction. Data were shown as mean ± S.E.M. of triplicates from a representative of three independent experiments. Asterisk (*) represents a significant difference ($P < 0.05$) compared to normoxia reading.

Cell density is known to be a factor in the increased HIF activity in certain cell lines [23]. To test whether cell density influences the luciferase signal of C3, the cells were cultured at different cell numbers and then exposed to hypoxia. Results showed that at densities of 2.6 x 10^4, 3.3 x 10^4 and 4.0 x 10^4 cells/cm^2, no statistically significant variation was observed in the hypoxia-responsive luciferase signals (Fig. 3A). Hence, we concluded that the hypoxia-responsive signal produced by C3 remained consistent at the different cell densities tested. To test the ability of C3 to retain the signal strength over several passages, the hypoxia-responsive luciferase reading was measured over a range of C3 passages, beginning from passage one up to passage 42. Data showed that the fold difference fluctuated throughout the tested period (Fig. 3B). The lowest value was obtained at passage 23 while the highest was seen at passage 24. However, the average level of all the passage numbers remained 500-fold. To ensure that this signal is specific to HIF activities in the cells, the C3 was then subjected to treatments with HIF-inhibitory drugs.

A proof of concept study was conducted using known HIF inhibitors, Bortezomib [22, 24] and cisplatin [25, 26]. Treatment with bortezomib significantly reduced the hypoxia-induced luciferase signal in the C3 cells (Fig. 4A). At the lowest concentration used (50 nM), almost 70% of the luciferase signals were inhibited. At this concentration, the cells appeared to be completely viable. At 100 nM, the signal was further reduced. However, as the drug concentration increased, the inhibitory activity was slightly affected. This was accompanied by a gradual reduction in cell viability. Besides bortezomib, cisplatin was also shown to inhibit HIF. However, the inhibition mechanism was achieved via repression of HIF1-1α protein expression [26]. In the present study, the addition of cisplatin at 25 μM concentration led to almost 60% inhibition of luciferase signals in hypoxic C3 (Fig. 4B). The viability of the cells, however, was also slightly reduced. As the drug concentration increased, both the luciferase reading and cell viability decreased.

This study describes the establishment of a stable reporter cell line that measures the activity of the HIF transcription factor. This cell line is useful in the screening of HIF-regulatory compound libraries for drug discovery. Drug candidates identified with this technology could potentially address therapies for various conditions and diseases, including ischemia and cancer. The development of cell-based HIF assay systems has been previously reported [27, 28]. However, up to now, only a number of HIF assay cell lines are commercially available [29, 30]. In the present study, we established a highly sensitive cell-based assay system that can monitor HIF activity using the luciferase signal. We utilized the use of four copies of the HRE of EPO gene [19], which is one of the targets of HIF [31], to drive the expression of the luciferase reporter protein.

The presence of the increased copy number of the HRE in our system tremendously improved the sensitivity of the system. The signal ratio of hypoxia versus normoxia for the commercially-available system CellSensor™ HRE-bla ME-180 Cell Line (Invitrogen) is 13-fold [29]. While another system developed by Ji et al., (2008), gave 14-fold differences [28]. In this study, the mass population of the drug-resistant transfectant showed an 88-fold increase in hypoxia luciferase signal compared to its normoxia control. This is far superior compared to the previously reported HIF-assay cell lines [27, 28]. In this study, assays using each of the four single-cell clones obtained gave dramatically increased signal intensities. This increased sensitivity will highly improve the signal-to-noise ratio of various experimental conditions. The highest signal was seen in the clone labelled as C3. Its 517-fold difference contributes to a drastic increase in the assay sensitivity. The increase in

