Characterization of arginine methyltransferase PRMT8 in cells with increased plasticity

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Identification of therapeutically relevant molecules is necessary for the advancement of non-viral reprogramming of human cells for regenerative medicine. We have developed a novel non-viral model system that transforms primary human dermal fibroblasts into cells with induced regeneration competence (iRC). Low oxygen-mediated effects of fibroblast growth factor FGF2 lead to an increased cellular lifespan with a two fold increase in population doublings before senescence, remaining non-tumorigenic when injected into SCID mice while maintaining regeneration competence. This system allows us to study molecules that participate in increased cellular lifespan in a non-tumorigenic system. Analysis of chromatin modification enzymes by hybridization array, RT-PCR, and Western blots revealed upregulation of the arginine methyltransferase PRMT8 in iRC cells, challenging the paradigm that PRMT8 is solely expressed in brain tissue at the plasma membrane. Possibly leading to the erroneous conclusions that PRMT8 is brain specific at the plasma membrane is the fact that PRMT8 has several mRNA variants and protein isoforms. Here, I report expression of a novel PRMT8 variant in human dermal fibroblasts. Essential participation of PRMT8 in cellular proliferation was identified as a novel function for this enzyme through siRNA-mediated knockdown in both non-tumorigenic and tumorigenic cell lines. While other members of the PRMT family have known roles in cell cycle progression, I show for the first time that PRMT8 expression is reduced in both natural senescence and by premature induction of replicative senescence using sub-cytotoxic levels of hydrogen peroxide, implicating a correlation between PRMT8 expression and cell cycle progression. However, PRMT8 overexpression causes no significant change in the number of population doublings or the amount of time spent in culture prior to senescence, and does not alter the expression of key cell cycle regulatory genes. These results suggest that maintenance of PRMT8 expression is critical for cellular proliferation, but overexpression of PRMT8 alone is not sufficient to increase cellular lifespan. I determined that oxygen is the primary mediator of PRMT8 upregulation in the iRC system and therefore investigate histone occupancy of the PRMT8 promoter at hypoxia response elements. Through this analysis, I found bivalent occupancy regardless of culture conditions, indicating that PRMT8 maintains a state of poised readiness for transcriptional accessibility. The mechanism by which PRMT8 participates in cellular proliferation was investigated through binding partner identification. A binding partner of endogenous PRMT8 is identified here for the first time as FGF2 using co-IP and mass spectrometry. As iRC cells demonstrate a unique phenotype that uncouples the mechanisms of increased lifespan from tumorigenesis, I investigated the feasibility of PRMT8 as a cancer biomarker by mining publicly available data in light of our own. I showed that PRMT8 is not only expressed in a variety of cancers, but that its expression is amplified. Moreover, PRMT8 expression significantly correlates to patient survival in specific cancers, strengthening the feasibility of this molecule as a biomarker. Aberrant expression of most PRMT family members has been described in various cancers, and specific PRMT variants are currently being used as prognostic markers. As such, I analyzed variant-specific PRMT8 expression in primary cancer cell lines and show that tumorigenic glioblastomas express PRMT8 mRNA variant 2. These data suggest that PRMT8 is a viable candidate for further study as a prognostic cancer biomarker, specifically for brain cancer.
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Abbreviations

°: degrees
Δ: change
µL: microliter
µg/mL: microgram per milliliter

3D: three dimensional
5' RACE: five prime rapid amplification of complementary deoxyribonucleic acid ends

A: adenine
Ac: acetylation
aDMA: asymmetric dimethyl arginine
AdoHcy: S-adenosylhomocysteine
AdoMet: S-adenosylmethionine
AR: androgen receptor
ATCC: American Tissue Culture Collection

bp: base pairs
BSA: bovine serum albumin

C: cytosine
C: Celsius
Ca/Mg: Calcium and Magnesium
CAD: Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase
CARM: coactivator-associated arginine methyltransferase
CDK: cyclin-dependent kinase
cDNA: complementary deoxyribonucleic acid
ChIP: chromatin immunoprecipitation
CHO: Chinese hamster ovary
CMV: cytomegalovirus
CNA: copy number alteration
CNS: central nervous system
Ct: Cycle threshold

DMA: dimethyl arginine
DMEM: Dulbecco's Modified Eagle Medium
DNA: deoxyribonucleic acid
DNMT: deoxyribonucleic acid methyltransferase
DPBS: Dulbecco’s phosphate buffered saline

E. coli: Escherichia coli
EDTA: ethylene-diamine-tetraacetic acid
EMT: epithelial to mesenchymal transition
ER: estrogen receptor
ESC: embryonic stem cells
EWS: Ewing sarcoma

F12: Ham’s F12
FBXO: F-boxes other
FCIII: FetalClone III serum
FDA: Food and Drug Administration
FGF: fibroblast growth factor
FOXO: forkhead box O

G: glycine
G: guanine
GAR: glycine arginine rich motif
GFP: green fluorescent protein

H1: histone 1
H2A: histone 2A
H2B: histone 2B
H2O2: hydrogen peroxide
H3: histone 3
H3.3: histone 3 variant 3
H4: histone 4
HCl: Hydrochloric acid
HDAC: histone deacetylase
hDF: human dermal fibroblast
hESC: human embryonic stem cell
HIF: hypoxia inducible factor
HMW: high molecular weight
HRE: hypoxia response element

