CONSTRUCTION AND CHARACTERIZATION OF OC125 S INGLE-CHAIN ANTIBODY

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II. ABSTRACT

Le cancer ovarien est la principale cause de la mortalité attribuable aux cancers gynécologiques dans les pays occidentaux. De plus, pour la majorité des patientes (>70%), la maladie en diagnostiqué en phase tardive. Ces diagnostiques tardifs conduisent à un taux de survie de 15 à 30 pour cent après cinq ans. Dans le but d'accroître la qualité et la rapidité du dépistage, la découverte et l'utilisation de marqueurs spécifiques sont d'ungrand intérêt. Pour les cancers ovariens, la progression clinique de la maladie est en corrélation avec l'expression de l'antigène tumoral CA125, ce qui en fait un marqueur de choix. Cet antigène est actuellement utilisé en tant qu'indice de récurrence clinique des cancers ovariens. Aussi, l'antigène CA125 est employé pour vérifier l'efficacité des traitements de chimiothérapie pour ces cancers.

Des études antérieures ont montré que CA125 est une glycoprotéine de haut poids moléculaire avec des propriétés semblable à une molécule de mucine (mucin-like). La nature biochimique et moléculaire de cet antigène ainsi que sa fonction demeure peu ou pas connu. Dans cette étude, nous avons développé une approche basée sur l'expression d'anticorps à chaîne unique (ScFv) pour abroger sélectivement CA125 afin d'étudier son rôle dans les cancers ovariens chez l'humain.

L'ADNc qui code pour l'anticorps ScFv a été produit à partir des ARNm extraient de l'hybridome OC125. Cet hybridome exprime l'anticorps monoclonal spécifique à la partie extracellulaire de CA125. Les gènes codant pour la partie variable des chaînes lourdes (V_H) et légères (V_L), séparées par la séquence codant pour le peptide $(Gly_4Ser)_3$, ont été clonés dans le même vecteur. L série de gènes résultants, contenant les ScFvs, a

été clonée dans le vecteur d'expression procaryote pCANTAB 5E. Cette banque d'ADNc a été criblée par les essais de "phage-display" et "colony-lift". La capacité des ScFvs de lier CA125 a été validée par ELISA. Six ScFvs positifs ont été choisis parmi 176 candidats. La séquences en acides nucléiques montre que la région V_L et la majeure partie des peptide de liaison ont été éliminées dans l'anticorps ScFv #148. Cet anticorps a démontré la meilleure affinité pour CA125 en ELISA. Par la suite, celuici a été sous-cloné dans le vecteur d'expression eucaryote pSTCF.KDEL contenant le peptide signal pour le transport de la protéine au réticulum endoplasmique. Un système d'expression transitoire de la protéine encodé par le plasmide pSTCF.KDEL dans des lignées cellulaires d'ovaires humains a été utilisé comme modèle. Nous avons ainsi démontré une diminution significative de #148 ScFv dans les cellules d'une lignée co-exprimant l'antigè CA125 (OVCAR3: cellules tumorales d'ovaries humains) par rapport à une lignée n'exprimant pas CA125 (PA-1). Par contre, si l'on compare avec d'autres ScFvs ne liant pas CA125 en ELISA, dans ces deux mêmes lignées cellulaires, leurs concentrations demeurent équivalentes. Ces résultats suggèrent que la protéine ScFv #148 peut lier CA125 dans le réticulum endoplasmique des cellules OVCAR3. Néanmoins, des expériences de l'immunoprecipitation n'ont pas démontré une interaction directe entre la protéine de ScFv #148 et CA125.

En conclusion, dans cette étude nous avons construit un ScFv contre l'antigène CA125. Cet anticorps lorsque exprimé chez *E.coli* est soluble et reconnaît spécifiquement la protéine CA125 en ELISA. Lorsque le ScFv fut sous-cloné dans le vecteur eucaryote pSTCF.KDEL et ensuite transfecté dans des cellules ovariennes humaines, un niveau d'expression élevé a été obtenu.

III. ABSTRACT

Ovarian cancer is the leading cause of death from gynecological cancers in Western countries. Over 70% of the patients present with late stage disease, the five-year survival rate for these patients remains at best of 15-30%. Tumor antigen CA125 expression has been shown to correlate with the clinical course of the disease, and CA125 is currently used as a predictor of clinical recurrence in ovarian cancer and to monitor response to chemotherapy treatment.

Previous studies showed that CA125 is a high molecular weight glycoprotein having properties of a mucin-type molecule. The biochemical and molecular nature and the function of this antigen are poorly understood. In this study, we have developed an approach based upon intracellular expression of single-chain antibodies (ScFvs) to achieve selective abrogation of CA125, thus to study the role of CA125 in human ovarian cancer cells.

The cDNA coding ScFv was generated from mRNA extracted from the hybridoma cell line OC125, which expresses monoclonal antibodies that specifically recognize an epitope located in the extracellular portion of CA125. Genes coding for the V_H and V_L were cloned into the same vector, separated by a linker sequence that coded the peptide $(Gly_4Ser)_3$. The constructed ScFv library was cloned into the procaryotic expression vector pCANTAB 5E and the cDNA library was screened by phage-display and colony-lift assay. CA125 antigen-binding activity of ScFvs was tested by ELISA, and six positive ScFvs were selected among 176 candidates. Sequencing analysis showed that the V_L region and most of the linker were deleted from CA125-positve #148 ScFv, which

has the strongest binding activity in ELISA. After subcloning #148 OC125 ScFv into the ER-targeting eukaryotic expression vector pSTCF.KDEL, in which the protein was targeted into ER, and using a transient expression system, we showed that the expression level of #148 ScFv was significantly lower in the human ovarian cancer cell line OVCAR3, which expresses CA125, than in the cell line PA-1, which does not express CA125. Meanwhile, the control ScFv proteins, which did not bind to CA125 in ELISA, displayed equal expression levels in both cell lines. These results suggest that #148 OC125 ScFv protein may bind to CA125 in the ER of OVCAR3 cells. However, immunoprecipitation experiments did not demonstrate a direct interaction between #148 ScFv and the CA125 protein.

In conclusion, the study shows that a ScFv against the CA125 antigen was constructed, and that the soluble antibodies bind to CA125 protein in ELISA when expressed in *E.coli*. When the ScFv was subcloned into the eukaryotic vector pSTCF.KDE, which targets cloned genes to the ER, high levels of expression were obtained in mammalian cells. An interaction between OC125 ScFv and CA125 protein was not demonstrated in this study.

IV. GLOSSARY OF ABBREVIATIONS

2xYT-AG: 2xYT medium containing 100μg/ml ampicillin and 2% (v/v) glucose

2xYT-AK: 2xYT medium containing 100μg/ml ampicillin and 50μg/ml kanamycin

2xYT-AI: 2xYT medium containing 100μg/ml ampicillin and 1mM IPTG

4-CN: 4-chloro-1-naphthol

AA: amino acid

ATCC: American Type Culture Collection

bp: base pair

kb: kilo-base pair

CA125: cancer antigen 125

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CDR: complementary determining region

C_H: heavy chain constant region

C_L: light chain constant region

CMV: cytomegalovirus

dNTP: deoxyribonucleic triphosphate

DMEM: dulbecco's modified eagle's medium

E.coli: *Escherichia coli*

EDTA: ethylene-diaminetetra-acetic acid disodium salt

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

EOC: epithelial ovarian cancer

ER: endoplasmic reticulum

Fab: fragment of antibody

FBS: fetal bovine serum

Fv: fragment of variable region

FW: framework

g: gram

mg: milligram

μg: microgram

ng: nanogram

pg: picogram

g3p: gene III minor coat protein

HRP: horsradish peroxidase

Ig: immunoglobulin

IPTG: isopropyl-1-thio-β-D-galactopyranoside

kDa: kilodalton

kV: kilovolt

L: liter

ml: milliliter

μl: microliter

LMP1: latent membrane protein 1

M: mole

mM: millimole

MAb: monoclonal antibody

NFDM: non-fat dry milk

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

RT PCR: reverse transcription polymerase chain reaction

P.E.: periplasmic extraction

PEG: polyethylene glycol

pfu: plaque-forming unit

PVDF: polyvinylidene difluoride

RNA: ribonucleic acid

mRNA: messenger ribonucleic acid

rpm: revolutions per minute

RS primer: restriction site primer

ScFv: single-chain variable region fragment

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SS: disulfide cross-links

TGF-α: transforming growth factor alpha

U: unit

μF: microfarad

V_H: heavy chain variable region

V_L: light chain variable region

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CHAPTER 1 INTRODUCTION

The objective of this study is to identify the role of tumor antigen CA125 in human ovarian cancer cells using ScFv. This study includes three major parts: construction and screening of a OC125 ScFv, expression of the recombinant ScFv plasmid and analysis of ScFv protein's binding activity to the CA125 tumor antigen. The introduction covers four parts: human ovarian cancer (section 1.1), CA125 (section 1.2), strategies to be used to study the function of protein (section 1.3) and structure of ScFv (section 1.4).

1.1 Human ovarian cancer

Ovarian cancer represents the fifth most significant cause of cancer-related death for women and is the most frequent cause of death from gynecological neoplasia in the Western world. Ovarian cancer causes more deaths than any other cancer of the female reproductive system. Most (80-90%) ovarian tumors are epithelial in origin and arise from the coelomic epithelium. The remainders arise from germ cell or sex cord/stromal cells. A hereditary component in the latter group is rare, but includes granulose-cell tumors in patients with Peutz-Jeghers syndrome (Ferry, J.A. et al, 1994) and autosomal dominant inheritance of small-cell carcinoma of the ovary (Lamovec, J., et al., 1995; Longy, M., et al., 1996).

Over 70% of ovarian cancer patients present with late stage disease, the majority of which cannot be completely resected by surgery. Chemotherapy has become the primary adjunct to surgery in obtaining a clinical remission or enhancing disease free survival in ovarian cancer patients. Although response to initial chemotherapy treatment approaches 70%, most are transient and approximately 80% of patients will recur and eventually die of the disease. A variety of salvage agents and strategies have been investigated, but none have demonstrated long-term effectiveness. The five-year survival rate of the patients with a stage III epithelial ovarian cancer remains, at the best, at 30% (American Cancer Society Surveillance Research, 1997). This poor prognosis is largely due to the fact that 75% of cases present with extra-ovarian disease, which in turn, reflects the absence of symptoms in early-stage disease. Advanced stage ovarian cancer (stage IV) has a five-year survival rate of approximately 10% whereas early stage (stage I) ovarian cancer has a five-year survival rate of at least 85%. These data suggest that there may be a survival benefit from the detection of ovarian cancer at an early stage.

To be able to develop appropriate screening strategies for ovarian cancer, it is necessary to understand the processes of carcinogenesis and tumor progression. More than 90% of epithelial ovarian cancers are clonal neoplasms that arise from the progeny of a single cell (Jacobs, I.J., et al, 1992). Given the clonality of most ovarian cancers, multiple genetic alterations must occur in the progeny of a single cell to permit progression from a normal epithelial phenotype to that of a malignant cell capable of uncontrolled proliferation, invasion, and metastasis. Somatic mutations have been found in sporadic ovarian cancers that activate oncogenes or that result in loss of tumor suppressor gene

function. Different ovarian cancers can also exhibit aberrant autocrine and/or paracrine growth regulation with alteration in the expression of growth factors and their receptors. Certain changes in oncogenes, tumor suppressor genes, growth factors, and their receptors occur in a significant fraction of epithelial ovarian cancers, whereas others are uncommon.

Numerous of tumor suppressor genes have been identified in cancers and subsequently evaluated in ovarian cancers, including RB, VHL, WT, and p53 (Donehower, L.A., 1992). Tumor suppressor genes normally function to inhibit or to slow down the cell growth and division cycle; they function to prevent the development of neoplasia. Mutations in tumor suppressor genes cause the cell to ignore one or more of the components of the network of inhibitory signals, removing the brakes from the cell cycle and resulting in a higher rate of uncontrolled growth of cancer. Loss of p53 function is observed in more than 50% of advanced ovarian cancers, but in only 15% of stage I lesions (Marks, J.R., et al., 1991). Mutation of p53 is only occasionally observed in ovarian cancers with low malignant potential and is rarely detected in benign ovarian tumors. Consequently, abnormalities of p53 have been considered a "late change" in tumor progression, associated with the acquisition of metastatic potential.

Activation of several oncogenes has been reported in subsets of ovarian cancers, including erb-B2 (Bergmann, C., et al., 1986; Berchuck, A., et al., 1990; Felip, E., et al., 1995), cyclin D-1 (Baserga, R., 1994), and EGFR (epidermal growth factor receptor) (Warenius, H.M., et al., 1996; Hochhauser, D., et al., 1996). Oncogenes are mutated

forms of proto-oncogenes whose functions are to encourage and promote the normal growth and division of cells. Deregulation of proto-oncogenes may result in overproduction of growth factors; flooding of the cell with replication signals; uncontrolled stimulation in the intermediary pathways; and/or unrestrained cell growth driven by elevated levels of transcription factors. Normal ovarian surface epithelium expresses EGFR detected by immunohistochemical techniques and this expression is lost in approximately 30% of ovarian cancers, associated with a slightly better prognosis (Berchuck, A., et al, 1991). Activation of EGFR can occur through truncation of its extracellular domain and this variant has been observed in some ovarian cancers (Huang, H.J.S., et al, 1997; Hekis, J.V., et al, 1997).

Growth of ovarian cancers can be stimulated by several peptide and lipid growth factors. Peptide ligands that bind to the EGFR are produced by ovarian cancers including EGF, transforming growth factor alpha (TGF-α), and amphiregulin (Stromberg, K., et al, 1994). Antibodies against TGF-α can inhibit the growth of ovarian cancer cell lines that continue to express EGFR, consistent with autocrine growth stimulation (Stromberg, K., et al, 1992). Paradoxically, it has been difficult to document activation of EGFR in ovarian cancer cell lines, challenging the functional importance of this particular receptor for autocrine growth stimulation (Ottensmeier, C., et al, 1996).

1.2 CA125 tumor antigen

Tumor markers are used for multiple purposes in clinical care, including screening asymptomatic subjects, differential diagnosis of symptomatic patients, treatment planning, prognosis during and immediately following treatment, and monitoring for recurrence. CA125 is the most widely used ovarian tumor marker, and is currently approved in the United States and Canada for monitoring of disease to determine if second-look surgery is required. The main concern about using CA125 as a first-line test for ovarian cancer is its apparent lack of sensitivity for early-stage disease (Helzlsouer, K.J., et al, 1993). Only 50% of stage I ovarian cancer-patients have elevated CA125 preoperative serum levels. The traditional cutoff level for a positive CA125 test is 30 or 35U/ml. It is important to note that this level was established for patients with clinically established disease (Bast, R.C., et al, 1983), and was not recommended as the appropriate cutoff level for screening asymptomatic populations.

CA125 is a mullerian duct differentiation antigen that is overexpressed in epithelial ovarian cancer cells and released into the blood, although its expression is not entirely confined to ovarian cancer. Ovarian cancer CA125 antigen was first detected by Bast and Knapp in 1981, using the monoclonal antibody OC125 that was raised against the human ovarian carcinoma cell line OV433 (Bast, R.C., et al, 1981). These investigators subsequently developed a radioimmunoassay for the antigen and showed that serum CA125 levels are elevated in about 80% of patients with epithelial ovarian cancer (EOC) but in less than 1% of healthy women (**Table 1**) (Bast, R.C., et al, 1983). The usefulness

of CA125 levels in monitoring the progress of patients with EOC has been proved by a variety of studies. Most reports indicate that a rise in CA125 levels precedes clinical detection by about 3 months. During chemotherapy, changes in serum CA125 levels correlate with the course of the disease. It is why today the CA125 assay is the most useful diagnostic test in the management of ovarian cancer. It is currently used as a predictor of clinical recurrence in ovarian cancer and to monitor response to chemotherapy treatment (**Figure 1**) (Niloff, J.M., et al., 1986; Fish, R.G., et al., 1987; Makar, A.P., et al., 1993; Rustin, G.J.S., et al., 1996).

