Methylation of EGFR by Arginine Methyltransferase PRMT1 Enhances EGFR Signaling and Cetuximab resistance

Hsin-Wei Liao
Methylation of EGFR by Arginine Methyltransferase PRMT1 Enhances
EGFR Signaling and Cetuximab resistance

By

Hsin-Wei Liao, B.S., M.S.

APPROVED:

______________________________
Mien-Chie Hung, Ph.D., Supervisor

______________________________
Dihua Yu, M.D., Ph.D.

______________________________
Hui-Kuan Lin, Ph.D.

______________________________
Liuqing Yang, Ph.D.

______________________________
Pierre D. McCrea, Ph.D.

APPROVED:

______________________________
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston
Methylation of EGFR by Arginine Methyltransferase PRMT1 Enhances EGFR Signaling and Cetuximab resistance

A DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Hsin-Wei Liao, B.S., M.S.

Houston, Texas

August, 2015
Dedication

This dissertation is dedicated to my parents and all of my family and friends who provided me their unconditional support and love during my years at UT MD Anderson Cancer Center, Houston, Texas, United States.
Acknowledgments

I would like to express my greatest appreciation to all those who have assisted, supported, and encouraged me during my Ph.D training. First, I would like to thank my mentor, Dr. Mien-Chie Hung for accepting me to join his excellent lab. With his fully support and guidance, I learned about not only scientific thinking but also how to face challenges optimistically, which will definitely benefit my future career and life. Second, I would like to thank all of my committee members for their valuable advice and encouragement, Dr. Dihua Yu, Dr. Zhen Fan, Dr. Hui-kuan Lin, Dr. Min-Gyu Lee, Dr. Ho-Young Lee, Dr. John E. Ladbury, Dr. Liuqing Yang, Dr. Pierre D. McCrea, and Dr. Mong-Hong Lee. Third, I would like to thank my friends and colleagues who have helped me throughout my graduate study, especially to Dr. Jun-Mao Hsu, Dr. Jennifer Hsu, Dr. Weiya Xia, Dr. Ying-Nai Wang, Dr. Hirohito Yamaguchi, Dr. Chao-Kai Chou, Dr. Heng-Huan Lee, Dr. Hong-Jen Lee, Dr. Stefan T. Arold, Dr. Pei-Hsiang Tsou, and Dr. Yueh-Fu Fang. Also, I thank Su Zhang, Zhenbo Han, Jian Guan Shi, and Jin-Fong Lee for their daily assistance. Last but not least, I would like give my sincere appreciation to my parents and all my family for their encouragement and support.
Methylation of EGFR by Arginine Methyltransferase PRMT1 Enhances EGFR Signaling and Cetuximab resistance

Hsin-Wei Liao, B.S., M.S.

Advisory Professor: Mien-Chie Hung, Ph.D.

Protein modifications of epidermal growth factor receptor (EGFR) intracellular domain are well known regulators of EGFR functions whereas those of its extracellular domain remain relatively unexplored. Here, we report that methylation at R198 and R200 of EGFR extracellular domain by protein arginine methyltransferase 1 (PRMT1) upregulates its binding to EGF and subsequent receptor dimerization and signaling activation. Methylation-defective EGFR mutant reduced tumor growth in mouse orthotopic xenograft model. Importantly, increased EGFR methylation sustains its signaling activation and cell proliferation in the presence of therapeutic EGFR monoclonal antibody, cetuximab. EGFR methylation level also correlates with higher recurrence rate after cetuximab treatment and poorer overall survival in colorectal cancer patients. These data suggest that R198/R200 methylation plays important role in regulating EGFR functionality and resistance to cetuximab treatment.
Table of Contents

Approval sheet ........................................................................................................................................ i
Title page ........................................................................................................................................... ii
Dedication........................................................................................................................................... 1
Acknowledgments............................................................................................................................... 2
Abstract ............................................................................................................................................... 3
Table of Contents ................................................................................................................................. 4
List of Figures ....................................................................................................................................... 7

CHAPTER 1. INTRODUCTION ................................................................................................. 11

1.1 Colorectal cancer and epidermal growth factor receptor (EGFR) target therapy .............. 11

1.2 Epidermal Growth factor receptor and its regulation ......................................................... 14

1.3 Protein arginine methylation ............................................................................................. 17

1.4 Rationale ..................................................................................................................................... 19

CHAPTER 2. MATERIAL and METHODS................................................................................. 20

2.1 Constructs, reagents, peptides and antibodies ................................................................... 20

2.2 Cell culture ............................................................................................................................... 21

2.3 shRNA construct and transfection .................................................................................... 21

2.4 Mass spectrometry .................................................................................................................. 22

2.5 In vitro methylation assay ...................................................................................................... 22

2.6 In vivo protein interaction by Duolink assay ........................................................................ 22

2.7 Cell proliferation assay ............................................................................................................ 23
2.8 Anchorage-independent growth assay for colony formation ................................. 23
2.9 Dimerization assay .................................................................................................. 23
2.10 Orthotopic colon cancer mouse model ............................................................... 24
2.11 Immunohistochemical staining (IHC) .................................................................. 24
2.12 Statistics ............................................................................................................... 25
2.13 Saturation binding assay ......................................................................................... 25
2.14 Clonogenic assay ................................................................................................... 26
2.15 Study approval ....................................................................................................... 26

CHAPTER 3. RESULTS .................................................................................................. 27
3.1 PRMT1 methylates EGFR at R198 and R200 ....................................................... 27
3.2 PRMT1 interacts with and methylates EGFR before its translocation to the cell membrane ........................................................................................................... 39
3.3 PRMT1 upregulates EGFR signaling and cell proliferation in colorectal cancer cell lines ............................................................................................................ 47
3.4 PRMT1 upregulates EGFR dimerization, activation, and EGFR-dependent cell proliferation through R198/200 methylation ......................................................... 57
3.5 Methylation of EGFR increases tumorigenesis in orthotopic colorectal cancer mouse model and correlates with poorer clinical outcomes of colorectal cancer patients ............................................................................................................. 69
3.6 EGFR methylation enhances EGF binding to EGFR and correlates with higher recurrence rate of colorectal cancer patients after cetuximab treatment ............ 75

CHAPTER 4. DISCUSSION ........................................................................................... 93
4.1 Conclusion and significance ...................................................................................... 93
4.2 EGFR target therapy and predictive markers for drug resistance ...................... 93
4.3 Cross talk between PRMT family members ...................................................... 97
4.4 Extracellular methylation of membrane receptors and their target therapies .... 97
4.5 Locations where extracellular domain modification takes place ..................... 98
4.6 Future direction .................................................................................................. 99

Bibliography ................................................................................................................... 103

Vita .................................................................................................................................. 124
List of Figures

Figure 1. Methylation on EGFR extracellular domain Arg53. ......................................... 29

Figure 2. Methylation on EGFR extracellular domain Arg98. ......................................... 30

Figure 3. Methylation on EGFR extracellular domain Arg198 and Arg200. ................. 31

Figure 4. Methylation on EGFR extracellular domain Arg285. ....................................... 32

Figure 5. Methylation on EGFR extracellular domain Arg497. ....................................... 33

Figure 6. Illustration of EGFR extracellular and intracellular domains. ....................... 34

Figure 7. In vitro methylation assay of EGFR ................................................................. 35

Figure 8. In vitro methylation assay of WT EGFR and methylation-site single mutants. 36

Figure 9. In vitro methylation assay of WT EGFR and R198/200K double mutant. ...... 37

Figure 10. Sequence alignment of PRMT1 substrates along with EGFR showing a potential GAR motif on EGFR extracellular domain 2. ........................................ 38

Figure 11. Dot blot showing specificity of EGFR me-R198/200 Ab. .............................. 41

Figure 12. Immunoblots comparing EGFR methylation level in SKCO1 cells. .......... 42

Figure 13. EGFR is methylated before membrane translocation............................. 43

Figure 14. Isolation of ER organelle.......................................................... 44

Figure 15. Reciprocal co-immunoprecipitation of EGFR and PRMT1...................... 45

Figure 16. Duolink assay of SKCO1 cells......................................................... 46
Figure 17. PRMT1 upregulates EGFR signaling in SKCO1 cell. ................................. 48

Figure 18. PRMT1 upregulates EGFR signaling in GEO cell................................. 49

Figure 19. PRMT1-mediated upregulation of EGFR signaling can be stimulated by TGFα. ......................................................................................................................... 50

Figure 20. PRMT1-mediated upregulation of EGFR signaling is enzymatic activity dependent. ............................................................................................................. 51

Figure 21. Immunoblots of SKCO1 cells expressing first PRMT1 shRNA.............. 52

Figure 22. Immunoblots of SKCO1 cells expressing second PRMT1 shRNA. ......... 53

Figure 23. Cell proliferation assay of SKCO1 cells. .................................................. 54

Figure 24. Cell proliferation assay of GEO cells....................................................... 55

Figure 25. Anchorage-independent growth of SKCO1 cells................................. 56

Figure 26. PRMT1 upregulates EGFR activation through R198/200 methylation. .... 60

Figure 27. PRMT1 upregulates EGFR activation through R198/200 methylation in HT29 cells. .................................................................................................................. 61

Figure 28. PRMT1 EGFR-dependent cell proliferation through R198/200 methylation. 62

Figure 29. Structural analysis of EGFR............................................................... 63

Figure 30. Structural analysis of EGFR Arg 198 and Arg 200............................... 64

Figure 31. PRMT1 upregulates EGFR dimerization. .............................................. 65
Figure 32. Knocking down of PRMT1 attenuates EGFR dimerization ability. .......... 66

Figure 33. PRMT1 upregulates EGFR dimerization through R198/200 methylation..... 67

Figure 34. EGFR methylation does not affect its cell surface expression level. ........... 68

Figure 35. Methylation of EGFR increases tumorigenesis in orthotopic colorectal cancer mouse model ............................................................................................................. 71

Figure 36. Characterization of me-R198/200 antibody. .............................................. 72

Figure 37. Methylation of EGFR correlates with poorer overall survival rate of colorectal cancer patients .................................................................................................................. 73

Figure 38. Methylation of EGFR correlates with higher recurrence rate of colorectal cancer patients .................................................................................................................. 74

Figure 39. EGFR methylation regulates EGF binding to EGFR. ................................. 78

