CHAPTER IV

DISCUSSION AND CONCLUSION

Gene therapy holds considerable potential for the treatment of both hereditary genetic disorders and infectious disease. Human gene therapy is defined as the introduction of new genetic material into the cells of an individual with the intention of producing a therapeutic benefit for the patient. Gene therapy requires the introduction of genes designed to specifically block or inhibit the gene expression or function of gene products, such that the function of cull surface protein is blocked or inhibited. The transfer of genetic material into the target cells and tissue has been identified as critical for the study.

Classical methods of gene transfer, such as transfection, are insufficient and limited mainly to delivery into actively proliferating cells *in vitro*. The development of viral vector gene delivery systems is beginning to circumvent these initial setbacks. Several kinds of viruses, including retrovirus, herpes simplex virus, adenovirus and adeno-associated virus, have been manipulated for use in gene transfer and gene therapy applications. As different viral vector systems have their own unique advantages and disadvantages, they each have applications for which they are best suited. This study mentions to adenovirus vector which have the many advantages including, the ability to infect a wide variety of cell types, infection of dividing and non-dividing cells, high efficiency of gene transfer, no integration into the host genome, relatively large transgene capacity, easy manipulation, and high titers (Stone *et al*, 2000). The recombinant adenovirus vector are mostly based on the human

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adenovirus serotypes 5 and have been deleted E1 gene region to allows the insertion of foreign DNA into the adenoviral genome and hold back the viral replication. To allow the insertion of larger transgenes, the E3 gene region was also deleted. Because an adenoviral vector without the E1 gene region can not replicate, the recombinant virus have to be propagated in a cell line expressing the E1 region such as HEK 293A cells (Hitt *et al*, 1997; He *et al*,. 1998; Russell, 2000). Adenovirus can be utilized for many applications such as cancer therapy to deliver genes that will lead to tumour suppression and elimination, gene therapy to deliver genes to tissue to augment defective genes and supplementary therapy to deliver genes to combat disease processes (Rosenfeld *et al*, 1991; Hamada *et al*, 1996; Clayman *et al*, 1999; Schuler *et al*, 2001; Song, 2005).

The use of antibodies in medicine and research depends on their specificity and affinity in the recognition and binding of individual molecules. Advances in molecular cloning technology have allowed the manipulation of genes encoding antibodies into many forms, creating modified antibodies that retain the binding specificity of the parent antibody while deleting or adding additional functions (Marasco and Dana Jones, 1998; Sanz *et al*, 2005). The simplest forms of engineered antibodies are those which contain only the antigen binding domain. These can be engineered as single chain antibodies that contain the variable regions connected by a small flexible interchain linker (Marasco and Dana Jones, 1998), as shown in Figure 3.7a. With a molecular weight five times smaller than a full-length antibody, the single chain antibody or single chain Fv fragment (scFv) has been the recombinant antibody format most widely used to express inside cells, called intracellular antibodies or intrabodies (Kontermann, 2004). The specificity and affinity that can be

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obtained in intrabodies make these molecules effective modulators of viral or cellular targets. The successful using of scFv as an intrabody was achieved by blocking the interaction between the intracellular target and other partners to disrupt the biological processes in which it is involved or trapping and retaining their antigen in a wrong cellular compartment by the intrabodies (Lobato and Rabbitts, 2004; Boldicke, 2007). There are several ways to obtain antibody fragments suitable for intracellular use and it is often necessary to employ selection methods because many scFv do not seem to fold correctly *in vivo*, due to the adverse reducing conditions inside cells.

