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Hypoxia-Sensitive Reporter System for High-Throughput Screening

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The induction of anti-hypoxic stress enzymes and proteins has the potential to be a potent therapeutic strategy to prevent the progression of ischemic heart, kidney or brain diseases. To realize this idea, small chemical compounds, which mimic hypoxic conditions by activating the PHD-HIF-*α* system, have been developed. However, to date, none of these compounds were identified by monitoring the transcriptional activation of hypoxia-inducible factors (HIFs). Thus, to facilitate the discovery of potent inducers of HIF-*α*, we have developed an effective high-throughput screening (HTS) system to directly monitor the output of HIF-*α* transcription. We generated a HIF-*α*-dependent reporter system that responds to hypoxic stimuli in a concentration- and time-dependent manner. This system was developed through multiple optimization steps, resulting in the generation of a construct that consists of the secretion-type luciferase gene (*Metridia* luciferase, MLuc) under the transcriptional regulation of an enhancer containing 7 copies of 40-bp hypoxia responsive element (HRE) upstream of a mini-TATA promoter. This construct was stably integrated into the human neuroblastoma cell line, SK-N-BE(2)c, to generate a reporter system, named SKN:HRE-MLuc. To improve this system and to increase its suitability for the HTS platform, we incorporated the next generation luciferase, Nano luciferase (NLuc), whose longer half-life provides us with flexibility for the use of this reporter. We thus generated a stably transformed clone with NLuc, named SKN:HRE-NLuc, and found that it showed significantly improved reporter activity compared to SKN:HRE-MLuc. In this study, we have successfully developed the SKN:HRE-NLuc screening system as an efficient platform for future HTS.

Keywords: chemical screening; high-throughput screening; hypoxia responsive element; hypoxia stress; reporter cell system
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Introduction

The molecular mechanism through which hypoxic stress regulates gene expression has been the focus of intense research in recent years, culminating in the identification of the hypoxia-inducible factors (HIFs), which consist of HIF-*α* and HIF-*β* proteins (Semenza et al. 1994; Wang et al. 1995; Semenza 2003). HIF-*α* is hydroxylated by prolyl hydroxyl domain-containing proteins (PHD1, PHD2 and PHD3) and FIH (factor inhibiting HIF) under normoxic conditions, resulting in its rapid degradation through the proteasome pathway (Kallio et al. 1994; Mahon et al. 2001; Semenza 2001). In contrast, under hypoxic conditions, HIF-*α* escapes from this degradation and thus transactivates genes that facilitate cellular adaptation to hypoxic stress (Iyer et al. 1998).

It has previously been shown that a pre-conditioning strategy, through which the expression of anti-hypoxic stress enzymes and proteins is increased prior to the hypoxic insult, is an effective strategy to prevent the progression of ischemic heart, kidney and brain diseases (Fraisl et al. 2009). Thus, small chemical compounds that induce pre-conditioning, such as inhibitors of PHDs, have been developed for this purpose (Nangaku et al. 2007; Fraisl et al. 2009; Vachal et al. 2012).

Traditionally, two strategies have been utilized to identify chemical compounds that regulate the activities of transcription factors: structure-based screening and reporter-

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based screening. For the latter screening approach, construction of an effective reporter cell system to monitor transcriptional activity is critical. While several screening systems for HIF-α-inducing chemicals have previously been described (Woldemichael et al. 2006; Ehrismann et al. 2007; Pappalardi et al. 2011; Vachal et al. 2012), the compounds that were identified previously were not found through direct monitoring of the HIF-mediated transcriptional activation. In addition, the selection of cell lines that give rise to an efficient response is also important. In most cases, specific information related to the reporter system and responsive cell lines is limited, which means that researchers who wish to identify chemical compounds that modulate the activity of specific transcription factors must also carry out laborious optimization studies. We surmise that this may be the reason that elaborate reporter systems for the screening of HIF modulating chemicals are currently limited (Smirnova et al. 2011). Since phenotype screening, including transactivating reporter gene assays, is an important approach to identify lead compounds, in this study we aimed to develop an effective and highly sensitive screening system applicable for the high-throughput screening (HTS) of lead compounds for the hypoxia-response.

Materials and Methods

Cell culture

SK-N-BE(2)c human neuroblastoma cell line, Hepa1c1c7 mouse hepatic carcinoma cell line, and RL34 rat liver epithelial cell line were all purchased from American Type Culture Collection (ATCC). IRPTC immortalized rat proximal tubule cell line was obtained from Dr. J.R. Ingelfinger (Tang et al. 1995). SK-N-BE(2)c and IRPTC cells were cultured in RPMI1640 medium containing 10% charcoal/resin absorbed heat-inactivated fetal bovine serum (CF-FBS, MP Biomedicals) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Hepa1c1c7 and RL34 cells were cultured in DMEM (High glucose, Nacalai) supplemented with 10% CF-FBS and antibiotics. All cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Generation of HRE reporter constructs