IP: immunoprecipitation
iPSC: induced pluripotent stem cell
iRC: induced regeneration competent cell

JMJD: Jumonji domain-containing protein

K: lysine
kD: kilodalton
KD: knock down
KM plot: Kaplan-Meier plot

L-Gln: L-Glutamine
LB: Lysogeny broth
LD: long distance
LiCl: lithium chloride
LMW: low molecular weight

M: molar
mm: millimeter
Me: methylation
MEF: mouse embryonic fibroblast
mL: milliliter
mM: milimolar
MMA: monomethyl arginine
mRNA: messenger ribonucleic acid
NaCl: sodium chloride
NaHCO₃: sodium bicarbonate
NCBI: National Center for Biotechnology Information
ng: nanogram

ORF: open reading frame

P: phosphorylation
PADI: peptidylarginine deiminases
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD: population doubling
PGM: proline, glycine, methionine motif
PIC: protease inhibitor cocktail
PR: progesterone receptor
PRMT: protein arginine methyltransferase
PTM: post-translational modification
PVDF: polyvinylidene fluoride

qRT-PCR: quantitative reverse transcription polymerase chain reaction

R: arginine
RB: retinoblastoma
RFP: red fluorescent protein
RNA: ribonucleic acid
RTK: receptor tyrosine kinase
RT: reverse transcriptase
RT-PCR: reverse transcription polymerase chain reaction

S: serine
SA-β-gal: senescence associated β galactosidase
SCID: severe combined immunodeficiency disease
SCNT: somatic cell nuclear transfer
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
sDMA: symmetric dimethyl arginine
shRNA: short hairpin ribonucleic acid
S.O.C.: Super Optimal broth with Catabolite repression
Su: sumoylation

T: threonine
T: thymidine
TAE: Tris base, acetic acid, and ethylene-diamine-tetraacetic acid
TBS: Tris-buffered saline
TBST: Tris-buffered saline with tween
TCGA: The Cancer Genome Atlas
TdT: terminal deoxynucleotidyl transferase
THW: threonine, histidine, tryptophan
TPR: tetratricopeptide repeats

Ub: ubiquination
**UTR**: untranslated region

**Y**: tyrosine
CHAPTER 1
Introduction and background
Personalized medicine

Sir William Osler (1849-1919), one of the founding members of Johns Hopkins Hospital, understood the impact of an individual’s own genetic signature on disease presentation and treatment when he noted that “variability is the law of life, and as no two faces are the same, no two bodies are alike, and no two individuals react alike, and behave alike under the abnormal conditions we know as disease” (1). While interest in personalized medicine has undoubtedly increased over time, the concept of taking individual variability into account for medical applications is not new. In 1901 Karl Landsteiner discovered ABO blood groups, for which he was awarded the 1930 Nobel Prize for Medicine (2). While transfusions had been practiced without precision since the late 1600s, Landsteiner’s discovery was one of the first instances of using an individual’s physiological individuality to develop a treatment plan, a true leap forward for personalized medicine.

Personalized medicine has given way to precision medicine, an initiative backed by both President Obama and NIH Director Francis Collins in 2015 (3). The precision medicine initiative has two components – a near-term focus on oncology, and a long-term focus on generating advances for health and disease as a whole. In the short-term, the initiative aims to gain insight into an individual’s genomic signature to tailor diagnostics and therapeutic strategies for specific molecular drivers of that patient’s disease. In the long-term, the initiative aims to develop methods that allow for better assessment of disease risk to enable administration of optimal treatment strategies for a variety of diseases.

Cellular reprogramming

While the aims of the precision medicine initiative are expected to take time, many of the tools necessary to aid in this endeavor have already been created. Arguably, more than any other discovery in recent history, cellular reprogramming holds promise for the development of autologous cell therapies for regenerative medicine and accurate modeling of human disease (4,5). Like personalized medicine,
cellular reprogramming is not a new concept, although the most notable advancements have been made during the 21st century. As early as 1958, experiments were being performed to test the stability of the terminally differentiated state using somatic cell nuclear transfer (SCNT) (Fig. 1.1A) (6). As a Ph.D. student, John B. Gurdon transferred the nucleus from a somatic cell to an unfertilized *Xenopus laevis* oocyte, resulting in the development of normal tadpoles with no differences from their wild-type siblings (7,8). From this work, the field was able to conclude that factors within the oocyte contain reprogramming factors sufficient to dedifferentiate somatic nuclei to a pluripotent state. These advancements in amphibians led to SCNT experiments in mammals, and in 1996 the first mammal was cloned: Dolly the sheep (9,10). However, normal development of nuclear transplant embryos is
significantly lower than with normal reproduction. While approximately 95% of children born by normal reproduction develop without defects, only up to 3% of cows born from SCNT develop without defects (11), and only 1% of mice (12-15). While these experiments have demonstrated reprogramming can be done, they also demonstrate that mechanisms required for cellular reprogramming are not mimicked efficiently solely by nuclear transfer.

In 2002, a second method of cellular reprogramming was described – cell fusion (16). As stem cell research gained traction, so did work describing the innate plasticity maintained by even terminally differentiated cells. Advancements in stem cell therapy led to the discovery that bone marrow transplantation can lead to cell fusion, the combination of a differentiated cell with a progenitor or stem cell which can cause reprogramming into a new cell type (Fig. 1.1B) (16-19). While these types of cells are of interest as a tool to study cellular reprogramming, specifically at the epigenetic level, they will likely always lack therapeutic transferability due to their tetraploid nature (18).