Adenoviral vector was widely used to deliver the scFv gene into the mammalian cells. For example, Whittington *et al.* (1998) have applied this technique to generated two recombinant adenoviruses expressing the scFv fused to murine GM-CSF. The scFv used are MFE-23 which binds to a human tumour-associated marker carcino embryonic antigen (CEA). Cell lines transduced with this adenoviral vector in vitro express and secrete high levels of the scFv.GM-CSF fusion proteins. Using scFv to target GM-CSF to tumour cells not only reduce the systemic toxicity of GM-CSF but also retain its ability as a cytokine to induce systemic immune responses to tumour targets (Whittington *et al*, 1998). More recently, Arafat et al. (2002) have developed an approach to express scFv in vivo. Adenoviral vector expressing anti-erbB2 scFv was constructed for secretion by eukaryotic cells. The secreted scFv could bind to its target and specifically suppress cell growth of erbB2-positive cells in vitro. *In vivo* gene transfer via the anti-erbB2 scFv encoding adenovirus also showed anti-tumour effects. These approaches have the potential to realize effective scFv-based approaches using the adenoviral vector (Arafat *et al*, 2002).

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Here, two versions of adenovirus vector expressing scFv-M61B9 in cytosolic and ER compartment were constructed to produce the scFv-M61B9 in HeLa cell cytosol and secretory system by using the AdEasy system. The strategy of recombinant adenoviral production involves three major steps. First, the scFv-M61B9 gene is cloned into a shuttle vector pAdTrack-CMV. Second, the resultant construct is cleaved with *Pme*I restriction endonuclease to linearize it and transformed into *E. coli* strain BJ5183 containing an adenoviral backbone vector pAdEasy. Because the *E. coli* strain BJ5183 is not *recA*-mutated bacteria, it contains the enzymes that mediate recombinant in bacteria and was chosen for honomogous recombination. Recombinants were selected with kanamycin and screened by *Pac*I restriction endonuclease digestion. Third, the recombinant adenoviral construct was cleaved with *Pac*I to expose its inverted terminal repeats and transfected into HEK 293A packaging cells. The process of viral production can be directly followed in the transfected cells by visualization of the GFP reporter.

Intrabodies that are targeted to the lumen of the ER provide a simple and effective mechanism for inhibiting the transport of plasma membrane or secreted proteins to the cell surface. The ER is a specialized compartment where folding occurs for the proteins that are destined for secretion to an extracellular environment or the plasma membrane and other organelles in the secretory pathway. The ER lumen is more vesemble to the extracellular space than the cytosol with respect to high oxidizing potential. It contains various types of chaperones and folding factors not only to assist efficient folding of scFv but also to play a quality control role. In this study, three different forms of scFv-M61B9 gene were designed. The scFv-M61B9ER that are intended for localization in the ER are generally equipped with a leader

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peptide and a C-terminus ER retention signal (the KDEL amino acid motif – Lys-Asp-Glu-Leu). As a result, the leader peptides allow the scFv-M61B9 to enter ER compartment and can be retained in the ER lumen through the use of the ER retention signal (KDEL) at their carboxyl termini. The binding activity and specificity of these scFv were shown by the indirect ELISA and the western immunobloting. In comparison, the scFv-M61B9 which was mutated at the lysine sequence (AAA) of the KDEL region to create the stop codon (TGA) was significantly secreted out of the cell, there by allowing them to perform their function.

The expression of scFv-M61B9 in the cytoplasm has been achieved by simply removing the leader sequence. Nevertheless, one of the main problems associated with intrabodies is that most scFv are not able to fold under the reducing conditions of the cell cytosol, where most of the interesting targets are located. This is thought to be due to the limited stability of scFv after the two conserved disulfide bonds are reduced, as occurs in the cell cytosol. In addition, even if a scFv-M61B9 is indeed stable enough in its reduced form to be expressed and active in the cytoplasm, other parameters such as protease susceptibility or folding kinetics may also influence the final fate of scFv activity. The misfolded scFv-M61B9 in the cell cytosol may be degrade and turnover by the ubiquitin-proteasome system which is now believed to be the principal system for turnover of short-lived, misfolded or truncated proteins in eukaryotic cells (Lee and Yu, 2005; Gao and Hu, 2008).

In conclusion, the adenovirus expression system for production of a scFv in each mammalian cell compartment was established. This study demonstrates the construction of adenoviral vector for the production of recombinant adenovirus which can be expressed the scFv-M61B9 in ER compartment by using AdEasy system.

