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ESTABLISHMENT AND VALIDATION OF A MONOCLONAL CELL-BASED REPORTER ASSAY FOR SCREENING OF HYPOXIA-INDUCIBLE FACTOR ACTIVITIES

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By

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Cell-based assay is a powerful tool in the fields of drug discovery. One of the main targets of current drug development is transcription factor. Hypoxia-inducible factors (HIFs) are transcription factors, which involve in cellular oxygen homeostasis and association with disease pathophysiology. Hence, manipulation of HIF activities in HIF-related diseases by HIF regulators is claimed to be an ideal and effective therapeutic therapy. Currently, a highly sensitive cell-based assay to measure HIF activity is still lacking in the market. Hence, the main objective in this study was to establish a highly sensitive HIF cell-based assay system that can quantitate HIF activity in high-throughput screening (HTS) format. This study described the development of a monoclonal, highly sensitive and stable reporter cell line for monitoring the activity of HIF by using luciferase reporter constructs. Four copies of hypoxia response element (HRE) of the erythropoeitin (*EPO*) gene, which is one of the targets of HIF, were used to drive the expression of the luciferase reporter. One of the monoclonal stable reporter cell lines, designated as Clone 3 (C3) 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 usefulness of C3 HIF assay system in measuring HIF activities was then confirmed by the proof of concept study using known HIF-regulated drugs. After the establishment of the C3 HIF bioassay system, the bioassay system was validated on the HTS suitability factors rigorously in various experimental conditions. Data obtained in various HTS parameters such as robustness, linearity, specificity, intermediate precision and solvent compatibility of C3 HIF bioassay system were statistically controlled by coefficient of variation (CV) lower than 20% throughout the study, indicative of C3 HIF bioassay a reliable and controllable assay system for HTS format. The quality of C3 HIF bioassay was graded as excellent level by exhibiting Z-factor (Z') values that range from 0.524 to 0.954. Apart from the establishment and validation of this HIF assay system, the application of measuring HIF activities by C3 HIF assay system was evaluated using traditional medicinal plants in Malaysia in this study. Specific cytotoxic effects of traditional medicinal plant

extracts under hypoxic conditions were observed in this study. Among nine tested traditional medicinal plants, methanolic crude *C. papaya* leaves extracts exhibited potent HIF inhibition. The dose-dependent HIF inhibitory effect was further confirmed by the use of C3 HIF bioassay system. Interestingly, *C. papaya* extracts were observed to be specific in targeting the hypoxic cancer cells by delineating significant lowered IC_{50} of cell proliferation compared to normoxic cancer cells. These findings highlight the importance of identifying specific traditional medicinal plant extracts which can be promising candidates for new approaches to eradicate hypoxic cancer cells in solid tumors. Thus, the establishment of this highly sensitive C3 HIF cell-based assay is concluded to be a promising approach to expedite research in the discovery of HIF regulatory drugs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBINAAN DAN PENGESAHAN ASAI BERASAS SEL UNTUK PEMERIKSAAN AKTIVITI FAKTOR INDUKSI HIPOKSIA