Various HRE reporter plasmids were generated by combining consensus HIF-binding sites with different minimal promoter sequences. Five to seven tandem repeats of a 25-bp HRE (hypoxia responsive element) sequence (5′-CAG TGC AT**A CGT G**GG CTC CAA CAG G-3′) or a 40-bp HRE sequence (5′-CCA CAG TGC AT**A CGT G**GG CTC CAA CAG GTC CTC TGG ATC T-3′) were generated as follows. Two oligonucleotides were synthesized (for 25-bp HRE, 5′-CAG TGC AT**A CGT G**GG CTC CAA CAG G-3′ and 5′-ACT GCC TGT TGG AGC C**CA CGT** ATG C-3′; and for 40-bp HRE, 5′-CCA CAG TGC AT**A CGT G**GG CTC CAA CAG GTC CTC TGG ATC T-3′ and 5′-GTG GAG ATC CAG AGG ACC TGT TGG AGC C**CA CGT** ATG CAC T-3′), and their 5′-ends were phosphorylated with T4 Polynucleotide Kinase (Takara). Each pair of oligonucleotides was annealed and ligated by using a Ligation High kit (Toyobo). The resulting ligation products were subcloned into pBlue-Script II SK(−) vector (Agilent Technologies) and sequenced. We selected clones that have five to seven tandem repeats of HRE sequence. For the promoter sequences, the SV40 minimal promoter (Gorman et al. 1982) from the pGL3-promoter vector (Promega) and the artificial mini-TATA (mT) promoter from the pGL4-promoter vector (Promega) were used. The various combinations of HIF binding sites with minimal promoter sequences used in this study are shown in Fig. 1A. These sequences were ligated and inserted upstream of luciferase in the pMetLuc vector (Clontech). The most effective reporter construct was designated pMetLuc-7xHRE (40)-mT. The 7xHRE (40 bp)-mT fragment was also introduced into pNL1.3 (Promega). To generate stable cell lines, the neomycin-resistant cassette was inserted into BamHI and SalI restriction enzyme sites of pNL1.3 prior to the insertion of the 7xHRE (40 bp)-mT reporter. The successful construct was named pNL-7xHRE (40)-mT.

Transient reporter assay

SK-N-BE(2)c cells were plated onto 12-well plates (2×10^5 cells per well), and incubated for 16 hours. The cells were transiently transfected with various HRE luciferase reporter plasmids (800 ng) using LipofectAMINE 2000 (Life Technologies). After the transfection, cells were split into 6 wells of a 96-well plate and cultured for 16 hours. Where indicated, the effect of exogenously added $CoCl_2$ on the HRE reporter activity in the SK-N-BE(2)c cells was determined. In such instance, the transfected cells were treated with $CoCl_2$ (100 μM) or vehicle at 37°C for 12 hours. For analysis of HRE reporter activity, the culture supernatant was harvested and the luciferase activity was measured with the reagents and protocols provided in the Ready-To-Glow Secreted Luciferase Reporter Assay kit (Clontech) or Nano-Glo Luciferase Assay System (Promega) using a multi-plate reader (PheraSTAR FS, BMG Labtech). To standardize for cell number, the protein concentrations of the cells lysed with RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS) were measured using BCA protein assay reagent (Thermo Scientific). The degree of increases in the luciferase activity after $CoCl_2$ or hypoxia treatment is presented as fold luciferase unit that was calculated by dividing each luciferase activity of treated cells with a respective luciferase activity of vehicle-treated cells or cells incubated under normoxia.

Generating the stable HRE reporter cell line

SK-N-BE(2)c cells were plated into a 3-cm dish (1×10^6 cell per dish). After 16-hour incubation, the cells were transfected with the pMetLuc-7xHRE (40)-mT or pNL-7xHRE (40)-mT luciferase reporter plasmid (4 μg) using LipofectAMINE 2000 reagent. The cells were split and reseeded into four 10-cm dishes and cultured with 500 μM G418 (Roche) containing medium for 2 weeks. The resultant colonies were picked up with 0.25% trypsin soaked filter membrane and cultured in 24-well plate supplemented with 500 μM G418. The sensitivity of the clones to $CoCl_2$ stimulation was determined by the Ready-To-Glow Secreted Luciferase Reporter Assay kit or Nano-Glo Luciferase Assay System as described above.

Determination of the assay quality

The most efficient stable clones were transformed with pMetLuc-7xHRE (40)-mT or pNL-7xHRE (40)-mT (*i.e.,* clone #15 in Fig. 3 and clone #8 in Fig. 4 B, C), and were designated SKN:HRE-MLuc and SKN:HRE-NLuc, respectively. SKN:HRE-MLuc or SKN:HRE-NLuc cells were seeded into 96-well plates (4.0×10^4 cells/well) cultured for 16 hours. The first and last wells of each vertical lane were treated with vehicle as a control, while the remaining wells were treated with 100 μM $CoCl_2$ dissolved in fresh complete media. After

24-hour incubation, the culture medium was harvested and the luciferase activity was analyzed as described above. The values of assay quality were indicated with coefficient of variation, CV in negative control or 100% positive control, signal and noise (S/N), signal and background (S/B), and Z′ factor (Zhang et al. 1999). CV (%) = standard deviation ($SD_{0\% \text{ or } 100\%}$)/average ($AV_{0\% \text{ or } 100\%}$). S/B was calculated $AV_{100\%}/AV_{0\%}$. S/N was calculated $(AV_{100\%}/AV_{0\%})/SD_{0\%}$. Z′ factor was calculated $1 - (3 \times SD_{100\%} + 3 \times SD_{0\%})/(AV_{100\%} - AV_{0\%})$.

