ENGINEERED CHO CELL LINE WITH COXSACKIE AND ADENOVIRUS RECEPTOR GENE ENHANCE THE SUSCEPTIBILITY OF ADENOVIRUS INFECTION

SAYANG BINTI BABA

UNIVERSITI TEKNOLOGI MALAYSIA
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SAYANG BINTI BABA

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Alhamdullilah...

Segala puji-pujian kehadrat Allah s.w.t
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Gene-based therapies promise the potential to target the explicit gene delivery and expression to target cell populations. Adenoviral vectors are presently being tested clinically as a new strategy for the treatment of cancer. However, an important determining factor for the successful entry of such adenoviruses into target cells is expression of the Coxsackie virus and Adenovirus Receptor (CAR) at the cell surface. This raises the possibility that those gene-based therapy face the greatest therapeutic challenge might be the least susceptible to infection with therapeutic adenoviruses. If effective strategies can be implemented to boost CAR expression and hence the presence of primary receptor at the cell surface, this could prove most useful to adenovirus-based gene transfer. Therefore, this study aimed to possibly boost the expression of CAR specifically on CAR-negative Chinese Hamster Ovary (CHO) cell lines and evaluate their biological function by examining their susceptibility to transduction and infection of Adenovirus type 5. Initially, CHO cell line were transfected with human CAR cDNA fragment tagged with red fluorescent protein (DsRed) and sequentially selected with G418 antibiotic to generate stable cell line containing CAR-DsRed. Several transfection reagents such as GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen) and Xtreme HP plasmid DNA (Roche) were used for transfection study using flow cytometry. In addition, the expression of CAR on CHO-CAR-DsRed cell was determined by inverted fluorescent microscope, qRT-PCR and western blotting and antibody-blocking assay to verify the role of CAR in CHO-CAR-DsRed cells. CHO-CAR-DsRed cells were further examined by infecting wild-type adenovirus type 5 (wt-Ad5) and transduction recombinant adenovirus type 5 tagged with enhanced green fluorescent protein (Ad5EGFP) and their susceptibility were observed by using cytopathic effect and Giemsa staining. Flow cytometry analysis, showed that transfection efficiency of Xtreme HP (39.25±1.00 MFI) was higher than Lipofectamine™ 2000 (17.1±1.00 MFI) and GeneJuice (11.1±1.00 MFI). The stable CHO-CAR-DsRed cells were estimated at average of 68.9±1.12 MFI by transduction of Ad5EGFP and supported by evidence of infectibility of wt-Ad5 from Giemsa staining. Moreover, blocking of CAR expression on CHO-CAR-DsRed showed negative susceptibility to Ad5EGFP. These findings suggested that the strategy could be implemented to augment CAR expression and enhance the presence of primary cell surface receptor, as this could ornament the cell’s susceptibility to adenovirus infection and beneficial for adenovirus-based gene therapy.
ABSTRAK