Yamanaka then made major advancements in personalized medicine in 2006 when lineage differentiation was reversed through viral addition of stem cell-associated transcription factors, reprogramming terminally differentiated fibroblasts into induced pluripotent stem cells (iPSCs) (Fig. 1.1C) (20-26). However, translation of this methodology for personalized medicine applications is handicapped by viral addition of reprogramming factors, low reprogramming efficiency, genetic and epigenetic abnormalities, and tumorigenicity (5,27-34). Reprogramming factors have since moved away from the initial four (OCT4, SOX2, KLF4, and c-MYC) so that cells can be trans-differentiated across lineages without first passing through the pluripotent state (Fig. 1.2) (35-38). Although, the more distantly related the cell types are, the more difficult the lineage conversion tends to be. Alternatively, microenvironment-driven reprogramming has also been demonstrated, where cellular reprogramming occurs through microenvironmental cues, such as the addition of small molecules with the ability to regulate signaling pathways for induction of pluripotency (39).
Figure 1.2 Multiple cellular lineages and cell types achieved from transcription factor-mediated cellular reprogramming.

Summary of the various cell types derived from human dermal fibroblasts by lineage reprogramming. From fibroblasts, the transcription factors listed in the center ring were used to produce lineage conversions to the cell types listed in the outer ring (20,36-38,40-51). Figure adapted from Vierbuchen and Wernig, 2012 (52).

Induced regeneration competent cells

Because of limitations currently seen with iPSC derivation, it is imperative to understand the molecular mechanisms that lead to cellular reprogramming for development of effective and controlled methods for personalized medicine. We have reported reprogramming using a novel, non-viral, in vitro system from primary human dermal fibroblasts (hDFs), termed induced regeneration competent (iRC)
cells. iRC cells are defined by exogenous addition of human fibroblast growth factor FGF2 and culture
under reduced oxygen tension. In vitro environmental cues such as FGF2 addition and reduction in
oxygen concentration have been shown to influence cellular phenotype in other systems. FGF2 is a
critical component of stem cell cultures; it is a mitogen required for the maintenance of pluripotency
(53,54). In iRC cells, the addition of FGF2 to reduced oxygen conditions induces the expression of
specific stem cell-associated genes (55).

Reduction in oxygen concentration has been shown to increase cellular lifespan and to regulate
epigenetic changes (56,57). Cell culture is routinely performed at atmospheric oxygen levels (between
19% to 20%) even though physiological levels tend to be substantially lower (ranging from 0.5% to 10%,
depending on the tissue type) (58-61). Oxygen concentration was reduced in our model system to more
closely match the physiological state. Due to defined changes in culture conditions, iRC cells display
increased cumulative population doublings, increased time to cellular senescence, and at the same time
lack the potential for tumor formation when injected into SCID mice (55). At a mouse skeletal muscle
wound site, iRC cells engraft and aid in regeneration of muscle tissue (62). Reprogramming of these cells
at the wound site was characterized molecularly by expression of the myogenic marker PAX7.

Chromatin modification enzymes

Chromatin modification enzymes, regulators of epigenetic changes, are known mediators of cellular
reprogramming. Chromatin is the combination of DNA and proteins called the nucleosome that protects
genes by packaging DNA around histone subunits of a protein core. Each nucleosome is an octamer made
up of two each of the four core histones, histone H2A, histone H2B, histone H3, and histone H4, that
wrap around 147 nucleotides (63). Histone H1 is one of the five main histones, but is not a component of
the nucleosome “bead.” Histone H1 is a “linker” protein, which secures the wound DNA around the
nucleosome. The N-terminal “tails” of the nucleosome-associated histones protrude from the
predominantly globular structure and amino acids within the tails can be modified in various ways to
influence transcription, replication, or repair of a gene (Fig. 1.3). The most common types of histone
modifications are acetylation (ac), methylation (me), phosphorylation (P), ubiquitylation (ub), and sumoylation (su). Lysine (K) is the most diversely modified amino acid with the potential to be acetylated, methylated, ubiquitinlated, and sumoylated. However, methylation is not reserved solely for lysine residues, as arginine (R) residues can also be methylated. Expression can be further nuanced by multiple additions of a methyl group to a single amino acid, with each variation controlling transcriptional permissiveness differently. For example, a single lysine residue can be methylated once (mono), twice (di), or three (tri) times.

The highly dynamic orchestra of epigenetic modification is conducted by three major types of chromatin modification enzymes: 1) writers, which deposit a specific modification, 2) readers, which determine the modification present at a certain location, and 3) erasers, which remove a specific modification. All chromatin modification enzymes fall into one of these three categories, and each type of

Figure 1.3 Nucleosome occupancy of DNA.