Statistical analyses

All data were analyzed using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test using GraphPad Prism software.

Results

Selection of an efficient HRE reporter construct for screening of chemicals

We wished to screen chemicals that induce HIF-α activity in order to identify new potential treatments for ischemic diseases. In order to identify an efficient HRE reporter construct with which to monitor HIF-α activity, we prepared a series of reporter constructs harboring multiple copies of an artificial HIF-binding sequence (HRE) placed in front of two minimal promoters (Fig. 1A). We selected HRE of either 25- or 40-bp in length, both of which con-

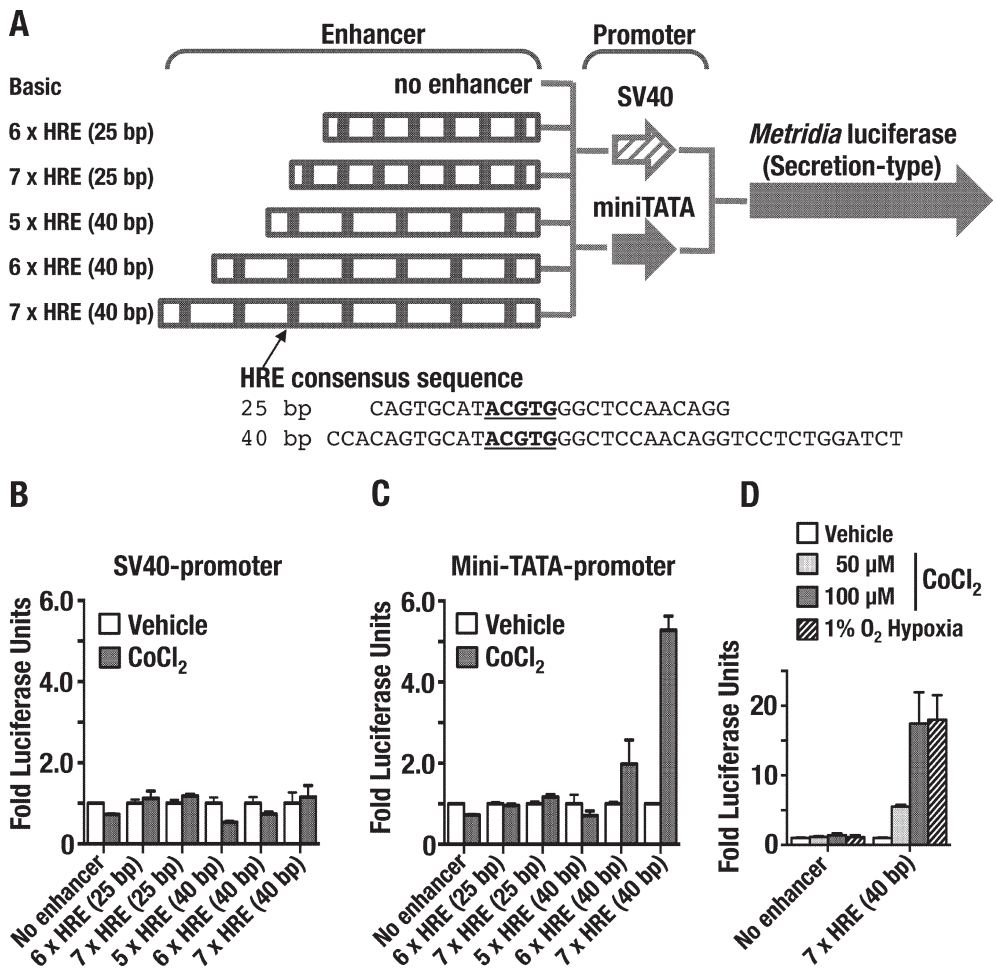

Fig. 1. Identification of the suitable enhancer-promoter pair for hypoxia stress response.
(A) Construction of suitable enhancer-promoter pair for hypoxia stress response. Either 25- or 40-bp HRE consensus sequence was multiplied 5 to 7 times and used as enhancers. Hypoxia responsive element (HRE) sequences are depicted in bold and underlined characters and the locations the HREs within the enhancer are indicated with grey lines. Two promoter sequences, *i.e.,* SV40 T-antigen promoter or mini-TATA (mT) promoter were ligated to secretion-type *Metridia* luciferase (MLuc). Total 10 reporter constructs and 2 control promoter-less constructs were generated. (B and C) Luciferase activity of SV40 and mT promoter-based reporter constructs. SKN-BE(2)c cells were transiently transfected with the reporter constructs, and cells were then stimulated with vehicle or 100 μM $CoCl_2$ for 24 hours. Luciferase activity in each culture medium was measured and normalized for protein concentrations. The degree of induction is presented as fold luciferase units, with the value from vehicle-treated cells set as 1. Note that 7 × HRE (40 bp) ligated to mT promoter gave rise to strong induction upon the $CoCl_2$ treatment. (D) Luciferase reporter activity in response to hypoxia. SK-N-BE(2)c cells were transfected with pMetLuc-7xHRE (40)-mT or control pMetLuc-mT construct, and cells were treated with $CoCl_2$ or hypoxia (1% O_2) for 24 hours. Luciferase activity in each culture medium was measured, and the degree of induction was calculated, as described above.

tained a HRE consensus motif (5′-ACGTG-3′). We designed these two HRE oligonucleotides mainly from the human VEGFA regulatory sequence. In order to determine the optimum HRE copy number for monitoring HIF-α activity, we prepared plasmids containing 6xHRE (25 bp), 7xHRE (25 bp), 5xHRE (40 bp), 6xHRE (40 bp) or 7xHRE (40 bp). In addition, we compared the activities of two different promoter sequences: the SV40 promoter (Gorman et al. 1982) and a mini-TATA promoter. As a control, we also prepared basic