CHAPTER IV

DISCUSSION

CD147 is a member of the Ig superfamily that plays a crucial role in several tissues, but is particularly dense on the surface of activated T-lymphocytes (Kasinrerk et al., 1992; Koch et al., 1999) and several malignant tumor cells (Muraoka et al., 1993; Suzuki et al., 2004; Riethdorf et al., 2006). In T cells a negative regulatory signal arises from cross-linking of CD147 molecules and T cell regulation has been demonstrated (Igakura et al., 1996; Koch et al., 1999; Staffler et al., 2003; Chiampanichayakul et al., 2006). Recently, Chiampanichayakul *et. al.* demonstrated that two clones of anti-CD147 mAbs (M6-1E9 and M6-1B9), which react with the membrane-distal Ig domain, inhibited OKT3-induced T cell proliferation (Chiampanichayakul et al., 2006). These mAbs inhibited cell proliferation by delivery of a negative signal through CD147 to suppress CD25 and IL-2 expression (Chiampanichayakul et al., 2006). We thus decided to generate the monomeric scFv-M6-1B9 to investigate the extracellular effect of monomeric scFv-M6-1B9 on OKT3-induced T cell proliferation compared to its parental antibody (M6-1B9). We also generated an intrabody against CD147 to evaluate the intracellular function of scFv against CD147 on various cell types including 293A, HeLa and Jurkat cells. Diminishing the expression of CD147 on the cell surface could serve as a step towards exploring the significance of CD147 in cellular functions and tumor growth.

Prior to generating the scFv-M6-1B9, the critical residues implicated in antigen binding from a given antibody paratope are important for the construction of different antibody formats. The deduced amino acid residues responsible for paratope in CDR regions of the scFv-M6-1B9 were identified *via* the WAM algorithm (Whitelegg and Rees, 2000) and can be numbered following Kabat's rule (Kabat et al., 1976b; Kabat et al., 1976a). These confirmed the precise cloning of the immunoglobulin variable domains of anti-CD147 mAb, M6-1B9, into the phagemid vector (**Figure 3.1**).

Two antibody formats, Fab and scFv, were exploited for antibody phage display. To assess whether Fab-M6-1B9 or scFv-M6-1B9 on gpIII of phage particles have exclusive activities, both antibody formats were generated and evaluated. The expression of phage-displayed scFv-M6-1B9 was significantly greater than phage-displayed Fab-M6-1B9, and both antibody fragments could recognize the CD147 protein (**Figure 3.2A**). Noticeably, the scFv format exhibited a greater binding activity compared to the Fab format (**Figure 3.2B**). The expression level of scFvs in *E. coli* is typically higher than Fabs and generates a more efficient antibody display on the phage particle. The interdomain disulfide bond at the C-terminal of the constant region, which plays an important role in Fab stabilization, is predisposed to provide lower production yield than scFvs (Rothlisberger et al., 2005).

Recognition of the amber stop codon between the scFv and gpIII genes that occurs during the expression of pComb3X-scFv-M6-1B9 in the non-suppressing HB2151 *E. coli* strain resulted in the production of soluble scFv-M6-1B9 (Barbas, 2001; Su et al., 2003). This antibody fragment was specifically targeted both

recombinant CD147 (non-glycosylated form) (**Figure 3.3B**) and native CD147 (glycosylated form) expressed on the surface of U937 cell (**Figure 3.3D**). In addition, the inhibition of soluble scFv-M6-1B9 by the original antibody, M6-1B9 was demonstrated (**Figure 3.4**). These data show that the soluble antibody fragment contained a properly folded, bioactive paratope which recognized both non-glycosylated and glycosylated forms of CD147. CD147 is a highly glycosylated membrane protein. The variation in its molecular weight, ranging between 30 and 66 kDa, arises from different glycosylation patterns (Biswas et al., 1995). Targeting of both glycosylated and non-glycosylated CD147 molecules *via* scFv-M6-1B9 will be useful as a tool to knockdown the molecules in various cell types.

Moreover, soluble scFv-M6-1B9, which contains only one binding site, had the same inhibitory effect as observed for the parental mAb (**Figure 3.5**). These results indicate that the monomeric scFv form of M6-1B9 is a biologically active protein that is able to activate CD147 and regulate T cell activation. These results also confirm the successful production of a recombinant scFv-M6-1B9 retaining a CD147-specific paratope that binds to its epitope. This result is in agreement with the anti-CD3 scFv-B7.1 fusion protein expressed on the surface of HeLa cells which bound to T lymphocytes and strongly induced T-cell activation (Yang et al., 2007b). Furthermore, many reports have described the same binding specificity and affinity of scFv as the monomeric form of their parental antibody (Bedzyk et al., 1990; Pantoliano et al., 1991; Kim et al., 2002). These studies indicate that in the absence of cross-linking and covalent dimerization of parental CD147 mAb, the monomeric form of anti-CD147 molecules can mediate signal transduction

and exhibit the biological activity in T cells through their antigen-specific domain. Even though hybridoma cells can produce monoclonal antibodies for years, but production involves a labor-intensive multistep process limited by the constant risk of contamination, often requires feeder cells, and may be genetically unstable (Harlow, 1998). Thus, our scFv format has a favorable circumstance to preserve the genetic source of the antibody fragment by cloning it into a phagemid vector and utilizing relatively simple production in bacteria. Furthermore, the small molecular size of scFv should provide high tissue penetration efficiency (Kioi et al., 2008; Liu et al., 2008; Stirnemann et al., 2008), and a lack of significant toxicity (Korn et al., 2004; Yang et al., 2007a; Nam et al., 2008) while giving rapid plasma clearance (Mao et al., 1999; Hamilton et al., 2002; Olafsen et al., 2004; Pavoni et al., 2006), which makes such an antibody a potentially suitable candidate for clinical application.