Terapi berasaskan gen menjanjikan potensi untuk sasaran penyampaian gen yang tepat dan ekspresi kepada sasaran populasi sel. Vektor Adenoviral kini sedang diuji secara klinik sebagai strategi baru untuk rawatan kanser. Walau bagaimanapun, penentuan kejayaan Adenovirus masuk ke dalam sel sasaran adalah ekspresi “Coxsackie virus and Adenovirus Receptor” (CAR) di permukaan sel. Ini meningkatkan kemungkinan bahawasanya terapi berasaskan gen terapeutik menghadapi cabaran terbesar boleh jadi kurangnya penerimaan terhadap jangkitan adenoviruses terapeutik. Jika strategi yang berkesan boleh dilaksanakan bagi meningkatkan ekspresi CAR dan dengan kehadiran reseptor utama di permukaan sel, sekaligus memberi pembuktian yang sangat berguna bagi terapi gen berasaskan Adenovirus. Oleh itu, matlamat kajian ini bagi berkemungkinan meningkatkan ekspresi CAR pada negatif CAR iaitu sel Ovari Hamster Cina (CHO) dan memeriksa kecenderungan mereka kepada transduksi dan jangkitan Adenovirus jenis 5. Pada mulanya, sel CHO transfeksi dengan jujukan CAR manusia cDNA dilabelkan dengan protein merah pendarfluor (DsRed) dan pemilihan berterusan dengan G418 antibiotik untuk menjana barisan sel stabil yang mengandungi CAR-DsRed. Beberapa reagen transfeksi seperti GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen) dan Xtreme HP plasmid DNA (Roche) reagen transfeksi digunakan bagi kajian sitometri aliran. Di samping itu, ekspresi CAR pada sel CHO-CAR-DsRed boleh ditentukan oleh mikroskop pendarfluor songsang, qRT-PCR, pewarnaan western dan esei penyekatan-antibodi untuk mengesahkan peranan CAR dalam sel CHO-CAR-DsRed. CHO-CAR-DsRed seterusnya dikaji dengan menjangkkiti adenovirus kumpulan 5 jenis-liar (wt-Ad5) dan trasnduksi rekombinan Ad5 berlabel dengan peningkatan fluoresen protein berwarna hijau (Ad5EGFP) dan penerimaan jangkitan telah diperhatikan telah menggunakan kesan cytopathic (CPE) dan perwarnaan oleh Giemsa. Analisis sitometri aliran, memperlihatkan bahawa kebolehan transfeksi Xtreme HP (39.25 ± 1.00 MFI) adalah tertinggi berbanding kepada Lipofectamine™ 2000 (17.1 ± 1.00 MFI) dan GeneJuice (11.1 ± 1.00MFI). Sel-sel CHO-CAR-DsRed yang stabil telah dijangkakan pada purata 68.9% ± 1.12 MFI oleh transduksi daripada Ad5EGFP dan disokong dengan pembuktian oleh jangkitan daripada jenis liar-Ad5 melalui pewarnaan Giemsa. Selain itu, penyekatan CAR menunjukkan pemerhatian yang negatif terhadap kecenderungan mana-mana Ad5EGFP. Kepentingan penemuan ini dapat memberi kesimpulan bahawa strategi ini boleh dilaksanakan untuk menghadirkan ekspresi CAR dan meningkatkan kehadiran reseptor primer di permukaan sel, justeru memudahkan penerimaan jangkitan adenovirus terhadap sel dan sekaligus bermanfaat untuk terapi gen berasaskan Adenovirus.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SUPERVISOR’S APPROVAL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DECLARATION ON COOPERATION</td>
<td></td>
</tr>
<tr>
<td></td>
<td>THESIS TITLE</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>ACKNOWLEDGEMENT</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>xviii</td>
</tr>
<tr>
<td></td>
<td>LIST OF ABBREVIATIONS</td>
<td>xxii</td>
</tr>
<tr>
<td></td>
<td>LIST OF APPENDICES</td>
<td>xxv</td>
</tr>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1 Background of Research</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.2 Problem Statement</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.3 Research Objectives</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.4 Scope of Research</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.5 Significance of Study</td>
<td>5</td>
</tr>
</tbody>
</table>
2 LITERATURE REVIEW