DNA is tightly packaged around a core of histone proteins called the nucleosome, made up of histones H2A, H2B, H3, and H4. Histone H1 acts as a “linker”, securing the DNA around the nucleosome core. Histone “tails”, made up of amino acid “strings” that can be modified protrude from the core structure. Modification of residues within these tails promotes conformational changes allowing for, or preventing, association of DNA binding proteins, including transcription factors, with DNA. It is the tight packaging of DNA around nucleosomes that allows for chromatin compaction into chromosomes.
Table 1.1 Classes of chromatin modification enzymes responsible for writing, reading, and erasing epigenetic modifications.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Writers</th>
<th>Readers</th>
<th>Erasers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>Acetylase</td>
<td>Bromodomain</td>
<td>Deacetylases</td>
</tr>
<tr>
<td>Methylation</td>
<td>Methylase</td>
<td>Chromodomain, PHD domain</td>
<td>Demethylase</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Kinase</td>
<td>14-3-3 domain</td>
<td>Phosphatase</td>
</tr>
</tbody>
</table>

modification has its own writers, readers, and erasers (Table 1.1). These enzymes deposit acetyl or phospho groups on histone tails to induce changes in charge that influence chromatin compaction; methylation effects chromatin compaction via steric interaction. It is changes between the internucleosomal contacts that organize higher-order chromatin structure, thus allowing or preventing accessibility of the transcriptional machinery to the associated gene (64-67). Chromatin in a transcriptionally permissive state is referred to as euchromatin, and in a transcriptionally repressive state is referred to as heterochromatin. Typically euchromatin is highly acetylated and is methylated at specific histone residues commonly associated with transcriptional permissiveness, such as histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36), and histone H3 lysine 79 (H3K79). Conversely, heterochromatin typically has low levels of acetylation and is methylated at specific histone residues commonly associated with transcriptional repression, such as histone H3 lysine 9 (H3K9), histone H3 lysine 27 (H3K27), and histone H4 lysine 20 (H4K20) (63). Changes in charge within histone tails also serve to encourage or occlude non-histone proteins from binding specific domains. The most pertinent example of transcriptional permissiveness of change in charge influencing non-histone protein binding is acetylation enrichment at specific sites within a promoter to recruit the polymerase complex necessary for transcription (68-72).

The variety of combinations of histone modifications allows for precise control of functional responses defined by signaling conditions within the cell, which can appear and disappear within minutes, enabling fast, dynamic control over gene expression. Various modifications within the same nucleosome, or even on the same histone, enables “crosstalk” between modifications – the ability of a specific
modification to antagonize another modification (63) (Fig. 1.4). There are many examples of binding disruption (65), compromise of catalytic activity (73), and more effective substrate recognition (74) all due to a modification on a nearby histone residue.

Chromatin occupancy is further complicated by the existence of bivalent domains – the simultaneous occupation of a single nucleosome with classically active and repressive marks, allowing for maintenance of a state of poised activation to remain ready for the cues of a cell to either activate or repress transcription of the associated gene (75,76). Bivalent domains were first described in undifferentiated cells, which require transcriptional access to most genes as developmental fate determines lineage commitment by restricting expression to genes specific to the desired differentiated cell type. Upon differentiation, bivalent domains only preserve either the repressive or the active mark.

Figure 1.4 Epigenetic “crosstalk” between modified residues of a single nucleosome.

Epigenetic “crosstalk” is the ability of a specific modification to antagonize another modification, either positively or negatively. The charge change produced by specific epigenetic modifications influences chromatin compaction to either prevent or promote access to certain residues by chromatin modification enzymes. K refers to lysine residues, S to serine, and R to arginine. Me refers to methylation of a residue (blue circle), P to phosphorylation (yellow circle), Ac to acetylation (green circle), and Ub to ubiquitination (purple circle). Numbers below specific residues refer to the position of a specific amino acid within the specified histone tail. H2A refers to histone H2A, H2B to histone H2B, H3 to histone H3, and H4 to histone H4. Figure adapted from Kouzarides, 2007 (63).
**The influence of epigenetics on cellular reprogramming**

As cells divide, they inherit “epigenetic memory,” a stable phenotype maintained by epigenetic modifications acquired during developmental progression (77-79). Cellular reprogramming is, in essence, abolishment of existing somatic epigenetic memory followed by establishment of a new cell type-specific epigenetic signature. So while current cellular reprogramming makes use of transcription factors, it is the modification of the cell’s epigenetic memory that actually induces pluripotency. Gaining precise understanding of the timing of expression of epigenetic molecules during reprogramming is the key to purposeful manipulation of cell lineage determination and for identifying molecules that can be exploited for therapeutic purposes.

While many epigenetic changes necessary for cellular reprogramming are still unknown, some have already been described. It is known that heterochromatin is exchanged for euchromatin through removal of repressive histone marks as well as through global DNA hypomethylation (76,80-88). Both of these processes appear to be critical, as maintenance of the repressive di- and trimethylated H3K9 marks, as well as DNA methylation, act as barrier to reprogramming (31,89-93). Other histone marks have also been associated with promotion or prevention of cellular reprogramming. Levels of di- and trimethylated H3K4 increase in the initial stages of reprogramming, marking promoters and enhancers of pluripotency- and differentiation-associated genes (94,95), trimethylation of H3K27 maintains progression of developmental lineage determination by repressing pluripotency-associated genes in somatic cells and differentiation-associated genes in iPSCs (96), and dimethylation of H3K79 acts as a barrier for reprogramming by preventing repression of lineage-specific genes (97).

Along with changes in DNA methylation and histone modifications, reprogramming is also influenced at the epigenetic level by expression differences in enzymes that promote such changes, such as histone and chromatin remodelers, as well as changes in histone variants. The histone variant H3.3 is perhaps one of the most studied variants regarding incorporation during cellular reprogramming. H3.3 promotes euchromatin conformation by counteracting chromatin compaction via association with the
linker histone H1 (98). H3.3 is also required for successful SCNT, as H3.3 down-regulation in oocytes leads to compromised reprogramming (99-101).

