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Improved soluble expression of a single-chain antibody fragment in *E. coli* for targeting CA125 in epithelial ovarian cancer

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ABSTRACT

Production of antibody fragments in heterologous hosts such as *Escherichia coli* provides a unique and cost-effective method to develop engineered vectors for tumor targeting. A single-chain Fragment variable (scFv) of the murine monoclonal antibody MAb-B43.13 targeting CA125 in epithelial ovarian cancer was previously developed, expressed, purified and proposed as a functional targeting entity for biomedical applications. However, the yields from its soluble expression in heterologous systems were very low for any practical use in preclinical translational research; leave alone the defeated objective of convenient and cost-effective production. In the present work, the anti-CA125 scFv gene was re-organized and subcloned into pET-22b(+) vector to be in frame with the pelB leader peptide for periplasmic localization and C-terminal hexa-histidine tag to facilitate downstream purification. Six variants of the scFv were constructed to investigate the impact of variable domain orientations, inter-domain peptide linker sequences and codon optimization on the soluble expression of the scFv using Rosetta 2(DE3) as the *E. coli* host supplemented with tRNAs for rare codons. Expression in shake flask cultures under the control of an inducible T7 promoter and subsequent purification by cobalt based immobilized metal affinity chromatography yielded differential amounts of high purity scFv for all constructs. Here, we report up to 14-fold increase in the soluble expression of the scFv primarily as a result of codon optimization with minor effects from inter-domain peptide linkers and variable domain orientation in the anti-CA125 scFv molecule. All the scFv constructs expressed and purified were found to be immunoreactive for *in vitro* targeting of CA125 antigen.

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Introduction

Recombinant technology has expanded the scope for engineering antibodies which have evolved from being sentinels of the immune system to becoming key components in targeted diagnostic and therapeutic applications. One of the most popular format of an engineered antibody is the single-chain Fragment variable (scFv[1]). Comprised of the heavy (V_H) and light (V_L) chain of an immunoglobulin's variable domains that are connected by a flexible peptide linker, this class of recombinant molecules are often referred to as the smallest yet complete active component of the immunoglobulin capable of binding to a target antigen [1,2].

Some advantages of the scFv format over full-length antibodies are (a) minimal immunogenicity due to the lack of F_c regions; (b) faster *in vivo* clearance and better tissue penetration owing to a relatively smaller molecular size; (c) cost effectiveness due to ease of production in simple host systems such as *Escherichia coli* and (d) their amenability to recombinant engineering [3]. Furthermore, the possibilities to screen immune libraries for isolation of high affinity scFv binders to practically any antigen by phage display [4], ribosome display [5] and yeast display [6] technologies have expanded scientific capabilities through ease of discovery, synthesis and production of engineered antibodies. Nevertheless, having access to the parent hybridoma cell for a monoclonal antibody (MAb) can facilitate the isolation of its derivative scFv with retained immunoreactivity and specificity [7,8]. This gains translational relevance when the scFv is sought from a clinically approved MAb [9]. However, there are challenges with the expression of murine antibody sequences in heterologous prokaryotic hosts such as *E. coli* in order to yield soluble and functional scFv.

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[1] *Abbreviations used:* scFv, single-chain Fragment variable; MAb, monoclonal antibody; CA125, Cancer Antigen 125; EOC, epithelial ovarian cancer; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; WT, wild-type; IMAC, immobilized metal affinity chromatography; S, serine; G, glycine; L, leucine; K, lysine; R, arginine; CAI, codon adaptation index; I, isoleucine; P, proline.

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Cancer Antigen 125 (CA125) is a mucinous glycoprotein that serves as a USFDA approved tumor biomarker for the diagnosis of epithelial ovarian cancer (EOC) [10,11]. CA125 can be detected in immunoassay techniques as an antigen shed in the serum of patients presenting in the clinic with pelvic masses [12]. The immunoassay formats and targeting applications have been greatly facilitated by the production of several antibodies against CA125 [13–16]. Among those reported, MAb B43.13 has shown great translational benefit in the immunotherapy of EOC [17]. Interestingly, there are not as many scFv molecules developed against this tumor-associated antigen [18–22]. Prior literature indicates that the anti-CA125 B43.13 scFv was purified as a secreted protein from yeast [18], as a secreted bi-specific scFv from murine myeloma NS0 cells [20], and engineered to be produced as a bi-functional fusion construct in *E.* coli, albeit with low yields obtained from soluble expression [21]. Considering its previously demonstrated potential for biopharmaceutical application to target CA125 [20,21,23], the production of this scFv in multi-milligram amounts could be a starting point for further engineering and expansion of its utility as an EOC targeting vector for immunodiagnostics, drug delivery and potential immunotherapy [24].

In the present work, we investigated the effects of variable domain orientation, different inter-domain linkers and codon optimization to increase the yields of soluble protein from heterologous expression of anti-CA125 B43.13 scFv in *E. coli*. The scFv was isolated from soluble fractions of recombinant cell lysates with high purity using a single step purification via immobilized metal affinity chromatography and was found to be biochemically active for binding to target antigen CA125. Codon optimization was found to be the most important factor to positively impact soluble expression of anti-CA125 scFv resulting in up to 14-fold higher yields than all the other murine scFv variants examined in this study.