2.1 ADENOVIRUSES

2.1.1 INFECTIBILITY OF ADENOVIRUSES (Ads)

2.1.1.1 Adenovirus vector entry uptake into human

2.1.1.2 Adenoviral DNA replication: Early and Late transcription

2.1.2 ADENOVIRUS RECEPTORS

2.1.2.1 COXSACKIE AND ADENOVIRUS RECEPTOR (CAR)

2.1.2.1.1 Expression and Distribution

2.1.2.1.2 Biology function as adhesion protein

2.1.2.2 INTEGRINS

2.1.2.3 OTHER RECEPTORS FOR ADENOVIRUS

2.2 GENE THERAPY

2.3 ADENOVIRUSES AS A GENE THERAPY VECTOR

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Reagents

3.1.2 Vectors

3.1.3 Bacterial Strain

3.1.4 Viruses

3.1.5 Cell Cultures

3.1.6 Antibodies
3.3 METHODS

3.3.1 GENERAL FLOW METHODOLOGY 24

3.3.2 CONSTRUCTION OF pCAR-DsRed 25

3.3.2.1 Mini Preparation Plasmid DNA Isolation (Mini-Prep Isolation, Qiagen) 25

3.3.2.2 Agarose Gel Electrophoresis (Stellwagen, 1998) 26

3.3.2.3 Amplification of pCAR and pDsRed-Monomer-N1 using Polymerase Chain Reaction (PCR) (Pfx DNA Polymerase) 27

3.3.2.4 PCR Product Purification (GeneJet PCR Purification Kit) 28

3.3.2.5 Restriction Endonucleases and Alkaline Phosphatase Treatment for Vector Plasmid DNA (Thermo Scientific FastDigest® Enzyme) 29

3.3.2.6 DNA Fragments Extraction from Gel (PureLink® Quick Gel Extraction Kit) 30

3.3.2.7 DNA Insert (pCAR) Ligation into Vector plasmid DNA (pDsRed-Monomer-N1) (Thermo Scientific) 31

3.3.2.8 Preparation of Chemically Competent E. coli DH5α (Chung et al., 1989) 32

3.3.2.9 Transformation of DNA plasmid into competent E. coli DH5α (Chung et al., 1989) 32
3.3.2.10 Analysis of Transformants (pCAR-DsRed) by using PCR (2X TopTaq Master Mixture, Qiagen) 33

3.3.2.11 E. coli Storage in Glycerol Stock (Laible, 2011) 34

3.3.3 TRANSFECTION OPTIMISATION OF pCAR-DSRED INTO CHO CELL LINE 34

3.3.3.1 Mammalian Cell Lines Maintenance 34

3.3.3.2 Transfection of Plasmid DNA by using GeneJuice, Lipofectamine™ 2000 and Xtreme HP plasmid DNA Transfection Reagent (Novagen, Invitrogen and Roche) 35

3.3.3.3 Selection of Stable Transfected Clone Cells via G418 Antibiotic-Titration Killing Curve (Ambion Inc.) 36

3.3.4 CAR (CHO-CAR-DSRED) EXPRESSION ANALYSIS 36

PART I CAR mRNA Level Analysis

3.3.4.1 RNA Isolation (GeneJET™ RNA Purification Kit) 36

3.3.4.2 Preparing for cDNA molecules from RNA Isolated (Maxima™ Reverse Transcriptase) 38

3.3.4.3 Quantitative Real-Time PCR (qRT-PCR) Amplification (Maxima™ SYBR Green/Fluorescein qPCR Master Mix (2X)) 39

PART II CAR Protein Level Analysis 41
3.3.4.4 Protein Preparation – Cell Lysis
by using
Radioimmunnoprecipitation Assay (RIPA) Buffer (abcam) 41

3.3.4.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Walker, 2009) 41

3.3.4.6 Western Blotting (Mini Trans-Blot® Bio-Rad) 42

3.3.4.7 Indirect Immunofluorescence Analysis of CAR Expression on the Mammalian Cell Lines (Bacallao et al., 2006) 43

3.3.5 ADENOVIRUS TYPE 5 (Ad5) TECHNIQUES 45

3.3.5.1 High Titer of rAd5 by using Adenovirus Replication Assay (Q-Biogene) 45

3.3.5.2 Purification of Ad5 (Vivapure® AdenoPACK™ 20) 45

3.3.5.3 Viral Particle Titration by using Optical Density (OD) 260 nm Viral Particle per mL (VP/mL) (Maizel et al., 1968) 47

3.3.6 BIOLOGICAL FUNCTION
ANALYSIS OF CHO-CAR-DSRED 48
PART I FLUORESCENCE
ANALYTICAL METHODS
3.3.6.1. Transfected Cells Analysis by Flow Cytometry (Hunt et al., 2010) 48
3.3.6.2 Virus Infectivity Assays Analysis by Flow Cytometry (Murakami et al., 2010) 49

PART II MICROSCOPIC ANALYSIS

3.3.6.3 Transduction of Ad5EGFP on engineered stable CHO-CAR-DsRed by using Live-Cell Imaging Microscopic (Murakami et al., 2010) 49