Despite the widespread use of CA125 as a clinical marker of ovarian cancer, the biochemical and molecular nature and the function of this antigen is poorly understood. At the time we started this study, the gene had not been cloned and its sequence was therefore unknown. Just before I finished my project, Dr. Kenneth Lloyd, who discovered CA125, recently reported the isolation of a long, but partial, cDNA that corresponds to the CA125 antigen. A rabbit polyclonal antibody produced to purified CA125 antigen was used to screen a λZAP cDNA library from OVCAR-3 cells in E.coli. The longest insert from the 54 positive isolated clones had a 5965 bp sequence containing a stop codon and a poly A sequence but no clear 5' initiation sequence. Northern blotting showed that the level of MUC16 mRNA correlated with the expression of CA125 in a panel of cell lines. But since it has an incomplete structure, it only can be considered as the best candidate for CA125.

Group	Total Number Tested	Antigen Level	
		> 35 U/ml	> 65 U/ml
		no. (%)	
Apparently healthy controls	**888**	**9 (1.0)**	**2 (0.2)**
Males	537	4 (0.7)	2 (0.4)
Females	351	5 (1.4)	0 (0.0)
Patients with benign diseases	**143**	**9 (6.3)**	**3 (2.1)**
Patients with nongynecological cancer	**200**	**57 (28.5)**	**44 (22.0)**
Pancreatic	29	17 (58.6)	13 (44.8)
Lung	25	8 (32.0)	6 (24.0)
Breast	25	3 (12.0)	2 (8.0)
Colorectal	71	16 (22.5)	12 (16.9)
Misc. gastrointestinal	30	8 (26.7)	6 (20.0)
Misc. non gastrointestinal	20	5 (25.0)	5 (25.0)
Patients with ovarian carcinoma	**101**	**83 (82.2)**	**75 (74.3)**

Table 1. Detection of CA125 antigen in serum: serum CA125 levels are elevated in about 80% of patients with epithelial ovarian cancer (EOC) but in less than 1% of healthy women.

--Adapted from Bast, R.C. et al. (1983) *New England Journal of Medicine*, 309:883-887

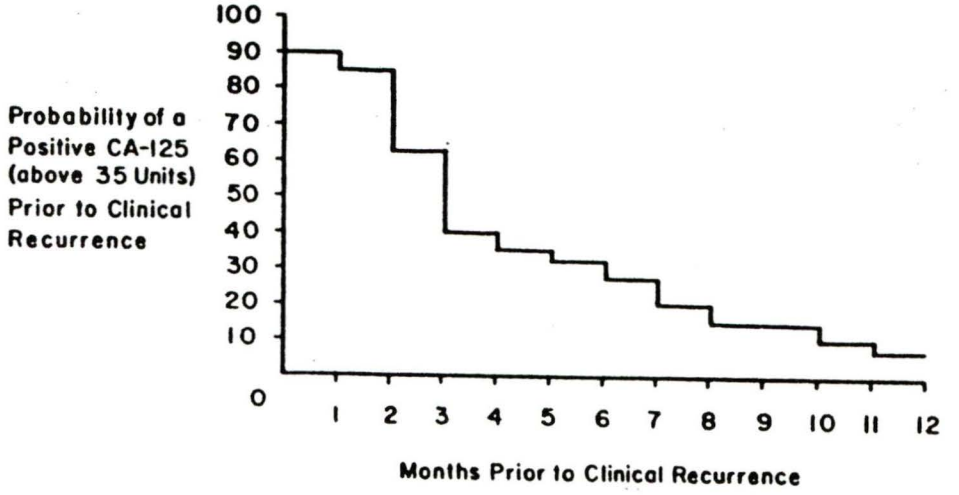


Figure 1. 35 of 55 patients had a clinical recurrence after a second-look operation. Serial CA125 levels rose to >35 U/ml in 94% of these patients before their recurrences became clinically detectable. The median lead-time before clinical recurrence was 3 months, and in over 90% of patients was at least 2 months. Among the 20 of 55 patients who did not recur clinically, CA125 levels were always <35 U/ml in serum.

--Niloff, J.M. et al. (1986) *American Journal of Obstetrics and Gynecology*, 155:56-60

Previous studies showed that the CA125 epitope is carried on a large glycoprotein with a molecular weight in the range of 200-2000 kDa, while other studies have reported that CA125 consists of many subunits of 50-200 kDa (De Los Frailes, M.T., et al., 1993; Kobayashi, K., et al., 1993; Matsuoka, Y., et al., 1987; Nagata, A., et al., 1991; Yu, Y.H., et al., 1991; Zurawski, V.R., et al., 1988). The study of Lloyd et al. has shown that CA125 is a high molecular weight glycoprotein having properties of a mucin-type molecule (Lloyd, K.O., et al., 1997). Mucins, or mucin-type glycoproteins, can be defined as large extended molecules with a high percentage (50%-90%) of their molecular mass made up of carbohydrate that is attached via O-glycosidic linkage through N-acetylgalactosamine to serine and/or threonine. They can be subdivided into the classical secretory or soluble mucins and the membrane-associated mucins(Spicer, A.P., et al, 1995). In this regard, it has been suggested that mucin-like proteins could play a role in the increased invasiveness of tumor cells and cell/cell-matrix adhesion (Guddo, F., et al., 1998; Yonezawa, S. et al., 1997). For instance, the Muc-1 mucin protein, one of the most characterized mucins, has been found to be expressed in all invasive types of pancreas, bile duct and breast carcinomas but were not frequently detected in the non-invasive types (Suwa, T., et al., 1998). In addition, the growth rate of primary breast tumors was found to be significantly reduced in Muc-1 deficient mice, and the percentage of animals with metastases was lower in Muc-1 deficient mice than in Muc-1 positive mice (**Figure 2**), suggesting that Muc-1 plays an important role in the progression of mammary carcinoma (Spicer, A.P., et al., 1995).

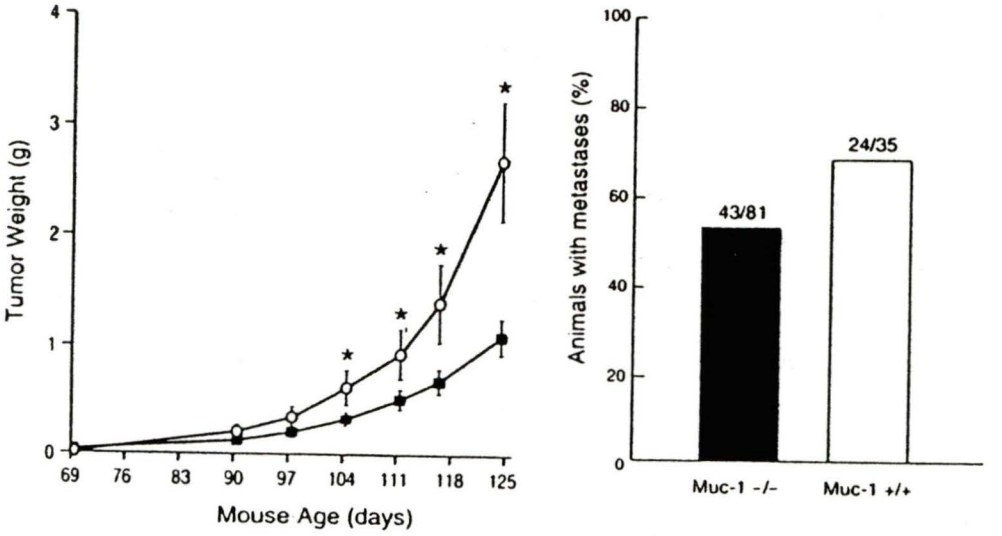


Figure 2. Primary tumor growth rate is reduced in Muc-1 deficient mice. A, graph showing growth rate of polyoma middle T-induced mammary tumors in Muc-1 -/- (filled square) and Muc-1 +/+ (open square) mice. At 104 days, Muc-1 -/- mice had significantly smaller tumors than did Muc-1 +/+ mice ($p<0.05$). By the 124-day end point, differences in tumor size were highly significant ($p<0.001$). Asterisks indicate statistical significance. B, graph showing the percentage of Muc-1 +/+ and -/- mice with metastatic lesions in the lung at 124 days. The trend toward decreased rates of tumor metastasis in Muc-1 -/- mice suggested that the lack of Muc-1 was showing some effects. The sample size was not sufficiently large to reach statistical significance.

--Spicer, A.P. et al (1995) *The Journal of Biological Chemistry*, 270(50):30093-30101

CA125 expression has been detected in over 80-90% of ovarian cancer (Bast, R.C., et al., 1983), but has never been found in the ovary during development or in the adult. Because CA125 seems to be a mucin-like protein, it might play an adhesive role, similar to MUC-1, by presenting carbohydrates as ligands for selectin-like molecules and thus aiding metastatic dissemination. In addition, evidence show that CA125 production and/or release can be regulated by several hormones (Bischof, P., et al., 1986; Karlan B.Y., et al., 1988; Brumsted J.R., et al., 1990), growth factors (Marth, C., et al., 1990) and cAMP(Ishiwata I., et al., 1986). Taken together, these data suggest that the deregulation of CA125 expression may play a role in the pathogenesis of ovarian cancer.

1.3 Strategies to be used to study the function of proteins

A variety of genetic strategies have been used to study the function of a protein in transformation, these methods usually involved either gain or loss of protein function.

In the first instance, studies to define the role of a gene in the transformation process were done using single gene transfer experiments. In this context, the cDNA coding the protein of interest is transfected into selected cells in order to analyze any new phenotypes associated with overexpression of this protein. One obvious limitation of this approach is the necessity of cloning the target gene prior to conducting gene transfer experiments.

As an alternative to the gain-of-function studies, the use of gene therapy strategies to specifically knockout a protein of interest in transformed cells offers new means to define its function. In this regard, loss of a critical protein in a transformed cell may be more relevant to study its mediated pathway. The most common method is the use of antisense molecules. These molecules are designed specifically to target sense (coding) sequences to block the transcription and translation of the encoded genetic information (Gura, T., 1995). Thus, this method relies on the prior knowledge of the coding sequence of the target gene. In addition, severe limitations, including non-specific effects, instability, difficulty to achieve adequate intracellular levels, have precluded widespread use of antisense molecules (Gewirtz, A.M., et al., 1996; Stein, T.A., 1995).

Ribozymes are RNA molecules that have catalytic activities. They function by binding to a specific RNA target through antisense and inactivate it by cleaving the phosphodiester backbone at a specific site (James, H.A., et al., 1998). Although ribozymes have been used to inhibit a variety of viral and cellular genes, the method also relies on the prior knowledge of the coding sequence of the target molecule.

One obvious common limitation to these approaches is the necessity of cloning the target gene prior to conducting the gain or loss of function experiments. Thus, these current strategies are of limited use to study the function of CA125 since the gene is not cloned and its sequence remains unknown.

1.4 Structure and properties of ScFvs

1.4.1 Monoclonal antibodies

Antibodies have long been used in biomedical science as in vitro tools for the identification, purification and functional manipulation of target antigens; they have been exploited in vivo for diagnostic and therapeutic applications as well. Recent advances in antibody engineering have allowed the genes encoding antibodies to be manipulated so that the antigen-binding domain can be expressed intracellularly. The specific and high-affinity binding properties of antibodies, combined with their ability to be stably expressed in precise intracellular sites inside mammalian cells, has provided a powerful new family of molecules for cancer gene therapy applications.

Typical monoclonal antibodies (**Figure 3A**) have a common structure consisting of two identical heavy and light chain polypeptides held together by disulfide bridges and non-covalent bonds. The DNA and amino acid sequence of the C region is relatively conserved within a given animal species while the V region sequence is antigen-dependent. Pairing of V regions of heavy and light chain creates an antigen-binding site (paratope), which recognizes the epitope of an antigen. Most protein antigens contain many potential epitopes, which are recognized by a correspondingly large number of specific antibodies that comprise a polyclonal antibody population.

The constant (C) region is located at the carboxy-terminus of the antibody chain. In the mouse, there are five classes of constant heavy (C_H) chain genes (α, δ, ε, γ, μ) and two classes of constant light (C_L) chain genes (κ and λ). DNA and amino acid sequences are relatively conserved within each class. Antibodies are grouped into five major classes according to their C_H region: IgA, IgD, IgE, IgG and IgM. An antibody is further distinguished by the class of light chain (κ or λ) paired with its heavy chain. The class of each chain can be identified immunologically or isotyped with commercially available reagents which are animal-species-specific.

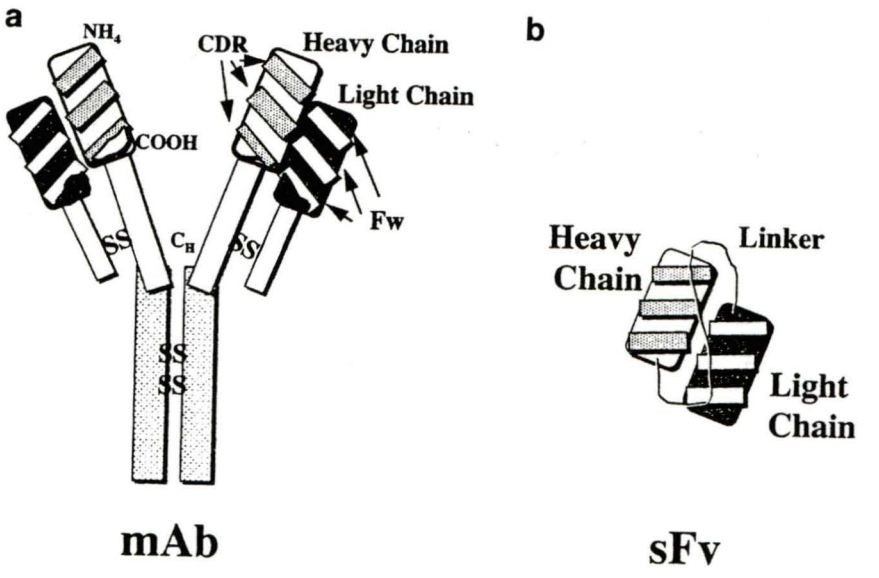


Figure 3. Structure of a monoclonal antibody and the single-chain antibody derived from it. The single-chain antibody is constructed by linking the variable heavy and light regions of the antibody using a small flexible peptide liker. The variable heavy and light regions are derived by PCR (polymerase chain reaction). CDR, complementary determining region; FW, framework; MAb, monoclonal antibody; C_H, heavy chain constant region; SS, disulfide cross-links; ScFv, single-chain antibody.

--Piché, A. and Rancourt, C. (1999) *Gene Therapy*, 6:1202-1209

The variable (V) region is located at the amino-terminus of the antibody chain. It consists of alternating framework (FW) and hypervariable, or complementarity-determining regions (CDRs). The greatest sequence diversity occurs in the CDRs, while the FW region sequences are more conserved. The J region (heavy and light chains) and the D region (heavy chain only) lie immediately upstream from the C region. The variability in the variable regions of both light and heavy chains is for the most part restricted to three small hypervariable regions in each chain. The remaining parts of the variable region, framework, are relatively constant. These findings led to the prediction that only the 5 to 10 amino acids in each hypervariable region form the antigen-binding site (Capra, J.D. et al., 1977; Wu, T.T. et al., 1970). Sequence variations within the J and D regions further influence the conformation of the site. The cumulative effect of the variations in these regions determines the antigenic affinity and specificity of an antibody, as well as its idiotype.