Oleh

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Asai berasaskan sel adalah alat yang berkuasa dalam bidang penemuan ubat. Salah satu sasaran utama untuk pembangunan ubat-ubatan semasa adalah faktor transkripsi. Faktor induksi hipoksia (HIFs) adalah faktor-faktor transkripsi yang terlibat dalam homeostasis oksigen sel dan juga berkaitan dengan penyakit patofisiologi. Oleh itu, manipulasi aktiviti HIF dalam penyakit-penyakit yang dikawal dan berkaitan dengan HIF merupakan terapi terapeutik yang sesuai dan berkesan. Pada masa ini, asai berasaskan sel yang sangat sensitif untuk mengukur aktiviti HIF masih kurang di pasaran. Maka, objektif utama dalam kajian ini adalah untuk membina satu sistem pengasaian HIF berasaskan sel yang sangat sensitif untuk mengukur aktiviti HIF dalam format penabiran celusan tinggi (HTS). Kajian ini menerangkan tentang pembinaan titisan sel pelapor monoklonal yang sangat sensitif dan stabil bagi memantau aktiviti HIF dengan menggunakan gen konstruk pelapor lusiferase. Empat salinan elemen respon hipoksia (HRE) dalam gen eritropoietin (*EPO*) yang merupakan salah satu sasaran HIF telah digunakan untuk mengekspreskan gen pelapor lusiferase. Salah satu titisan-titisan sel pelapor monoklonal stabil dinamakan sebagai Klon 3 (C3). Klon 3 menunjukkan peningkatan 500 kali ganda ekspresi lusiferase dalam keadaan hipoksia berbanding keadaan normoxia. Peningkatan drastik terhadap responsi hipoksia adalah penting untuk memastikan konsistensi dan kebolehulangan keputusan pengasaian HIF di samping dapat mengurangkan kos pengasaian keseluruhan. Penggunaan sistem pengasaian C3 HIF untuk mengukur aktiviti-aktiviti HIF telah disahkan oleh kajian bukti konsep dengan menggunakan ubat pengawal HIF. Selepas itu, pengesahan faktor-faktor kesesuaian HTS oleh sistem bioasai C3 HIF dalam pelbagai keadaan eksperimen. Data yang diperolehi dalam pelbagai parameter HTS seperti keteguhan, kelinearan, kekhususan, ketepatan pertengahan dan keserasian pelarut sistem bioasai C3 HIF dikawal secara statistik oleh koefisien variasi (CV) sepanjang kajian ini. CV yang lebih rendah daripada 20% sepanjang kajian ini telah menunjukkan sistem bioasai C3 HIF boleh dipercayai dan dapat dikawal dalam format HTS. Kualiti bioasai C3 HIF telah digredkan sebagai tahap yang sangat baik dengan menunjukkan faktor-Z (Z') yang bernilai dari 0.524 kepada 0.954. Selain daripada pembinaan dan pengesahan sistem pengasaian HIF, aplikasi untuk pengukuran aktiviti HIF oleh sistem pengasaian C3 HIF

dinilai dengan menggunakan tumbuh-tumbuhan perubatan tradisional di Malaysia juga dibincangkan. Kesan-kesan sitotoksik khususnya dalam ekstrak tumbuh-tumbuhan perubatan tradisional pada keadaan hipoksia diperhatikan dalam kajian ini. Antara sembilan tumbuh-tumbuhan perubatan tradisional yang diuji, ekstrak mentah metanol C. papaya daun betik ekstrak menunjukkan perencatan HIF yang kuat. Penggunaan sistem bioasai C3 HIF telah mengesahkan lagi kesan perencatan HIF adalah bergantung kepada dos. Menariknya, ekstrak *C. papaya* didapati khusus dalam mensasarkan sel-sel kanser hipoksia dengan menunjukkan penurunan IC_{50} dalam percambahan sel semasa berbanding dengan sel-sel kanser normoxia. Penemuan ini menekankan kepentingan mengenal pasti ekstrak tumbuh-tumbuhan perubatan tradisional tertentu sebagai pendekatan baru untuk memusnahkan sel-sel kanser hipoksia dalam tumor pepejal. Oleh itu, kita dapat menyimpulkan bahawa pembinaan sistem pengasaian C3 HIF berasaskan sel yang sangat sensitif ini adalah salah satu pendekatan yang dijamin dapat memajukan penyelidikan dalam penemuan ubat pengawal HIF.