3.3.6.4 Giemsa Staining (Suchman and Blair, 2007) 50

4 CONSTRUCTION OF COXSACKIE AND ADENOVIRUS RECEPTOR (CAR) TAGGED WITH pDSRED-MONOMER-N1 (pCAR-DsRed) 52

4.1 CONSTRUCTION OF pCAR-DSRED 52

4.2 RESULTS AND DISCUSSION 53

4.2.1 Cloning of pCAR into pDsRed-Monomer-N1 vector 53

4.2.1.1 Validation and Purification of pCMV-IRES2-CAR-EGFP and pDsRed-Monomer-N1 56

4.2.1.2 PCR amplification of pCAR using recombinant pCMV-IRES2-CAR-EGFP DNA as a template 57

4.2.1.3 Restriction Endonucleases (RE) Digestion of Vector (pDsRed-Monomer-N1) 58

4.2.1.4 Analyze Transformants (pCAR-DsRed) by using PCR Amplification and RE Digestion 60
ENGINEERED STABLE CELL LINE
CONTAINING pCAR-DSRED FOR
ADENOVIRUS TYPE 5 INFECTION AND
TRANSDUCTION

5.1 OPTIMISATION OF TRANSFECTION 68
5.2 RESULTS AND DISCUSSION 70
  5.2.1 Optimization of Transfection pDsRed-Monomer-N1 plasmid DNA via
      GeneJuice (Novagen), Lipofectamine™
      2000 Transfection Reagent (Invitrogen)
      and Xtreme HP DNA Transfection
      Reagent (Roche) 70
  5.2.2 Transfection pCAR-DsRed plasmid
      DNA via GeneJuice (Novagen),
      Lipofectamine™ 2000
      Transfection Reagent (Invitrogen) and
      Xtreme HP DNA Transfection Reagent
      (Roche) 73
  5.2.3 Selection Stable Transfected via G418
      Antibiotic 75
      5.2.3.1 Titration Killing Curve 75
      5.2.3.2 Selection Stable Engineered CHO-CAR-DsRed 76
5.3 CONCLUSION 83
### 6 CAR EXPRESSION ANALYSIS

6.1 DETECTION OF CAR EXPRESSION ON MAMMALIAN CELL LINES

6.2 RESULTS AND DISCUSSION

6.2.1 Quantitative Analysis of CAR Expression by using Real-Time PCR (qRT-PCR) Amplification

6.2.1.1 Efficiency of $\Delta \Delta CT$ Method

6.2.1.2 Quantification of CAR mRNA expression in various cancer cell lines

6.2.2 Western Blotting

6.2.2.1 Protein Level of CAR Expression

6.2.3 Indirect Immunofluorescence via Live Imaging Microscope

6.3 CONCLUSION

### 7 SUSCEPTIBILITY OF CHO-CAR-DSRED TOWARDS AD5EGFP TRANSDUCTION AND WT-AD5 INFECTION

7.1 INFECTIBILITY OF ADENOVIRUSES

7.2 RESULTS AND DISCUSSION

7.2.1 Viral Particle (VP) Titration

7.2.1.1 Optical Density (OD) 260 nm Viral Particle per mL (VP/mL) of recombinant Ad5 tagged with enhance green fluorescence protein (Ad5EGFP) and wild-type Ad (wt-Ad5)

7.2.2 Transduction of Ad5EGFP Assay

7.2.2.1 Fluorescence Analytical Analysis by using Flow Cytometry
7.2.2.2 Internalisation of Ad5EGFP
Observation by using Live-Cell Imaging Microscopic 99
7.2.3 Infectibility assay with wt-Ad5 101
7.2.3.1 Giemsa Staining Analysis 101
7.2.4 Blocking of Ad5EGFP Transduction Assay 103
7.2.4.1 Fluorescence Analysis by using Inverted Fluorescence Microscope 103
7.3 CONCLUSION 106