Figure 40. EGFR R198/200 methylation regulates EGF binding to EGFR. ................. 79

Figure 41. EGFR methylation regulates EGF binding to EGFR after cetuximab treatment. .............................................................................................................. 80

Figure 42. EGFR methylation regulates EGF binding to EGFR and correlates with higher recurrence rate of colorectal cancer patients after cetuximab treatment........ 81

Figure 43. EGFR methylation regulates colony formation after cetuximab treatment in HT29 cells .............................................................................................................. 82
Figure 44. EGFR methylation regulates colony formation after cetuximab treatment in GEO cells. ................................................................. 83

Figure 45. EGFR methylation regulates colony formation after cetuximab treatment in SW48 cells............................................................ 84

Figure 46. EGFR R198/200 methylation increases cetuximab resistance in GEO cells. 85

Figure 47. EGFR R198/200 methylation increases cetuximab resistance in SW48 cells. 86

Figure 48. Correlation between total EGFR expression with cetuximab response. ....... 87

Figure 49. Correlation between EGFR methylation level and cetuximab response in colorectal cancer patients. ........................................ 88

Figure 50. Correlation between PRMT1 expression and EGFR methylation level in colorectal cancer patients. ........................................ 89

Figure 51. Correlation between PRMT1 expression and EGFR methylation level in head and neck cancer patients................................. 90

Figure 52. Correlation between EGFR methylation level and cetuximab response in head and neck cancer patients................................. 91

Figure 53. Clonogenic assay of SKCO1 cells with cetuximab treatment .............. 92

Figure 56. Co-immunoprecipitation of EGFR and Her2 in cells overexpressing wild type or R198/200K mutant EGFR........................................ 96

Figure 57. Methylation percentage of endogenous EGFR from SKCO1 cell .......... 102
CHAPTER 1. INTRODUCTION

1.1 Colorectal cancer and epidermal growth factor receptor (EGFR) target therapy

Colorectal cancer (CRC) is the third leading cause of cancer deaths in the United States. The recommended treatment for early stage colorectal cancer is surgical removal, while the management of late stage colorectal cancer relies heavily on chemotherapy (1). Optimization of dosing and scheduling of chemotherapy agents have been developed to improve response and survival rate of patients. Meanwhile, rational targeting of molecular signaling pathways that are involved in the etiology of malignancies is currently one of the most promising strategies in novel anticancer drug development. The comprehensive molecular characterization of 224 CRC cases reported by Cancer Genome Atlas Network showed sixteen percent of them are hyper-mutated (2). WNT pathway alterations have been found in 93% of all tumors and involved 16 different genes in WNT signaling pathway. Tumor growth factor β signaling alterations are identified preferentially in the hyper-mutated tumors (87%). Moreover, ERBB family gene amplification or mutations are observed in 19% of tumors. Over all, 24 genes are significantly mutated and many of them are considered targetable.

Since the discovery of EGFR in 1962, members of the EGFR family and their downstream signals have become one of the most well characterized receptor tyrosine kinase (RTK) system. In addition to their function in normal development, aberrant expression of EGFR is involved in abnormal cell proliferation (3), reduced apoptosis (4), cell migration (5, 6), metastasis (7, 8), and angiogenesis (9) in cancer patients. Owing to the importance of EGFR’s role in tumorigenesis, new classes of drugs that target EGFR are among the most clinically advanced molecular-targeted therapies. Although EGFR
tyrosine kinase inhibitors combined with chemotherapy presented severe toxicity (10-12) and limited effects (13, 14), the combination of EGFR monoclonal antibody, such as cetuximab and panitumumab, with chemotherapy has shown efficacy in colorectal cancer treatment (15, 16).

Cetuximab was initially approved based on the BOND clinical trial, which compared cetuximab plus irinotecan with cetuximab alone in patients with fluoropyrimidine- and irinotecan resistant metastatic colorectal cancer (mCRC) (17). Response rate and progression free survival for the combination arm are significantly more effective than cetuximab only. Subsequently, the NCI-CO17 (Cetuximab and Best Supportive Care Compared With Best Supportive Care Alone in Treating Patients With Metastatic EGFR-Positive Colorectal Cancer) study confirmed an advantage for cetuximab in chemotherapy-resistant mCRC (18). Unfortunately, resistance to EGFR-targeted therapy has been recently observed, and many potential mechanisms have been proposed to explain the poor response to cetuximab, including activation of Her2 (19) or MET (20) signaling, mutation of PIK3CA (21) and BRAF (22), or status of PTEN (23). However, retrospective analyses revealed inconsistent and controversial findings (24). So far, the most accepted predictive marker for poor cetuximab response is mutant KRAS status due to its association with poor survival rate under cetuximab treatment in colorectal cancer clinical trials (25-28). Therefore, American Society of Clinical Oncology has recommended that all patients with metastatic colorectal carcinoma who are candidates for therapy with anti-EGFR antibodies should have their tumor tested for KRAS mutations, and only patients with wild-type (WT) KRAS are recommended for the cetuximab treatment (29). The predictive value of KRAS mutation status for benefit from
anti-EGFR monoclonal antibody therapy was similarly confirmed in the trial of panitumumab antibody therapy (30). In addition, panitumumab is as effective as cetuximab in a phase III clinical trial in patients with chemotherapy-resistant KRAS-wt mCRC. Nevertheless, KRAS mutation examination is limited to exon 2 codons 12 and 13 (31). Around 37% to 45% of CRCs carry activating KRAS mutations in exon 2 (32-35). The predictive value of individual exon 2 KRAS-mutation in mCRC is controversial. Although some reports have suggested a possible benefit from anti-EGFR monoclonal antibody therapy for patients with KRAS codon 13 (G13D) mutations (36, 37), a meta-analysis of three randomized phase III clinical trials of panitumumab in first-line, second-line, and refractory settings failed to confirm these findings (38). The observation that WT KRAS is not sufficient to confer sensitivity to cetuximab (39-41), while some patients with mutant KRAS are still sensitive to cetuximab (24, 36, 37, 42, 43), indicating the underlying mechanism of cetuximab resistance remains controversial and warrants further investigation to identify potential predictor of cetuximab response.

Over the last two decades, significant progress has been made in the treatment of mCRC. Overall survival has increased from approximately 12 months to nearly 30 months when treated with fluoropyrimidine monotherapy in recent clinical trials, especially in RAS wild type patients (22, 44). More importantly, a better understanding regarding molecular mechanism of CRC has largely improved patient prognostication and the launch of precision medicine in the treatment of mCRC. In the future, development of novel therapeutics for patients with RAS mutations or other defined molecular subgroups such as HER2 amplification, PIK3CA, BRAF mutations, would be important to improve treatment efficiency. In addition, to understand and overcome the
cause of resistance to anti-EGFR therapy in the patients with wild type RAS and wild type BRAF will further maximize the therapeutic benefit.

1.2 Epidermal Growth factor receptor and its regulation

EGFR family, which includes four members, EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, is one of the most well characterized receptor tyrosine kinase systems. Members in EGFR family are known oncogenic drivers in lung cancer, breast cancer, colorectal cancer and glioblastoma (45-47). EGFR, encoded within the 7p11.2 chromosomal locus, is the first receptor discovered with intracellular tyrosine kinase activity (48). The protein products are expressed in two isoforms of 145 and 165 kD, containing the extracellular (EC), transmembrane (TM), intracellular tyrosine kinase (TK), and intracellular C-terminal domains.

Seven activating ligands were identified to regulate activity of EGFR (49), including EGF, transforming growth factor α (TGF-α), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (ARG), epiregulin (EPR), and epigen (EGN). All these ligands contain an EGF-like domain, with six spatially conserved cysteines (that form three intramolecular disulfides), which is responsible for receptor binding and activation. The membrane-bound precursor EGFR ligands are cleaved by cell-surface proteases in a ligand-specific manner to generate the active growth factors (50). Although deficiency of EGFR affect a wide range of cellular processes, it still remains unclear which ligands are responsible in which context. Upon ligand stimulation, EGFR converts from an inactive monomeric form to an active homodimer or to a heterodimer with another EGFR family member. EGFR dimerization
leads to a conformational change that triggers autophosphorylation of C-terminal tyrosine residues and activates the TK domain. The interaction between EGFR-phosphorylated tyrosines and several intracellular proteins with phosphotyrosine binding SH2 domains conveys signal transduction (51). The downstream signaling pathways include phospholipase C (PLC), the signal transducer and activator transcription (STAT), phosphatidylinositol 3-kinase (PI3K/Akt), the mitogen-activated protein kinase (MAPK), and the SRC/FAK pathways (52).

Protein modifications play critical roles in regulating stability, localization, and signaling activation of EGFR. Cross talk between phosphorylation and PRMT5 (protein arginine methyltransferase 5)-mediated methylation on intracellular domain regulates downstream signaling activation (53), whereas ubiquitination by Cbl mediates EGFR stability, trafficking, and signal sustainability (54). In addition, EGFR acetylation enhances EGFR tyrosine phosphorylation and augmented its association with Src kinase. Acetylation-deficient EGFR mutant significantly reduces the activation of EGFR (55). While intracellular domain modifications of transmembrane proteins have been well studied, only a few types of extracellular domain modifications have been identified (56, 57). There is evidence that extracellular modifications of transmembrane proteins have important physiological functions. For example, extracellular domain phosphorylation of cadherin protein by intracellular Golgi kinase or ectokinase regulates cell adhesion, cell growth, and cell polarity (57, 58). In addition, glycosylation on the extracellular domain of RTK is critical for protein stabilization and subcellular localization (56, 59). However, other types of extracellular domain protein modifications besides phosphorylation and
glycosylation are still significantly unexplored. Interestingly, our mass spectrum analysis revealed several methylated arginines on EGFR extracellular domain.
1.3 Protein arginine methylation

Since the discovery of protein arginine methyltransferases (PRMTs), arginine methylation has been implicated in many biological processes and human diseases (60). During arginine methylation, PRMTs transfer methyl groups from S-adenosylmethionine (SAM) to the guanidine nitrogen of specific arginine residues on their target proteins, which in turn alter the protein structure (61), protein-protein interaction (62), protein localization (63) and enzyme activity (64) that are critical for various cellular functions. For example, signal transduction (65), RNA processing (66), DNA repair (64), and gene transcription (67), are regulated by arginine methylation. PRMT1 is the best characterized among the ten mammalian PRMT family members, catalyzes around 85% of all asymmetric arginine di-methylation events in mammalian cells (68), and is required for mammalian development and survival as PRMT1 null mice die around embryonic day 6.5. However, PRMT1 is dispensable for basic cellular processes such as gene expression and DNA replication, because embryonic stem cells are viable under cell culture conditions (69). Embryonic fibroblasts from PRMT1-deficient mice have higher incidence of chromosome losses, gains, polyploidy, and failure to divide, indicating that PRMT1 is essential for cell proliferation and survival (70).