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I certify that a Thesis Examination Committee has met on 17 April 2017 to conduct the final examination of Liew Sien Yei on her thesis entitled "Establishment and Validation of a Monoclonal Cell-Based Reporter Assay for Screening of Hypoxia-Inducible Factor Activities" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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AF	Aminoflavone
AKT	Protein Kinase b
ARNT	Aryl hydrocarbon receptor nuclear translocator
ARD1	Arrest-defective-1
BCA	Bicinchoninic acid
BHLH	Basic Helix-Loop-Helix
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRET	Bioluminescence resonance energy transfer
C3	Clone 3
CAD	Carboxyl-terminal transactivation domain
CAIX	Carbonic anhydrase IX
CBP/p300	Camp-responsive element binding protein-binding protein/ p300
CH1	Cysteine-histidine-rich domain 1
ChiP	Chromatin immunoprecipitation
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
CNS	Central nervous system
CO_2	Carbon dioxide
CoCl2	Cobalt chloride
CV	Coefficient of variations
DAPI	4',6-diamidino-2-phenylindole
DFO	Deferoxamine
DLR	Dual luciferase reporter assay
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophorectic mobility shifting assay
EPO	Erythropoietin
EtBr	Ethidium bromide
FBS	Fetal bovine serum

FDA	Food and Drug Administration
Fe^{2+}	Iron
FIH	Factor inhibiting HIF
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GLB	Glo Lysis Buffer
GLUT1	Glucose transporter 1
h	Hour
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
HRP	Horseradish peroxidase
HSP90	Heat shock protein 90
HTS	High-throughput screenin
IBS	Institute of Bioscience
kb	Kilobase
kDa	Kilodalton
LARII	Luciferase assay reagent II
LDHA	Lactate dehydrogenase A
mA	Milliampere
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning sites
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide)
min	Minute
N_2	Nitrogen
N803	Asparagine-803
N851	Asparagine-851
NAC	N-acetylcysteine
NAD	Amino-terminal transactivation domain
NCBI	National Centre for Biotechnology Information
NCI	National Cancer Institute
NO	Nitric oxide
O_2	Oxygen
OD	Optical density

ODD	Oxygen-dependent degradation
P402	Proline-402
P405	Proline-405
P531	Proline-531
P564	Proline-564
PAS	PER-ARNT-SIM
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PHD	Prolyl hydroxylases
PI3K	Phosphatidylinositol 3-kinase
PLB	Passive lysis buffer
pO_2	Partial pressure of oxygen
PVDF	Polyvinylidene difluoride
pVHL	von Hipple-Lindau tumor suppressor protein
PX-12	1-methypropyl-2-imidazolyl-disulphide
RCC	Renal cell carcinoma
RET	Resonance transfer energy
RIPA	Radioimmunoprecipitation assay
RLU	Relative light units
x*g*	Times gravity
rRNA	Ribosomal ribonucleic acid
RTCA	Real time cell analyzer
s	Second
SAHA	Suberoylanilide hydroxamic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SLR	Single luciferase reporter assay
S/N	Signal to noise
SUMO	Small ubiquitin-like modifier
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TU	Transducing units
V	Voltage
VEGF	Vascular endothelial growth factor
Z'	Z-factor

2OG	2-oxoglutarate
μ	Means
σ	Standard deviations

CHAPTER 1

INTRODUCTION

Recently, the use of *in vitro* cell-based assays expedites the process of modern drug discovery, including natural product-derived candidates such as microbes, plants and animals. These systems are direct to cellular responses, well represented the research biological models, and managed to mimic the real disease conditions. The success of discovering therapeutic drugs using bioassay systems evidences these systems are rigorous, practical and applicable. Thus far, numerous researches have been done to develop the reporter cell-based assays system that can monitor and assess the cellular responses in various oxygen tensions (Ji, Zhu, Ye, & Li, 2008; Woldemichael et al., 2006; Chau et al., 2005).