**Arginine methylation**

Arginine methylation is a posttranslational modification (PTM) representing about 0.5% of all arginine residues within the mammalian proteome at any given time (102,103) and participates in many critical biological process required for cellular reprogramming, such as transcriptional activation (104-106), transcriptional repression (107-109), mRNA splicing (110-113), subcellular compartmentalization (105,114,115), signal transduction (116-119), and more. Arginine methylation is conserved from fungi to vertebrates (120) and arginine methyltransferases are emerging as regulators of proliferation and differentiation and are established modulators of gene expression (121-126). Protein arginine methyltransferases (PRMTs) regulate gene expression through modification of arginines within histone tails. PRMTs act to methylate substrates by utilizing S-adenosylmethionine (AdoMet) as a methyl donor, resulting in S-adenosylhomocysteine (AdoHcy) and methylarginine (125,127). Similar to lysine methylation, arginine residues can be modified once (monomethylation) or twice (dimethylation). In mammals, all PRMTs can catalyze the intermediate modification monomethyl arginine (MMA), while, unlike lysine methylation, PRMTs that catalyze dimethyl arginine (DMA) are divided into two groups: type I catalyze asymmetric dimethyl arginine (aDMA), where two methyl groups are added to the same atom, and type II catalyze symmetric dimethyl arginine (sDMA), where two methyl groups are added to different atoms (Fig. 1.5) (128). Type III PRMTs can only catalyze the intermediate MMA, although no mammalian type III PRMTs have been described. There are also no described PRMTs that catalyze both aDMA and sDMA. Arginine methyltransferases typically methylate glycine arginine rich (GAR) motifs, where one or more glycine residues follow the methylated arginine residue (120,128-131).

Arginine methylation is a modification that was once thought to be irreversible. In 2007 the first putative arginine demethylase, Jumonji domain-containing protein 6 (JMJD6), was described (132).
Methylation of the arginine amino acid side chain is catalyzed by protein arginine methyltransferases (PRMTs). Methylation of nitrogen atoms within the side chain is catalyzed using S-adenosylmethionine (AdoMet) as a methyl donor, producing S-adenosylhomocysteine (AdoHcy) as a byproduct along with methylarginine. Arginine side chains can be methylated once, producing monomethyl arginine (MMA), or twice, producing dimethyl arginine (DMA). All currently described mammalian PRMTs are capable of catalyzing the intermediate MMA. PRMTs are classified into two groups regarding their capabilities of catalyzing DMA. Type I PRMTs catalyze asymmetric DMA (aDMA) from MMA. Type II PRMTs catalyze symmetric DMA (sDMA) from MMA. Methyl groups are annotated as CH₃ and are represented by orange stars.

**Figure 1.5 Structure of arginine methylation.**

Methylation of the arginine amino acid side chain is catalyzed by protein arginine methyltransferases (PRMTs). Methylation of nitrogen atoms within the side chain is catalyzed using S-adenosylmethionine (AdoMet) as a methyl donor, producing S-adenosylhomocysteine (AdoHcy) as a byproduct along with methylarginine. Arginine side chains can be methylated once, producing monomethyl arginine (MMA), or twice, producing dimethyl arginine (DMA). All currently described mammalian PRMTs are capable of catalyzing the intermediate MMA. PRMTs are classified into two groups regarding their capabilities of catalyzing DMA. Type I PRMTs catalyze asymmetric DMA (aDMA) from MMA. Type II PRMTs catalyze symmetric DMA (sDMA) from MMA. Methyl groups are annotated as CH₃ and are represented by orange stars.
However, conflicting reports exist regarding the true role of JMJD6 as an arginine demethylase (133-135). Arginine residues can also be converted to the unconventional amino acid citrulline through deimination by the enzymes peptidylarginine deiminases (PADIs) (136-138). However, PADIs are not demethylases as only non-methylated arginine residues can be converted to citrulline (139,140).

Arginine methylation regulates physiological functions of proteins by influencing protein shape and affinity to modulate binding partner interactions (141-146). Methylation of arginine residues within histone tails can promote or prevent transcriptional permissiveness either directly or indirectly by influencing the generation of other histone marks, specifically methylation of lysine residues (125). The relationship between the methylation status of specific arginine and lysine residues has been termed the “arginine/lysine-methyl/methyl switch”, referring to the specific combinations of residues where both sites are methylated, such as H3R2/H3K4, H3R8/H3K9, and H3R26/H3K27 (147,148). PRMTs can directly influence transcriptional permissiveness by methylating specific arginine residues of histones known to be associated with euchromatin. For example, asymmetric dimethylated H3R17 is associated with promoters of transcribed genes, and elevated levels have been associated with the tumor suppressor gene E2F1 and the pluripotency genes OCT4 and SOX2 (149,150).