promoter constructs, which did not include the HRE enhancer elements. Consequently, in total we prepared 12 combinations of enhancer-promoter constructs. These enhancer-promoter constructs were ligated to a secretion-type luciferase gene (*Metridia* luciferase, MLuc) (Markova et al. 2004) in the pMet-Luc vector (Fig. 1A). Importantly, the MLuc system enables us to monitor luciferase activity without terminating the assay.

To determine the activities of these reporter constructs, we introduced the plasmids into the human neuroblastoma cell line, SK-N-BE(2)c, and measured the luciferase activity in the culture medium after stimulation with 100 μM $CoCl_2$, which mimics hypoxia in cell culture conditions (Salnikow et al. 2004). In the SV40 promoter group, the basal luciferase activity was high even in non-stimulated conditions (data not shown), and significant elevation of luciferase was not observed upon $CoCl_2$ stimulation (Fig. 1B).

In contrast, in the mini-TATA promoter reporter group, multiple plasmids showed $CoCl_2$ responsiveness. Thus, the 6 × HRE (40 bp) and 5 × HRE (40 bp) constructs effectively drove luciferase gene expression when stimulated with $CoCl_2$. The 7xHRE (40 bp)-based vector responded to two different forms of hypoxic stress, both $CoCl_2$ and 1% O_2 hypoxia (Fig. 1D). On the contrary, neither of the 25-bp enhancer constructs, nor the 5xHRE (40 bp) constructs increased luciferase gene expression after $CoCl_2$ addition (Fig. 1C). Based on these results, we concluded that the most effective enhancer-promoter construct was the 7-time-repeated 40-bp HRE enhancer with mini-TATA promoter, and referred to this vector as pMetLuc-7xHRE (40)-mT.

HRE reporter activity is specifically enhanced in SK-N-BE(2)c cells

To examine whether the enhancer-promoter reporter constructs are active in multiple cell types, we transiently introduced the pMetLuc-7xHRE (40)-mT reporter construct into IRPTC rat proximal tubule cells, Hepa1c1c7 mouse hepatocarcinoma cells, and RL34 rat liver epithelial cells. IRPTC and Hepa1c1c7 cells were reported to effectively recognize hypoxia and $CoCl_2$ stimulations (Tanaka et al. 2005; Knaup et al. 2009) and RL34 cells were utilized as sensitive cells for oxidative stresses (McMahon et al. 2004). These transformants were treated with 100 μM $CoCl_2$ for 24 hours and the luciferase activity was measured in the culture medium in comparison with the vehicle treatment group. Despite the fact that these cell lines have a higher transfection efficiency compared with SK-N-BE(2)c cells, no significant elevation of luciferase activity was observed in IRPTC (Fig. 2A), Hepa1c1c7 (Fig. 2B) and RL34 (Fig. 2C) cells. Thus these results indicate that the SK-N-BE(2)c cell line is ideal for testing the HRE reporter activity.