The methyltransferase activity of PRMT1 toward different substrates is modulated by its interactions with TIS21 (tetradeconoyl phorbol acetate-inducible sequence 21) and BTG1 (B-cell translocation gene 1) (71). In addition, interaction between transcriptional regulator hCAF1 (CCR4-associated factor 1) and PRMT1 down-regulates the methylation levels of PRMT1 substrates, Sam68 (the Src-Associated substrate in Mitosis of 68 kDa) and histone H4 (72). In line with the
observation that PRMT1 exists in a high molecular weight complex in mammalian cells (73), dimerization of PRMT1 is required for its binding to the methyl donor S-adenosyl methionine (SAM) (74). Therefore, macromolecular complex formation of PRMT1 is critical for the regulation of its enzymatic activity and substrate specificity. Although PRMT1 frequently methylates arginine residues in the glycine- and argininerich (GAR) motifs or RXR sequences (75), arginine methylation of some PRMT1 substrates do not have these motifs. Identification and analysis of more methyl group acceptors will further elucidate critical elements of substrate recognition.

More recently, PRMT1 has been linked to cancers (76-78). For instance, in prostate cancer, H4R3 methylation by PRMT1 predicts the risk of cancer recurrence (79); in mixed lineage leukaemia (MLL), PRMT1 cooperates with MLL-EEN to promote self-renewal and colony formation ability of primary hematopoietic progenitors (80); in breast cancer, PRMT1 methylates estrogen receptor alpha, leading to subsequent activation of AKT and cell cycle progression (81). Importantly, overexpression of PRMT1 has been observed in tumor tissues of both breast and colorectal cancer (60). PRMT1 is also associated with poor clinical outcomes in colorectal cancer patients and has been proposed to serve as a predictive marker for patient prognosis (77). Although high PRMT1 expression has been shown to correlate with colorectal cancer progression (82), its role in colorectal cancer pathophysiology remains obscure.
1.4 Rationale

The observation of EGFR extracellular domain methylation prompts us to ask whether this modification affects EGFR functionality and the efficacy of extracellular domain-targeted therapeutic monoclonal antibody, cetuximab, in colorectal cancer treatment. To answer these clinically important questions, we first investigated which member in protein arginine methyltransferase family was responsible for extracellular domain methylation of EGFR. Second, we examined the effects of EGFR extracellular domain methylation on the receptor signal activation. Third, we studied how EGFR extracellular domain methylation affected the cellular and patient response to therapeutic monoclonal antibody, cetuximab.
CHAPTER 2. MATERIAL and METHODS

2.1 Constructs, reagents, peptides and antibodies.

EGFR and PRMT1 constructs were prepared as described previously (53). Four extracellular domains, juxtamembrane domain, kinase domain, and C-terminal tail of EGFR were further subcloned into pGEX vector for the preparation of truncated EGFR recombinant proteins. EGFR (R198/200K) mutagenesis was generated using the QuickChange Site-Directed Mutagenesis Kit according to the manufacturer’s protocol (Stratagene). Epidermal growth factor (EGF; Sigma) was prepared according to the manufacturers’ instructions. Unmodified (Ac-QCSGRCRGKSPSD-C), asymmetric di-methylated (Ac-QCSG(asymmetric dimethyl-R)C(asymmetric dimethyl-R)GKSPSD-C), symmetric di-methylated (Ac-QCSG(symmetric dimethyl-R)C(symmetric dimethyl-R)GKSPSD-C), and mono methylated (Ac-QCSG(mono methyl-R)C(mono methyl-R)GKSPSD-C) EGFR peptides were chemically synthesized by Lifetein for antibody production in mice and dot blot and peptide competition assays. Anti-EGFR antibody (06-847, 1:2,000; Millipore) was used to detect full-length EGFR. Antibodies against phospho-Tyr 1086 and -Tyr 1148 (Abcam) were used for detection of EGFR activation. Antibodies to ERK (1:5,000; Millipore) and phospho-ERK (1:5,000; Cell Signaling) were used to detect the EGFR downstream signaling activation. Anti-PRMT1 (Cell Signaling) was used to detect PRMT1 level after overexpression or knockdown. Anti-tubulin antibody was purchased from Sigma.
2.2 Cell culture.

SKCO1 (KRAS G12V) and SW48 (KRAS wild type) were purchased from ATCC. GEO (KRAS G12A) and HT29 (KRAS wild type) cells were kind gifts from Dr. Zhen Fan (MD Anderson cancer center). GEO, HT29, and SW48 cells were cultured in DMEM/F-12 with 10% fetal calf serum. SKCO1 cells were cultured in MEM with 10% fetal calf serum. All cell lines were characterized as mycoplasma negative and validated by STR DNA fingerprinting using the AmpF_STR Identifiler kit according to manufacturer's instructions (Applied Biosystems cat 4322288). The STR profiles were compared to known ATCC fingerprints (ATCC.org), and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/) (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526). The STR profiles matched known DNA fingerprints or were unique. Before EGF stimulation, 80% confluent cells were serum-starved for 20 hr, and then stimulated with 50 ng/ml EGF for indicated time or 50ng/ml TGFα for 20 min.

2.3 shRNA construct and transfection

Lentiviral-based pLKO.1 PRMT1 shRNA vector was obtained from Academia Sinica (Taipei, Taiwan). The pLKO.1 scrambled shRNA vector was purchased from Addgene. (Cambridge, MA). The PRMT1-targeting shRNA sequences used in the lentiviral construct were: 5’-CCGCGCGGCAGTACAAAGACTACCTCGAGTTGTAGTCTTTGTACTGCCGGGTTTTTG-3’ (shRNA #1) and 5’-CCGGGCAAGTGAGCTAAGCTACTACACTCGAGTAATTCCGCTTCACTTGCTTTTTTGG-3’ (shRNA #2). For lentiviral production, PLKO.1 PRMT1 shRNA vector, packaging (pCMV-dr8.Z
and envelope (pCMV-VSV-G) plasmids were co-transfected into 293T cells using Lipofectamine Reagent (Invitrogen, Carlsbad, California). After 48-hr transfection, colon cancer cells were infected with viral particles. Stable knockdown clones were selected by culturing cells in medium with 4 µg/ml puromycin for 1 month.

2.4 Mass spectrometry

EGFR was isolated by immunoprecipitation with anti-EGFR antibody and then was analyzed by SDS-PAGE. The protein band corresponding to EGFR was excised and subjected to in-gel digestion with trypsin. After isolation by immobilized metal affinity chromatography, the enriched methyl-peptides were analyzed by micro-liquid chromatography/tandem MS.

2.5 In vitro methylation assay

GST-tagged PRMT1 and GST-tagged EGFR fragments were expressed in *E. coli* individually and purified using *glutathione sepharose* 4B. They were then incubated together in the presence of 2.2 Ci S-adenosyl-L-[methyl-³H] methionine (85 Ci/mmole from a 0.55 mCi/ml stock solution; MP Biomedicals) for 1 hr at 30 °C in a final volume of 50 µl of phosphate-buffered saline. After incubation, samples were separated by SDS-PAGE and transferred to PVDF membrane. Methylation levels were examined by fluorography.

2.6 In vivo protein interaction by Duolink assay

Cells were seeded in 8-well chamber slides. When harvesting cells, cells were washed with cold PBS twice and fixed with 4% paraformaldehyde at 4°C for 2 hr. After
two more PBS washes, cells were permeabilized by cold 0.2% Triton X-100 for 30 min at room temperature and subjected to Doulink assay (Olink Bioscience) according to the manufacturers’ instructions.

2.7 Cell proliferation assay

Cells were seeded in 6-well plates (triplicate), and fresh medium (with or without gefitinib or cetuximab) were added every day. Cells were then trypsinized and cell numbers counted on a daily basis.

2.8 Anchorage-independent growth assay for colony formation

The base layer of cell growth matrix containing DMEM/F12 medium, 10% FBS, and 0.5% agar was paved in 6-well plates (1.5 ml per well). After solidification of the base layer, the top layer (1.5 ml per well) containing DMEM/F12 medium, 10% FBS, and 0.35% agarose, and cells was plated. Culture medium (1 ml) was added to each well and changed every 3 days. After 4-week culture, colonies were stained by 0.005% crystal violet. Colonies with a diameter larger than 0.5 mm were counted.

2.9 Dimerization assay

Cells were starved in serum free medium for 24 hr. After starvation, cold PBS containing 50 ng/ml EGF was added onto plates for 30 min at 4°C. Then, cells were washed with cold PBS (137 mM NaCl, 0.67 mM KCl, 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄) three times and incubated for 2 hr at 4°C with 5 mM cross linker BS³ (bis[sulfosuccinimidyl] suberate; Thermo scientific) in PBS. After washing three times with cold PBS, cross linking reactions were stopped by incubating cells in 50 mM Tris
buffer (pH 7.5) for 15 min at ambient temperature. Cells were subsequently lysed and cell lysates analyzed by Western blotting.

2.10 Orthotopic colon cancer mouse model

All animal experiments were carried out in accordance with approved protocol from Institutional Animal Care and Use Committee (IACUC) at MD Anderson Cancer Center. Nude female mice at 4-5 weeks of age were maintained at the MD Anderson Animal Facility for 1 week prior to injection of cancer cells. The cecum was exteriorized through a small midline laparotomy and $10^7$ GEO cells expressing WT EGFR, EGFR methylation-site mutant, or vector control were injected into the cecal wall. After injection, the abdominal wall was closed by wound clips. One month after surgery, tumors were harvested and tumor weight measured.

