Chapter 1

Introduction

1.1. Cell Sorting in Cancer Research

Cancer is a heterogeneous complex system often composed of different clonal cell subpopulations[1]. Increasing reports of intratumor heterogeneity and its augmentation for the selective pressure of tumor microenvironment, and recent achievements in cancer therapeutics requires the investigation and tracking of the subpopulations of cancer cells[2]. Hence, it is important to develop a multiplex and high-throughput technology for sorting cells to diminish the heterogeneity of cancer cells.

As the current state of the art, fluorescent activated cell sorting (FACS) has been widely used since its invention in early 1970s[3] due to its multiplexity and ability to sort small subpopulations (cells with frequency around 0.03%). However, its sorting speed cannot be over one million cells per hour, and it requires expensive instrumentation as well as a well-trained operator. Furthermore, rare cells, such as circulating tumor cells, cannot be sorted by FACS because of their extremely low frequency.

With the recent advance in microfluidics technology, powerful techniques have been developed to overcome the limitation of FACS. High-throughput cell sorting has been achieved by Wolff and co-workers, who described the 'on-chip' system allowing sorting of 12,000 cells per second at 100-fold enrichment[4], and by Simmonet and Groisman, whose microfluidic system is reported to sort 17,000 cells per second at 83-fold enrichment with 40% of recovery[5]. In the case of rare cells, Toner and co-workers

have managed to sort circulating tumor cells from peripheral blood by using a microfluidic chip with a large number of microposts[6].

1.2. Proteomics for Cancer Diagnosis

In contrast to DNA, which is subject to one major form of modification as methylation, proteins can be altered in many ways after their translation, such as by phosphorylation, acetylation, and glycosylation, which can bring a functional shift that potentially affects disease development, progression and response to clinical therapies. Therefore, a pressing need exists for improved and innovative technologies to profile oncoproteins for better understanding and treatment of cancer.

Conventionally, there are two approaches to proteomics for cancer diagnosis. Serum-based proteomics has been focused on for its non-invasiveness and opportunity to reduce time for assaying. As in the case of pancreatic cancer, with antibody arrays, signatures of sera distinguish cancer patients from healthy controls[7]. Serum proteomics is also used in monitoring breast cancer patients to detect early local recurrence or metastatic deposits[8]. In addition to the serum-based proteomics, tissue-based proteomics gives opportunities to improve diagnostic sensitivity and disease classification. As Kashani-Sabet and co-workers reported, tissue proteomics through immunohistochemistry using five markers increases the sensitivity and specificity of the assay to 91% and 95%, respectively, for the diagnosis of melanoma[9]. In the case of lung cancer, Ring et al. described a five-marker diagnostic assay that distinguishes adenocarcinoma from squamous cell carcinoma[10].

1.3. Metabolomics for Cancer Diagnosis

Besides the abnormal expression and post-modification of oncoproteins, cancer cells can be identified for their specific profiles of metabolites associated with the alteration of a particular metabolic pathway[11]. In the case of the breast cancer, compared with healthy tissue or benign tumors, breast biopsy samples shows reliably elevated total choline-containing compounds (tCho), low glycerophosphocoline and low gluce through NMR studies[12,13]. Similar to breast cancer, prostate cancer represents particular metabolic profile, especially high level of tCho, phosphocholine, lactate and alanine[14].

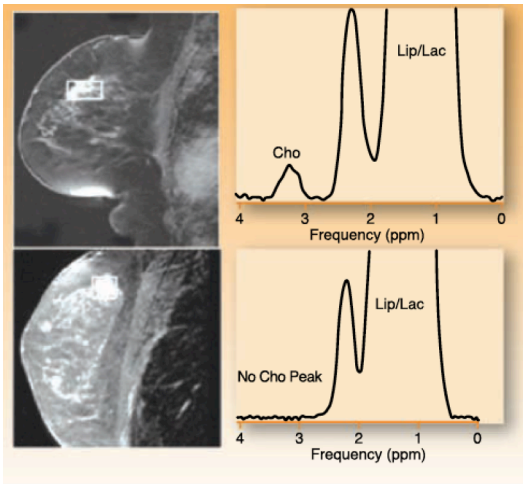

Fig. 1.1. Diagnosis of breast cancer with metabolites. **(Top) Breast tumor with corresponding Cho peak. (Bottom) Benign tissue without Cho peak. Adapted from *Clin. Cancer Res.* (2009)**