1.4.2 Shortcomings of monoclonal antibodies

One of the major failures of conventional cancer therapy has been the inability to achieve a sufficient differential in toxicity between normal and neoplastic tissues. Since hybridoma technology was established, monoclonal antibodies have been raised against a variety of tumor-associated antigens to overcome the lack of specificity. Unfortunately, MAbs have for the most part, failed to fulfill the expectations of being the desired "magic bullets" capable of directing cytotoxic effects strictly to malignant cells. The notable exception has been in the treatment of B-cell lymphomas that have exhibited an exciting

response rate when treated with unconjugated (Brown, L., et al, 1989) and radiolabeled (Kaminski, M.S., et al, 1993) MAbs. However, similar progress has not been observed in the treatment of ovarian carcinoma and other solid malignancies. These tumors contain disordered vasculature and lack draining lymphatics, resulting in elevated interstitial pressure. In this environment, the diffusion of a relatively large, 150 kDa IgG molecule is limited to 1mm in 2-3 day. In contrast, smaller molecules such as 50 kDa Fab fragments exhibit faster movement through the tumor interstitium, on the order of 1 to 2 mm per day (Jain, P.K.,1989).

1.4.3 Single-chain antibodies (ScFvs)

Using recombinant techniques, we can obtain Fab (fragment of antibody), Fv (fragment of variable region) and ScFv (single chain variable region fragment) (**Figure 3B**). Pastan et al. have developed methods to derive cDNAs, which encode the variable regions of specific immunoglobulins (Pastan, I., et al., 1993). Specific primers to the framework 1 and 4 of the variable regions of an IgG gene are used to amplify the variable heavy (V_H) and light (V_L) chains of an antibody, the two regions are artificially joined together with a neutral linker and expressed as a single polypeptide chain. This enables the V_H and V_L regions to associate intramolecularly and stabilizes variable domain combinations that interact weakly. Most ScFv contain a single 15-amino acid linker $(Gly_4Ser)_3$. This construction facilitates chain pairing and minimizes refolding and aggregation problems encountered when the two chains are expressed individually. The affinity and stability of ScFv antibodies containing the $(Gly_4Ser)_3$ residues are generally comparable to those of

the native antibody (Huston, J.S. et al, 1988; Whitlow, M. and Filpula, D., 1991; Takkinen, K. et al, 1991).

The recombinant ScFv retains the tumor specificity of its original mAb with little or no decrement in binding affinity (Huston, J.S. et al, 1988). ScFv with their small size of approximately 30-35 kDa should exhibit an excellent penetration into solid tumors. These molecules present the functional monovalent antigen-binding site of an antibody containing six antigen-binding loops, or complementary determining regions (CDRs), flanked by the supporting framework regions. Furthermore, ScFv consistently exhibit highly specific tumor retention and diffuse tumor penetration in their terminal distribution phase in immunodeficient mice bearing subcutaneous human tumor xenografts. However, the small size of these molecules also allows rapid elimination through the kidneys (Gregory, P.A. and Robert S., 2000). These factors, coupled with the monovalent nature of the ScFv's interaction with its target antigen lead to significantly lower quantitative tumor retention than is achievable with the parent MAbs.

The intracellular antibody is a strategy to obtain phenotypic knock-out of selected gene products in mammalian cells (Biocca, S. et al, 1990; Cattaneo, A. et al, 1997; Richardson, J.H and Marasco, W.A., 1995). This technology uses the ectopic expression of recombinant antibodies targeted to different intracellular compartments in order to neutralize intracellular antigens. The function of many antigens has been successfully inhibited by expressing antibodies in the cytoplasm, the nucleus and the secretory

pathway of animal and plant cells (Biocca, S. et al, 1993; Biocca, S. et al, 1994; Tavladoraki, P. et al, 1993; Duan, L. et al, 1994; Mhashilkar, A.M. et al, 1995; Marasco, W.A. et al, 1993). Although some ScFvs are soluble cytoplasmic proteins, others are highly concentrated in granular structures whose number, shape and size vary for each ScFv and are typical for each antibody (Cardinale, A. et al, 2001). The construction of a ScFv does not require cloning of the gene encoding the target protein, and derivation of a ScFv relies only on the availability of a hybridoma cell line producing the parental antibody. Thus, the rationale for using ScFvs to achieve selective abrogation of a protein has been established. Intracellular ScFvs can be employed to sequester a target protein in a specific subcellular compartment, to decrease the availability of protein for receptor/ligand binding at the cell surface and to inhibit the function of an oncogene. It has been recently shown in our lab that the abrogation of cell surface expression of the latent membrane protein 1 (LMP1) of Epstein-Barr virus, a transmembrane protein, was mediated by an ER-targeted anti-LMP1 ScFv (Piché, A., et al., 1998). These findings suggest that intracellular ScFvs can be employed to selectively abrogate the function of a membrane-associated protein, such as CA125, thereby enabling definition of its role in human ovarian cancer cells. It is thus our hypothesis that a ScFv derived from the OC125 monoclonal antibody can be constructed and used intracellularly in human ovarian cancer cells to achieve the phenotypic knockout of the CA125 tumor antigen in order to identify the role of this antigen in ovarian cancer.

CHAPTER 2 MATERIALS AND METHODS

2.1 Derivation of ScFv constructs

The mRNA extracted from the murine hybridoma cell line OC125 was used to generate ScFv cDNA, and the ScFv constructs were constructed with the Recombinant Phage Antibody System (Amersham Pharmacia Biotech) according to the instructions provided by the manufacturer (**Figure 4**).

2.1.1 mRNA isolation and purification

The QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech) was used to isolate and purify hybridoma mRNA.

Approximately $5x10^6$ OC125 hybridoma cells were pelleted by spin for 5 minutes at 1000g, then 1.5ml of Extraction Buffer was added to resuspend the cell pellet and the cells were lysed by gently vortexing. Three ml of Elution Buffer was added to the extract and mixed by vortexing, then after centrifugation at full speed for 10 minutes at room temperature, 4ml of the supernatant was transferred onto the surface of the resin of a Oligo (dT)-Cellulose Spun Column. The column was mixed for 10 minutes by gently inverting, then the resin was separated from the suspension by spin at 350g for 2 minutes. Then the supernatant was discarded and 3ml of High-Salt Buffer was used to resuspend the matrix. The matrix was pelleted by spin at 350g for 2 minutes.

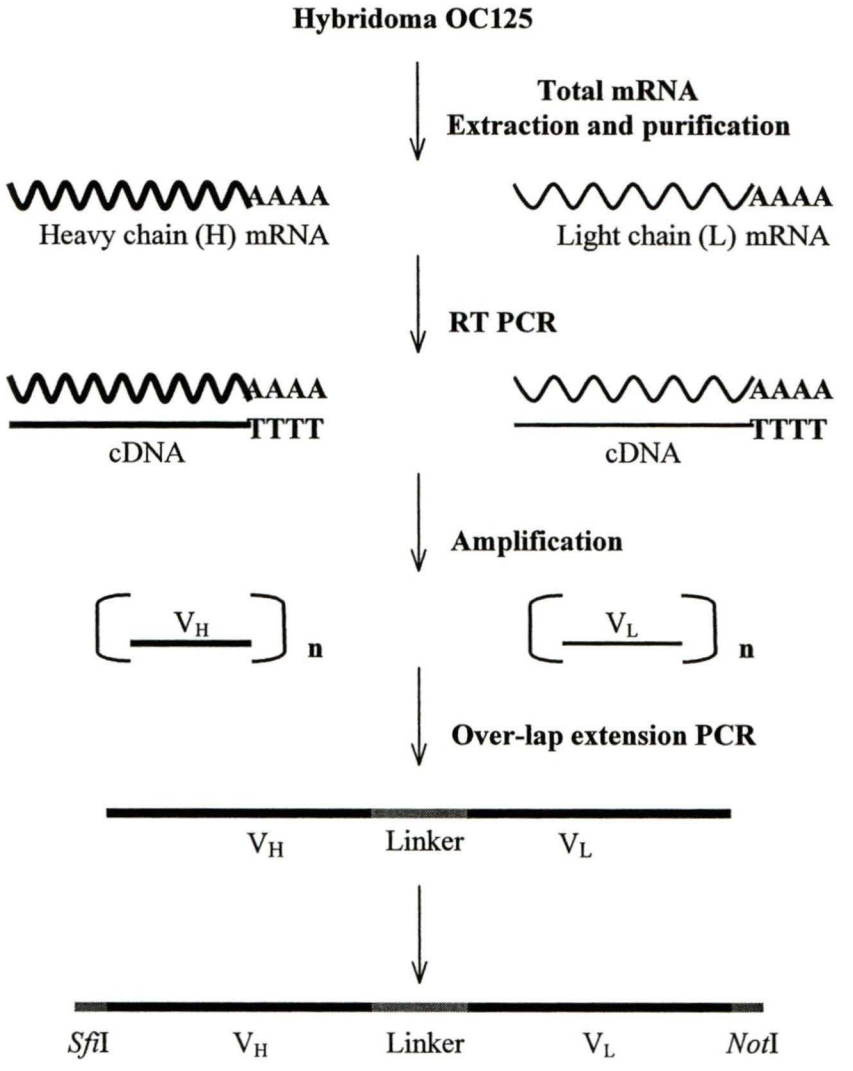


Figure 4. Derivation of ScFv constructs: The murine hybridoma cell line OC125 was used to generate cDNA from purified mRNA by reverse transcription PCR. The V_H and V_L chains were amplified from the cDNA by PCR using mouse variable region primers. The V_H and V_L DNA fragments were linked together by overlap extension PCR using a $(Gly_4Ser)_3$ linker to generate a 750-bp ScFv construct with flanking *Sfi*I and *Not*I restriction sites.

The matrix was washed two more times, then resuspended using 3ml of Low-Salt Buffer. Spinning at 350g for 2 minutes and discarding the supernatant, 0.75ml of 65°C Elution Buffer was used to elute the bound poly $(A)^+$RNA by centrifuge at 350g for 2 minutes. The mRNA (750µl total volume) was ethanol-precipitated by adding15µl of Glycogen Solution (10mg/ml), 1/10 volume of 2.5M potassium acetate (pH5.0) and 2.5 volumes of -20°C 95% ethanol. Spinning at full speed for 10 minutes at room temperature, the precipitate was washed twice with cold 95% ethanol, dried and then resuspended in 20µl of RNase-free water for storage at -70°C.

2.1.2 First-strand cDNA synthesis

M-MuLV reverse transcriptase was used in the first-strand cDNA reaction to generate cDNA from the mRNA (from section 2.1.1). The mRNA was heated at 65°C for 15 minutes and chilled immediately on ice. For each sample, the reaction was prepared in duplicate. One tube was labeled as light chain and the second tube as heavy chain. Each reaction contained 10µl of mRNA, 11µl of RNase-free Water, 11µl of Primed First-Strand Mix and 1µl of DTT Solution. The mixture was incubated for 1 hour at 37°C.

2.1.3 Primary PCR amplification

The first-strand antibody cDNA (from section 2.1.2) was used as a template for PCR amplification to generate suitable quantities of antibody heavy and light chain DNA for cloning. Each PCR reaction contained 33µl of First-strand reaction, 2µl of Light Primer

Mix, and 64μl of Sterile distilled water (or 33μl of First-strand reaction, 2μl of Heavy Primer 1, 2μl of Heavy Primer 2, and 62μl of Sterile distilled water). After 5 minutes of denaturation at 95°C, 1μl of AmpliTaq DNA polymerase was added, followed by 30 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C, and at the end of cycling an incubation of 20 minutes at 72°C.

2.1.4 Purification of primary PCR products

Prior to performing the assembly reaction, the amplified heavy and light chain PCR fragments were isolated from the other reaction components by electrophoresis on 0.8% agarose gel. The 340bp heavy chain and 325bp light chain fragments were cut out from the gel, from which the amplified cDNA was extracted using Gel Extraction Kit (QIAGEN), according to the instructions provided by the manufacturer.

2.1.5 Assembly and fill-in reactions

The ScFv cDNAs were generated by assembly of purified V_H, the $(Gly_4Ser)_3$ linker, and V_L fragment. A mixture containing 50ng of heavy chain product, 50ng of light chain product, 4μl of Linker-primer Mix, 5μl of 10xPCR Buffer, 2.5μl of dNTP Mix (20mM of each dNTP), and 5μl of 25mM MgCl2, was made. Sterile distilled water was added to a total volume to 49μl, after 5 minutes of denaturation at 94°C, 1μl (5U) of AmpliTaq DNA polymerase was added, followed by 7 cycles of 1 minute at 94°C, 4 minutes at 63°C.

2.1.6 Second PCR amplification and purification

In the second PCR, the assembled ScFv DNA was amplified and restriction sites were added. A primer mixture was made, which containing 1μl (5U) of AmpliTaq DNA polymerase, 5μl of 10xPCR Buffer, 1μl of dNTP Mix (20mM of each dNTP), 4μl of RS Primer Mix (Restriction Site Primers, which contain either *Sfi* I or *Not* I restriction sites. Its sequence is not given in the manufacturer's instruction.), and 39μl of sterile distilled water. The primer mixture was mixed briefly and added to the assembly reaction (from section 2.1.5), followed by 30 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C. The reaction was hold at 4°C if necessary.

The second PCR product was transferred to the top of the compacted resin of a prepared MicroSpin Columns, which was put in a sterile 1.5ml microcentrifuge tube, and spun at 735g for 2 minutes. The column was discarded. The effluent in the tube contained the purified assembled ScFv DNA that was ready for quantitation on an agarose gel alongside the ScFv Marker.

The ScFv genes were digested with *Sfi* I and *Not* I, agarose gel-purified, and then ligated into the prokaryotic expression vector pCANTAB 5E (Pharmacia Biotech) that had been cut with the same restriction enzymes. Screening of recombinant clones expressing a ScFv against the CA125 protein was accomplished by phage-display and colony-lift assay, as will be described in section 2.5 and 2.6.

2.2 Plasmids

The phagemid pCANTAB 5E (**Figure 5**) which contains the OC125 ScFv DNA under the control of the IPTG inducible and glucose repressible *lac* promoter; a g3p leader sequence that directs transport of the protein to the inner membrane/periplasm of *E.coli* where the main g3p domain attaches the fusion protein to the tip of the assembling phage. pCANTAB 5E also contains a sequence encoding an E-tag, which allows easy immunological detection of ScFv protein expression, followed by an amber translational stop codon at the junction between the cloned ScFv and the sequence for the g3p. When a suppressor strain of *E.coli*, such as DH11S, is transformed with this recombinant vector, translation continues through the amber stop codon to produce the ScFv-g3p fusion protein which can be displayed on the phage tip. In nonsuppressor strains, such as HB2151, the stop codon is recognized, protein synthesis is aborted at the stop codon, the g3p fusion protein is not made, and the resulting ScFv protein is transported and accumulated into the periplasmic space.