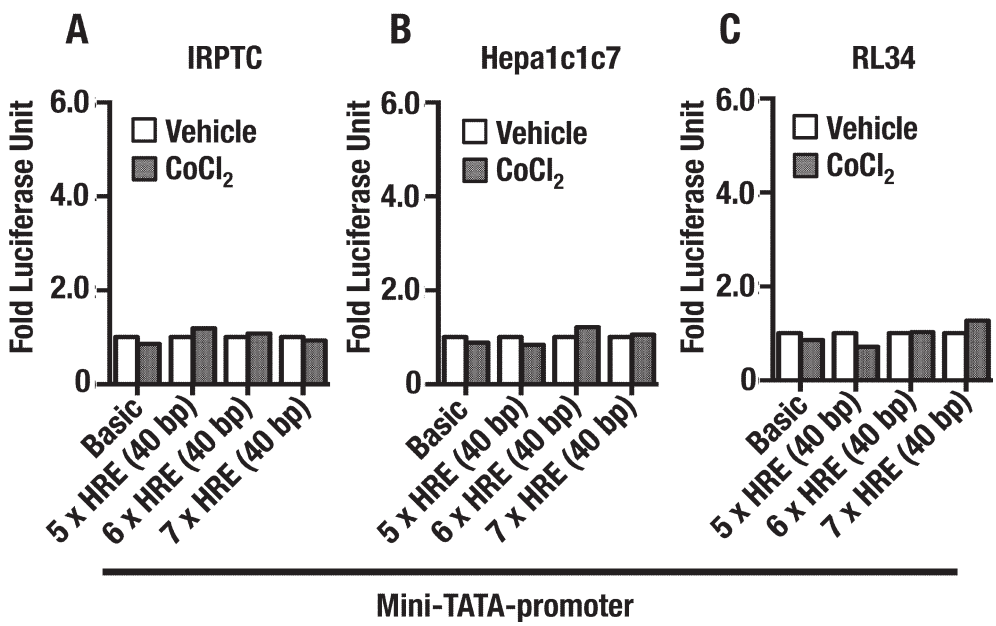

Fig. 2. Activity of pMetLuc-7xHRE (40)-mT reporter in cultured cell lines.
The reporter activity of pMetLuc-7xHRE (40)-mT and related constructs in IRPTC, Hepa1c1c7 and RL34 cells. The reporter constructs were introduced into IRPTC (A), Hepa1c1c7 (B), and RL34 (C) cells, and cells were stimulated with vehicle or 100 μM $CoCl_2$ for 24 hours. The degree of induction is presented as fold luciferase units, with the value from vehicle-treated cells set as 1 in each cell line.

Generation of the stable HRE cell lines

For future chemical screening, we decided to generate stably integrated transformants of the pMetLuc-7xHRE (40)-mT plasmid using the neomycin-resistant gene cassette within the pMetLuc vector. To this end, SK-N-BE(2)c cells were transfected with pMetLuc-7xHRE (40)-mT. Stable transformants were selected using G418 treatment (500 μM) for 2 weeks. The resultant colonies were picked up and cultured in media containing 500 μM G418 (Fig. 3A). We selected 15 colonies, of which 3 colonies (#4, #15 and #19) were maintained. The other clones did not grow normally.

We found that upon treatment with $CoCl_2$, luciferase activity was effectively induced in clone #15 in a dose- and time-depended manner, while that of clone #4 and #19 was only marginally induced (Fig. 3B). Therefore, we decided to use clone #15 hereafter, and this reporter cell line was referred to as SKN:HRE-MLuc. The luciferase activity of SKN:HRE-MLuc could also be induced by 1% O_2 treatment (Fig. 3B).

To determine the optimal culture density of SKN:HRE-MLuc cells for the hypoxic induction of luciferase activity in each well of a 96-well plate, we seeded 1.0×10^4, 2.0×10^4, 3.0×10^4, 4.0×10^4 and 5.0×10^4 SKN:HRE-MLuc cells into a 96-well plate and treated the cells with 100 μM $CoCl_2$ (Fig. 3C). We found that the luciferase activity reached a plateau at a cell density of 4.0×10^4/well. Since a cell density of 5.0×10^4 cells/well was close to confluency, we decided to seed 4.0×10^4 cells to one well for the chemical screening.

In order to check whether this SKN:HRE-MLuc stably-transformed reporter system is suitable for automatic HTS, we seeded 4.0×10^4 cells to each well of a 96-well plate and treated the cells with vehicle or 100 μM $CoCl_2$ for 24 hours (Fig. 3D). Using whole relative luciferase unit data from the plate, we calculated CV (coefficient of variation) of vehicle treatment ($CV_{0\%}$) and $CoCl_2$ treatment ($CV_{100\%}$). We also calculated S/B (signal/background), S/N (signal/noise) and Z′ factor as described in Materials and Methods. Importantly, S/B, S/N and Z′ factor of this analysis were 17.7, 73.4 and 0.62, respectively (Fig. 3E). These values conform to the criteria, which recommended that the S/B and S/N should be greater than 3.0, and Z′ factor should be greater than 0.5 (Zhang et al. 1999). Based on these results, we conclude that the SKN:HRE-MLuc system satisfies the criteria for HTS to screen HIF inducing chemicals.