As a form of *in viv*o metabolomics, positron emission tomography (PET) imaging has been widely used in clinics to diagnose cancer before the introduction of metabolomics. Glucose, among many others, is the most important metabolite in PET imaging due to the "Warburg effect"[15], the phenomenon in cancer cells that cellular energy is generated through aerobic glycolysis instead of mitochondrial oxidative

phosphorylation. Although the exact mechanism is not yet well understood at present, most cancer cells uptake a high level of glucose compared with their normal counterpart, and their glucose uptake can be visualized by the measurement of radioactivity from fluorodeoxyglucose (FDG), a radioactive glucose analog[16].

1.4. Thesis Overview

This thesis presents the development of biotechnologies for *in vitro* cancer diagnostics. In Chapter 2, I will introduce my contribution to the development of cell sorting technology based on DNA-encoded p/MHC complexes for antigen-specific T cells. Through the development of genetic engineering, T cells are able to recognize cells presenting particular antigens, including cancer-specific antigens. Exploiting this characteristic of T cells selecting target populations of cells, researchers has been developed a new way of cancer treatment, so called T cell immunotherapy. By the injection of engineered and activated cancer-specific T cells to patients, the regression of tumor has been reported in subsets of patients with metastatic cancers. However, it was not easy to characterize the antigen-specific T cells during the treatment due to their small populations. To achieve highly multiplexed T cell detection, Chapter 2 will introduce the technique named as "Nucleic Acid Cell Sorting (NACS)". By employing p/MHC tetramers site-specifically conjugated with single-stranded DNA oligomers (ssDNA), specific target T cells from cellular suspensions can be captured and immobilized via hybridization to a complementary-printed substrate. The immobilization of T cells is optimized by engineering streptavidin to have cysteine residue at a specific site for the formation of ssDNA-p/MHC tetramer guaranteeing the superiority of NACS over conventional spotted arrays. With restriction enzymes and sophisticatedly designed ssDNA, the selective release of sorted T

cells is also investigated. Finally, the capability of NACS as a clinical application is studied by monitoring a cancer-specific T cell population from a melanoma patient undergoing T cell immunotherapy. Chapter 2 has been taken in part from *J. Am. Chem. Soc.*, **2009**, *131*(28), 9695–9703.

In order to clinical treatments for cancer cells, it is highly important to understand their protein-protein signaling network. With the development of single-cell barcode chip (SCBC), the measurement of multiple proteins in single-cells became available, but more studies were needed for the quantitative understanding of protein-protein network to predict the effect of perturbation, especially induced by targeted cancer therapy. In Chapter 2, I will also explain briefly about my contribution to develop experimental and theoretical methods for measuring protein secretion level of single or few cells and analyzing the results quantitatively. As a model system, mimicking innate immune response of human macrophages to Gram-negative bacteria, macrophages stimulated with lipopolysaccharide are employed. The secreted proteins are measured with SCBC to generate a covariance matrix for the analysis through a quantitative version of Le Chartelier principle, derived from information theory. Importantly, through the quantitative analysis, it is possible to rank the contributions of proteins to the entire network as well as to generate the diagram of protein-protein correlation network. Lastly, without actual experiments, the role of perturbation in the network can be theoretically predictable from the fluctuation of protein secretions. This prediction is then validated with the experiments with cells treated neutralizing antibodies to eliminate the secretion of certain proteins, which shows great accordance with the experimental result. Chapter 2 has also been taken in part from *Biophys. J.,* **2011**, *100(10),* 2378-2386.

In addition to proteins, metabolites can be used to diagnose cancer. Due to their high proliferation rate, in general, cancer cells uptake huge amount of glucose compared to their normal counterpart. For the monitoring of metabolites in small number of cells, in Chapter 3, a new platform, composed of a beta-particle camera and a microfluidic chip, will be introduced. The microfluidic chip, so called "Radiopharmaceutical Imaging Chip(RIMChip)" is sophisticatedly designed for the visualization of radio probes inside of cells with a small sample size (10 to 100 cells). The performance of RIMChip is validated with the uptake of [F-18]fluorodeoxyglucose (FDG), a glucose analog, in adherent and suspension cell lines. This chip is then utilized to study the response kinetics of lymphoma and glioblastoma cell lines under gemcitabine and erlotinib treatment, respectively. Within short time (~ 1 hour), the decrease of FDG uptake in those treated cells is observed, correlated to the arrest of cell-cycle (gemcitabine treatment to lymphoma cells) or the decreased signaling in epidermal growth factor receptor (EGFR) pathway (erlotinib treatment to glioblastoma cells) confirmed by separate cell-based assays. Chapter 3 has been taken in part from *J. Nucl. Med.*, **2013**, *in press*. By applying technique, clinical researches have been also proceeding to understand the glycolytic metabolism in primary glioblastoma cells. The results of ongoing study, imaging of glucose uptake in glioblastoma cells according to their expression level of oncoprotein (EGFRvIII) or in gliolastoma cells under different types of drug treatments, are briefly presented in Chapter 5.