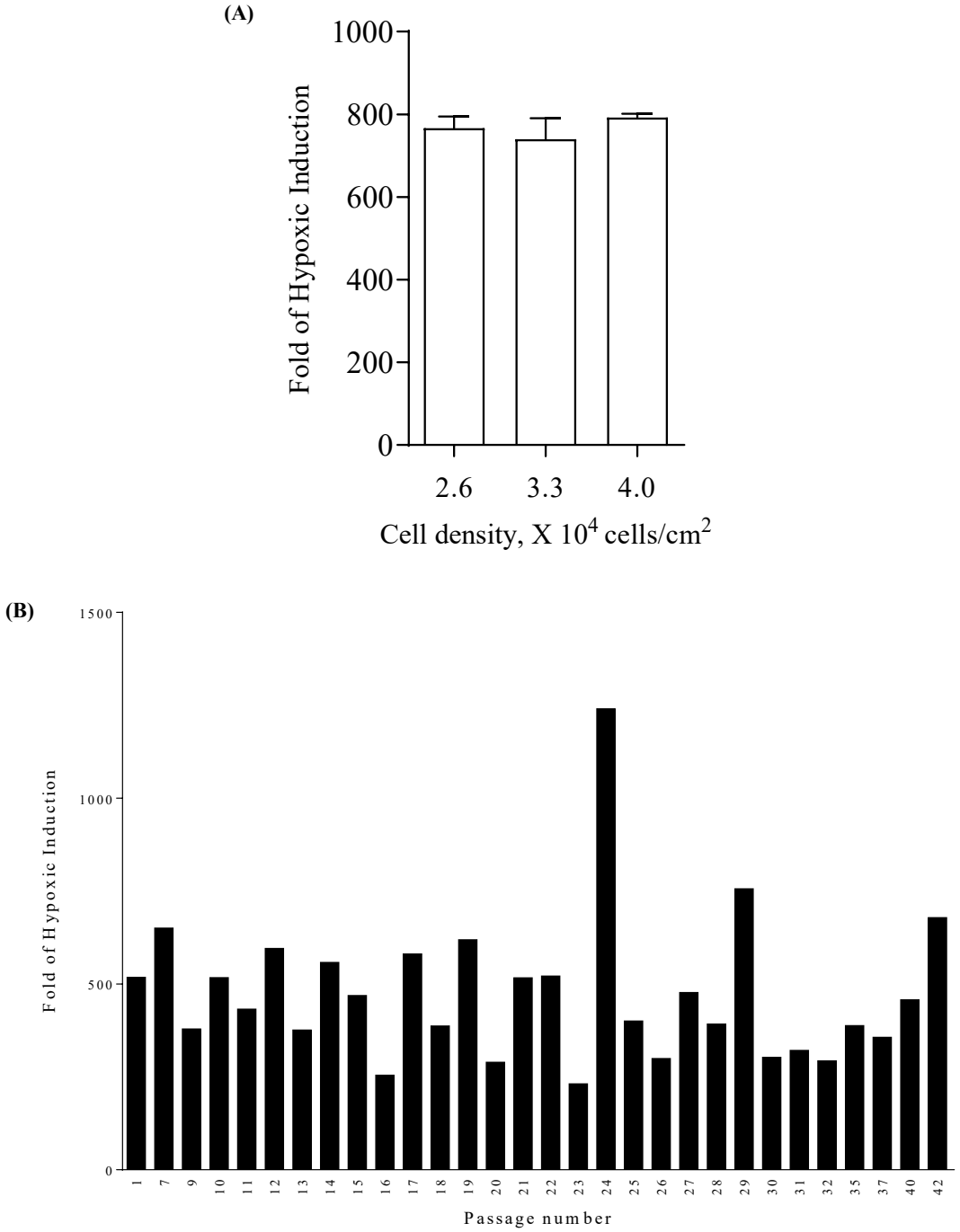

Figure 3. Passage numbers and cell density affected C3 luciferase signal intensity. (A) Different cell numbers gave a similar fold difference in bioluminescence intensity following hypoxia induction. (B) Cell passage numbers influence the sensitivity of the hypoxia-responsive luciferase. Data were shown as mean ± SEM of triplicates from a representative of three independent experiments. Asterisk (*) represents a significant difference ($P < 0.05$) compared to the normoxia reading.