**Protein arginine methyltransferases**

As early as 1967 it was demonstrated that arginine could be methylated, but only within the past two decades has arginine methylation begun to be investigated in great detail (105). As their name suggests, PRMTs catalyze the methylation of arginine residues within both histone and non-histone proteins. PRMTs are generally ubiquitously expressed and are mediators of critical cellular functions. Although eleven PRMTs have been described, only nine have been characterized in vertebrates. PRMTs 1, 2, 3, 4 (also known as coactivator-associated arginine methyltransferase 1, CARM1), 6, and 8 having described type I activity, catalyzing aDMA, and PRMTs 5, 7, and 9 having described type II activity, catalyzing sDMA (151-153). While all family members harbor the same core motifs, none so far appear functionally redundant, as knockout mice and knockout cell lines display unique and often dramatic
phenotypes. For example, PRMT1 null mice only maintain viability to embryonic day 6.5 (154), and while CARM1 (PRMT4) null mice survive embryogenesis, they display developmental defects and die shortly after birth (155). Though no functional redundancy has been reported, PRMTs can share substrates in vitro, specifically PRMTs 1, 3, 6, and 8 (148,155-159).

Arginine methylation has been shown to play an important role in differentiation and proliferation. CARM1 has been linked to adipocyte differentiation (123), chondrocyte proliferation (122), and pulmonary epithelial proliferation and differentiation (124). Aberrant expression of PRMT family members is associated with cardiovascular and pulmonary diseases and various types of cancers, including lung, bladder, colon, and breast cancers (160-163). While the correlation between PRMTs and cancer is relatively new, their known roles in cellular proliferation and growth validate recent work linking these enzymes to tumorigenesis and metastasis (164). For example, methylation of the RNA-binding protein HuD by CARM1 blocks p21 and p27 transcript decay by preventing HuD binding, thereby preventing G1 cell cycle arrest (165). Also, PRMT6 directly methylates the promoters of p16 and p21, thereby preventing transcription and arrest in G1, allowing for progression of cell cycle (166). As the number of reports implicating PRMTs as vital contributors to tumorigenic phenotypes increases (164,167-169), publications identifying PRMT inhibitors with therapeutic promise increase concomitantly (170-173).

Adding layers of complexity to the role of PRMTs in various diseases is the more recent finding that alternatively spliced PRMT variants demonstrate tissue-specific expression with unique substrate specificity and tissue distribution (163,174-177). Generally, protein diversity can be regulated by transcription of multiple mRNA variants from a single gene or post-translational processing, by, for example, alternative transcription initiation and termination sites, splicing, and polyadenylation (178-180). Alternative polyadenylation was found to produce a second PRMT2 transcript that positively correlates with estrogen receptor α (ERα) status in breast cancer patients (181). ERα overexpression is frequently observed in early stage breast cancer and is used as a disease biomarker for certain subsets of breast cancer (182). PRMT2 is not the only PRMT family member with variant-specific biological
functions, as PRMT1 has seven known protein isoforms with unique expression patterns and differing correlation to patient prognosis (163,174-177). Studies such as these underscore the importance of not only understanding the biological functions of PRMT family members, but also identifying and defining functions for alternative variants and isoforms of members of the PRMT family.

PRMT1 was the first identified family member (183) and is the primary arginine methyltransferase, catalyzing about 85% of all protein arginine methylation (184). PRMT1 is ubiquitously expressed and is found in both nuclei and cytoplasm (117,185,186). PRMT1 has wide substrate specificity, although it has a preference for methylating arginine residues within GAR motifs (128,131). It is also a transcriptional coactivator and is recruited to promoters by transcription factors (105). PRMT1 methylates tumor suppressors, such as forkhead box O1 (FOXO1) (187), and PRMT1 overexpression has been linked to cancer progression and has been correlated to poor prognosis (162,188,189). Arginine methylation by PRMT1 also regulates telomere length and stability (190). One of the major telomere regulating complexes is shelterin. This complex consists of six proteins that protect the end of the single-stranded telomere so that it is not read by cellular machinery as a double stranded break (190). One of the six shelterin proteins in vertebrates, TTAGGG repeat binding factor 2 (TRF2), is regulated by arginine methylation catalyzed by PRMT1. It was shown that arginines within TRF2 are required for preventing telomere doublets and are important for the binding of shelterin to the telomere, and that PRMT1 depletion resulted in growth arrest and telomere doublets (190). Interestingly, the relationship between TRF2 and PRMT1 does not exist in cancer cells, indicating control of cellular proliferation by PRMT1 is likely cell type-specific. Uncovering the mechanism, or specific arginine methyltransferase that regulates TRF2 in cancer cells would be of interest as a therapeutic target.

PRMT2 was initially believed to not possess enzymatic activity (105), but it has since been demonstrated to catalyze aDMA (191). Like some other PRMT family members, PRMT2 is a coactivator of gene expression. Specifically, PRMT2 is a coactivator for the androgen receptor (AR) and estrogen receptor α (ERα) (192,193). Additionally, PRMT2 controls the nuclear export of IκB-α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) to inhibit NF-κB, thus regulating the
inflammatory response and promoting apoptosis (194,195). PRMT2 is also involved in other cellular processes, such as leptin signaling (196) and regulation of Wnt signaling (197). PRMT2 participates in cell cycle regulation by binding the tumor suppressor retinoblastoma (pRb) to regulate function of the E2F transcription factors (198). While all PRMTs share conserved domains, some PRMTs harbor unique motifs (Fig. 1.6). For example, PRMT2 harbors an N-terminal SH3-domain necessary for association with RNA-binding proteins (199).