2.11 Immunohistochemical staining (IHC)

IHC of methylated EGFR was performed using homemade me-R198/200 antibody. Colorectal cancer tissue microarrays were purchased from National Cancer Institute Cancer Diagnosis Program. Head and neck cancer samples from patients treated with cetuximab were collected from Taipei Veterans General Hospital (N = 38) and University of Pittsburgh Medical Center (N = 21). Samples were deparaffinized and rehydrated. Antigen retrieval was performed by using 0.01 M sodium-citrate buffer (pH 6.0) in a microwave oven. The sections were treated with 1% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After 1 hr preincubation in 10% normal serum to prevent nonspecific staining, the samples were incubated with primary antibodies at 4°C overnight. The sections were then treated with biotinylated
secondary antibody, followed by incubations with avidinbiotin peroxidase complex solution for 1 hr at room temperature. Color was developed with the 3-amino-9-ethylcarbazole solution. Counterstaining was carried out using Mayer's hematoxylin. All immunostained slides were scanned on the Automated Cellular Image System III (ACIS III) (Dako, Denmark) for quantification by digital image analysis. A total score of protein expression was calculated automatically from the percentage of immunopositive cells and immunostaining intensity.

2.12 Statistics

Statistical analyses were performed using SPSS software. The association between the expression level of methyl-EGFR and PRMT1 was analyzed by Spearman’s rank correlation test. Survival curves were plotted using Kaplan-Meier method, and log-rank tests were performed to evaluate prognostic differences between groups for categorical variables. For all analyses, two-sided tests of significance were used. A p value of < 0.05 was considered statistically significant.

2.13 Saturation binding assay

ELISA 96-well plates were captured with 3 µg/ml anti-EGFR antibody (Abcam) in 0.2 M sodium phosphate buffer (pH 6.5) at 100 µl/well overnight at room temperature. The plates were then rinsed three times with PBS with 0.05% Tween-20 (PBST) and blocked with 200 µl/well of 1% BSA solution at 37°C for 2 h. After rinsing three times with PBST, 100 µl/well of HT29-RIPA lysates or RIPA buffer only as a negative control were added and incubated at 37°C for 1.5 h. The plates were then washed with 400 µl/well of PBST three times, followed by addition of recombinant human biotin-EGF at a
series of diluted concentrations in RIPA buffer. After incubation at 37°C for 1.5 h, wells were washed with 400 μl/well of PBST three times, added by 100 μl/well of streptavidin-conjugated HRP (1:2,000 in blocking buffer), and incubated for 30 min at room temperature. The wells were washed again with PBST three times and 100 μl/well of TMB as a peroxidase substrate were added and incubated for 30 min at room temperature. The reaction was terminated by addition of 50 μl/well of stop solution. The optical density was determined at 450 nm, corrected by subtraction of readings at 570 nm, using a BioTek Synergy™ Neo multi-mode reader. The dissociation constant (Kd) was estimated by the above binding data and then transformed to create a Scatchard plot with GraphPad Prism program (version 6; Prism Software Inc., San Diego, USA).

2.14 Clonogenic assay

Cells (5,000 per well) were seeded in 24-well plates. Culture medium was changed every three days. After 10 days of culturing, cells were washed by cold PBS twice and fixed by 4% paraformadehyde for 1 hr. Cells then were stained by 0.005% crystal violet at 4°C overnight. After ddH₂O wash, colonies with a diameter larger than 0.5 mm were counted.

2.15 Study approval

This study was approved by the IACUC of the University of Texas MD Anderson Cancer Center and adhered to NIH guidelines for the use of experimental animals. Human colorectal cancer and head and neck cancer tissues with cetuximab treatment were obtained under protocols approved by the University of Texas MD Anderson Cancer Center IRB.
CHAPTER 3. RESULTS

3.1 PRMT1 methylates EGFR at R198 and R200

Mass spectrum analysis of immuno-purified endogenous EGFR proteins from SKCO1 colorectal cancer cells demonstrated several methylated arginines on the extracellular domain of EGFR, including R53, R98, R198, R200, R285 and R497 (Fig. 1-Fig. 5). Among them, missense mutation of R198 and R285 were reported in colorectal adenocarcinoma (TCGA database), and both happen to be located on EGFR extracellular domain 2 (D2) (Fig. 6), suggesting functional importance of these arginine residues in colorectal cancer biology. To understand whether methylation of EGFR extracellular domain arginines affect its function, we began by first interrogating which protein in PRMT family (PRMT1-8) is responsible for methylation of these arginines. Results from in vitro methylation screening assay by using D2 as substrate revealed that only PRMT1 generated strong methylation signal when co-incubated with D2 (Fig. 7). Notably, among various functional domains of EGFR (Fig. 6), PRMT1 methylated only extracellular domain 2 (D2) (Fig. 7). Next, we individually mutated all three arginines, which were positive for methylation as determined by mass spectrum analysis, on D2 to lysine to determine which of these are methylated by PRMT1. Mutation of both R198 and R200 but not R285 to lysine reduced methylation signals compared with wild type EGFR (WT) as demonstrated by an in vitro methylation assay (Fig. 8). Methylation signal was completely abolished for the R198/200K double mutant, suggesting that these two arginines in D2 are the major targets of PRMT1-mediated EGFR methylation in vitro (Fig. 9). PRMT1 frequently methylates arginine residues found within glycine-arginine rich (GAR) domains (83, 84) and generates asymmetric dimethylated arginine. Sequence
alignment between the GAR domains of known PRMT1 substrates and D2 of EGFR revealed a putative GAR domain between amino acids 196 and 204 with two arginine residues, R198 and R200 (Fig. 10), supporting our observation that R198/200 are the target sites of PRMT1 in vitro.
Figure 1. Methylation on EGFR extracellular domain Arg53.

Mass spectrum analysis showing R53 methylation on immunopurified endogenous EGFR.
Figure 2. Methylation on EGFR extracellular domain Arg98.

Mass spectrum analysis showing R98 methylation on immunopurified endogenous EGFR.
Figure 3. Methylation on EGFR extracellular domain Arg198 and Arg200.

Mass spectrum analysis showing R198 and R200 methylation on immunopurified endogenous EGFR.
Figure 4. Methylation on EGFR extracellular domain Arg285.

Mass spectrum analysis showing R285 methylation on immunopurified endogenous EGFR.
Figure 5. Methylation on EGFR extracellular domain Arg497.

Mass spectrum analysis showing R497 methylation on immunopurified endogenous EGFR.
Figure 6. Illustration of EGFR extracellular and intracellular domains.

Illustration of EGFR extracellular and intracellular domains that were individually subcloned with GST tag and were purified for \textit{in vitro} methylation assay.
Figure 7. *In vitro* methylation assay of EGFR.

*In vitro* methylation assay showing methylation signal from each GST-tagged EGFR domain after incubation with purified GST-tagged PRMT1. Methylation signal were examined by fluorography.

<table>
<thead>
<tr>
<th>GST-PRMT1</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-EGFR</td>
<td>D1</td>
<td>D1</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>D2</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>D3</td>
</tr>
<tr>
<td></td>
<td>D4</td>
<td>D4</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>D5</td>
</tr>
</tbody>
</table>

* : D1-associated bacterial protein
** : Methylated D2
Figure 8. *In vitro* methylation assay of WT EGFR and methylation-site single mutants.

<table>
<thead>
<tr>
<th>GST-PRMT1:</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-EGFR D2:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Coomassie blue**

**Fluorography**
Figure 9. *In vitro* methylation assay of WT EGFR and R198/200K double mutant.

**Table 1:**

<table>
<thead>
<tr>
<th>GST-PRMT1:</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>WT</td>
<td>R198/200K</td>
</tr>
<tr>
<td>R198/200K</td>
<td>WT</td>
<td>R198/200K</td>
</tr>
</tbody>
</table>

*Coomassie Blue*

*Fluorography*
Figure 10. Sequence alignment of PRMT1 substrates along with EGFR showing a potential GAR motif on EGFR extracellular domain 2.

<table>
<thead>
<tr>
<th>PRMT substrate</th>
<th>GAR motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>196-SCRCRGKSP-204</td>
</tr>
<tr>
<td>p53</td>
<td>331-QIRGRERFE-339</td>
</tr>
<tr>
<td>p80 coilin</td>
<td>411-GMRGERGER-419</td>
</tr>
<tr>
<td>Sm D1</td>
<td>98-RGGERGERGER-106</td>
</tr>
<tr>
<td>Sm D3</td>
<td>110-RGGERGERGER-118</td>
</tr>
<tr>
<td>Histone H4</td>
<td>2-SCRGKGGKGG-10</td>
</tr>
<tr>
<td>Histone H3</td>
<td>-TARKSTGGK-15</td>
</tr>
</tbody>
</table>
3.2 PRMT1 interacts with and methylates EGFR before its translocation to the cell membrane

To analyze the methylation status of endogenous EGFR, we generated an anti-me-R198/200 methylation-specific antibody by using a synthesized asymmetric dimethylated EGFR peptide. Dot blot assay showed that this me-R198/200 antibody recognized asymmetric dimethylated R198/200 EGFR peptide but not unmodified R198/200 EGFR peptide, other methylation forms of R198/200 EGFR peptide, or asymmetric dimethylated histone H4 peptide (Fig. 11), validating the specificity of the antibody. The me-R198/200 antibody was later used to detect the methylation level of endogenous EGFR. We found that the methylation status of endogenous EGFR was proportional to PRMT1 expression, and the methylation signal detected by the me-R198/200 antibody was specifically depleted by methylated but not by unmodified peptides (Fig. 12), supporting that EGFR R198/200 methylation is regulated by PRMT1 in cells.