Materials

All chemicals including antibiotics used were purchased from Sigma Aldrich unless otherwise specified. Phusion high-fidelity DNA polymerase (Finnzymes, F-530S), and oligonucleotide primers (Integrated DNA Technologies) were used for PCR amplification of the scFv domains. *Nco* I (NEB, R0193S), *Not* I (NEB, R0189S) and T4 DNA Ligase (NEB, M0202S) were used for directional cloning of genes encoding the scFv domains. pET-22b(+) vector (Novagen, 69744) and *E. coli* Rosetta™ 2(DE3) (Novagen, 71400) were used for expression of the scFv. Bacto tryptone (BD, 212750), Yeast extract (BD, 211705) and sodium chloride (Fisher Scientific, 7647-14-5) were used to prepare the 2× YT medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Fisher Scientific, BP1755-10) was used for induction of recombinant scFv expression. BugBuster Master Mix (Novagen, 71456) was used for cell lysis of harvested *E. coli* cultures. TALON® Superflow resin (Clontech, 635506) was used for immobilized metal affinity chromatography. Glycine (Bio-Rad, 161-0718) and Coomassie brilliant blue R-250 (Bio-Rad, 161-0400), Pre-stained SDS–PAGE standards – Low Range (Bio-Rad, 161-0305) were used for protein analysis by gel electrophoresis. Amicon Ultra-15, 10K MWCO filters (EMD Millipore, UFC901024) were used to concentrate the purified proteins. Fetal bovine serum (Life Technologies, 12483-020), penicillin–streptomycin (10,000 U/ml) (Life Technologies, 15140-122) and recombinant human insulin (SAFC Biosciences, 91077C) were used for culturing ovarian cancer cell lines. CelLytic™ M (Sigma, C2978) Trans-Blot nitrocellulose membrane (Bio-Rad, 162-0115), Amersham Hyperfilm ECL (GE Healthcare, 28906839), Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, RPN2132), mouse anti-β actin IgG (Sigma, A1978), goat anti-mouse HRP conjugate (Sigma, A4416), 6X His MAb-HRP conjugate (Clontech, 631210) were used for immunoblotting. Alexa-fluor® 488 goat anti-mouse antibody (Life Technologies, A-11001) and Penta·His Alexa Fluor 488 conjugate (Qiagen, 35310) were used in immunofluorescence and flow cytometry studies. Anti-CA125 MAb was isolated from hybridoma B43.13 and anti-RANK scFv was generously provided by Dr. Michael Doschak, University of Alberta, Canada.

Methods

Cloning of anti-CA125 scFv variants

The genes encoding the variable domains of the anti-CA125 scFv were amplified by polymerase chain reaction (PCR) using a previously reported plasmid construct pWET8 [20] as the template. Engineering of inter-domain linkers and production of different orientations of the single chain domains was performed by splice-overlap extension PCRs to create the following constructs viz. V_H-$(G_2S)_5$-V_L; V_L-$(G_2S)_5$-V_H; V_H-$(G_4S)_3$-V_L; V_L-$(G_4S)_3$-V_H; V_L-(218)-V_H, (Fig. 1) using *Nco* I and *Not* I as unique 5′ and 3′ cloning sites respectively for ligation into pET-22b(+) expression vector.

Based on demonstrated outcomes suggested in prior reports for other scFv molecules [25–27], the V_L-(218)-V_H scFv gene was codon-optimized for expression in *E. coli* and synthesized by GeneArt® (Life Technologies). The codon-optimized scFv gene was subcloned into pET-22b(+) vector between *Nco* I and *Not* I sites. This construct is hereafter designated as GA218 in this article, whereas the murine anti-CA125 scFv coding sequences will be referred to as the wild-type (WT) sequence. The scFv DNA cloned in each recombinant construct was verified by Sanger sequencing on a 3730 DNA analyzer (Applied Biosystems, Life Technologies) prior to transformation in the bacterial expression host.

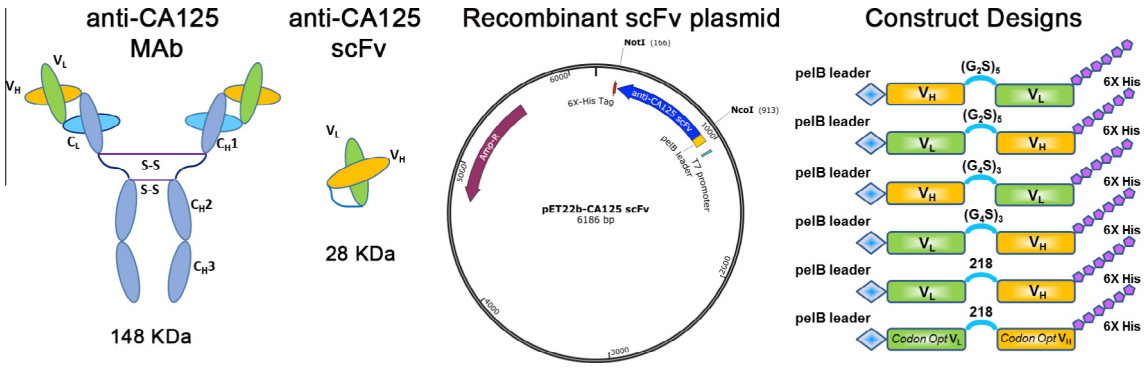

Fig. 1. Schematic representation of the anti-CA125 MAb and its derivative scFv cloned into pET-22b(+) vector in different orientations for heterologous expression in *E. coli*.

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