8 CONCLUSION AND RECOMMENDATION 107
8.1 CONCLUSION 107
8.2 RECOMMENDATION 108

REFERENCES 109

APPENDICES
A - E 138 - 148
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Advantages and disadvantages of non-viral vectors</td>
<td>17</td>
</tr>
<tr>
<td>2.2</td>
<td>Advantages and limitations of viral vectors</td>
<td>18</td>
</tr>
<tr>
<td>3.1</td>
<td>Specific antibodies used in this study</td>
<td>23</td>
</tr>
<tr>
<td>3.2</td>
<td>PCR mixture for RCR reactions</td>
<td>27</td>
</tr>
<tr>
<td>3.3</td>
<td>Optimal condition for PCR reactions</td>
<td>27</td>
</tr>
<tr>
<td>3.4</td>
<td>The primers used for PCR amplification</td>
<td>28</td>
</tr>
<tr>
<td>3.5</td>
<td>Mixture for single restriction enzyme digestion</td>
<td>29</td>
</tr>
<tr>
<td>3.6</td>
<td>Mixture for double restriction enzyme digestion</td>
<td>30</td>
</tr>
<tr>
<td>3.7</td>
<td>Ligation mixture for pCAR (insert) and pDsRed-Monomer-N1 (vector)</td>
<td>31</td>
</tr>
<tr>
<td>3.8</td>
<td>PCR reaction mixture of 2X Top Taq Polymerase</td>
<td>33</td>
</tr>
<tr>
<td>3.9</td>
<td>The primers used for qRT-PCR amplification</td>
<td>40</td>
</tr>
<tr>
<td>3.10</td>
<td>Cycling conditions for two-step qRT-PCR by using SYBR Green/Fluorescein qPCR Master Mix (2X)</td>
<td>40</td>
</tr>
<tr>
<td>3.11</td>
<td>Preparation of blank dilutions for Optical Density (OD) 230 nm Viral Particle per mL (VP/mL)</td>
<td>47</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.12</td>
<td>Preparation of viral dilutions for Optical Density (OD) 230 nm</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Viral Particle per mL (VP/mL)</td>
<td></td>
</tr>
<tr>
<td>3.13</td>
<td>Serial viral dilutions for Optical Density (OD) 230</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>nm Viral Particle per mL (VP/mL)</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>Absorbance of the serial dilution of viral particle (VP/mL) of Ad5EGFP</td>
<td>94</td>
</tr>
<tr>
<td>7.2</td>
<td>Absorbance of the serial dilution of viral particle (VP/mL) of wt-Ad5</td>
<td>94</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic model of an adenoviral particle (adapted from Russell, 2009)</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Binding and internalization of Adenovirus (Adapted from Q-Biogene)</td>
<td>9</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic Adenoviral infection model (adapted from Greber et al., 1993)</td>
<td>10</td>
</tr>
<tr>
<td>2.4</td>
<td>Early and Late transcription of Adenoviral DNA (Vorburger et al., 2002)</td>
<td>11</td>
</tr>
<tr>
<td>2.5</td>
<td>Schematic structure of the hCAR protein (adapted from Freimuth et al., 2008)</td>
<td>13</td>
</tr>
<tr>
<td>2.6</td>
<td>Adenovirus fiber structure and binding sites (adapted from Zhang and Bergelson, 2005)</td>
<td>16</td>
</tr>
<tr>
<td>2.7</td>
<td>Vectors used in Gene Therapy Clinical Trials (data was provided by the Journal of Medicine)</td>
<td>19</td>
</tr>
<tr>
<td>4.1</td>
<td>Diagram illustrates of restriction map and Multiple Cloning Site (MCS) of pDsRed-Monomer-N1 vector (Clontech)</td>
<td>54</td>
</tr>
<tr>
<td>4.2</td>
<td>Schematic representation of the construction of pCAR-DsRed</td>
<td>55</td>
</tr>
<tr>
<td>4.3</td>
<td>Mini preparation isolation of pCMV-IRES2-CAR-EGFP and pDsRed-Monomer-N1 plasmid DNA</td>
<td>57</td>
</tr>
</tbody>
</table>
4.4 PCR amplification of pCAR using DNA from a recombinant pCMV-IRES2-CAR-EGFP as a template.