The next generation luciferase gives a high potential to SKN:HRE-MLuc reporter cells

One aspect that can be improved upon in the SKN:HRE-MLuc system is the short half-life of *Metridia* luciferase. *Metridia* luciferase has a 5-min half-life, making its use in HTS by means of a robotic dispenser system inefficient. To overcome this issue, we decided to use a next generation luciferase, Nano luciferase (NLuc), in our reporter system, as NLuc has a longer half-life of 2.5 hours. Therefore, we introduced the 7xHRE (40 bp)-mT reporter sequence into the pNL1.3-neo vector harboring a secretion-type NLuc reporter gene, and named the construct pNL-7xHRE (40)-mT (Fig. 4A).

Exploiting the pNL-7xHRE (40)-mT construct, we repeated the preparation of stable reporter cell lines as described above, establishing five cell lines (clone number #5, #6, #8, #9 and #10; Fig. 4B). Further antibiotic selection was conducted on the most effective clones (clone number #5 and #8), after which we found that the luciferase activity of clone #8 was efficiently induced in both dose- and time-depended manners when treated with $CoCl_2$ (Fig. 4C). Of these two clones, we chose to use clone #8 hereafter, and this reporter cell line was referred to as SKN:HRE-NLuc. The luciferase activity of SKN:HRE-NLuc could also be induced by even mild 5% O_2 treatment (Fig. 4C). That means SKN:HRE-NLuc can recognize mild hypoxic stress compared with SKN:HRE-MLuc.

We then seeded 4.0×10^4 SKN:HRE-NLuc cells into a 96-well plate, the number of which was optimum for luciferase induction by 100 μM $CoCl_2$ (data not shown), and treated the cells with vehicle or $CoCl_2$ for 24 hours. Of note and in keeping with our expectation, the SKN:HRE-NLuc stably-transformed reporter system gave rise to a ten-fold higher luciferase activity than the SKN:HRE-MLuc reporter (Fig. 4C) and also had lower background levels of luciferase activity (data not shown).

To further define the efficacy of the SKN:HRE-NLuc activity for HTS, we calculated values of $CV_{0\%}$, $CV_{100\%}$, S/B, S/N and Z′-factor value (Fig. 4D). We found that the SKN:HRE-NLuc reporter system gave rise to the S/B and S/N values significantly higher than SKN:HRE-MLuc, *i.e.,* S/B 17.7 to 183 and S/N 73.4 to 1137, respectively. The Z′-factor value of the SKN:HRE-NLuc reporter system was greater than 0.5. These results thus clearly demonstrate that we have established an efficient HRE reporter system applicable for automatic HTS, and the SKN:HRE-NLuc stably-transformed reporter cell system will be a powerful tool to isolate HRE modulator compounds.

Discussion

Small chemical compounds that induce the hypoxic response have been the subject of extensive research (Ivan et al. 2002; Warshakoon et al. 2006a, b, c; Tegley et al. 2008), but monitoring the transactivation activity of the HIFs *per se* is difficult, and an effective monitoring systems with which to do this has not been reported. In this study, we developed two HRE reporter systems, SKN:HRE-MLuc and SKN:HRE-NLuc, which allow us to efficiently monitor the transactivation of HIF factors by hypoxic stress.

To generate the SKN:HRE-MLuc and SKN:HRE-NLuc reporter systems, we tested various HRE enhancer constructs in combination with two promoter systems. Through these processes, we obtained the optimum results with the enhancer containing 7 copies of a 40-bp HRE coupled with the mini-TATA (mT) promoter, designated as the