PRMT1 is an intracellular protein and less likely to contact with R198 and R200, which are located on the extracellular domain of EGFR, when EGFR is transported to the cell membrane. Protein modifications on the extracellular domain of transmembrane proteins, such as phosphorylation and glycosylation can occur during biosynthesis before the transmembrane proteins are transported to cell membrane (56, 57, 59). Therefore, we speculated that PRMT1 methylates R198/200 through a similar mechanism, before EGFR is transported to the cell membrane. To investigate this possibility, we examined methylation status by treating cells with or without tunicamycin, an N-linked glycosylation inhibitor. During EGFR protein translation and maturation, glycans are added onto its extracellular domain before it is transported to the cell membrane.
Addition of tunicamycin disrupted glycosylation, resulting in the accumulation and retention of immature, unglycosylated EGFR (130 kDa) in the endoplasmic reticulum (ER)-Golgi compartments (85). In the presence of tunicamycin, we detected EGFR methylation using the me-R198/200 antibody in both newly synthesized (130 kDa) and old (170 kDa) EGFR. EGFR methylation signals were reduced when we knocked down PRMT1 (Fig. 13, top). To rule out the possibility that PRMT1 methylates EGFR during cell lysis, PRMT inhibitor, AMI1, was added into the cell lysis buffer (86). Detection of methylation signals both in the presence or absence of AMI1 indicated that the methylation event occurred in cells before cell lysis. The efficacy of AMI1 was validated by in vitro methylation assay in which the addition of AMI1 blocked methylation reaction of PRMT1 toward GST-GAR, a methyl-accepting substrate (Fig. 13, bottom). To further validate that newly synthesized EGFR is methylated, we isolated ER organelle and showed that the newly synthesized EGFR in the isolated ER was indeed methylated as detected by the me-R198/200 antibody (Fig. 14). In contrast, knocking down of PRMT1 abolished methylated EGFR signals. Next, we validated the interaction between PRMT1 and EGFR by reciprocal co-immunoprecipitation (Fig. 15). Additionally, in vivo Duolink assay showed that the PRMT1-EGFR interaction occurred mainly in the intracellular space rather than on the cell membrane (Fig. 16). Taken together, the results suggest that PRMT1 interacts with and methylates EGFR before its translocation to the cell membrane.
Figure 11. Dot blot showing specificity of EGFR me-R198/200 Ab.

H3R4: Histone H4 arginine 3 asymmetric dimethylated peptide. Asym di-me: EGFR peptides asymmetric dimethylation on indicated sites. Sym di-me: EGFR symmetric dimethylated R198/200 peptide. Mono-me: EGFR mono methylated R198/200 peptide. Scrambled: peptide with the same amino acid composition as the EGFR R198/200 peptide with the amino acids scrambled while maintaining the position of the two methyl-arginines.
Figure 12. Immunoblots comparing EGFR methylation level in SKCO1 cells.

Immunoblots comparing EGFR methylation level in SKCO1 cells exogenously expressing PRMT1 (left), PRMT1 shRNA (right), or control vector with EGFR methylation-specific antibody, me-R198/200 Ab.
Figure 13. EGFR is methylated before membrane translocation.

Top: Immunoblots of indicated proteins of SKCO1 cells expressing control vector or PRMT1 shRNA in the absence or presence of tunicamycin (2 µM, 24 hr) or AMI1 (100 µM). Bottom: *In vitro* methylation assay showing methylation signal of GST-GAR after incubation with purified GST-tagged PRMT1 in the absence or presence of AMI1 (100 µM). Methylation signal were examined by fluorography.
Figure 14. Isolation of ER organelle.

Immunoblots of indicated proteins after ER isolation of SKCO1 cells expressing control vector or PRMT1 shRNA. Calnexin: ER marker; Lamin b1: nuclear marker; HSP60: mitochondrial marker.

<table>
<thead>
<tr>
<th>Fraction:</th>
<th>Control</th>
<th>shPRMT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td>ER</td>
<td>ER</td>
</tr>
<tr>
<td>EGFR</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>me-R198/200</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>PRMT1</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Calnexin</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>Lamin b1</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
<tr>
<td>HSP60</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 15. Reciprocal co-immunoprecipitation of EGFR and PRMT1.

Reciprocal co-immunoprecipitation of SKCO1 cells with the indicated antibodies.
Figure 16. Duolink assay of SKCO1 cells.

Red spots represent the interaction between PRMT1 and EGFR. Phase contrast image shows cell boundary.
3.3 PRMT1 upregulates EGFR signaling and cell proliferation in colorectal cancer cell lines

Next, we asked how PRMT1 affects EGFR signaling. EGFR activation status was evaluated upon EGF stimulation by measuring specific tyrosine phosphorylation and two main downstream signaling, ERK and AKT, in both SKCO1 and GEO colorectal cancer cells expressing exogenous PRMT1 or vector control. Interestingly, EGFR activation was stronger in PRMT1-expressing than in vector control cells upon stimulation by EGF (Fig. 17 and Fig. 18) and another EGFR ligand, transforming growth factor alpha (TGFα), which is also highly expressed in colorectal cancer (87) (Fig. 19). Notably, exogenous expression of catalytically inactive mutant PRMT1 did not enhance EGFR activation or its downstream signaling, which indicates that upregulation of EGFR signaling by PRMT1 requires its enzymatic activity (Fig. 20). In contrast, knockdown of PRMT1 by two different shRNAs severely blocked EGF-induced EGFR, ERK, and AKT activation (Fig. 21 and Fig. 22). Although high PRMT1 expression has been shown to correlate with colorectal cancer progression (82), its role in colorectal cancer pathophysiology remains obscure. We therefore measured cell proliferation and anchorage-independent cell growth of stable transfectants that ectopically expressed PRMT1 with or without treatment of EGFR tyrosine kinase domain inhibitor, gefitinib. In line with upregulated EGFR signaling by PRMT1, gefitinib significantly inhibited cell proliferation (Fig. 23 and Fig. 24) and anchorage-independent growth (Fig. 25) in both PRMT1-overexpressing and vector control (expressing endogenous PRMT1) cells, supporting the concept that PRMT1 upregulates EGFR signaling in response to ligand stimulation and increases cellular transformation.
Figure 17. PRMT1 upregulates EGFR signaling in SKCO1 cell.

Immunoblots comparing pEGFR, pERK, and pAKT level upon EGF stimulation for indicated time in SKCO1.
Figure 18. PRMT1 upregulates EGFR signaling in GEO cell.

Immunoblots comparing pEGFR, pERK, and pAKT level upon EGF stimulation for indicated time in GEO cells expressing PRMT1 or control vector.
Figure 19. PRMT1-mediated upregulation of EGFR signaling can be stimulated by TGFα.

Immunoblots comparing pEGFR and pERK levels upon TGFα stimulation for 20 min in GEO cells expressing PRMT1 or control vector.
Figure 20. PRMT1-mediated upregulation of EGFR signaling is enzymatic activity dependent.

Immunoblots comparing pEGFR and pERK levels upon EGF stimulation for 20 min in SKCO1 cells expressing control vector, wild type or catalytically inactive mutant PRMT1.
Figure 21. Immunoblots of SKCO1 cells expressing first PRMT1 shRNA.

Immunoblots evaluating pEGFR, pERK and pAKT level upon EGF stimulation for indicated time in SKCO1 cells expressing first PRMT1 shRNAs or control vector.
Figure 22. Immunoblots of SKCO1 cells expressing second PRMT1 shRNA.

Immunoblots evaluating pEGFR, pERK and pAKT level upon EGF stimulation for indicated time in SKCO1 cells expressing second PRMT1 shRNAs or control vector.
Figure 23. Cell proliferation assay of SKCO1 cells.

Cell proliferation assay of SKCO1 cells expressing PRMT1 or vector control with or without gefitinib treatment. $P < 0.05$, t-test.
Figure 24. Cell proliferation assay of GEO cells.

Cell proliferation assay of GEO cells expressing PRMT1 or vector control with or without gefitinib treatment. \( P < 0.05 \), t-test.
Figure 25. Anchorage-independent growth of SKCO1 cells.

Anchorage-independent growth of 1,000 SKCO1 exogenously expressing PRMT1 and control vector with or without gefitinib treatment. **$P < 0.005$, t-test. All quantitative data were generated from a minimum of three replicates. Data are expressed as mean ±SD.
3.4 PRMT1 upregulates EGFR dimerization, activation, and EGFR-dependent cell proliferation through R198/200 methylation

To understand whether PRMT1 upregulates EGFR signaling through R198/200 methylation, we constructed a full-length EGFR R198/200K mutant that cannot be methylated by PRMT1 for comparison with WT EGFR. Since both pEGFR and pERK were significantly changed by ectopic expression or knockdown of PRMT1 (Fig. 17, 18, 21 and 22), we used them as markers to monitor EGFR signaling. Notably, EGFR and ERK phosphorylation were significantly reduced in GEO cells expressing the R198/200K mutant compared with those expressing the WT EGFR, suggesting the importance of EGFR R198 and R200 methylation for PRMT1-upregulated EGFR signaling (Fig. 26).

To further confirm that stronger signaling activation of WT EGFR was a result of R198/200 methylation by PRMT1, we knocked down PRMT1 in both WT EGFR and EGFR R198/200K expressing HT29 colorectal cancer cells. Consistently, HT29 cells expressing exogenous WT EGFR had stronger EGFR and downstream ERK activation than those expressing exogenous EGFR R198/200K mutant (Fig. 27, left). Notably, knocking down of PRMT1 reduced signaling activation of exogenous WT EGFR but not exogenous EGFR R198/200K mutant (Fig. 27, middle and right). In line with EGFR activation, HT29 cells expressing exogenous WT EGFR also had higher cell proliferation rate (Fig. 28) compared with EGFR R198/200K-expressing cells. Knocking down of PRMT1 in WT EGFR-expressing cells significantly reduced the EGFR-dependent cell growth rate to a similar level observed in EGFR R198/200K-expressing cells, indicating that PRMT1 upregulates EGFR-dependent cell growth via R198/200 methylation.
R198 and R200 are situated in the hinge region between D1 and D2. Available crystallographic structures show that EGFR dynamically transitions between an inactive monomeric ‘tethered’ conformation and an active dimeric ‘extended’ conformation (Fig. 29) (88, 89). In the inactive form, the R198 side chain inserts into a narrow pocket provided by D1. Asymmetric dimethylation would increase the volume of R198 sufficiently to cause unfavorable steric clashes with D1, and hence disfavor the inactive conformation (Fig. 30A, left). In the active form, the same binding pocket is substantially enlarged (Fig. 30A, right) and provides sufficient space and hydrophobic surface patches (green) to accommodate asymmetric dimethylation. The relative orientation of domains D1 and D2 is conserved in all dimeric EGFR forms, including ligand-free forms from the drosophila EGFR homolog (90, 91), suggesting that our analysis holds for all dimeric conformations. Meanwhile, in the inactive form, R200 compensates for the charge of D206 (Fig. 30B, left), and the backbone carbonyl of D206 binds to the backbone nitrogen of R200. In the active form, the side chain of D206 is rotated away and exposed to the solvent. The D206 carbonyl forms a hydrogen bond with R200, bringing the R200 guanidinium moiety close to the hydrophobic surface of P219 (Fig. 30B, right). Consequently, methylation of R200 appears to favor the active conformation, because it provides a less charged and more hydrophobic environment for R200. Our structural analysis therefore suggests that R198/200 methylation predisposes EGFR to assume an active conformation, and hence increases ligand-stimulated downstream signaling, supporting the observation of upregulated EGFR signaling and EGFR-dependent cell growth by R198/200 methylation.
The effect of EGFR R198/200 methylation by PRMT1 on EGFR signaling and subsequent cell growth prompted us to investigate how this extracellular modification affects intracellular downstream signaling of the receptor. Given that D2 of EGFR is critical for the receptor dimerization and subsequent downstream signaling activation (92), we further examined the effect of R198/R200 methylation on EGFR dimerization. Interestingly, upon EGF stimulation, endogenous EGFR of PRMT1-expressing SKCO1 cells showed higher receptor dimerization ability than cells expressing control vector (Fig. 31). In contrast, EGFR in PRMT1 shRNA-expressing cells had lower EGF-stimulated dimer formation than cells expressing control vector (Fig. 32). Consistently, loss of methylation of EGFR R198/200K mutant significantly reduced its dimerization ability compared with WT EGFR in GEO cells (Fig. 33). Knocking down of PRMT1 in WT EGFR-expressing cells significantly reduced the dimer formation of WT EGFR to a similar level observed in EGFR R198/200K-expressing cells, indicating that PRMT1 upregulates EGFR dimerization via R198/200 methylation (Fig. 33). Notably, while glycosylation was reported to affect the expression level of cell surface EGFR (93), our data indicated that methylation did not (Fig. 34). These results support a model that methylation at R198/200 of EGFR by PRMT1 enhances its EGF-induced dimerization ability and provide an explanation for PRMT1-upregulated EGFR signaling and cell proliferation.
Figure 26. PRMT1 upregulates EGFR activation through R198/200 methylation. Immunoblot comparing EGFR and downstream ERK activation level of GEO cells expressing control vector, WT and methylation-site mutant EGFR upon EGF stimulation.
Figure 27. PRMT1 upregulates EGFR activation through R198/200 methylation in HT29 cells.