PRMT3 is localized exclusively in the cytosol (200), thus preventing its direct participation in epigenetic regulation. However PRMT3 is an active methyltransferase and uses the zinc-finger domain harbored at its N-terminus for substrate recognition of RNA-associated substrates (200,201). PRMT3 is essential for ribosome maturation through methylation of the ribosomal protein S2 (RPS2), which is a component of the 40s ribosomal subunit (125,201-203).

CARM1, also referred to as PRMT4, was first identified as a steroid receptor coactivator (204). Given that most PRMTs methylate GAR motifs, CARM1 has unique substrate specificity in that it does not methylate GAR motifs but rather proline, glycine, methionine rich (PGM) motifs (112,131). CARM1 expression may be an underlying pathological mechanism in breast cancer, as CARM1 is a coactivator of multiple cancer-associated transcription factors, such as NF-κB, p53, E2F1, and ERα (151). Compared to normal breast tissue, CARM1 expression is higher in metastatic breast tumor tissue, specifically in triple negative tumors lacking ERα, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (205). The therapeutic potential of CARM1 was demonstrated when 90% knockdown of methyltransferase activity was sufficient to sustain wild-type methylation by CARM1 (206), showing that decreased CARM1 levels can be tolerated physiologically, thus a reduction of increased levels that are a result of tumorigenesis may have therapeutic relevance.

PRMT5 is one of the most well-characterized PRMT family members and is the predominant type II arginine methyltransferase. PRMT5 methylates GAR motifs as well as PGM motifs (112) and is critical for survival, as loss of PRMT5 is non-viable at either the cellular level or organismal level in mouse
PRMT5 regulates gene expression through association with the nucleosome remodeling complex SWI/SNF to methylate histones H3 and H4, and it also regulates cellular growth and proliferation by controlling expression of the tumor suppressors ST7 (suppressor of tumorigenicity 7) and NM23 (nonmetastatic 23) (208). PRMT5 also acts to regulate cell cycle progression through repression of Cyclin E and CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase), a gene necessary for pyrimidine biosynthesis (107,108). Gene silencing is carried out by the addition of methyl groups to DNA by de novo DNA methyl transferases (DNMT) 3A and 3B. DNMT3A is recruited for

![Figure 1.6 Structures of PRMT family members.](image)

PRMTs contain two binding domains, a methyl donor-binding and a substrate-binding domain, the latter of which is a barrel-like domain. These two domains make up the highly conserved PRMT core region (grey box) that harbors three signature motifs, I, II, and III, along with a post-I domain (black boxes) (210). When folded into their 3D conformations, the threonine, histidine, tryptophan (THW) loop (blue box) lies between the binding domains, forming the active site which holds the arginine residue to be modified (211). Some PRMT family members harbor unique motifs: PRMT2 harbors an SH3 domain (orange box), PRMT3 a zinc-finger domain (green box), PRMT8 a myristoylation motif (red coil), PRMT9 has two tetratricopeptide repeats (TPRs) (purple boxes), and PRMTs 7 and 9 contain two methyltransferase domains.
gene silencing by symmetric dimethylated H4R3 catalyzed by PRMT5 (209), which is required for subsequent DNA methylation and is a direct binding target of DNMT3A. This implicates a direct role for PRMT5 in gene silencing.

PRMT6 is a type I arginine methyltransferase, conferring aDMA, but it preferentially catalyzes dimethylation of already monomethylated substrates (159,185,212). PRMT6 is the primary enzyme that catalyzes methylation of H3R2 in mammalian cells. As H3R2 methylation counteracts transcriptional activation by trimethylated H3K4, PRMT6 has reported functions as a transcriptional repressor (148,158,159). A later report demonstrated that PRMT6 expression decreases as cells undergo senescence and accumulate p21. This was found to be the result of the repressive activity of PRMT6 at H3R2, which induces cell proliferation by blocking senescence through regulation of the cyclin-dependent kinase (CDK) inhibitors p21 and p16 (166). Methylation of R29 on histone H2A is another modification catalyzed by PRMT6, which is enriched at repressed genes (213). Like many other PRMT family members, PRMT6 tends to be overexpressed in a variety of cancers (168). It is hypothesized that PRMT6 is necessary for tumor growth and progression, which is supported by studies that have shown PRMT6 knockdown prevents growth and viability of tumor cell lines (168,214). Other work has shown this result may be due to the relationship between PRMT6 and p21, as PRMT6 causes cytoplasmic accumulation of p21 (215), which has an anti-apoptotic effect (216).

PRMT7 is one of the few described type II arginine methyltransferases (217) and is one of only two PRMT family members to harbor two copies of the methyltransferase domain as a result of gene duplication (218). The little that is known about PRMT7 indicates that it has a vital role in development, as it participates in gene imprinting during male germ line development via H4R3 methylation (219), and its expression is lost during differentiation, mimicking that of the pluripotency marker OCT4 (220). Expression of PRMT7 is also elevated in breast cancer, where it mediates the epithelial-to-mesenchymal transition (EMT) by repressing E-cadherin expression (221). E-cadherin loss is a hallmark of EMT, which is a shift in cell phenotype from an epithelial to mesenchymal state, a process known to enable metastasis.
PRMT7 has also been shown to regulate embryonic stem cell pluripotency (220), again indicating the importance of arginine methylation and PRMT family members in cellular plasticity.