To assess whether scFv-M6-1B9 generated by mammalian cells possesses the same activity as scFv-M6-1B9 generated by bacterial cells, we evaluated the binding ability of scFv-M6-1B9 intrabody produced from 293A cells to CD147. This intrabody was specifically targeted to both recombinant CD147 (non-glycosylated form, **Figure 3.10B**) and native CD147 expressed on the surface of HeLa cells (**Figure 3.11**). Our results demonstrated that the soluble antibody fragment produced from bacteria and mammalian cells had correct folding and could recognize both the native and recombinant forms of CD147.

Generation of adenoviral recombinants carrying scFv-M6-1B9 intrabody in 293A cells was deemed successful, since the cell surface expression of CD147 on these transduced cells declined (**Figure 3.14**). This indicates that an

intrabody with a carboxyl-terminal ER retention signal (KDEL) was retained in the membrane of the ER compartment. This sequestration resulted in the binding of intrabody to the newly synthesized CD147 and retained this molecule inside the cells, as confirmed by three-dimensional imaging (**Figure 3.15**).

While non-viral strategies demonstrate low transduction efficiency and transient transgene expression, viral transfer methods seem to have a greater potential for a successful gene transfer (Yamaoka et al., 2005; Doebis et al., 2006; Boldicke, 2007). Intrabody expression in HeLa and Jurkat cells using the adenoviral system was achieved in this study and resulted in decreasing of CD147 cell surface expression on these transduced cells (**Figure 3.16B** and **3.18B**). Colocalization between scFv-M6-1B9 intrabody and CD147 was demonstrated *via* confocal microscopy (**Figure 3.17D** and **3.17E**). This supports the starting assumption that the specific interaction between CD147 and scFv-M6-1B9 specific intrabody leads to abate the surface CD147 expression. In other studies, targeting of intrabodies to the ER has been successfully applied to knockdown major histocompatibility complex I (MHC-I) expression (Mhashilkar et al., 2002; Beyer et al., 2004; Zdoroveac et al., 2008) and inactivate various neoplastic cell-surface receptors (Wheeler et al., 2003; Jendreyko et al., 2005; Peng et al., 2007). Additionally, the same strategy was used to block cell surface expression of the CCR5 receptor which is important for cell entry of HIV-1 (Steinberger et al., 2000). Thus the KDEL signal may be essential for the single-chain antibody protein to abate the expression of cell surface molecules. The remaining CD147 could be due to the incomplete knockdown effect resulting from transient expression of the scFv-M6-1B9 intrabody.

Overexpression of CD147 promotes invasion, metastasis, growth and survival of malignant cells (Yan et al., 2005). In previous studies CD147 has been successfully downregulated by RNA interference (RNAi) technology in several cell lines, including prostate cancer (Wang et al., 2006), human malignant melanoma A375 (Chen et al., 2006), human ovarian cancer cell line HO-8910pm (Zou et al., 2007), and human Jurkat T-lymphoma (Chen et al., 2008). Knockdown of CD147 by siRNA resulted in decreased tumor cell proliferation and invasion activity *in vitro*. In addition, CD147 siRNA also downregulated the expression of VEGF at mRNA and protein levels in A375 cells (Chen et al., 2006). The urokinase-type plasminogen activator (uPA) system including uPA, its specific receptor uPAR and its primary inhibitor plasminogen activator inhibitor-1 (PAI-1) was also downregulated at the mRNA and protein levels in human breast epithelial cell line, NS2T2A. In human breast epithelial cell lines, MDA-MB 231 and Malme-3M, uPA was downregulated at the mRNA and protein levels (Quemener et al., 2007) and MCT4 was downregulated at the protein level (Gallagher et al., 2007). Intrabodies demonstrate an alternative strategy of gene inactivation that targets genomic DNA or mRNA. Unlike RNAi technology, intrabodies act at the posttranslational level and can be directed to relevant subcellular compartments (Lobato and Rabbitts, 2004; Heng et al., 2005; Boldicke, 2007). Because of their high affinity, high specificity and stability, intrabodies present an attractive alternative to modulate protein function and many protocols utilizing intracellular antibodies to neutralize the function of target proteins have been developed for cancer research (Cao and Heng, 2005; Heng et al., 2005). We feel that downregulating CD147 on cancer cells using intrabody technology is

available strategy for studying the role of CD147 in tumor invasion and metastasis. And furthermore, this methodology could prove to be a promising approach for cancer therapy.

In summary, we demonstrated the advantages of recombinant antibody technology and provided an alternative strategy to engineer low-cost antibodies with desirable affinity and specificity by enabling one to manipulate the basic domain structure of the immunoglobulin molecule. We have shown that soluble scFv-M6-1B9 binds to CD147 resulting in an inhibition of OKT3-mediated T cell proliferation. Furthermore, the scFv-M6-1B9 intrabody produced after adenoviral gene transfer was able to suppress the expression of CD147 on 293A, HeLa and Jurkat cells. These novel findings could prove to be a promising strategy for the study of the function of CD147 and provide ways to treat cancer in the near future.