4.5 Restriction endonuclease digestion of pDsRed-Monomer-N1.

4.6 Screening of the insert (pCAR) from selective transformant colonies by using PCR amplification.

4.7 Restriction endonucleases digestion analysis of a pCAR-DsRed vector containing pCAR insert.

4.8 Alignment of Forward and Reverse sequencing of nucleic acid of the pCAR (pCAR-DsRed) PCR product and human CAR cDNA sequence (accession Y07593).

4.9 Alignment of Forward and Reverse sequencing of nucleic acid of the DsRed (pCAR-DsRed) PCR product and synthetic pDsRed cDNA sequence (accession EU827527.1).

5.1 CHO cells transiently transfected with the pDsRed-Monomer-N1 plasmid were incubated for 24 hours.

5.2 DNA titration. Cells were analyzed 24 hours post transfection for DsRed expression and cell viability.

5.3 CHO cells were transfected with selected optimum concentrations of a pCAR-DsRed expression plasmid using GeneJuice, Lipofectamine™ 2000 and Xtreme HP transfection reagent respectively.

5.4 CHO cells were treated with G418 antibiotic at range of 0.84 - 4.0 mg/mL for 15 days and cells were analyzed for every 3 days selection.
5.5 a) FACS analysis of CHO cells (untransfected) as a control.

5.5 b) FACS analysis of CHO-CAR-DsRed cells with selection 1.31 mg/mL G418 antibiotic for 24 days.

5.6 Transfected CHO-CAR-DsRed cells were selected with 1.31 mg/mL G418 antibiotic for 24 days and reading were taken for every 3 days selection for CAR-DsRed expression.

5.7 Image of CHO-CAR-DsRed cells with selection 1.31 mg/mL G418 antibiotic for 24 days.

6.1 Standard curve of CAR (TG). Amplification of the standard dilution series of the cDNA target sequence is carried out in separate wells (n=3).

6.2 Standard curve of β-actin (HKG). Amplification of the standard dilution series of the cDNA target sequence is carried out in separate wells (n=3).

6.3 Comparisons of different amplification efficiencies between C_T value of β-actin (HKG) and CAR (TG).

6.4 Detection of mRNA levels of CAR expressions on mammalian cell lines.

6.5 Protein levels of CAR expressions on various types of cell lines.

6.6 Composite images of indirect immunofluorescence.

7.1 Fluorescence microscopy of transduction of Ad5EGFP in MDA-MB-231 and CHO cells.

7.2 Histograms analysis of Ad5EGFP-mediated transduction in a MDA-MB 231 and CHO cell lines.
7.3 Histograms analysis of Ad5EGFP-mediated transduction in a CHO-CAR-DsRed cell lines.

7.4 The sequential images of live transduction of Ad5EGFP to CHO-CAR-DsRed.

7.5 Giemsa staining of wt-Ad5-mediated infection in a HER911, CHO and CHO-CAR-DsRed cell lines.

7.6 Transduction of Ad5EGFP at 25 MOI before and after MDA-MB-231 and CHO cells was blocked with anti-CAR polyclonal (H300).

7.7 Transduction of Ad5EGFP at 25 MOI before and after CHO-CAR-DsRed cells was blocked with anti-CAR polyclonal (H300).
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>SYMBOLS</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>abs</td>
<td>absorbances</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Ads</td>
<td>Adenoviruses</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus serotype 5</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ATCC</td>
<td>Animal Tissue Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie and adenovirus receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
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<td>CO₂</td>
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<td>CPE</td>
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<tr>
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<tr>
<td>DNA</td>
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<td>DsRed</td>
<td><em>Discosoma</em> Red Fluorescence Protein</td>
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<td>EGFP</td>
<td>Enhance green fluorescent protein</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence proteins</td>
</tr>
<tr>
<td>IF</td>
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</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
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<td>internal ribosome entry site</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>MOI</td>
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<td>nm</td>
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<tr>
<td>PBST</td>
<td>Phosphate Buffer Saline with 0.05% Tween 20</td>
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<td>Cephalomycarditis virus plasmid</td>
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<td>Phenylmethylsulfonate</td>
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<td>quantitative Real-Time PCR</td>
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<td>rAd</td>
<td>recombinant Adenovirus</td>
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<td>RGD</td>
<td>Arg-Gly-Asp peptide motif</td>
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<td>rpm</td>
<td>revolution per minute</td>
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<td>S.O.C</td>
<td>Super Optimal broth with Catabolite repression</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>----------------------------------</td>
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<td>Tris-acetate-EDTA</td>
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<td>V</td>
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## LIST OF APPENDICES