The endoplasmic reticulum (ER)-targeting eukaryotic vector pSTCF. KDEL (**Figure 6**) contains a CMV promoter; an IgK leader sequence that directs the ScFv protein into ER, a *myc* epitope coding an easily detected *myc* tag, while the KDEL sequence located at the C-terminus causes the protein to be retained in the ER. The pSTCF.cyto vector (**Figure 6**), which directs cytoplasm expression, only has a CMV promoter and a myc tag sequence. In most cases, ScFv expressed in a cytoplasm vector are not stable (Willianms, G.T. et al, 1990), and in this study it is used as a negative control for ScFv expression.

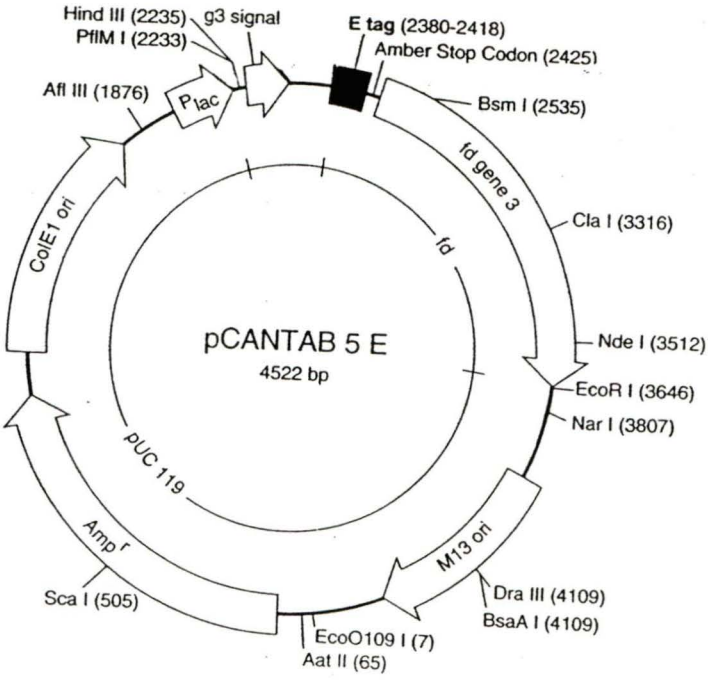


Figure 5. pCANTAB 5E vector showing control regions. This prokaryotic vector is designed such that ScFv genes can be cloned between the leader sequence and the main body of the M13 gene 3. The g3p leader sequence directs transport of the protein to the periplasm of *E.coli*. The ScFv is expressed as a fusion protein with the E-tag peptide to allow easy detection. The vector also contains an amber translational stop codon at the junction between the cloned ScFv and the sequence for the g3p.

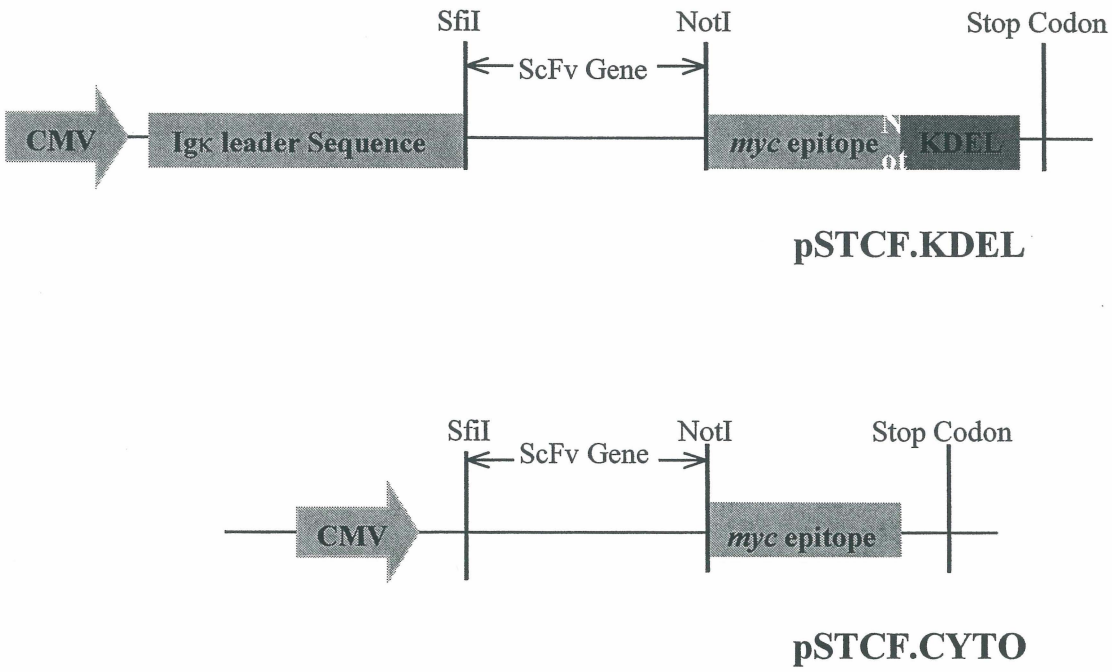


Figure 6. pSTCF.KDEL eukaryotic vector expressing ScFv genes. Expression of the ScFv protein is driven by the CMV promoter. The ScFv cDNA is introduced between the *Sfi*I and *Not*I restriction sites. The Igκ leader sequence directs the ScFv protein to the ER, and the KDEL signal at the COOH terminus leads to retention of the fusion protein in this cellular compartment. The ScFv open reading frame is also fused with a c-myc epitope to allow easy detection by Western-Blot. In pSTCF.cyto vector, the ScFv protein expression is targeted to the cytoplasm.

--Piché, A., Grim, J., Curiel, D.T. et al. (1998) *Cancer Research*, 58:2134-2140

2.3 Preparation of the ScFv cDNA library

One µl of ligated pCANTAB 5 vector was transformed into 40µl of competent *E.coli* XL1-Blue by electroporation. After incubating at 37°C for 1 ho

ur with shaking at 250rpm, the transformed cells were plated onto a LB-1.5% agar (For 1L: 10g Bacto-tryptone, 5g Yeast extract, 5g NaCl and 15g agar) plate containing 100µg/ml of ampicillin, and incubated overnight at 37°C. The next day, 5ml of LB medium containing 100µg/ml of ampicillin was used to suspend all of the colonies from the plate. This suspension was incubated for 6 hours at 37°C with shaking at 250rpm. A miniprep was made with 2.5ml of the culture using the QIAprep Spin Plasmid Miniprep kit (QIAGEN) and the remaining 2.5ml of culture was kept at -80°C as a frozen stock.

PCR was performed to confirm that the insert was present in the recombinant plasmid library, and was run on PTC-200 Peltier Thermal Cycler (MJ Research). One µl of 1:500 diluted pCANTAB.OC125 ScFv library (~5ng of DNA) was mixed with 5µl of 10xPCR buffer, 1µl of dNTP Mix (20mM of each dNTP), 4µl of RS Primer Mix, and 38µl of sterile distilled water. After 4 minutes of denaturation at 94°C, 1µl (5U) of AmpliTaq DNA polymerase was added, followed by 30 cycles of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C. Then the PCR procuct was loaded on 0.8% agarose gel, alongside with DNA marker and ScFv marker.

2.4 CA125 antigen

The CA125 antigen was obtained from 10-fold concentrated conditioned media of human ovarian cancer OVCAR3 cells. Fifteen ml of media was centrifuged at 1000rpm in a Millipore BioMax 100 Unit (Millipore), until the volume was reduced to about 750µl. This concentrated media was removed and the BioMax unit was washed with 750µl of PBS. The concentrated media and the washing buffer were transferred and combined together in a 1.5ml microcentrifuge tube. The protein concentration was 25.25mg/ml.

2.5 Phage display

2.5.1 Transformation

The library of pCANTAB 5E vector containing the OC125 ScFv insert was transformed into competent *E.coli* DH11S by heat-shock. Plasmid pUC19 (Amersham Pharmacia Biotech) was used as a control plasmid to determine the transformation efficiency. One µl of 1:8000 diluted miniprep DNA extracted from the ScFv cDNA library was mixed with 100µl of competent cells, the mixture was placed on ice for 45 minutes, incubated in a 42°C water bath for 2 minutes, and then chilled briefly on ice for 90 seconds. The mixture was diluted with 900µl of room temperature SOC (10g Bacto-tryptone, 2.5g yeast extract, 1ml 5M NaCl, 1.25ml 1M KCl, 10ml 1M $MgSO_4$, 5ml 1M $MgCl_2$, 2.5ml 20% glucose, for 500ml) medium and incubated for one hour at 37°C with shaking at 250rpm. This preparation was diluted ten-fold and then 100µl aliquots of the diluted

cells were plated onto a 2xYT-AG plate (17g Bacto-tryptone, 10g Yeast extract, 5g NaCl, and 15g agar, for 1L, containing 100μg/ml ampicillin and 2% glucose) to determine the transformation efficiency. The plate was incubated overnight at 37°C.

2.5.2 Phage rescue

The remaining 900μl of undiluted transformed cells (section 2.5.1) was added to 9.1ml of 2xYT-AG medium, and incubated at 37°C with shaking at 250rpm, until an OD_{600} of 0.5 was reached. At this point, 1ml of the culture was kept to make a frozen stock. Then, $4x10^{10}$ pfu (plaque forming unit) of M13KO7 helper phage (Amersham Pharmacia Biotech) was added to the 9ml of culture. After another one hour of incubation, the cells were spun for 15 minutes at 3500 rpm. The pellet was gently resuspended with 10ml of 2xYT-AK medium (17g Bacto-tryptone, 10g Yeast extract, and 5g NaCl, for 1L), which contained 100μg/ml ampicillin (to select for cells with phagemid) and 50μg/ml kanamycin (to select for cells infected with M13KO7). The suspension was incubated overnight at 30°C with shaking at 250rpm. The cells were pelleted by spinning at 2500rpm for 20 minutes at 4°C. The supernatant, which contained the recombinant phage, was used for the panning reaction.

2.5.3 Panning

The library was subjected to five rounds of panning. The 10ml supernatant (from section 2.5.2), which contained recombinant plasmid in phage particles having the ScFv expressed on their surface, was mixed with 2ml of ice-cold PEG/NaCl (200g Polyethylene glycol 8000, 146.1g NaCl, for 1L), and incubated on ice for 60 minutes. The suspension was centrifuged at 12,000rpm in a Beckman SS-34 rotor for 45 minutes at 4°C, and the pellet containing the phage was resuspended with 16ml of 2xYT medium.

A 25cm^2 tissue culture flask (FALCON) was coated overnight at 4°C with 5ml of CA125 antigen (2mg of protein) diluted in 0.05M Na_2CO_3 (pH9.6), and blocked by filling the flask with 4% NFDM (Non-fat Dry Milk) (BioRad) in 1xPBS for 1 hour at 37°C. The 16ml of PEG-precipitated recombinant phage was diluted with 14ml of blocking buffer (containing 0.01% sodium azide) and incubated at room temperature for 15 minutes. Twenty ml of the diluted recombinant phage was added to the 4% NFDM-blocked flask, and the flask was incubated for 2 hours at 37°C with rocking. The flask was washed 20 times with 1xPBS/0.1% Tween and 20 times with PBS only.

2.5.4 Reinfection of *E.coli* with enriched phage clones

Ten milliliters of log-phase DH11S cells were added to the flask (from section 2.5.3), and incubated for 1 hour at 37°C. Then 100µl of cell suspension was removed to prepare ten-fold dilutions in 2xYT medium (1:10, 1:100, 1:1000). One hundred µl of undiluted cells

and 100µl of each dilution were plated onto separate SOBAG plates (For 1L, add 20g Bacto-tryptone, 5g Yeast extract, 0.5g NaCl, 10ml 1M MgCl2, 55.6ml 2M glucose, and 15g agar, containing 20mg/ml ampicillin). After incubating overnight at 30°C, individual well-isolated colonies were transferred to separate tubes containing 400µl of 2xYT-AG medium, and were incubated overnight at 30°C with shaking at 250 rpm. These recombinant phage-infected cells contain the pCANTAB 5E phagemid with the ScFv gene insert, and do not contain complete, infectious phage genomes.

2.5.5 Screening for recombinant phage antibodies in enriched clones

Forty µl of overnight culture (from section 2.5.4) was transferred to 400µl of 2xYT-AG medium containing $2x10^8$ pfu of M13KO7 helper phage, and incubated for 2 hours at 37°C with shaking at 150 rpm. The culture was centrifuged for 20 minutes at 2500rpm at room temperature, and the pellet was resuspended with 400µl of 2xYT-AK medium (no glucose). The suspension was incubated overnight at 37°C with shaking at 250 rpm. The overnight culture was centrifuged as described above, and 320µl of supernatant (which contained recombinant plasmid in phage particles expressing the ScFv) was mixed with 80µl of 4% NFDM in 1xPBS. The mixture was incubated for 10 minutes at room temperature. An ELISA was performed as described in section 2.5.7 using mouse anti-M13 antibody (Amersham Pharmacia) to detect the phage which display antibodies on its tips.

2.5.6 Screening for expression of soluble antibodies in enriched clones

Forty µl of overnight culture (of section 2.5.4) was transferred to a tube containing 400µl of 2xYT-AG medium, and incubated for 2 hours at 30°C with shaking at 250rpm. The culture was centrifuged at 2500rpm for 20 minutes at room temperature, and the pellet was resuspended with 400µl of 2xYT-AI medium (containing 1mM IPTG). The cell suspension was incubated for 3 hours at 30°C with shaking at 250rpm. The suspension was centrifuged as described above, and 320µl of supernatant (which contained soluble recombinant antibodies) was mixed with 80µl of 4% NFDM in 1xPBS. The mixture was incubated for 10 minutes at room temperature. An ELISA was performed as described in section 2.5.7 using anti-E tag antibody (Amersham Pharmacia Biotech).

2.5.7 Binding analysis by ELISA

An ELISA was performed to detect ScFv either expressed on the surface of phage particles or free in bacterial extracts. The CA125 antigen was applied to a 96-well microtiter plates (SARSTEDT) at a concentration of 5µg/ml in 0.05M Na_2CO_3 (pH9.6) at 4°C overnight. After being washed 6 times with 1xPBS-0.1%Tween, the wells were blocked with 4% NFDM in 1xPBS for 1 hour at 37°C. Then, 50µl/well ScFv phage extract (from section 2.5.5) or soluble ScFv extract (from section 2.5.6) was applied and incubated for 1 hour at 37°C. After washing, phage that carried ScFv on their surface were detected by adding 50µl of horseradish peroxidase (HRP)-conjugated mouse anti-M13 (Amersham Pharmacia) 1:10,000 diluted in blocking buffer and incubating for 45 minutes at 37°C. For the ELISA detection of soluble ScFv, 50µl of HRP-conjugated

anti-E tag Ig 1:5,000 diluted in blocking buffer was added and incubated for 45 minutes at 37°C. For revelation, 100µl/well of TMB substrate (Pierce) was used, and the absorbance was read at 450nm after incubation for 20 minutes at room temperature in the dark.

Once the CA125-positive clones had been identified by ELISA, 2µl of phage supernatant (of section 2.5.5), which contained positive recombinant antibodies, was used to infect 400µl of log phase *E.coli* HB2151. The culture was incubated for 30 minutes at 37°C with shaking. SOBAG plates were inoculated with a loopful of the infected culture and incubated overnight at 30°C. Individual colonies were transferred into 15ml centrifuge tubes, which contained 5ml of 2xYT-AG medium, for production of soluble antibodies as described in section 2.6.3.