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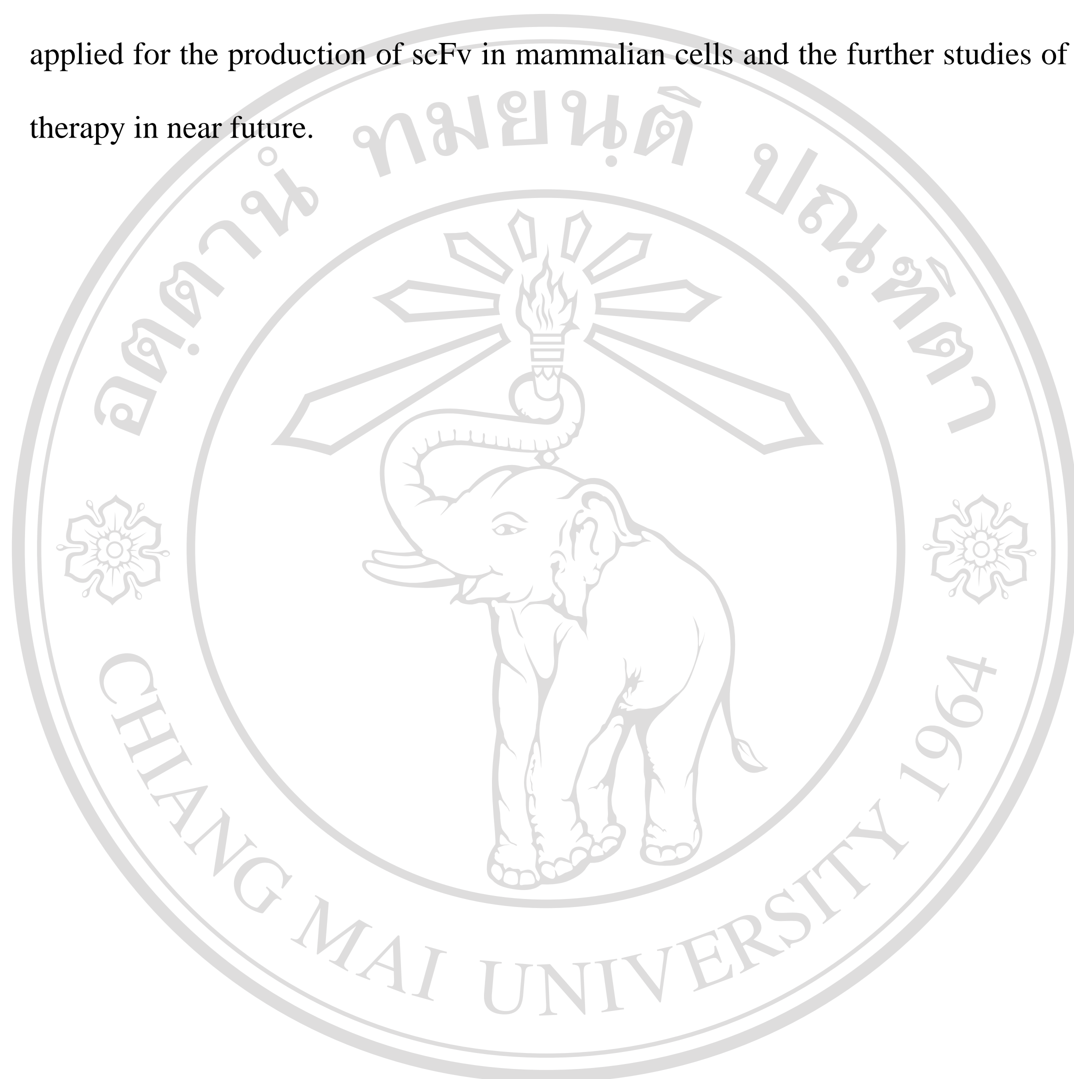
These produced scFv can be secreted outwards the extracellular milieu through the secretory system. The results from this study provide the promising method to further applied for the production of scFv in mammalian cells and the further studies of gene therapy in near future.



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