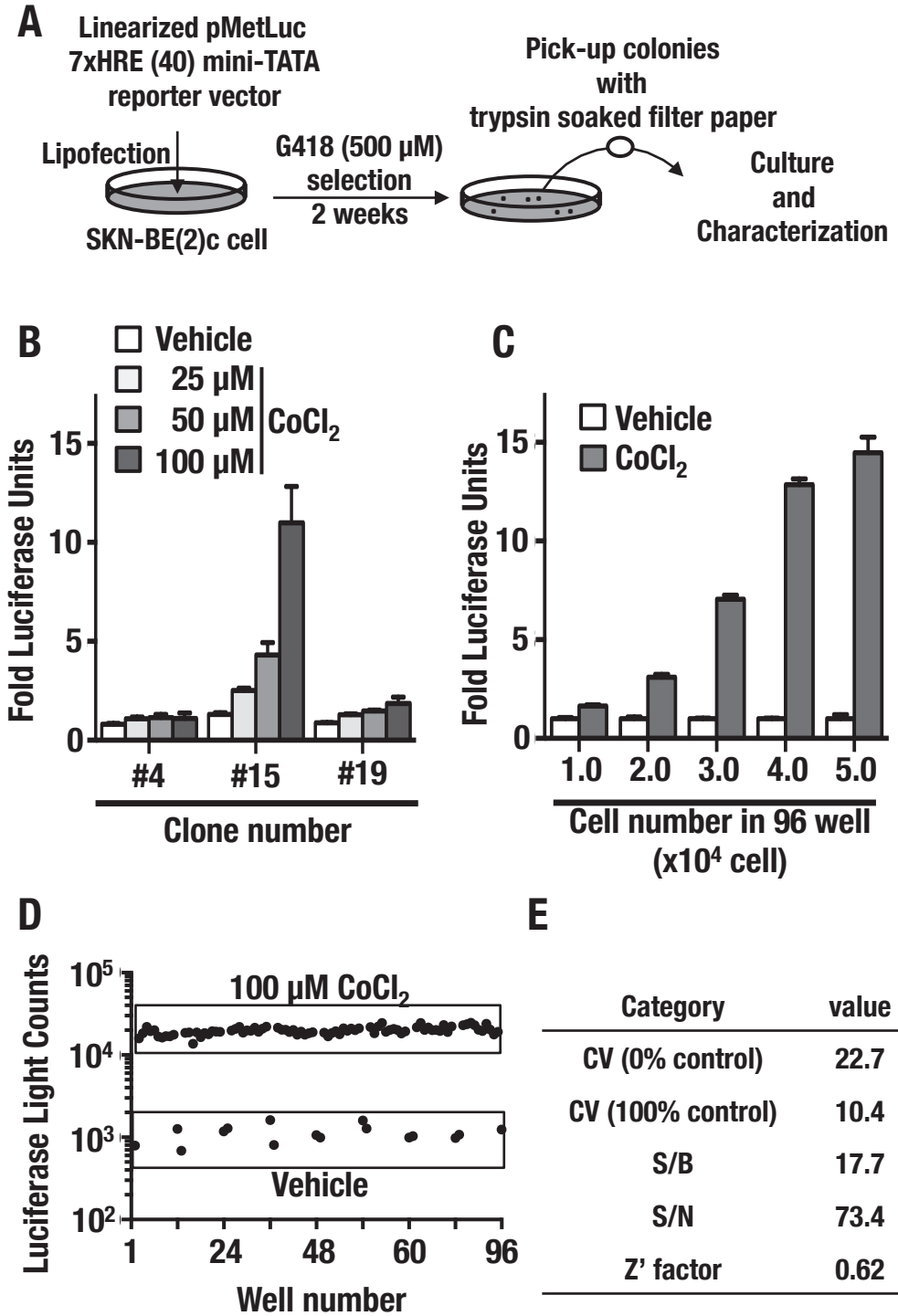

Fig. 3. Generation of stable HRE reporter cell lines.

(A) Schematic presentation of stable HRE-reporter cell line generation. G418 was used for the selection of transfected cells. (B) Reporter activities of individual HRE reporter cell lines. Three stable transformants with pMetLuc-7xHRE (40)-mT reporter construct were treated with 25, 50 or 100 μM $CoCl_2$ or incubated under hypoxia (1% O_2) for 24 hours. The luciferase activity in each culture medium was measured. Hereafter, the stable clone #15 was referred to as SKN:HRE-MLuc. The degree of induction is presented as fold luciferase units, with the value from vehicle-treated cells set as 1 in each cell line. (C) Optimization of the cell density to recognize $CoCl_2$ stimulation. SKN:HRE-MLuc cells were seeded into indicated numbers into a 96-well plate. The degree of induction is presented as fold luciferase units, with the value from vehicle-treated cells set as 1 in each cell density. (D) Validation of SKN:HRE-MLuc cells in a 96-well plate. SKN:HRE-MLuc cells (4.0×10^4) were seeded into each well and treated with vehicle (0% control, first and last lines) or 100 μM $CoCl_2$ (100% control, left lines). The luciferase activity in each culture medium was measured. (E) Calculated validation summary for HTS using SKN:HRE-MLuc reporter cells.