2.6 Colony-lift assay

2.6.1 Transformation

The ligated pCANTAB 5E vector containing the OC125 ScFv insert was transformed into competent *E.coli* HB2151 by electroporation: 40µl of the cells were transferred to a pre-chilled 0.2cm cuvette, 1µl of salt-free DNA containing 20ng pCANTAB/ScFv cDNA library was added and shaken to the bottom of the cuvette, which was then placed on ice for 1 minute. The electroporator (BioRad) was programed to give 25µF, 2.5kV at 200 ohms. After electroporation, 1ml of SOC medium was immediately added to resuspend the cells, and the suspension was transferred to a 15ml tube and incubated for 1 hour at

37°C with shaking at 250rpm. The cells were centrifuged for 5 minutes at 3000rpm, and 1ml of 2xYT-G medium was used to resuspend the cell pellet. Each 100µl of this 1ml suspension was used to plate a 2xYT-AG plate, then these 10 plates were incubated overnight at 30°C.

2.6.2 Colony lift

A piece of dry Butterfly-Membrane (Schleicher & Schuell) was used to lift the well-isolated colonies (~300/plate) in each plate. After lifting, the membrane with colony-side face up was either put on the top of 2xYT-AI plate (containing 100µg/ml ampicillin and 1mM IPTG) with (1^{st} protocol) or without (2^{nd} protocol) a CA125 antigen-coated membrane beneath (**Figure 7**). The plate was incubated overnight at 30°C. The second day, after washing with 1xPBS/0.1%Tween, the antigen-coated membrane (of the 1^{st} protocol) or the colony-membrane (of the 2^{nd} protocol) was blocked with 4% NFDM in 1xPBS for 1 hour at room temperature with rocking. The membranes were probed with anti-E Tag 1:1000 diluted in blocking buffer for 2 hours at room temperature with rocking. After washing, the same membrane was probed with HRP-conjugated anti-mouse Ig (Sigma) 1:10,000 diluted in blocking buffer for 45 minutes at room temperature with rocking. For detection, 8ml/membrane of 4-CN (4-chloro-1-naphthol) substrate (Sigma) was added and incubated for ~ 30 minutes with rocking in the dark. When blue dots (~ 1mm diameter) were detected on the membrane, the development was stopped with water. The corresponding colony was picked from the original plate to inoculate 5ml of 2xYT-AG medium and incubated overnight at 30°C with shaking at 250rpm.

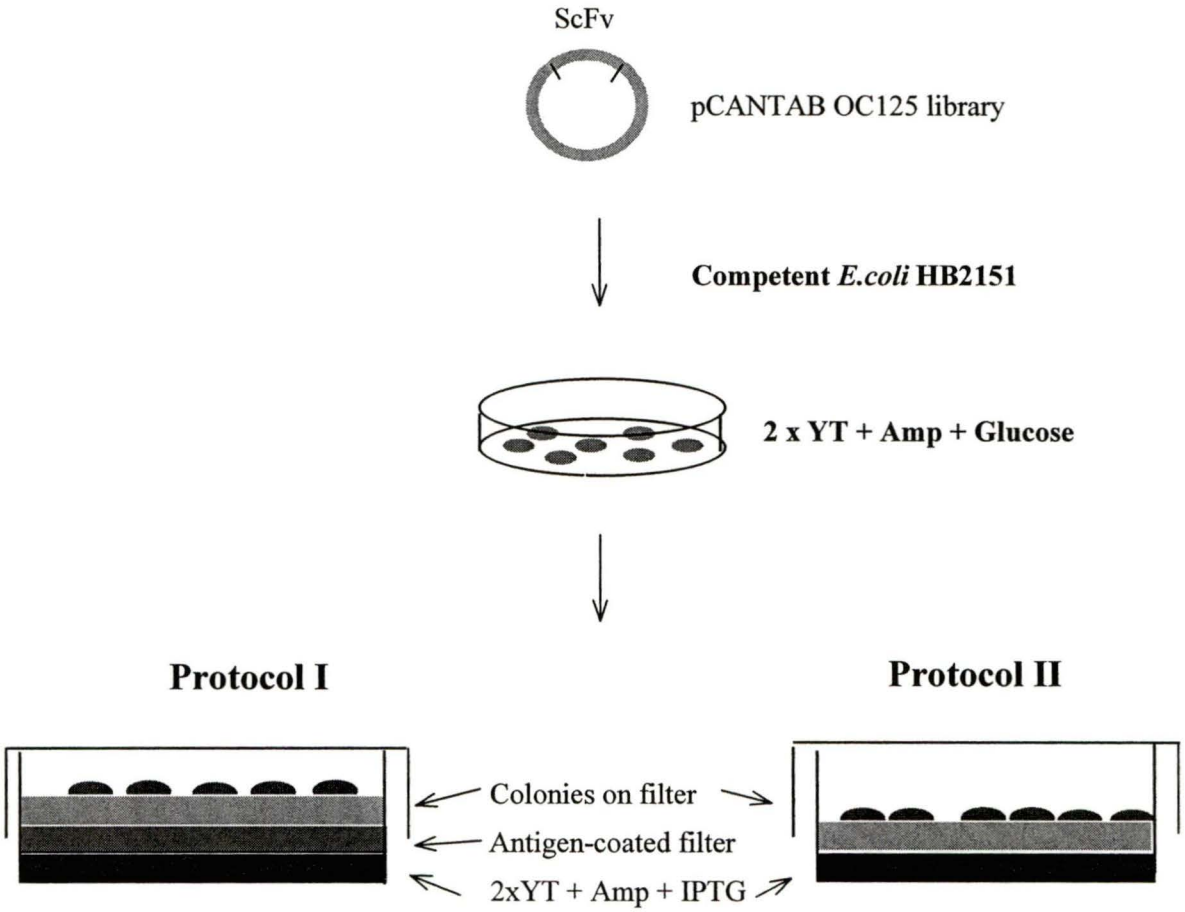


Figure 7. ScFv library screening using Colony-lift assay. ScFv cDNA library was transformed into competent *E.coli* HB2151 and grown on 2xYT-AG plate. A butterfly-membrane was used to lift the colony. Protocol I: a CA125 antigen-coated membrane was put on the 2xYT-AI agar surface, and the colony-lift membrane was put on this antigen-coated membrane with the colony-side face up. Protocol II: the colony-lift membrane was put directly on top of 2xYT-AI agar, with the colony-side face up. For both protocols, the membrane was developed using 4-CN substrate. Colonies corresponding to the blue dots were picked and grown in liquid medium.

--Rodenburg, C. and Bilbao, G. (1996) *Scientific Tools of Pharmacia Biotechnology*, 1:10-11

2.6.3 Periplasmic Extract

Five ml of overnight culture (from section 2.6.2) was added to 15ml of 2xYT-AG medium, and incubated for 1 hour at 30°C with shaking at 250rpm. The culture was centrifuged for 15 minutes at 3,500rpm at 4°C, and the pellet was resuspended with 30ml of 2xYT-AI (without glucose) and incubated for 3 hours at 30°C with shaking at 250rpm. After centrifuging the culture for 20 minutes at 4,000rpm at 4°C, 750µl of ice-cold 0.2xTES buffer (40mM Tris-HCl pH8.0, 0.1mM EDTA, and 0.1M sucrose, filter sterilized) was used to resuspend the pellet by vortexing. The suspension was incubated on ice for 30 minutes, and then the contents were centrifuged at full speed for 10 minutes at 4°C. The supernatant, which contains the soluble antibodies from the periplasm, was transferred to a clean tube and stored at -20°C until needed.

2.7 Cell lines

The human ovarian cancer cell line PA-1 and OVCAR3 were obtained from the ATCC (American Type Culture Collection). PA-1 cells were maintained in DMEM/F12 medium (Bio Media) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone Laboratories), 1% (v/v) L-glutamine, 1% antibody and 1% fungizone (Gibco). OVCAR3 cells were maintained in RPMI-1640 medium (Bio Media) supplemented with 20% (v/v) FBS, 1% (v/v) L-glutamine, 1% antibody, 1% fungizone and 0.01mg/ml bovine insulin.

2.8 Transient transfection

PA-1 or OVCAR3 cells (~300,000/well) were plated in 6-well plates (FALCON) 24 hour prior to transfection in 3ml of growing medium, and the cells were incubated in the incubator with 5% CO_2 at 37°C. For transient transfection experiments, cells were transfected with plasmid DNA with the liposomal transfection reagent SuperFect (QIAGEN). Plain cell growth medium (no supplements) was used to dilute 2μg of DNA (dissolved in TE buffer, pH7.4) to a total volume of 100μl. After mixing the diluted DNA, 10μl of SuperFect Transfection Reagent was added to the DNA, and mixed by pipetting up and down 5 times. After incubating the sample for 10 minutes at room temperature to allow complex formation, 0.6ml of complete growth medium was added to the 100μl of complex. During the complex formation, the cells were washed once with PBS. The total volume of the reaction was transferred to the cells in the 6-well plate, and the cells were incubated with the complexes for 5 hours with 5% CO_2 at 37°C. After removing the medium and washing the cells with PBS, 3ml of fresh cell growth medium was added to the cells.

After variable times of incubation, cells were lysed using Promega lysis buffer (Promega). The protein concentration of the lysates was measured by the Bradford method with BioRad Protein Assay according to the manufacturer's instruction (BioRad).

2.9 Immunoblot analysis

Equal amounts of protein (~35μg) were loaded and separated by SDS-PAGE (12%) with a stacking gel concentration of 4%. After transfer onto Hybond-P PVDF membranes (Amersham Pharmacia Biotech) (300mA, 90 minutes, 4°C) and blocking with 3% NFDM, the membranes were probed with either 1:1,000 anti-E Tag antibody or1:2,000 anti-c-*myc* antibody (Invitrogen). After washing with 1xPBS-0.1%Tween, 1:10,000 diluted (in blocking buffer) HRP-conjugated rabbit anti-mouse antibody was used. The immunoblots were developed using ECL or ECL PLUS system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To ensure equivalent protein loading, the membrane was also cut and probed with 1:7,000 anti-tubulin antibody (Sigma).

2.10 Sequence analysis of ScFvs

The nucleotide sequences of the ScFvs having different expression levels and antigen binding activities were determined using DNA Symo Sequencing technology and run on a Li-Cor sequencer by BioS&T Inc. (Montreal, Quebec). Samples were prepared with QIAprep Miniprep Kit (QIAGEN). The pCANTAB5-S1 and S6 primers were used:

pCANTAB5-S1 primer: CAACGTGAAAAAATTATTATTCGC

pCANTAB5-S6 primer: GGAGTATGTCTTTTAAGTAAATG

2.11 Immunoprecipitation

ScFv transfected OVCAR3 cells were washed twice with cold 1xPBS and lysed on ice using 1xlysis buffer (28.3mM KCl, 10mM $MgCl_2$, 50mM Hepes, 4mM EGTA and 0.6% NP-40). In each immunoprecipitation experiment, the cell lysates containing approximately 300μg of protein were incubated with 5 μl of polyclonal anti-*c-myc* antibody for 1 hour on ice without rocking. Thirty μl of Protein G-agarose beads (Boehringer Mannheim) were added into the protein-antibody mixture and incubated for 1 hour at 4°C with rocking. The beads were pelleted by centrifugation for 5 minutes at 5000 rpm and washed with 500μl of 1xlysis buffer. After the last wash, the beads were resuspended in 40 μl of loading buffer and boiled for 5 minutes at 95°C. The beads were pelleted by spining at 5000 rpm for 10 minutes and the supernatant was saved. The precipitated proteins were separated by SDS-PAGE and transferred as described in section 2.9. The precipitated ScFv proteins were detected using 1:2,000 anti-*c myc* antibody and the co-precipitated CA125 proteins were detected using 1:1,000 anti-CA125 OC125 monoclonal antibody.

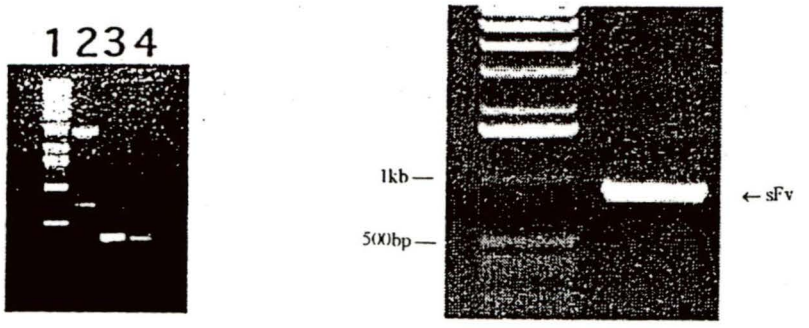
CHAPTER 3 RESULTS

3.1 Construction of the OC125 ScFv cDNA library

The mRNA extracted from the murine hybridoma cell line OC125 was used to generate ScFv cDNA, because this cell line expresses a monoclonal antibody that specifically recognize an epitope located in the extracellular portion of CA125. The cDNA encoding the V_H and the V_L domains was generated from the mRNA by reverse-transcription. After amplification and purification, the V_H and the V_L of this antibody were linked together as described in "Materials and Methods" and the full-length ScFv construct (**Figure 8A**) was cloned into *SfiI*/*Not*I-digested bacterial expression vector pCANTAB 5E (**Figure 5**). A PCR was performed to validate the 750bp insert of the ScFv cDNA library (**Figure 8B**).

3.2 ScFv cDNA library screening

Once the OC125 ScFv cDNA library was constructed and verified, strategies for screening were necessary to select the ScFvs that have antigen-binding activity to the CA125 protein. Both phage display and colony-lift assay were tried. Following are the results of different assays.



A B

Figure 8. Validation of amplified V_H, V_L, and assembled ScFv. About 1 µg of DNA was loaded on 0.8% agarose gel, alongside a DNA marker. **A)**, agarose gel showing amplified V_H, V_L fragments, and the assembled ScFv. Lane 1, DNA marker; Lane 2, ScFv marker; Lane 3, V_H; Lane4, V_L. **B)**, pCANTAB S1 and S6 primers were used in a PCR to verify the ScFv insert in the pCANTAB vector. Lane 1, 1 Kb DNA ladder; lane 2, PCR product obtained by using the S1 and S6 primers.

3.2.1 Phage display

Transformation efficiency. Approximately 125pg of pCANTAB.OC125 ScFv library were transformed into competent *E.coli* DH11S, and 50pg of pUC19 plasmid was also transformed as a control to determine the efficiency of transformation. The transformed cells were grown on 2xYT-AG plates. After overnight incubation, 1,200 colonies were detected on OC125 ScFv plate while 300 colonies appeared on pUC19 plates indicating that the pCANTAB.OC125 ScFv library produced $9.6x10^7$ colonies per μg of DNA, while pUC19 plasmid produced $6x10^8$ colonies per μg of DNA, suggesting that the transformation efficiency was around 16%.

Screening. After 5 rounds of panning against the CA125 antigen, the recombinant phage re-infected-DH11S cells were plated onto a SOBAG plates. After overnight incubation, 100 well-isolated colonies were transferred to individual tubes containing 400μl of 2xYT-AG medium for further incubation with shaking. The phage bound antibodies and the soluble antibodies were prepared as described in sections 2.5.5 and 2.5.6 of "Materials and Methods".

ELISA plates were coated with either phage, on the tip of which the antibodies were expressed, or CA125, and probed with HRP-conjugated anti-M13 antibody or anti-E tag antibody. When the plate was coated with phage antibodies, strong binding to anti-M13 antibody was detected (OD_{450} ~2.0), suggesting that DH11S cells were infected with phage and complete phage particles were produced and released in the media. When the plate was coated with CA125, and incubated with soluble antibody samples, after probing

with anti-E tag antibody, no binding was detected (data not shown). It meant that no CA125-binding phage-displayed single-chian antibody was selected. Since phage-display did not work in this study, a colony-lift assay was then used to do the screening.