Oxygen level is one of the crucial factors in the body. Hypoxia, a condition of lower oxygen concentration than normal level in human body is reputed to be associated with many pathobiological diseases, including ischemic diseases (Semenza, 2014; Simonides et al., 2008), infectious diseases (Bhandari & Nizet, 2014; Zinkernagel, Johnson, & Nizet, 2007), heart diseases (Ke & Costa, 2006), chronic kidney diseases (Nangaku, Rosenberger, Heyman, & Eckardt, 2013; Gunaratnam & Bonventre, 2009), vascular diseases (Kasivisvanathan et al., 2011), inflammatory diseases (Hirota, Beck, & Macdonald, 2009), neurodegenerative diseases (Zhang, Yan, Chang, ShiDu Yan, & Shi, 2011; Correia & Moreira, 2010; Schubert, Soucek, & Barbara, 2009) and advanced cancer progressions (Semenza, 2013; Poon, Harris, & Ashcroft, 2009). The causative factors of hypoxia in the human body can be due to insufficient of blood flow or compromised lung. Furthermore, hypoxic microenvironment inside tumors is owing to the irregular growth of blood vessels and vasculature system (Krock, Skuli, & Simon, 2011). To the best of knowledge, prolonged hypoxia brings lethality to cells and harmful to the human body. In addition to cell death, hypoxic tumors or cancer cells exhibit higher resistance to current cancer treatment modalities, including radiotherapy and chemotherapy, resulting in poor prognosis and increased mortality (Brown, 2007).

The master regulators that help cells to survive in hypoxia are hypoxia-inducible factors (HIFs). Basically, HIFs are transcription factors that consist of an oxygen-dependent α subunit and a constitutively-expressed β subunit. There are three isoforms of 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 (Melillo, 2006). When cells are exposed to hypoxia condition, HIF will be stabilized and specifically interacted with the hypoxia response element (HRE) existing in the promoter regions of HIF target genes. This specific binding leads to 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 particularly in ischemia and cancer progression. Hence, HIF is ideal to be therapeutically targeted for drug discovery and development in recent decades (Semenza, 2010a; Onnis, Rapisarda, & Melillo, 2009).

In sight of the core role of HIF in master regulating various physiological and pathological cellular mechanistic pathways, identification of its activity in cells or tissues is crucial. In this context, advancement in developing small scale yet high-throughput preclinical bioassay systems accelerates the screening and identification of new chemotherapeutic drugs, including natural product-based HIF regulators and synthetic chemical compounds (Melillo, 2007; Nagle, Zhou, Mora, Mohammed, & Kim, 2004). A few Food and Drugs Administration (FDA)-approved chemotherapeutic drugs have been successfully developed with the aid of primary screening using available HIF cell-based assay system (Nordgren & Tavassoli, 2011). This desired and attractive outcome has driven the markets for cell-based assays development. The high demand for cell-based assays in global markets had been valued at $12.1 billion in 2013 and was expected to grow to $15.2 billion in 2015 and $21.6 billion in 2018 at an annual growth rate of 12.4% (TransWorldNews, 2014). This market research report has shown the demand for cell-based assays to quantitate HIF activities is tremendous.

Though animal studies are claimed to be closer in drug response in comparison to the *in vitro* method, the low-throughput, expensive, time-consuming and controversial ethical issues make the animal studies unfavorable (Zang, Li, Tang, Wang, & Yang, 2012). The success of discovery of HIF regulators using high-throughput screening (HTS) cell-based assay system, which is considered as more robust, reliable, economical, and has raised the interests of researchers in this direction. Thus, this demand necessitates the discovery of more robust cell-based assay systems to measure HIF activities, particularly in HIF-related drug screening. Unfortunately, the currently available systems are inefficient, costly, time consuming and most importantly, provide limited sensitivity (Ji et al., 2008; Yamazaki, Egawa, Nose, Kunimoto, & Takeuchi, 2003; Rapisarda et al., 2002). Hence, a discovery of a new, highly sensitive and robust cell-based assay system will satisfy this demand. Moreover, the developed assay systems have to be rigorously validated and proper controlled in order to achieve continuous success in HIF regulators screening.

Therefore, the main objective of this study was to develop a highly sensitive cell-based assay for the screening of HIF activity. The study will be performed with the following specific objectives:

1. To develop a highly sensitive monoclonal cell-based assay for the screening of HIF activity through clonal selection.

2. To validate the HTS suitability factors (*i.e.*, robustness, linearity, specificity, intermediate precision and solvent compatibility) of the HIF bioassay system.

3. To test the application of the HIF bioassay system by a screening of traditional medicinal plant extracts.

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