Chapter 4 describes the ongoing project to develop novel agents for in vivo imaging of c-MET, a receptor tyrosine kinase largely amplified in prostate cancer cells.

The protein-catalyzed capture agents, PCCs, are synthesized from artificial amino acids by iteratively screening one-bead-one-compound combinatorial library method. Compared to commercial antibodies, PCC is inexpensive and stable for long-term storage at room temperature. The 1st generation of PCC for c-MET is developed and its performance is validated with cell-based fluorescent imaging assays for the quantitative comparison with commercial anti c-MET antibody.

Lastely, for reference, *J. Am. Chem. Soc.*, **2009**, *131*(28), 9695–9703 and *Biophys. J.,* **2011**, *100(10),* 2378-2386 are added as Appendix A and B.

1.5. References

1. Fisher, R., Pusztai, L. & Swanton, C. Cancer heterogeneity: implications for targeted therapeutics. *Br J Cancer* **108,** 479–485 (2013).
2. Barteneva, N. S., Ketman, K., Fasler-Kan, E., Potashnikova, D. & Vorobjev, I. A. Cell sorting in cancer research-Diminishing degree of cell heterogeneity. *BBA-Reviews on Cancer* **1836,** 105–122 (2013).
3. Sklar, L. A. & Finney, D. A. Analysis of ligand-receptor interactions with the fluorescence activated cell sorter. *Cytometry* **3,** 161–165 (1982).
4. Wolff, A. *et al.* Integrating advanced functionality in a microfabricated high-throughput fluorescent-activated cell sorter. *Lab Chip* **3,** 22–27 (2003).
5. Simonnet, C. & Groisman, A. High-throughput and high-resolution flow cytometry in molded microfluidic devices. *Anal. Chem.* **78,** 5653–5663 (2006).
6. Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* **450,** 1235–1239 (2007).
7. Orchekowski, R. *et al.* Antibody microarray profiling reveals individual and combined serum proteins associated with pancreatic cancer. *Cancer Res* **65,** 11193–11202 (2005).

8. Carlsson, A. *et al.* Serum protein profiling of systemic lupus erythematosus and systemic sclerosis using recombinant antibody microarrays. *Mol. Cell Proteomics* **10,** M110.005033 (2011).
9. Kashani-Sabet, M. *et al.* A multi-marker assay to distinguish malignant melanomas from benign nevi. *P Natl Acad Sci USA* **106,** 6268–6272 (2009).
10. Ring, B. Z. *et al.* A novel five-antibody immunohistochemical test for subclassification of lung carcinoma. *Mod. Pathol.* **22,** 1032–1043 (2009).
11. Spratlin, J. L., Serkova, N. J. & Eckhardt, S. G. Clinical applications of metabolomics in oncology: a review. *Clin Cancer Res* **15,** 431–440 (2009).
12. Bathen, T. F. *et al.* MR-determined metabolic phenotype of breast cancer in prediction of lymphatic spread, grade, and hormone status. *Breast Cancer Res Treat* **104,** 181–189 (2007).
13. Sitter, B. *et al.* Comparison of HR MAS MR spectroscopic profiles of breast cancer tissue with clinical parameters. *NMR Biomed* **19,** 30–40 (2006).
14. Swanson, M. G. *et al.* Quantitative analysis of prostate metabolites using 1H HR-MAS spectroscopy. *Magn Reson Med* **55,** 1257–1264 (2006).
15. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* **324,** 1029–1033 (2009).
16. Phelps, M. E. Positron emission tomography provides molecular imaging of biological processes. *P Natl Acad Sci USA* **97,** 9226–9233 (2000).