3.2.2 Colony lift assay

The OC125 ScFv cDNA library was transformed into competent *E.coli* HB2151, and the transformed cells were grown on individual 2xYT-AG plates. Once colonies were detected on a selection 2xYT plate containing glucose, two different protocols (as described in section 2.6.2) were used to select CA125-positive colonies.

The corresponding 176 colonies to the blue dots on the developed membrane were picked and grown in liquid medium. After inducing with IPTG, periplasmic extracts were prepared as described in section 2.6.3. Then these extracts, which should contain soluble ScFv proteins, were used in an ELISA to analyze their antigen binding activity, and in a Western blot to validate the expression of the ScFvs. The results are shown in **Table 2.**

3.3 Antigen binding assay using periplasmic extracts

Using the first protocol of colony-lift assay, in which ScFv secreted by positive colonies was captured on a membrane with bound CA125 antigen, around 2,500 colonies were analyzed and a few faint positive reactions were detected after developing the CA125 antigen-coated membrane. But when the antigen binding activity of those corresponding colonies was determined by ELISA, they were all negative. While using the second

protocol, in which ScFv was directly detected in the bacterial colonies, so it was only selective for soluble ScFvs, six CA125-positive clones (#91, #103, #148, #150, #151, #155) were obtained among 176 candidates by ELISA (**Table 2**), but only one of them (#148) had strong binding activity.

In the ELISA, the anti-E tag antibody (1:1000 diluted in blocking buffer) was used to detect the presence of E-tagged soluble ScFv antibodies. Periplasmic extracts of non-transformed HB2151 were used as a negative control and monoclonal antibody OC125 (1:1000 diluted in blocking buffer) was used as positive control. When the wells of an ELISA plate were coated with CA125 antigen and incubated with the periplasmic extracts (#58, #91, #103, #148, #150, #151, #155), after reading the plate at 450nm, the data shown in **Figure 9** indicate that only #148 ScFv protein has almost the same antigen binding activity as its parental monoclonal antibody OC125. If the wells were coated with each periplasmic extracts (#58, #91, #103, #148, #150, #151, #155), and monoclonal antibody OC125 was used to coat one well as a positive control, after probed with the HRP anti-E tag antibody, the OD_{450} data showed that E-tagged soluble OC125 ScFv proteins were detected in the periplasm of all of these colonies, even #58 ScFv, which was known to be negative for CA125 antigen binding in ELISA of colony-lift assay(**Figure 9**). Thus, we have derived an anti-CA125 ScFv (OC125 #148 ScFv) that, when expressed in a prokaryotic system, binds to the CA125 protein in ELISA. And since #58 ScFv protein was highly expressed in the bacterial, but has no CA125 binding activity, it was used as a negative control in all of the rest experiments.

Using Protocol I

	Experiment 1	Experiment 2	Experiment 3
Number of colonies	750	750	1000
Candidates	6	8	12
Positive colonies after ELISA	0	0	0

Using Protocol II

	Experiment 1	Experiment 2
Number of colonies	1500	2500
Candidates	10	166
Positive colonies after ELISA	0	6

Table 2. Summary of results of the colony-lift assay. Using the second protocol, which was selective for expression of the Etag, six CA125-positive clones (#91, #103, #148, #150, #151, #155) were obtained among 176 candidates. (**Candidate** means the colony which expresses E-tagged ScFv; **Positive colony** means the expressing ScFv of which has the CA125 antigen-binding activity in ELISA.)

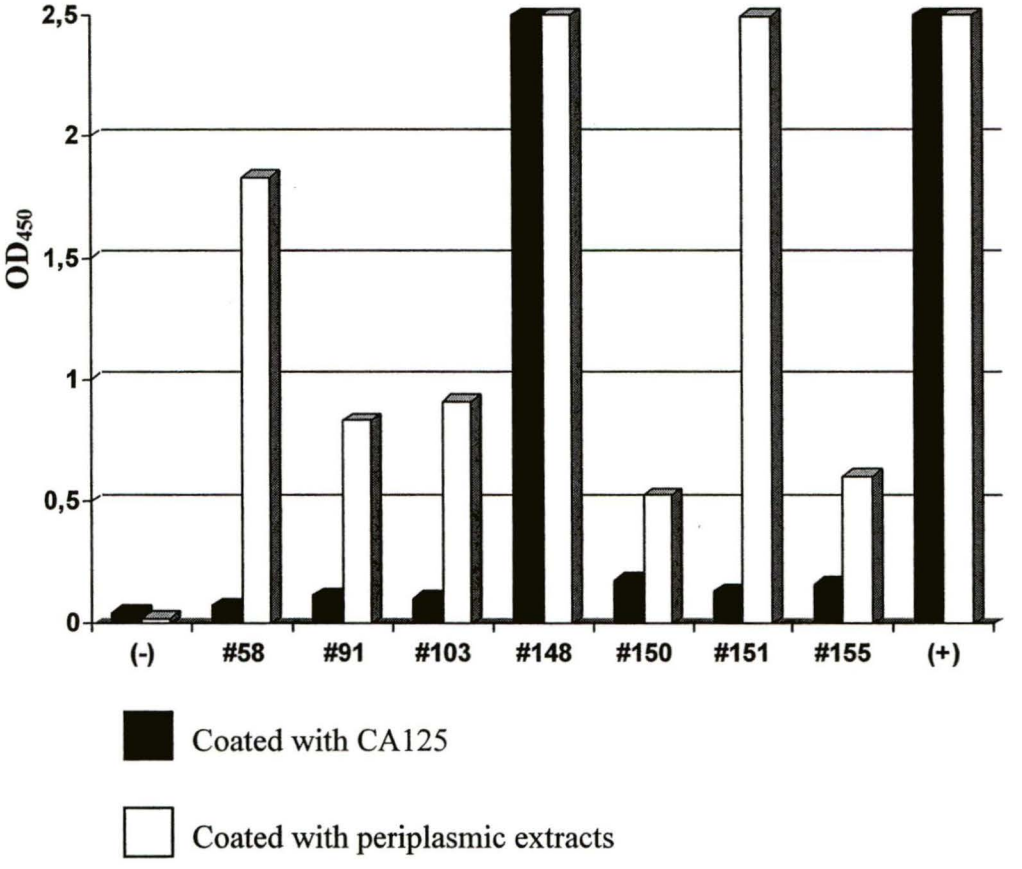


Figure 9. Expression and antigen binding activities of ScFvs in periplasmic extracts. **1)**, ELISA plate was coated with CA125 (*filled square*), incubated with periplasmic extracts and probed with anti-E tag antibody. **2)**, ELISA plate was coated with the periplasmic extracts (*open square*), and probed with anti-E tag antibody. Negative control: periplasmic extract of non-transformed *E.coli* HB2151, positive control: monoclonal antibody OC125.

3.4 Expression of ScFvs in periplasmic extracts

The same extracts of section 3.3 were also used to do a Western blot. Anti-Bcl-2 ScFv protein (Piché, A. et al, 1998-1), from Dr. Alain Piché, was used as positive control in this experiment, because it has the size of an ScFv protein of ~34 kDa. Expression of the OC125 ScFvs was validated by probing with anti-E tag antibody. As shown in **Figure 10**, most of these E-tagged OC125 ScFvs were expressed at high levels. The ScFvs were expressed at the expected size of ~34 kDa proteins, with the only exception of #148 ScFv, which has the strongest antigen binding activity in ELISA. It was expressed as a 19 kDa protein.

3.5 Confirmation of ScFv cDNA

To confirm the existence of these ScFvs, plasmids (pCANTAB.Bcl-2, #58, #148,#150, #151 and #155) were digested with *Sfi*I and *Not*I, the digested plasmids were loaded on 0.8% agarose gel. As shown in **Figure 11**, all of these clones displayed the expected 750-bp ScFv DNA fragment or a slightly smaller one, with the exception of #148 ScFv. The 350 bp small fragment confirmed the small size OC125 #148 ScFv protein, which was shown in **Figure 10**.

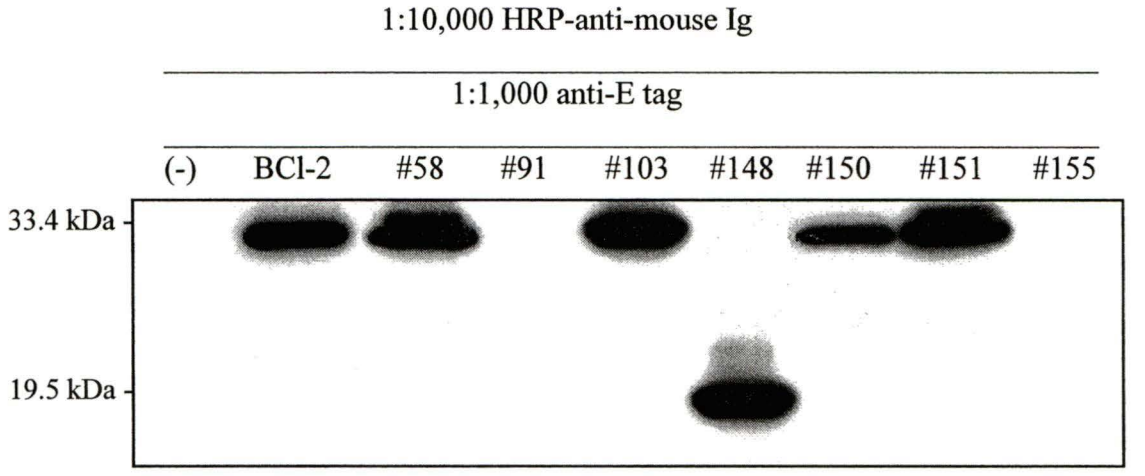
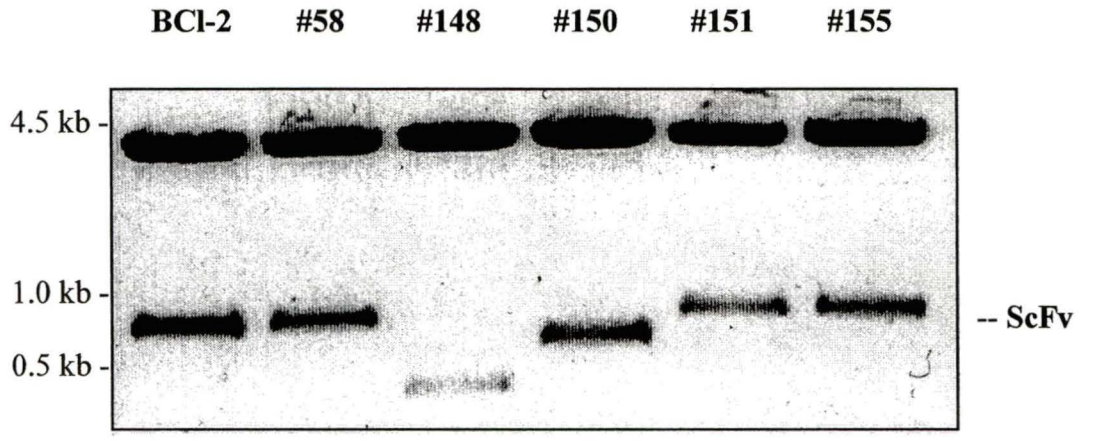


Figure 10. Immunoblot analysis to detect the expression of ScFvs in periplasmic extracts. Periplasmic extracts of pCANTAB.OC125 ScFvs were loaded on 12% SDS-PAGE. After transferring the proteins to a PVDF membrane, the membrane was probed with anti-E tag antibody. Negative control: untransformed *E.coli* HB2151; Positive control: pCANTAB.BCl-2 ScFv protein.



* It is the negative image of the original gel picture, to make the #148 ScFv more visiable.

Figure 11. pCANTAB.OC125 ScFvs were digested with *Sfi*I / *Not*I to verify the presence of ScFv inserts. The digested plasmids were loaded on 0.8% agarose. Positive control: pCANTAB.Bcl-2 ScFv. All of these clones displayed a DNA band of 500-bp to 750-bp, with the exception of 350-bp #148 ScFv.

3.6 ScFv sequence analysis

To determine if there were any differences among the sequences of these ScFvs, and in which domain were these differences located, the sequences of OC125 #58, #148 and #151 ScFvs were analyzed. It was suggested from **Figure 9** that all of these ScFvs were highly expressed in *E.coli*, but only #148 ScFv had strong CA125-antigen-binding activity, while #58 ScFv did not bind to CA125 and #151 ScFv had very low CA125-binding activity. It was the reason why we choose these three ScFvs to do the sequence analysis.

The alignment of protein sequences of these ScFvs is shown in **Figure 12.** Comparing the V_H sequence of #58 and #151 ScFv with #148 ScFv, there were only three amino acid differences, two of them located at framework 1 (FW1) and one at framework 4 (FW4), while there were no sequence differences in the CDR regions among these ScFvs. When we examined the consensus AA of mouse V_H FW1 and FW4, we found that Glutamine (Q) in the consensus sequence of FW1, had been replace by Lysine (K) in clone #148 and #58. Whereas a Threonine (T) in FW1 had been replaced by Proline (P) in clone #58 and #151, but not in clone #148. Comparing the V_L and linker sequences of these ScFvs, there were big differences: #148 ScFv had lost most of the V_L domain, from FW1 to within FW4 was deleted. Also 12 amino acids were deleted from the linker of #148 ScFv. The #58 and #151 ScFvs had the same complete V_L sequence and complete linker $(Gly_4Ser)_3$. The results suggest that a V_H domain alone can exhibit antigen-specific binding, and the existence of V_L may affect the antigen-binding activity of ScFv.

V_H	**FW1**	QV**K**LQ**E**SGAELVKPGTSVKLSCKASGYTFT	--#148
		QV**K**LQ**Q**SGAELVKPGTSVKLSCKASGYTFT	--#58
		QV**Q**LQ**E**SGAELVKPGTSVKLSCKASGYTFT	--#151
		EVQLQEGGELVKPG	-- Consensus AA
	CDR1	SYWMH	--#148
		SYWMH	--#58
		SYWMH	--#151
	FW2	WVKLRPGQGFEWIG	--#148
		WVKLRPGQGFEWIG	--#58
		WVKLRPGQGFEWIG	--#151
	CDR2	EINPSNGDTNYNERFKR	--#148
		EINPSNGDTNYNERFKR	--#58
		EINPSNGDTNYNERFKR	--#151
	FW3	KATLTVDKSSRTAYMQLSSLTSEDSAVYYCTR	--#148
		KATLTVDKSSRTAYMQLSSLTSEDSAVYYCTR	--#58
		KATLTVDKSSRTAYMQLSSLTSEDSAVYYCTR	--#151
	CDR3	ALGRDSAMDY	--#148
		ALGRDSAMDY	--#58
		ALGRDSAMDY	--#151
	FW4	WGQGT**T**VTVSSESGRS	--#148
		WGQGT**P**VTVSSESGRS	--#58
		WGQGT**P**VTVSS	--#151
		WGGGTTVTVSS	-- Consensus AA

V_L	**FW1**	----------	--#148		
		DIELTQSQKIMSTSVGDRVSFSC	--#58		
		DIELTQSQKIMSTSVGDRVSFSC	--#151		
	CDR1	----------	--#148		
		KASQNVRTTVA	--#58		
		KASQNVRTTVA	--#151		
	FW2	----------	--#148		
		WYQQKPGQSDKPLIY	--#58		
		WYQQKPGQSDKPLIY	--#151		
	CDR2	----------	--#148		
		LASNRHT	--#58		
		LASNRHT	--#151		
	FW3	----------	--#148		
		GVPERFTGSGSGTDFTLTVSNVQSEDLADYFC	--#58		
		GVPERFTGSGSGTDFTLTVSNVQSEDLADYFC	--#151		
	CDR3	----------	--#148		
		LQHWSYPYT	--#58		
		LQHWSYPYT	--#151		
	FW4	---TKLEIK	--#148	**Linker**	GGG---------- -- #148
		FGGGTKLEIK	--#58		GGGGSGGGGSGGGGS -- #58
		FGGGTKLEIK	--#151		GGGGSGGGGSGGGGS -- #151

Figure 12. Sequence analysis of OC125 ScFvs. #148 ScFv, which had the strongest CA125 binding activity in ELISA, was sequenced with control ScFv #58 and #151. The alignment of the protein sequences of these ScFvs shows that the V_L region and most of linker sequence were deleted from #148 ScFv. There were only three amino acid differences between CA125-binder #148 ScFv and its control ScFv in the V_H region, and these amino acid replacements occured within the FW1 and FW4 of the V_H region, which is not supposed to be related to antigen-binding.