Immunoblot comparing EGFR and downstream ERK activation level of HT29 cells expressing control vector, WT and R198/200K mutant EGFR with or without PRMT1 knocking down upon EGF stimulation.
Figure 28. PRMT1 EGFR-dependent cell proliferation through R198/200 methylation.

Cell proliferation assay of HT29 cells expressing control vector, WT and R198/200K mutant EGFR with or without PRMT1 knocking down.
**Figure 29. Structural analysis of EGFR.**

Left: Inactive ‘tethered’ conformation of EGFR. The structure was prepared based on the crystal structure of the inactive human EGFR (PDB accession 1IVO). Domains 1-4 (D1-4) are color-coded; the weakly bound EGF is in cyan. R198 and R200 are highlighted. Right: Active dimerized form of EGFR (based on the crystal structure of human EGFR, PDB accession 1NQL). The second EGFR and EGF molecules are shown in gray and black, respectively.
**Figure 30. Structural analysis of EGFR Arg 198 and Arg 200.**

Zoom onto R198 (A) and R200 (B) shown as stick figures. The molecular surface of EGFR (with the exception of the region 198-200) is colored in blue, positively charged atoms; red, negatively charged atoms; green, hydrophobic atoms; salmon, polar oxygens; marine, polar nitrogens; yellow, sulfur). EGFR domains are indicated on the surfaces, as is the location of D206.
Figure 31. PRMT1 upregulates EGFR dimerization.

Top: Dimerization assay of SKCO1 cells exogenously expressing vector control and PRMT1. Anti-EGFR antibody was used to detect EGFR monomer and dimer. Bottom: Quantification of EGFR dimer form.
Figure 32. Knocking down of PRMT1 attenuates EGFR dimerization ability.

Top: Dimerization assay of SKCO1 cells expressing vector control and PRMT1 shRNA.

Bottom: Quantification of EGFR dimer form.
Figure 33. PRMT1 upregulates EGFR dimerization through R198/200 methylation.

Dimerization assay of GEO cells expressing WT and R198/200K mutant EGFR with or without knocking down of PRMT1.
Figure 34. EGFR methylation does not affect its cell surface expression level.

A and B, Biotinylated cell surface EGFR from A, SKCO1 and B, GEO cells were captured on streptavidin-agarose beads and detected by immunoblot.
3.5 Methylation of EGFR increases tumorigenesis in orthotopic colorectal cancer mouse model and correlates with poorer clinical outcomes of colorectal cancer patients

Our current data support a model in which PRMT1 enhances EGFR dimerization and activation through methylation of EGFR at R198/200. Subsequently, upregulated EGFR signaling promotes cell proliferation and anchorage-independent growth of colorectal cancer cells. To further address the pathological relevance of this model, we examined whether PRMT1-mediated EGFR methylation contributes to tumorigenesis in an orthotopic colon cancer mouse model. One month after injection, HT29 cells expressing WT EGFR generated significantly larger tumors than those expressing R198/200K EGFR or vector control (Fig. 35). Knocking down PRMT1 substantially reduced tumor growth, supporting the positive role of EGFR R198/200 methylation in colorectal cancer tumorigenesis. To further address the clinical relevance of EGFR R198/200 methylation, we characterized me-R198/200 antibody for its ability to detect EGFR R198/200 methylation in immunohistochemical staining (IHC). GEO cells expressing WT EGFR, R198/200K EGFR or vector control were fixed on slides by cytopsin and stained by me-R198/200 antibody. As shown in Fig. 36A, IHC staining signals were significantly stronger in WT EGFR expressing cells than R198/200K EGFR or vector expressing cells. The staining signals in WT EGFR expressing cells were blocked by synthesized asymmetric dimethylated R198/200 EGFR peptide, but not by unmodified R198/200 EGFR peptides or asymmetric dimethylated histone H4 peptide, validating the specificity of the me-R198/200 antibody. Similarly, me-R198/200 antibody was able to detect EGFR methylation signals in patient tissue samples and these
methylation signals were blocked specifically by asymmetric dimethylated R198/200 EGFR peptide, but not by other forms of peptides (Fig. 36B). A retrospective study of clinical colorectal cancer specimens further indicated that EGFR R198/200 methylation level as detected by me-R198/200 antibody was elevated in tumor tissue compared with paired adjacent normal tissue. Also, higher EGFR R198/200 methylation level coincided significantly with poorer overall patient survival (Fig. 37) and higher recurrence rate (Fig. 38). Collectively, these results suggest that PRMT1-mediated EGFR R198/200 methylation contributes to tumorigenesis in vivo and the methylation status of EGFR has the potential to serve as a predictive marker for patient prognosis.
Figure 35. Methylation of EGFR increases tumorigenesis in orthotopic colorectal cancer mouse model

*In vivo* orthotopic colon tumor growth of HT29 cells expressing vector control, wild type (WT), or R198/200K mutant (Mut) EGFR with or without knockdown of PRMT1 (N = 5 per group). Top: Representative tumors from each group in the fourth week after inoculation.
Figure 36. Characterization of me-R198/200 antibody.

A, Immunochemistry staining of GEO cells expressing WT, R198/200K EGFR or vector control by me-R198/200 Ab competed without or with synthesized unmodified R198/200 EGFR peptide, asymmetric dimethylated histone H4R3 peptide or asymmetric dimethylated R198/200 EGFR peptide. B, Immunochemistry staining of colon cancer tissue by EGFR methylation antibody competed without or with indicated peptide.
Figure 37. Methylation of EGFR correlates with poorer overall survival rate of colorectal cancer patients.

Kaplan-Meier plot of overall survival of 215 colorectal cancer cases with low or high methyl-EGFR level detected by me-R198/200 Ab. $P < 0.05$. 

![Kaplan-Meier plot of overall survival of 215 colorectal cancer cases with low or high methyl-EGFR level detected by me-R198/200 Ab. P < 0.05.](image)
Figure 38. Methylation of EGFR correlates with higher recurrence rate of colorectal cancer patients.

Kaplan-Meier plot of recurrence rate of 120 colorectal cancer cases with low or high methyl-EGFR level detected by me-R198/200 Ab. $P < 0.05$.  

![Graph showing Kaplan-Meier plot of recurrence rate](image)
3.6 EGFR methylation enhances EGF binding to EGFR and correlates with higher recurrence rate of colorectal cancer patients after cetuximab treatment

Clinically, cetuximab is used to treat metastatic colorectal cancer by disabling the interaction between EGF ligand and EGFR and attenuating EGFR signaling (94-96). Cetuximab binds exclusively to D3 of EGFR in its inactive conformation (Fig. 29). Upon binding, cetuximab occludes the EGF-binding site and prevents EGFR from adopting the active conformation required for ligand binding and dimerization (97). By predisposing EGFR to assume an active EGF-bound conformation, R198/200 methylation is expected to enhance the interaction between EGFR and EGF. Therefore, we asked whether R198/200 methylation of EGFR affects its affinity for EGF and the efficacy of cetuximab. Dissociation constant ($K_d$) between EGFR and EGF in the absence or presence of cetuximab was measured in the EGFR stable transfectants in HT29 and SW48 colorectal cancer cells by saturation binding assay. In the absence of cetuximab, WT EGFR in HT29 (Fig. 39) and SW48 (Fig. 40) cells showed higher binding affinity (lower $K_d$) for EGF ($K_d = 16.03$ nM and $K_d = 16.21$ nM in HT29 and SW48, respectively) compared with the R198/200K mutant EGFR ($K_d = 52.16$ nM and $K_d = 51.79$ nM in HT29 and SW48, respectively). Knocking down PRMT1 in WT EGFR-expressing HT29 or SW8 cells significantly reduced the affinity of EGFR for EGF ($K_d = 44.32$ nM; $K_d = 46.65$ nM in HT29 and SW48, respectively) to a level comparable to that of the R198/200K mutant. The binding affinity between the R198/200K mutant EGFR and EGF was not affected by PRMT1 knockdown ($K_d = 55.09$ nM and $K_d = 54.54$ nM in HT29 and SW48, respectively). These results suggest that methylation of EGFR R198/200 by PRMT1 enhances its binding to EGF. In addition, in the presence of a relatively low concentration
of cetuximab (1 µg/ml), at which the binding affinity between WT EGFR and EGF did not change significantly compared to the absence of cetuximab (Fig. 41; Kd from 17.86 to 17.25 nM, without and with cetuximab, respectively), the affinity of R198/200K mutant EGFR for EGF was significantly reduced (Kd from 47.28 to 94.33 nM, without and with cetuximab, respectively). Together, these results suggest that methylated EGFR at R198/200 responds better to EGF binding and is more resistant to cetuximab treatment.