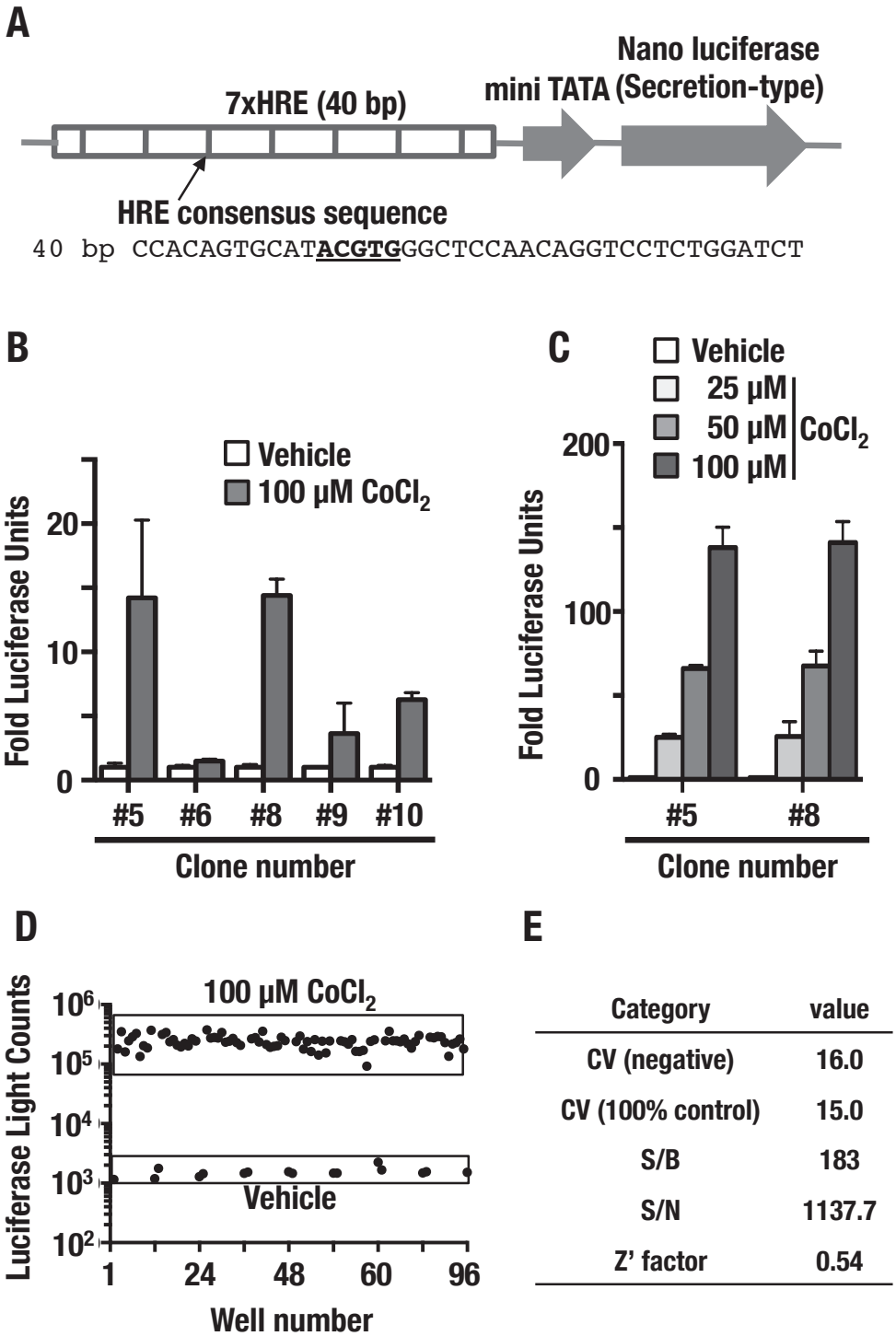

Fig. 4. Use of next generation luciferase significantly improves the HRE reporter activity.
(A) The schematic presentation of Nano luciferase reporter construct. The reporter constructs were changed to secretion-type Nano luciferase (NLuc). This reporter vector was designated as pNL-7xHRE (40)-mT. (B) Reporter activities on individual HRE reporter transformed cell lines. Five stable transformants of pNL-7xHRE (40)-mT reporters were generated, and the cells were treated with 100 μM $CoCl_2$ for 24 hours. The degree of induction is presented as fold luciferase units, with the value from vehicle-treated cells set as 1 in each cell line. (C) The most effective clone (clone number #8) was treated with 25, 50 or 100 μM $CoCl_2$ or incubated under 5% O_2 (hypoxia) for 24 hours. Hereafter the stable clone #8 was selected and renamed SKN:HRE-NLuc. The degree of induction is presented as fold luciferase units, with the value from each vehicle-treated cells set as 1. (D) Validation of stable SKN:HRE-NLuc stable cell line in 96-well plates. SKN:HRE-NLuc cells (4.0×10^4) were seeded into each well and treated with vehicle (0% control, first and last lines) or 100 μM $CoCl_2$ (100% control, left lines). The luciferase activity in each culture medium was measured. (E) Calculated validation summary of SKN:HRE-NLuc for HTS.

pMetLuc-7xHRE (40)-mT reporter. We then incorporated the 7xHRE (40)-mT reporter into a new generation luciferase-based construct and found that the SKN:HRE-NLuc reporter cell system is more effective at monitoring hypoxia stress.

We also explored which cell line is most suitable to monitor transactivation from the pMetLuc-7xHRE (40)-mT reporter. Initially, we examined cell lines originating from the kidney and liver, as these tissues are sensitive to the primary hypoxia response. However, unexpectedly, the human neuroblastoma cell line SKN-BE(2)c was found to be the most effective at driving expression of the HRE-reporter construct. As we have previously reported that neural tissues produce erythropoietin during embryonic development (Suzuki et al. 2013), the hypoxia stress response may also be fully functional in neuroblastoma cells. This observation is important, because traditionally, cells of hepatocyte origin, such as Hepa1c1c7, have been used to monitor the hypoxia. Our present result indicates that the SKN-BE(2)c cells may retain certain cellular machinery required to drive HRE-dependent gene transcription, unlike Hepa1c1c7 cells.

In summary, in this study we have established an efficient HRE reporter system, which is suitable for use in automatic HTS. The SKN:HRE-NLuc reporter cell system will be useful to screen chemical compound libraries for novel small-molecule modulators of the hypoxia response.

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Conflict of Interest

The authors declare no conflict of interest.

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