<table>
<thead>
<tr>
<th>APPENDIX</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Standard Buffers and Solutions</td>
<td>138</td>
</tr>
<tr>
<td>B</td>
<td>Sequencing of pCAR PCR fragments from recombinant pCAR-DsRed</td>
<td>143</td>
</tr>
<tr>
<td>C</td>
<td>Sequencing of pDsRed PCR fragments from recombinant pCAR-DsRed</td>
<td>145</td>
</tr>
<tr>
<td>D</td>
<td>Images of Reduction of Alamar Blue (AB) Solution</td>
<td>147</td>
</tr>
<tr>
<td>E</td>
<td>Formula Calculation of Percentage of Reduction of Alamar Blue (AB)</td>
<td>148</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Adenoviruses (Ads) vectors have been widely employed for therapeutic gene delivery for clinical trials (Räty et al., 2008; James et al., 2009; and Griesenbach et al., 2010). The most extensively studied and commonly used Adenovirus (Ad) serotype for gene delivery applications is human Adenovirus serotype 5 (hAd5). Promptly, from year 1989 to 2011, extensive pre-clinical and clinical studies using adenovirus-based vectors have been explored and conducted or are currently in progress (http://www.wiley.com/legacy/wileychi/genmed/clinical). Updated in June 2012, nearly 23.3% of 1843 gene therapy clinical trials were approved worldwide by utilizing the Ad-vector based. For example, it has been the first choice for the treatment of cystic fibrosis (Kovesdi et al., 1997; Vorburger and Hunt, 2002 and Price et al., 2007). Adenoviral vectors were also have been manipulated for use in gene therapy applications in glioma, prostate, hepatocellular, head and neck, gastric and oral leukoplakia cancers (Immonen et al., 2004; Li et al., 2007; Shirakawa et al., 2007; Khalighinejad et al., 2008 and Li et al., 2009).

Adenoviruses (Ads) have gained much attention due to their favourable features for gene therapy practices such as the ease to produce high titers with high purity and its ability to transduction a variety of proliferating and quiescent (inactive) cells. Ads also possess a large capacity of DNA cassette and high expression of transgenes. Furthermore, the main issue is the safety concern, adenovirus DNA is not incorporating into host genome (Russell, 2009) and their low pathogenicity for
humans (Glasgow et al., 2004). In addition, recombinants Ads are stable structurally, retains stability in vivo (Kanerva and Hemminki, 2005) such as transduction of Ad5Cox2Lluc and Ad5Cox2LlucUL into human fibroblasts (HS173We) and no mutations reported (McCelland et al., 2007).

Further study had revealed CAR as a prime docking site of all subgroups adenovirus except for subgroup B (Roelvink et al., 1998). The entry of Ads into target cell surface involves two diverse, chronological steps. An interaction between knob of the protruding fiber protein of particle of Ads with extracellular domain of CAR on the target cell surface leading toward internalisation via the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with secondary host-cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