In line with the higher EGF binding affinity and higher EGFR methylation level, PRMT1-overexpressing HT29 cells demonstrated higher EGFR and ERK activation after EGF stimulation, and the upregulated pEGFR and pERK remained relatively strong even in the presence of cetuximab in comparison to vector control cells (Fig. 42). To validate role of EGFR methylation in cetuximab response, cells expressing exogenous PRMT1 shRNA or PRMT1 were treated with cetuximab, and their clonogenic ability was evaluated (Fig. 43-47). The colony number was relatively lower in PRMT1 knocking down cells than control cells in the presence of cetuximab (Fig. 43-45). In contrast, cells expressing exogenous PRMT1 showed more resistance to cetuximab treatment (Fig. 46 and Fig. 47). To further investigate whether methyl-EGFR level is related to patient response to cetuximab, tumor tissues from cetuximab-treated metastatic colorectal cancer patients were collected and stained by me-R198/200 antibody. Consistent with previous clinical analyses (15, 17, 98), no association was found between total EGFR expression level and cetuximab response (Fig. 48); however, higher levels of methyl-EGFR in tumors from colorectal cancer patients correlated with higher recurrence rate after cetuximab treatment (Fig. 49). In addition, the expression level of methyl-EGFR correlated positively with PRMT1 expression (Fig. 50). Similarly, the correlation
between the expression level of methyl-EGFR and PRMT1 was also observed in patients with head and neck cancer (Fig. 51), another FDA-approved cancer type for cetuximab treatment. Importantly, higher methyl-EGFR level also correlated with poorer overall survival after cetuximab treatment in head and neck cancer patients (Fig. 52). Together, the results support the notion that PRMT1-mediated EGFR R198/200 methylation contributes to cetuximab resistance in colorectal and head and neck cancer patients.

Clinically, the status of *KRAS* mutation has been used as a biomarker to predict patient response to cetuximab (29), we therefore asked whether methylation-mediated cetuximab resistance is affected by *KRAS* mutation. Interestingly, independently of *KRAS* status, knockdown of PRMT1 in all WT *KRAS* (HT29 and SW48) (Fig. 43 and Fig. 45) and mutant *KRAS* (SKCO1: *KRAS* G12V and GEO: *KRAS* G12A) (Fig. 53 and Fig. 44) cell lines sensitized cells to cetuximab treatment. Notably, knockdown of PRMT1 in *KRAS* G12V mutant and cetuximab-resistant SKCO1 cells rendered cells more sensitive to cetuximab than vector control cells; In contrast, re-expression of shRNA-resistant PRMT1 (rPRMT1) in PRMT1-knockdown cells restored the observed cetuximab resistance (Fig. 53), suggesting that *KRAS* mutation does not play a role in PRMT1-mediated cetuximab resistance.
Figure 39. EGFR methylation regulates EGF binding to EGFR.

Top: Scatchard plot and binding curves (inset) which measured EGFR-EGF binding affinity of HT29 cells expressing wild type (WT) or R198/200K mutant (Mut) EGFR with or without knocking down of PRMT1. Bottom: Bar graph of Kd values, Red: WT EGFR, Blue: R198/200K mutant EGFR.
Figure 40. **EGFR R198/200 methylation regulates EGF binding to EGFR.**

Top: Scatchard plot and binding curves (inset) which measured EGFR-EGF binding affinity of SW48 cells expressing WT or R198/200K mutant EGFR with or without knocking down of PRMT1. Bottom: Bar graph of Kd values, Red: WT EGFR, Blue: R198/200K mutant EGFR.
Figure 41. EGFR methylation regulates EGF binding to EGFR after cetuximab treatment.

Top: Scatchard plot and binding curves (inset) which measured EGFR-EGF binding affinity of HT29 cells expressing wild type (WT) or R198/200K mutant (Mut) EGFR with or without cetuximab treatment. Bottom: Bar graph of $K_d$ values, Red: WT EGFR, Blue: R198/200K mutant EGFR.
Figure 42. EGFR methylation regulates EGF binding to EGFR and correlates with higher recurrence rate of colorectal cancer patients after cetuximab treatment.

Immunoblot assessing EGFR, ERK, and AKT activation levels of HT29 cells expressing control vector or PRMT1 upon EGF stimulation in the presence or absence of cetuximab.
Figure 43. EGFR methylation regulates colony formation after cetuximab treatment in HT29 cells.

Clonogenic assay of HT29 cells expressing control vector or PRMT1 shRNA under cetuximab treatment (N = 3). Data are expressed as mean ±SD. Expression levels of PRMT1 shown by immunoblot. Data shown are representative of at least 3 independent experiments.
Figure 44. EGFR methylation regulates colony formation after cetuximab treatment in GEO cells.

Clonogenic assay of GEO cells expressing control vector or PRMT1 shRNA.
Figure 45. EGFR methylation regulates colony formation after cetuximab treatment in SW48 cells.

Clonogenic assay of SW48 cells expressing control vector or PRMT1 shRNA.
Figure 46. EGFR R198/200 methylation increases cetuximab resistance in GEO cells.

Clonogenic assay of GEO cells expressing control vector or exogenous PRMT1.
Figure 47. EGFR R198/200 methylation increases cetuximab resistance in SW48 cells.

Clonogenic assay of SW48 cells expressing control vector or exogenous PRMT1.
Figure 48. Correlation between total EGFR expression with cetuximab response.

Kaplan-Meier plot of recurrence rate of 41 colorectal cancer cases with wild-type KRAS treated with cetuximab with low or high total EGFR level.
Figure 49. Correlation between EGFR methylation level and cetuximab response in colorectal cancer patients.

Kaplan-Meier plot of recurrence rate of 41 colorectal cancer cases with wild-type KRAS treated with cetuximab with low or high methyl-EGFR level detected by me-R198/200 Ab. $P < 0.05$, t-test.
Figure 50. Correlation between PRMT1 expression and EGFR methylation level in colorectal cancer patients.

Spearman’s rank correlation test for correlation between methyl-EGFR and PRMT1 in cetuximab-treated wild-type KRAS patient samples. (N = 41)

<table>
<thead>
<tr>
<th></th>
<th>Methyl EGFR low</th>
<th>Methyl EGFR high</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT1 low</td>
<td>11</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>PRMT1 high</td>
<td>10</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>total</td>
<td>21</td>
<td>26</td>
<td>41</td>
</tr>
</tbody>
</table>

$\chi^2 = 0.004$
Figure 51. Correlation between PRMT1 expression and EGFR methylation level in head and neck cancer patients.

Statistic analysis for expression correlation between methyl-EGFR and PRMT1 in 59 cetuximab-treated head and neck cancer patients.

<table>
<thead>
<tr>
<th></th>
<th>Methyl-EGFR low</th>
<th>Methyl-EGFR high</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT1 low</td>
<td>19</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>PRMT1 high</td>
<td>8</td>
<td>29</td>
<td>37</td>
</tr>
<tr>
<td>total</td>
<td>27</td>
<td>37</td>
<td>59</td>
</tr>
</tbody>
</table>

P = 0.0001
Figure 52. Correlation between EGFR methylation level and cetuximab response in head and neck cancer patients.

Kaplan-Meier plots of overall survival of 59 head and neck cancer cases after cetuximab treatment with low or high methyl-EGFR level detected by me-R198/200 Ab.
Figure 53. Clonogenic assay of SKCO1 cells with cetuximab treatment.

Clonogenic assay of SKCO1 cells expressing control vector, PRMT1 shRNA or re-expressing shRNA resistant PRMT1 under cetuximab treatment (N = 5). Data are expressed as mean ±SD.
CHAPTER 4. DISCUSSION

4.1 Conclusion and significance

Mutant KRAS is a widely recognized predictive marker for poor cetuximab response in colorectal cancer due to its association with poor patient survival. However, increasing evidence indicates that wild-type KRAS is insufficient to confer sensitivity to cetuximab and that some patients with mutant KRAS are still sensitive to cetuximab. Therefore, further investigation is warranted to elucidate the underlying mechanism of cetuximab resistance and identify other predictors of cetuximab response. Our data demonstrate that methylation of EGFR upregulates EGFR signaling, enhances tumorigenesis, and reduces cellular response to cetuximab. Patients with higher EGFR methylation level have higher recurrence rate after cetuximab treatment. Thus, methylated EGFR may serve as a biomarker to stratify colorectal cancer patients with the maximum benefit of cetuximab therapy.

4.2 EGFR target therapy and predictive markers for drug resistance

EGFR-targeted monoclonal antibodies have expanded the treatment options for colorectal cancer patients. Although these agents have great potential for individualized therapy, the reasons why some patients respond to treatment while others do not remain unclear. In this study, we demonstrate that PRMT1-mediated methylation of R198/200 on the extracellular domain of EGFR enhances receptor dimerization, EGFR signaling activation, cell proliferation, and reduces cellular response to cetuximab. Interestingly, although some studies have shown that mutant KRAS strongly represses EGF-stimulated activation of ERK phosphorylation in HCT116 and DLD1 colorectal cancer cell lines
Our data demonstrated that ERK was still activated by EGF stimulation in GEO (G12A) and SKCO1 (G12V) KRAS mutant cell lines (Fig. 17-21). The contradictory effect of EGF on ERK activation is somewhat expected as different cancer cell lines can behave differently. Moreover, knockdown of PRMT1 sensitized cells to cetuximab treatment regardless of KRAS mutation status (Fig. 43-45 and Fig. 53). High EGFR R198/200 methylation level correlated with higher recurrence rate in cetuximab treated patients, implying that EGFR R198/200 methylation has the potential to serve as a predictive marker for cetuximab resistance in clinical colorectal cancer therapy. It is worthwhile to mention that colorectal cancer patients carrying R497 polymorphism on EGFR extracellular domain 4 exhibit more unfavorable response to cetuximab than those carrying K497. Although we also observed endogenous EGFR R497 methylation from mass spectrum analysis (Fig. 5), suggesting that this arginine methylation event may be another factor that contributes to cetuximab resistance, PRMT1 is not the methyltransferase for R497 methylation (D4), at least from our in vitro methylation assay (Fig. 7). Thus, identification of the PRMT that is responsible for R497 methylation would be critical to address this issue in the future.