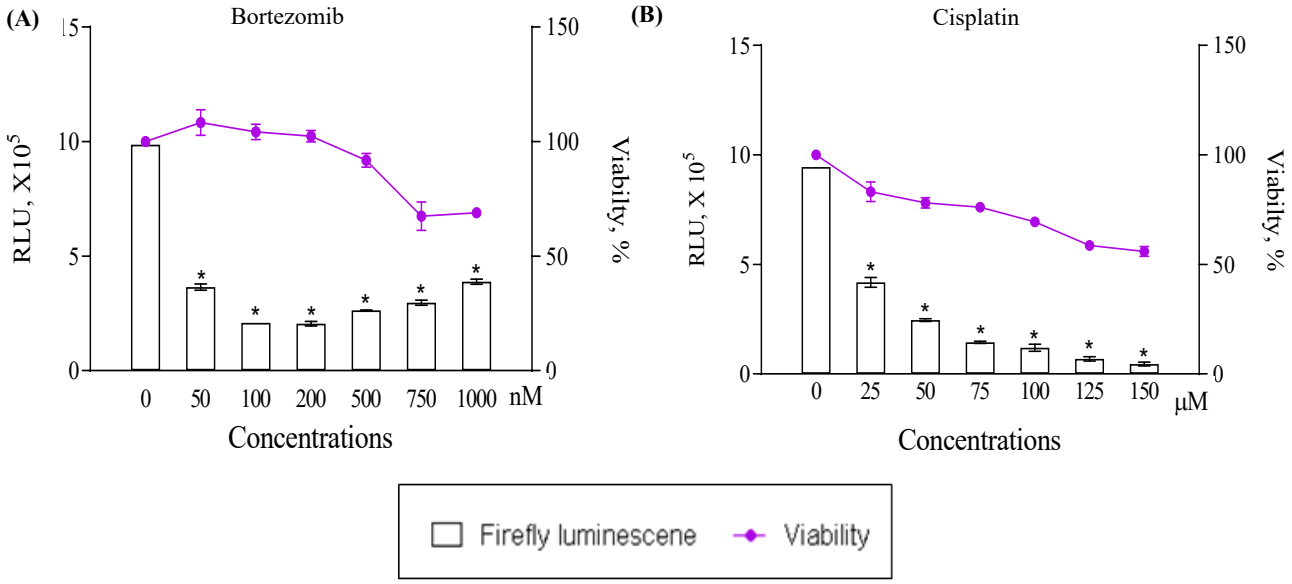

Figure 4. The hypoxia-induced luciferase expression was suppressed by Bortezomib and Cisplatin. Commercially available inhibitors of HIF activity, Bortezomib (A) and cisplatin (B), led to the reduction of reporter signal intensities and were associated with a gradual reduction in cell viability. Data were shown as mean ± SEM of triplicates from a representative of three independent experiments. Asterisk (*) represents a significant difference ($P < 0.05$) compared to the normoxia reading.

signal difference also translates to low background reading, allowing for a lesser amount of reagents used in the assays. In addition, the assay can be performed at small scales, requiring a reduced amount of precious drug target(s) of interest and allowing for the application of a high-throughput screening format.

Our proof of concept study using Bortezomib and cisplatin showed that the hypoxia-inducible signal in the C3 population is responsive to the inhibitory effects of these drugs, suggesting the reliability and sensitivity of the assay system. Bortezomib, a proteasomal inhibitor, was shown to inhibit the transcriptional activity of HIF-1 via the specific effect on the HIF1-1α C-terminal activation domain [23]. Previous studies have shown that cisplatin treatment resulted in HIF inhibition, but similar to our findings, it was also reported to induce cell death [32]. Due to its cytotoxicity effect that might influence the HIF-responsive luciferase readings, bortezomib would be the ideal control to be used in the HIF activity assay using the newly developed C3.

The added advantage of our newly-developed system is that we used the Saos-2 cell line, one of the most extensively studied human osteosarcoma cell lines in vitro [33, 34]. Our study screened several cancer cell lines, including human breast adenocarcinoma MCF7, cervical carcinoma HeLa, renal carcinoma 786-O and osteosarcoma Saos-2 cell lines (data not shown). Luciferase signals that were produced by our reporter construct upon hypoxia induction were highest in the Saos-2 cells. Based on these findings, we pursued the development of a stable cell line using the Saos-2. Extensive studies and reports available on the Saos-2 cell line will also allow for more efficient and comprehensive data analysis by potential users.

In conclusion, the establishment of this highly sensitive cell-based HIF assay cell line offers a great advantage over the currently available cell-based HIF assay systems. At the current state, the reporter cell line is able to measure total HIF activity in general, which includes the activities of HIF-1α and HIF-2α. It is known that these different HIF-α have separate sets of downstream target genes. Therefore, in certain conditions, a more specific assay system is required to measure the individual activity of the HIF-1α and HIF-2α. We are currently performing additional studies to establish this specific system. In addition, future studies should include a comparison with commercially available HIF assay cell lines. Despite this limitation, our present study has successfully established a superior cell-based assay that addresses concerns related to transfection efficiencies, ensures assay result reproducibility, and maintains cost-effectiveness.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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