However, it has been suggested that expression of Coxsackie and Adenovirus Receptor (CAR) is a rate-limiting factor for infectivity of adenoviral vectors; and subsequently shown to cause poor transduction of adenoviral vectors gene-delivery in clinical trials (Bauerschmitz et al., 2002 and Kanerva and Hemmiki, 2004). Pre-clinical studies revealed that CAR down-regulation on primary human tumor cells such as ovarian, cervical, prostate, head and neck, bladder, melanoma, glioma cancer and others may be a major hurdle for efficient Ad gene therapy utility (Breidenbach et al., 2004; Rein et al., 2004; Kanerva et al., 2002). Consequently, various approaches have been evaluated to modify or circumvent CAR deficiency, including genetic capsid modifications or retargeting complexes (Zhang and Bergelson, 2005; and Sharma et al., 2009). Scientists had also explored the possibility of finding new additional receptor for Ad other than its prime receptor (Short et al., 2006; Fleischli et al., 2007; Tuve et al., 2008).
By introducing CAR *ex vivo or in vitro* to little or no CAR expression host’s cell prior to adenovirus-based gene transfer may overcome the problem. Thus, it is crucial to investigate the possibility of cloning and expressing human CAR-receptor onto CAR-negative CHO cell lines that was tagged with *Discosoma* Red Fluorescent Protein (DsRed). Then, the transduction with recombinant adenovirus that expresses green fluorescence protein (EGFP) could be performed in order to evaluate the efficacy of the engineered CAR gene. The outcome could be used as a useful model to boost the presence of CAR expression on host’s cell surface and enhance the adenovirus-based transfer efficacy.

1.2 PROBLEM STATEMENT OF RESEARCH

The research was conducted to insure the successfulness of viral gene therapy based on adenoviral vector utilities into host’s cells. Therefore, an examination of structure and co-localization of receptor had been done intently due to fact that the absence of CAR expression in target cells hindered susceptibility of Ads (Wang *et al.*, 2007). Since the presence of CAR is a crucial factor to determine the efficacy of adenoviruses’ susceptibility into host’s cells. Thus, the absence or sparse of CAR expression on the cell surface will hinder the use of Ad based vector. If the presence of CAR can be boosted, subsequently the efficacy of Ad transduction can be enhanced and achieved.
1.3 RESEARCH OBJECTIVES

The objectives are:

1) To construct full-length of human Coxsackie and Adenovirus Receptor (pCAR) into pDsRed Monomer-N1 expression vector (DsRed)

2) To optimise transfection of CAR-DsRed into CHO cell line (CAR-negative) and evaluate of CAR-DsRed protein expression

3) To evaluate the infectibility of stable engineered CHO-CAR-DsRed cell line with wild-type Adenovirus serotype 5 and recombinant Adenovirus serotype 5 expressing enhance green fluorescence protein (Ad5-EGFP)

1.4 SCOPE OF RESEARCH

The scope of study for the first phase involves the construction of cDNA full-length CAR (pCAR) receptor fusion with pDsRed-Monomer-N1 vector with respective molecular cloning techniques. The second section focused on the transfection of CAR-DsRed onto negative-CAR mammalian cell lines namely Chinese Hamster Ovary (CHO) cells via several commercial chemical transfections such as GeneJuice (Novagen), Lipofectamine™ 2000 (Invitrogen) and Xtreme HP Plasmid DNA Transfection Reagent (Roche) according to the respective manufacturer’s protocol. Engineered stable CHO-CAR-DsRed cells were evaluated for CAR expression by using indirect immunofluorescence, quantitative Real-Time PCR (qRT-PCR), protein analysis such as western blotting and antibody-blocking assay were performed. Besides that, for the biological functions of engineered CHO-CAR-DsRed towards recombinant Adenovirus expressing enhance green fluorescent
(Ad5EGFP) was assessed by using flow cytometry. On the other hand, qualitative assessments of cytopathic effects were conducted by Giemsa staining and live imaging microscopic.

1.5 SIGNIFICANCE OF STUDY

This study focused on genetically engineered negative mammalian cell lines expressing stable CAR. Thus, the outcome of this study would infer the strategy to boost CAR expression and enhance the presence of primary receptor on cell surfaces. This could be useful tool to improve adenovirus-based gene transfer to CAR-deficient cells.
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Thomas, S. K., Liley, C. E., Latchman, D. S. and Coffin, R. S. (2002). A protein encoded by the herpes simplex virus (HSV) type 1 2-kilobase latency-associated transcript is phosphorylated, localized to nucleus, and overcomes the expression from exogenous promoters when inserted into the quiescent HSV genome. _Journal of Virology_. **76**: 4056-4067.