3.7 Intracellular expression of the OC125 ScFvs in eukaryotic cells

Subcloning. pCANTAB.OC125 ScFv #58, #148 and #151 plasmids were digested with *Sfi*I and *Not*I, and the insert ScFv cDNA fragments were obtained by running the digestion products on 0.8% agarose gel and extracting the cDNA using QIAGEN Gel Extracting Kit (QIAGEN). Then these purified ScFv cDNA were ligated into *Sfi*I / *Not*I digested pSTCF.KDEL (or cyto) vector, in which the expressed ScFv protein would be targeted into ER or cytoplasm. One μl of ligation reaction was used to transform competent *E.coli* XL-1 by electroporation. After incubating overnight the transformed cells on LB-agar medium containing ampicillin at 37°C, ~30 clones were obtained in each plate. Single clones were picked and grown with 3ml of LB-A medium overnight at 37°C with shaking at 250rpm. After performing miniprep of DNA of the overnight culture, the presence of the ScFv insert in the pSTCF vector was verified by *Sfi*I / *Not*I digestion of the DNA (data not shown).

Transient transfection. Once recombinant pSTCF.OC125 ScFvs were obtained, human ovarian cancer cell line OVCAR3, which is CA125 positive, and PA-1 cells, which are CA125 negative, were transiently transfected with either KDEL or cyto ScFv cDNA, as described in the section 2.8. Total cell lysates were prepared after 48 hours of transfection. These cells lysates were used to analyze the expression of ScFv by Western blot.

Expression of ScFvs in ovarian cancer cells. The same amount of cell-lysate (30μg of proteins) from each ScFv transfectant was loaded onto 12% SDS-PAGE, and separated by running the gel at 150V for 90 minutes. After transferring the protein to a PVDF membrane, ScFv expression was validated by probing the membrane with 1:1,000 anti-*myc* antibody, followed by an incubation with 1:10,000 HRP-conjugated anti-mouse secondary antibody. As shown in **Figure 13**, all OC125 ScFvs were expressed when cloned into the ER-targeted vector pSTCF.KDEL, but not in vector pSTCF.cyto. Therefore, as expected, expression of the ER-targeted OC125 ScFvs was achieved in mammalian cells using transient transfection.

The blot was also cut and probed with an anti-tubulin antibody to correct for the difference in protein loading between samples. The *Integration Data* show the percentage of each ScFv expression relative to tubulin expression. As shown in **Figure 13**, if we compare the *Integration Data* from the same ScFv in the two different cell lines, we find that #58 ScFv, anti-Bcl-2 ScFv, and #151 ScFv, have a similar level of expression regardless of the CA125 status of the cell line. We have already known that these ScFvs either did not bind to CA125, or the binding activity was very low (see **Figure 9**). However, #148 ScFv, which showed strong antigen binding in ELISA, was expressed approximately 2-fold less in OVCAR3 cells than in PA-1 cells. These results suggest that #148 ScFv may bind to CA125 antigen in OVCAR3 cells, but not in PA-1 cells.

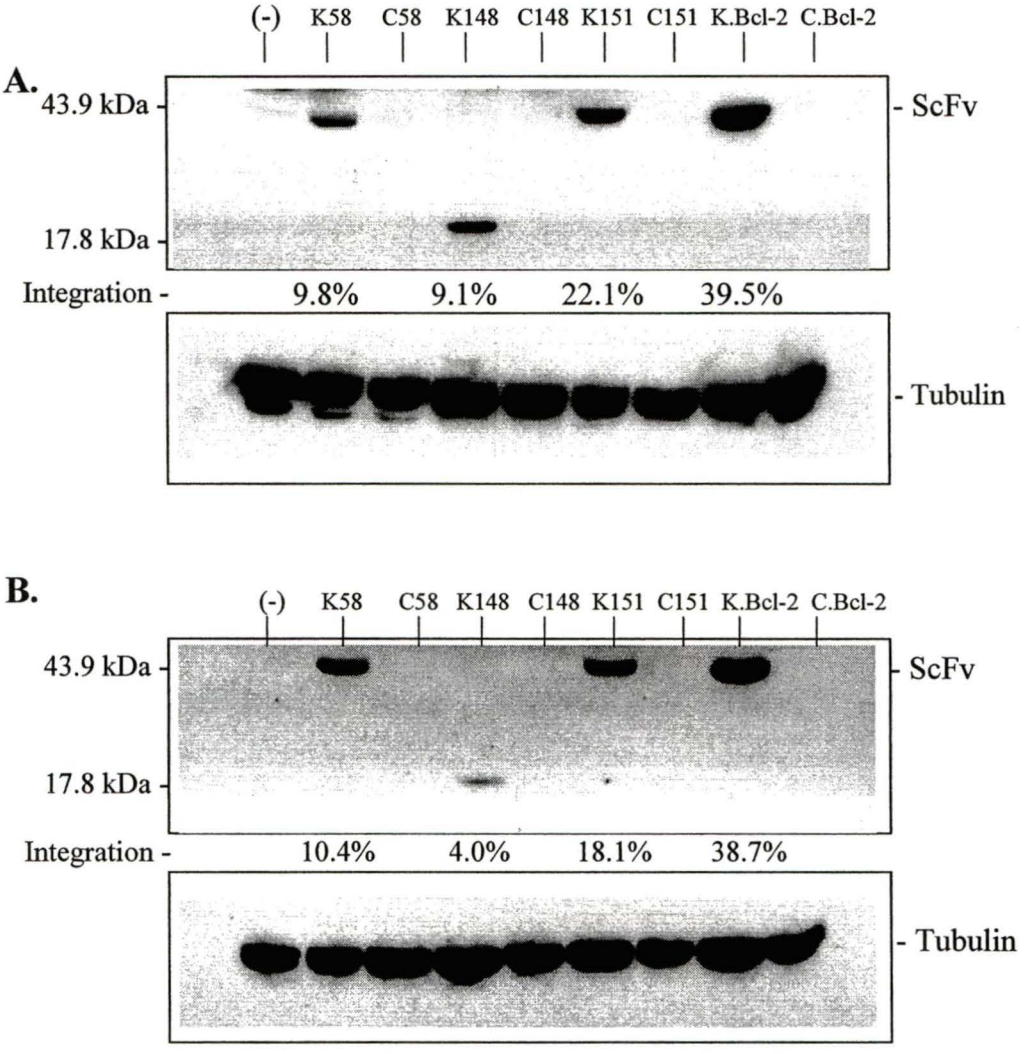


Figure 13. Expression of ScFvs in transiently transfected ovarian cancer cells. **A**, Western blot using PA-1 cell lysates; **B**, Western blot using OVCAR3 cell lysates. Cells were transfected with either pSTCF.KDEL or pSTCF.cyto ScFvs. Negative control: cell lysate of un-transfected cells. The cell lysates (30μg/well) were loaded on 12% SDS-PAGE, and the protein-transferred membranes were probed with anti-*myc* antibody to detect the expression of OC125 ScFv. The membranes were also cut and probed with anti-Tubulin antibody to correct for the difference in protein loading between each samples. The *Integration Data* show the percentage of each ScFv expression relative to the tubulin expression.

3.8 Immunoprecipitation

To confirm the binding of OC125 #148 ScFv to CA125 antigen intracellularly, an immuno-precipitation was performed to show the direct interaction between the pSTCF.KDEL#148 ScFv protein and the CA125 antigen.

The cell lysates of OVCAR3/#58 ScFv (used as negative control) and #148 ScFv transfectants were prepared, and each cell lysate was incubated with anti-*myc* antibody and protein G agarose. A complex, in which anti-*myc* antibody binds to both protein G agarose and the *myc*-tag of ScFv, was expected to be generated (**Figure 14**). When the cell lysate of OVCAR3/#148 ScFv transfectant was used to do this experiment, #148 ScFv was supposed to bind to CA125 antigen, which produced by OVCAR3 cells. However, if OVCAR3/#58 ScFv transfectant was used in the same experiment, CA125 antigen, which was produced by OVCAR3 cells, was not expected to be involved in the complex of **protein G agarose-anti-*myc*-ScFv**, since it was known that #58 ScFv did not bind to CA125 in ELISA (**Figure 9**). The complex was washed three times and was mixed with loading buffer, then protein G agarose was discarded after centrifugation. The supernatants, which contain anti-*myc* antibody, ScFv, and maybe CA125, were separated by 12% SDS-PAGE.

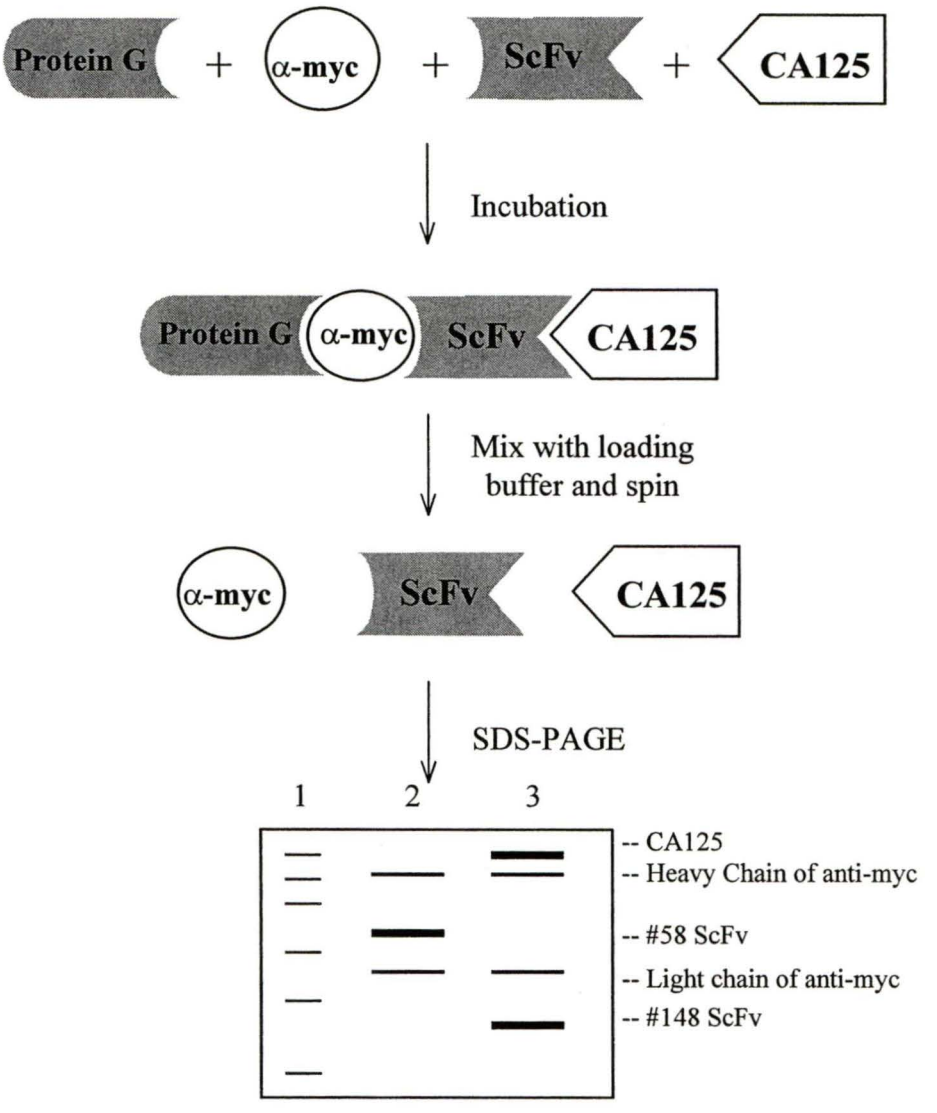


Figure 14. Strategy of immunoprecipitation. Freshly prepared ScFv transfected OVCAR3 cell lysates were incubated with protein-G agarose and anti-*myc* antibody, and a protein complex was formed as described in the figure. After incubation with loading buffer, protein-G agarose was removed by centrifugation, the supernatant which contains *myc* antibody, ScFv, and CA125, was separated on 12% SDS-PAGE. Lane 1, protein marker; Lane 2, #58 ScFv transfected cell lysates immunoprecipitated by *myc* antibody; Lane 3, #148 ScFv transfected cell lysates immunoprecipitated by *myc* antibody.

After Western blot, the membrane was probed with 1:1,000 anti-*myc* antibody to detect the expression of ScFv, as we can see from **figure 15A**, high-level expressed #58 ScFv and relatively lower-level expressed #148 ScFv were detected. These results suggest that both ScFvs were expressed in ER-targeted vector and the IP did work, since ScFvs were immunoprecipitated by anti-*myc* antibody.

When the same precipitates were probed with 1:1,000 OC125 monoclonal antibody, as shown in **Figure 15B**, unfortunately, CA125 protein could not be detected as expected. The immunoprecipitation was repeated several times, using different conditions. All the results suggested that, for some reason, CA125 was not co-immunoprecipitated by OC125 ScFv protein.

At the meantime, we still could see some of the #58 ScFv expression in **Figure 15B**. It is because the ScFv cDNA was derived from murine hybridoma cells, while the secondary antibody to be used in Western-Blot was anti-mouse HRP, so it is possible that ScFv protein could bind to the secondary antibody.