EGFR is a key regulator of proliferation and progression in human cancers. Five EGFR inhibitors, two monoclonal antibodies and three TKIs, have gained FDA approval on treatment of several cancer types (cetuximab for mCRC and head and neck cancer; panitumumab for mCRC; erlotinib for pancreatic cancer; gefitinib for non-small-cell lung cancer; and lapatinib for breast cancer). Interestingly, it has been shown that EGFR-negative colon tumours have the potential to respond to cetuximab therapies. EGFR target therapeutic strategies show tumor regressions in approximately 10–20% of
advanced cancer patients. However, many tumors eventually acquire resistance to therapy. A high-throughput screening study showed that cetuximab-resistant non-small-cell lung cancer cells manifested strong activation of HER2, HER3 and cMET. Additionally, HER2 signaling could mediate resistance to tyrosine kinase inhibitors in breast cancer cell lines owing to the activation of alternative EGFR family receptors (103). In accordance, EGFR promotes dimerization and elevated activation of HER2 in cells, which acquire cetuximab resistance, with consequent activation of downstream cascades and sustained proliferation (104). Based on these studies, we performed IP-western to examine the interaction between Her-2 and wild type EGFR in comparison with the binding with R198/200K EGFR. The result showed that wild type EGFR has increased interaction with Her-2 upon EGF stimulation, while abolishing R198/200 methylation by mutations of R198/200K significantly reduced its binding to Her-2 (Fig. 56). In line with other reports, the stronger interaction between methylated EGFR and Her-2 may contribute to one of the mechanisms of EGFR methylation-induced cetuximab resistance. In addition, it has been reported that acquired resistance to cetuximab is accompanied by dysregulation of EGFR-Her-2 heterodimer internalization/degradation and prolonged signal transduction (104, 105), suggesting the EGFR methylation may contribute to cetuximab resistance at least partially through enhancing EGFR-Her-2 heterodimer.
Figure 54. Co-immunoprecipitation of EGFR and Her2 in cells overexpressing wild type or R198/200K mutant EGFR.
4.3 Cross talk between PRMT family members

We previously reported methylation on EGFR intracellular domain mediated by PRMT5 inhibits downstream ERK activation in breast cancer cells (53). Here, methylations on EGFR extracellular domain regulated by PRMT1 enhance receptor function in colorectal cancer cells. Interestingly, in colorectal cancer patients, elevated level of PRMT5 was observed and coincided with poor prognosis (106). A molecular switch that governs the tumor suppressive or oncogenic activities of different PRMTs and their downstream target proteins in different cancer types remains to be further investigated.

It is known that some PRMTs may replace the role of another PRMT under some conditions, i.e. when the expression level of the major enzyme is low. We used in vitro methylation to know whether PRMT5, which also interacted with EGFR, could methylate R198/200, the screening analysis of PRMT1-8 showed only PRMT1 can methylate extracellular domain 2 R198/200 but not PRMT2-8, suggesting the particular methylation event of R198/200 may not be catalyzed by other PRMTs.

4.4 Extracellular methylation of membrane receptors and their target therapies

Several arginine methylated RTKs, such as EGFR and VEGFR-2, have been reported (53, 107). The current study demonstrates that arginine methylation on EGFR extracellular domain affects ligand-mediated signaling and may contribute to cetuximab resistance. Since many cell surface RTKs, including EGFR, HER2, and MET, are therapeutic targets and monoclonal antibodies against the extracellular domain of these RTKs are being used for therapy, this raises an interesting possibility that arginine
methylation of this region on other RTK may also play a role in the regulation of their activities and in response to their corresponding monoclonal antibody therapeutics.

### 4.5 Locations where extracellular domain modification takes place

Our results indicated that PRMT1 methylates EGFR before it reaches the cell membrane. However, the subcellular compartment in which PRMT1-mediated EGFR extracellular domain methylation occurs is interesting and remains unclear. Translation of type I transmembrane proteins, such as EGFR, begins on free ribosomes in the cytosol (108, 109). The nascent polypeptide chain, which includes the N-terminal signal sequence and extracellular domain, exposed to the cytosol during translation. Subsequent recognition of the signal sequence within the nascent polypeptide chain by the signal recognition particle (SRP) recruits the ribosome-nascent polypeptide chain complex to the Sec61 translocon complex on the ER membrane, leading to insertion of the nascent polypeptide into the ER lumen (108, 109). Thus, there are at least two possibilities regarding where methylation may occur. First, EGFR R198/200 may be methylated by cytosolic PRMT1. EGFR R198/200 may be exposed to the cytosol and methylated by PRMT1 in the cytosol before the free ribosome-nascent polypeptide chain complex is recruited to ER or before the nascent polypeptide is inserted into ER lumen. Second, EGFR may be methylated by PRMT1 in the ER since the presence of PRMT1 and SAM in the ER has been reported (110, 111), and EGFR and PRMT1 were both detected in the isolated ER (Fig. 14). A systematic approach would be required to further address this interesting mechanism in the future.
PRMT family members are constitutively active, and there is only little evidence showing that methylation event occurs specifically under specific cellular conditions such as the cell cycle or response to extracellular stimuli (112, 113). Therefore, most known PRMT substrates are methylated at any given time, and it is unlikely that methylation carries out a signal transduction like protein phosphorylation. Consistent with these reports, EGFR extracellular domain R198/200 methylation by PRMT1 cannot be regulated by EGF stimulation as shown in Fig. 42. This suggests that PRMT1 may predispose newly synthesized EGFR molecules to adopt the active EGF-binding conformation at the cell surface.

4.6 Future direction

Overall, the role of PRMT1-mediated EGFR methylation in colorectal cancer tumorigenesis and its correlation with poorer patient outcomes and cetuximab response by affecting the EGF-EGFR binding affinity and subsequent signaling activation, as demonstrated here, provide an insight into the response to EGFR-targeted therapy and also open an avenue toward the understanding of how arginine methylation regulates the function of RTKs.

The sub-cellular compartments where PRMT family members locate have not been well characterized yet. Interestingly, our study found that PRMT1 could be isolated with ER fraction. It is known that soluble endoplasmic reticulum (ER)–resident proteins usually contain a C-terminal KDEL-like motif, which prevents secretion of these ER resident proteins. Interaction between KDEL-like motifs of ER proteins with KDEL receptors, which localize in the intermediate compartment and Golgi apparatus, trigger
retrieval of ER resident proteins back to the ER via a coat protein I–dependent pathway (114). So far, three human KDEL receptors have been identified and each of them has a unique pattern of motifs with which it interacts, suggesting the specificity in the retrieval of human proteins that contain different KDEL variants. Intriguingly, a putative KDEL-like motif (KVEDL) was found in C-terminus of PRMT1. This KVEDL sequence is not conserved between PRMT family members, implying a unique function of PRMT1. Examining the localization of PRMT1 with mutations on KVEDL motif would be interesting to confirm whether PRMT1 indeed localizes and retains in ER compartment through ER retention motif. ER is a critical sub-cellular compartment where membrane receptors are synthesized and subsequently transported to the cell membrane. Identifying the ER-resident PRMT or extracellular domain modification enzymes would open an avenue regarding how intracellular proteins regulate cellular response to signals from extracellular challenges, such as tumor-targeting immune systems or tumor microenvironments.

Although PRMT family members are constitutively active (112, 113), based on our calculation of the percentage of methylated EGFR in cells, only around 10% of total EGFR are methylated (Fig. 57). In line with the report showing that ERα methylation is a dynamic process, which is methylated by PRMT1 and demethylated by arginine demethylase, JMJD6 (81, 115); global proteomic analysis also showed that arginine methylation sites in human cells are regulated dynamically by unknown arginine demethylase during transcriptional arrest (116). It is possible that arginine demethylation process is a general event regulating cellular arginine methylation level. So far, JMJD6 is the only known arginine demethylase and its demethylation activity has been debated for
a long time (81, 117-119). Since our study showed that high methylation level of EGFR R198/200 correlates with poor patient outcome, identifying the corresponding arginine demethylase and understanding the regulation between protein arginine methylation and demethylation would significantly broaden our knowledge about the field of arginine methylation and may guide the therapeutic strategy in the future.
Figure 55. Methylation percentage of endogenous EGFR from SKCO1 cell.

A, EGFR antibody was used to pull down endogenous EGFR protein from SKCO1 cells. Then, dot blot was performed by blocking peptide of total EGFR antibody (SANTA CRUZ BIOTECHNOLOGY, INC., EGFR (1005): sc-03) as standard and performed immunoblotting by total EGFR antibody to calculate the amount of EGFR protein (2 x dilution). B, R198/200 methylated peptide was used as standard and the immunoblotting was performed by me-R198/200 antibody to calculate amount of methylated EGFR protein. The result indicated there is around 10.5% (18.8/(2x 89.4)) of endogenous EGFR from SKCO1 cells were methylated.


44. P. M. Hoff, R. Ansari, G. Batist, J. Cox, W. Kocha, M. Kuperminc, J. Maroun, D. Walde, C. Weaver, E. Harrison, H. U. Burger, B. Osterwalder, A. O. Wong, R. Wong, Comparison of oral capecitabine versus intravenous fluorouracil plus


111. B. Meissner, T. Rogalski, R. Viveiros, A. Warner, L. Plastino, A. Lorch, L. Granger, L. Segalat, D. G. Moerman, Determining the sub-cellular localization of


Vita

Hsin-Wei Liao was born in Yunlin, Taiwan. She received the degree of Bachelor of Science with a major in Biology from National Chang-hua University of Education, Taiwan, in 2004 and the degree of Master of Science with a major in Microbiology from National Chiao Tung University, Taiwan, (mentor: Dr. Hui-Ling Peng) in 2006. In August 2008, she took her next step into Graduate School of Biomedical Sciences, the University of Texas Health Science Center at Houston to pursue her doctor of philosophy degree in cancer biology in Dr. Mien-Chie Hung’s lab at M. D. Anderson Cancer Center, Houston, Texas, United States.