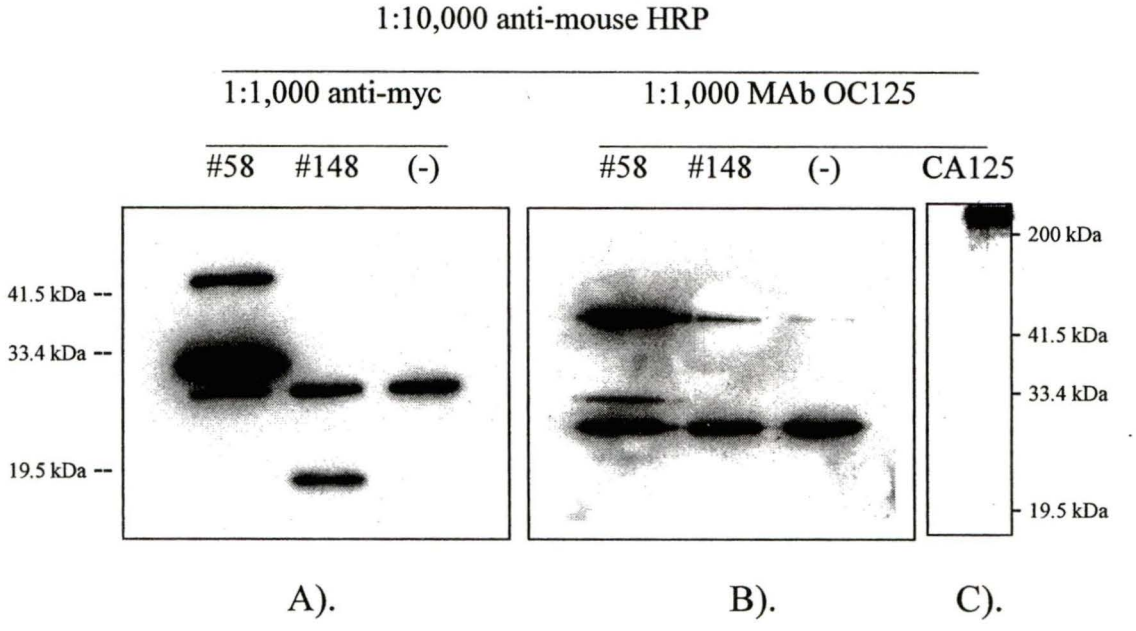


Figure 15. ScFv transfected OVCAR3 cell lysates immunoprecipitated with anti-*myc* antibody. The precipitates were loaded on 12% SDS-PAGE, the protein-transferred PVDV membrane was probed with different antibodies. Negative control: un-transfected OVCAR3 cell lysate. **A)**, the precipitates were probed with anti-*myc* antibody to detect the presence of ScFv proteins. **B)**, the same precipitates were probed with anti-CA125 monoclonal antibody OC125, to determine if CA125 expressed in OVCAR3 cells was co-immunoprecipitated by #148 ScFv. **C)**, Western blot using cell lysate of un-transfected OVCAR3, probed with MAb OC125 and anti-mouse HRP, showing the molecular weight of CA125.

CHAPTER 4 DISCUSSION

The tumor antigen CA125, an antigenic determinant which is recognized by the monoclonal antibody OC125 is associated with high molecular weight glycoproteins (Bast, R.C. et al, 1981). These antigens are expressed by more than 80% of epithelial ovarian tumors (Bast, R.C. et al, 1983). Decreasing concentrations have been shown to be associated with tumor regression, while increasing CA125 levels indicate resistance to treatment and disease progression (Marth, C. et al, 1998). Due to its clinical value in pre-operative diagnosis and monitoring of ovarian cancer, CA125 is the most popular used tumor marker in this disease. The function of the glycoproteins carrying CA125 is unknown and, because of their complex nature, only little information about the physical and immunological properties of these antigens is available.

In this study, we have reported the construction and intracellular expression of a single-chain antibody directed against the CA125 protein, and the expression level of this OC125 ScFv was selectively reduced in CA125-positive human ovarian cancer OVCAR3 cells. Intracellular immunization using ScFvs offers a novel strategy to achieve targeted abrogation of selected gene products, particularly when this gene is not cloned and the sequence is unknown. Intracellular immunization using ScFvs has been successfully applied to inhibit the function of intracellular target proteins in a variety of different biological systems (Tang, Y. et al, 1991; Marasco, W.A. et al, 1993; Biocca, S. et al, 1994; Tavladoraki, P. et al, 1993). These techniques should prove to be important for

studies of mammalian cells, where genetic approaches are more difficult. One obvious and simple application is to block the transport of proteins through the exocytic pathway. A potential therapeutic application of this method might be the targeting of antibodies against the human immunodeficiency virus type 1 (HIV-1) gp120 to the ER (Marasco, W.A. et al, 1993). This prevents the appearance of the viral protein on the plasma membrane and reduces the production of infectious virus. In our study, ER-targeted OC125 ScFv would act as a dominant negative inhibitor to cause a loss of function of CA125 protein, which is a membrane-associated glycoprotein (Nagata, A. et al, 1991), thus help to define the role of this tumor antigen in human ovarian cancer.

In recent years, a hybrid "phagemid" vector system has been developed which combines the advantages of phage and plasmid vectors (Vieira, J. et al, 1987). Phagemids can be conveniently grown as plasmids or alternatively packaged as recombinant M13 phage with the aid of a helper phage, such as M13KO7. To produce phage-displayed recombinant antibodies, competent *E.coli* DH11S cells were transformed with the phagemid library containing the OC125 ScFv genes. This host strain produces a suppressor tRNA which allows readthrough (suppression) of the amber stop codon located between the ScFv and gene 3 sequences of pCANTAB 5E (**Figure 5**). Since suppression of the amber stop codon in DH11S cells is only about 20% efficient, soluble antibodies will be produced in addition to phage-displayed recombinant antibodies. And since an accumulation of g3p is toxic to the cell, the *lac* promoter must be tightly controlled prior to infection with M13KO7 helper phage to avoid g3p expression, further repression can be achieved by growing the transformed cells more slowly at 30°C

(instead of 37°C) and in media that contain at least 2% of glucose. The glucose forces the transformed cells to shut down alternate metabolic pathways, which further represses the *lac* operon. Transformed cells were then infected with M13KO7 helper phage to rescue the phagemid with its ScFv gene insert. Recombinant phage that are produced contain a single-stranded DNA copy of the phagemid and antibody ScFv gene and display one or more copies of the recombinant antibody at their tips. During phage infection, glucose was removed from the medium and the *lac* promoter was induced to allow expression of the ScFv-g3p fusion protein.

Since the strategy of phage-display selects the ScFv which has CA125 binding activity, which is dependent on the combination of two DNA fragments (V_H and V_L), it needs a cDNA library larger than average, and it may be the reason why we were not able to selecte an appropriate clone by phage-display. To overcome the difficulty, a colony-lift assay was used as another screening strategy. In this assay, *E.coli* HB2151 was transformed with a recombinant pCANTAB.OC125 ScFv library. Since it is a nonsuppressor strain, soluble ScFv would be produced in each *E.coli* colony. In pCANTAB vector, the ScFv fragment is expressed as a fusion protein with a short detectable E-tag, so the CA125 positive E-tagged ScFv can be identified among a large population of colonies using this assay. The transformed HB2151 cells were grown on media containing ampicillin and glucose. The glucose forces the transformed cells to shut down alternate metabolic pathways, which represses the *lac* operon, since the soluble ScFv antibodies were not expected to be expressed at this step. Then, two different protocols were used to perform the screening. Both of these two protocols

(**Figure 7**) using a piece of dry "butterfly membrane" to lift colonies, and IPTG was used to induce the expression of soluble ScFv. The differences are: In the first protocol a CA125 antigen-coated membrane was needed, which can be bound with the expressed soluble antibody. This antigen coated membrane was probed with mouse monoclonal anti-Etag antibody, then with secondary antibody (HRP conjugated sheep anti-mouse antibody) and developed by 4-CN substrate. So, only when the soluble ScFvs was expressed and bound to the CA125 antigen, the color reaction would be detected on the membrane. Therefore, it was selective for soluble ScFvs that bind to CA125. While in the second protocol, after incubating on the selection plate containing IPTG, the colony-membrane was washed and probed with anti-E tag antibody and then developed as the first protocol. If there would be some color reaction after developing, it only means the soluble ScFvs was expressed, an Elisa was still necessary to verify their binding activity to CA125. This selects only for soluble ScFvs.

In this study, using colony-lift assay, six CA125-positive OC125 ScFvs were obtained among 176 candidates, but only one of them (#148 ScFv) showed strong antigen binding activity in ELISA. Sequence analysis was performed with #148 ScFv and two other ScFvs (#58, #151), to determine the relationship between the differences of their sequence and antigen-binding activity. The alignment of protein sequences of different ScFvs show that, in the region of V_H, there were only three AA differences, and all of these differences located in FW region. The AA replacements in FW1 and FW4 were not expected to result in any difference on antigen binding affinity. For a given antibody, the greatest sequence diversity occurs in the CDRs, which interact with the antigen to form

the core of an antigen-binding site, while the FW region sequences are more conserved. The cumulative effect of the variations in CDRs, J and D regions of antibody determines the antigenic affinity. It is why before sequencing, we expected there would be some differences in the domain of V_H CDRs. Comparison of the V_L and linker sequences of these ScFvs showed that there were big differences: #148 ScFv lost most of the V_L domain, and 12 amino acids were deleted from its linker, while the #58 and #151 ScFvs have the same complete V_L sequence, and they also have the complete linker $(Gly_4Ser)_3$.

The results may suggest that a V_H domain alone can exhibit antigen-specific binding, and the existence of V_L maybe affects the antigen-binding activity of ScFv. In fact, similar results were reported in 1997 (Cai, X.H., and Garen, A., 1997). These authors randomly got a truncated melanoma-specific ScFv molecule with most of the V_L domain deleted, the affinity of which was comparable to the affinity of the complete antibody. When different V_L domains from melanoma patients were conjugated to this ScFv to form complete single chain antibody, most of the V_L domains inhibited the antigen binding. It suggests that tumor-specific V_H might remain undetected in a ScFv library because the V_H and V_L domains are randomly paired and most V_L partners would probably be functionally incompatible; the compatible combinations might not be represented in a ScFv library of average size, which can encompass only a small fraction of the possible random combinations of V_H and V_L domains. It also might explain why after ScFv library screening we only got one ScFv with strong antigen binding activity, and only the V_H domain remained in its structure.

After the OC125 ScFv library was constructed and proved to be expressed in *E.coli*, we subsequently subcloned the OC125 ScFv into an eukaryotic vector that directs the ScFv to the ER, which is the component of the secretory pathway of CA125. We hypothesized that CA125 will be entrapped by the ScFv in the ER during synthesis and unable to normally localize at the cell surface, thus abrogating any function related to localization of CA125 at the cell surface. To determine if these ScFvs (#148, #150, #151) could be expressed in eukaryotic cells, and to determine the difference of binding activities of these ScFv proteins to CA125 antigen, OC125 #58, #148, and #151 ScFv were subcloned into a eukaryotic expression vector either pSTCF.KDEL or pSTCF.cyto. The recombinant ScFvs were then transiently transfected into human ovarian cancer cells PA-1 and OVCAR3. The immunoblot analysis using lysates of ScFv transfected cells showed that the expression level of #148 ScFv was reduced 2-fold in ovarian cancer OVCAR3 cells, when compared to PA-1 cells (**Figure 13**). The reason for the decrease in #148 ScFv protein level is not clear. It has been suggested that, quality control of newly synthesized proteins in the ER ensures that only correctly folded, processed, and completely assembled proteins exit this compartment for further transport through the secretory pathway. Most proteins that fail to reach this transport competent state are degraded (Finger, A. et al, 1993; Yuk, M.H. et al, 1993). The ER-associated protein degradation pathway is highly selective for specific unassembled and/or aberrant proteins, while the majority of ER resident and secreted proteins are quite stable. Evidence show that ER-associated degradation is predominantly mediated by the ubiquitin-proteasome system (Biederer, T. et al, 1996; Hiller, M.M. et al, 1996; Ward, C.L. et al, 1995). Ubiquitin-proteasome system detects and eliminates abnormal proteins

such as misfolded proteins or proteins that would otherwise be part of a larger complex but fail to find their partners (Jentsch, S. and Schlenker, S., 1995). So the possible explanation for the reduced OC125 ScFv expression level could be that the complex CA125/OC125 ScFv, when sequestered in the ER, was degraded as the result of the mislocalization of the CA125 protein. This phenomenon has also been observed with anti-Bcl-2 ScFv and anti-LMP1 ScFv in our lab previously, where the antigen-antibody complexes were degradated in ER (Piché, A. et al, 1998-1; Piché, A. et al, 1998-2). The later observations would suggest that #148 ScFv protein might bind to CA125 within ER.

Immunoprecipitation experiments did not give the demonstration of the direct interaction between #148 ScFv and the CA125 protein. One possibility is that, the CA125 protein can not be detected, because OC125 antibodies recognize only the glycosylated antigen proteins found on the membrane. The covalent addition of sugars to protein is one of the major biosynthetic functions of the ER. Most of the soluble and membrane-bound proteins that are made in the ER, including those destined for transport to the Golgi apparatus, lysosomes, plasma membrane, or extracellular space are glycoproteins. Proteins are further modified in the Golgi before transport to the cell surface (Hart, G.W., 1992). We do not know at what step the CA125 precursor protein becomes reactive to monoclonal OC125 (and then, probably also to ScFv #148). If the significant modification only occurs in the Golgi or late in the ER, then ScFv targetted to the ER maybe not react with it.

There are still some further studies to be taken into consideration:

To determine if the #148 OC125 ScFv can reduce cell surface expression of the CA125 antigen, the ovarian cancer cell line OVCAR3 will be transiently transfected with ScFv cDNA. And the consequence of intracellular ScFv expression on cell surface expression of CA125 can be evaluated by immunofluorescence. Transfected OVCAR3 cells will be fixed and incubated with monoclonal OC125 antibody. A second FITC-conjugated anti-mouse antibody will then be used, and cells will be analyzed using a fluorescent microscope to quantify the magnitude of CA125 cell surface modulation.

To analyze and characterize the expression of CA125 in ovarian cancer cell line OVCAR3 cells, ^{35}S labelling may be used. Previous studies showed that ^{35}S-methionine-labelled CA125 could be detected by 4 hours and reached maximal levels of radioactive incorporation in tissue culture medium by 12 hours when analyzed by immuno-precipitation with the M11 anti-CA125 monoclonal antibody and SDS-PAGE, followed by autoradiography (Fendrick, J.L., et al, 1993). In our study, we can transiently transfect #148 ScFv into ^{35}S-labelled-OVCAR3 cells, using monoclonal antibody OC125 to immunoprecipitate CA125 that expressed by OVCAR3 cells. Comparing with the CA125 expressing level in un-trasfected cells, try to determine if #148 ScFv could inhibit the CA125 synthesis and transport in OVCAR3 cells.

Also, monoclonal antibody VK8 can be considered to generate a novle anti-CA125 ScFv library, in order to screen antigen-positive single-chain antibodies. This monoclonal

antibody was obtained by immunizing mice with human ovarian cancer cell line OVCAR3. When tested on a panel of cultured ell lines, MAb VK8 was found to co-type with MAb OC125 and not with a number of other Mabs detecting antigens commonly expressed in ovarian cancer cells; Radioimmunoprecipitaion and SDS-PAGE analysis showed that both VK8 and OC125 reacted with similar high molecular weight antigens from [^{3}H]GlcN-labelled OVCAR3 cells. Application of the two-step immuno-precipitation technique, in which the antigen immunoprecipitated by one antibody was reisolated and reacted with a second test antibody, showed that VK8 epitopes was carried on the same macromolecules as CA125 epitopes (Lloyd, K.O., et al, 1997).

CONCLUSIONS

In conclusion, a ScFv cDNA library against the ovarian cancer CA125 antigen was constructed, using cDNA generated from the mRNA extracted from the murine hybridoma cell line OC125, which expresses monoclonal antibodies that specifically recognize the extracellular domain of CA125. The OC125 ScFvs were cloned into the procaryotic expression vector pCANTAB 5E and expressed in *E.coli* to high levels. After screening by colony-lift assay, one of the selected ScFv (#148) bound to CA125 antigen with high affinity in ELISA. The sequence analysis of #148 ScFv and the other ScFvs, which did not bind to CA125 in ELISA, suggests that the V_H domain of ScFv alone can exhibit antigen-specific binding activity, and the existence of V_L may affect this activity. The ScFvs were also subcloned into the ER-targeting eukaryotic expression vector pSTCF.KDEL and expressed in different human ovarian cancer cells. The expression of #148 ScFv in human ovarian cancer cells may suggest it bind to CA125 antigen, but we were not able to demonstrate the antigen-antibody interaction by immunoprecipitation.

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