PRMT9 is the most recently characterized arginine methyltransferase, and some controversy exists as to which protein the PRMT9 name actually refers. The PRMT9 designation here refers to the gene on chromosome 4q31, however other reports have also used the designation PRMT9 as well as the designation F-Box Protein 11 (FBXO-11) to refer to the gene on chromosome 2p16 (116,125,151,223). The structure of FBXO-11 indicates it is unlikely to be a true PRMT and possess methyltransferase activity. Recent reports have indicated PRMT9 on chromosome 4q31 has type II methyltransferase activity (223). PRMT9 harbors two tetratricopeptide repeats (TPRs) at the N-terminus and, like PRMT7, PRMT9 also contains a duplicated methyltransferase domain (223). The only currently-known function of PRMT9 is as a regulator of alternative splicing through modification of spliceosome-associated proteins (223).

PRMT8

PRMT8 was first described in 2005 because of sequence similarities with PRMT1 (116). Initial reports characterized PRMT8 as the only family member to demonstrate tissue specificity in expression. However in 2013, an in vivo zebrafish study found that PRMT8 is expressed ubiquitously during early development using whole-mount in situ hybridization and that PRMT8 is critical for embryonic and neural development using antisense morpholino oligonucleotides (224). Only after brain formation was PRMT8 expression restricted in a tissue-specific manner to the brain. This was the first evidence implicating a developmental role for PRMT8 as well as ubiquitous expression at least for some time during development, indicating PRMT8 is more similar to other PRMT family members than initially reported. This study also found that PRMT8 expression is critical for maintenance of genomic integrity, as knockdown results in premature death due to developmental defects (224).

Initial reports of PRMT8 also characterized it with a second unique qualifier, describing it as the only member of the PRMT family to localize to the plasma membrane (116). However, more recent
evidence suggests that endogenous mammalian PRMT8 localizes to cell nuclei (118,225). PRMT8 has four known protein isoforms with unique N-terminals (Fig. 1.7). Isoforms 1 through 3 are translated from the same mRNA but differ in length by a total of fifteen amino acids due to various in-frame start codons present within the mature mRNA. The second amino acid of isoform 1 is modified by a myristoylation motif, a post-translational hydrophobic moiety inducing localization to the plasma membrane (116,117,226). As isoforms 2 and 3 are truncated at the N-terminus, they lack the modified myristoylated residue and are thereby localized to cell nuclei (118). A fourth PRMT8 isoform translated from a second mRNA variant has only recently been studied experimentally (found in chapter 2 of this dissertation).

![Figure 1.7 PRMT8 protein isoforms.](image)

**Figure 1.7 PRMT8 protein isoforms.**

PRMT8 has four different protein isoforms that only differ at their N-termini. Isoforms 1, 2, and 3 are all translated from the same mRNA at different start codons. Isoform 1 contains a glycine residue that is post-translationally modified by a hydrophobic myristoylation moiety. The consequence of this moiety is localization to the plasma membrane. Isoforms 2 and 3 are truncated at their N-termini, only differing from isoform 1 by two and fifteen amino acids, respectively. Both isoforms 2 and 3 lack the glycine residue harboring the myristoylation motif and are consequently localized to cell nuclei. Isoform 4 is translated from a second mRNA variant with an alternative exon 1. Isoform 4 has only recently been studied experimentally (found in chapter 2 of this dissertation).

The N-terminal domain of PRMT8 suppresses its own enzymatic activity (117). This was discovered with experiments comparing the enzymatic activity of PRMT8 with its parologue PRMT1. While their sequences are similar, their reaction kinetics were quite different. To determine if the N-
The terminal region where the two proteins differ is responsible for their enzymatic differences, the first sixty amino acids of PRMT8 were removed to make its sequence more similar to PRMT1. This led to a significant increase in PRMT8’s activity, indicating that the N-terminal domain acts to regulate PRMT8 activity (117). PRMT8 can also self-regulate its activity by automethylation of its N-terminus (117), however the functional consequence of this remains unknown.

Binding partners for PRMT8 have only been identified using the purified, full-length, myristoylated isoform with overexpression techniques, limiting knowledge about true binding partners and substrates in vivo. In vitro methyl donor assays have identified histone H4, Ewing sarcoma (EWS), Dishevelled3 (DVL3), and nucleosome assembly protein 3 (NPL3) as substrates of full-length PRMT8 (116,227,228), however substrates for the endogenous isoform of PRMT8 have not yet been described. Most recently, NIFK (nucleolar protein interacting with the forkhead associated domain of Ki-67) was described as a substrate of a truncated isoform of PRMT8 missing the first sixty amino acids in a study that reported the crystal structure of PRMT8 (229).

**Thesis objectives**

The overall goal of the work presented here was to understand how epigenetics contribute to the iRC phenotype produced by changes in culture conditions, specifically by reduction in oxygen concentration and supplementation with FGF2. **The driving hypothesis for this work was that a shift occurs in the chromatin modification enzyme profile to allow for acquisition of increased plasticity,** evidenced by expression of specific stem cell-associated genes, increased life span, and acquisition of regeneration competence observed when human dermal fibroblasts are grown under iRC culture conditions.

The work in the following chapters extends previous observations published by our lab by characterizing expression of the chromatin modification enzyme PRMT8 within the iRC system, as well as providing a possible mechanism by which PRMT8 expression influences lifespan. The work described
here also proposes a useful translational application for iRC cells as precursors of a tumorigenic state, exploiting their demonstration of increased lifespan while remaining non-tumorigenic in SCID mice.

The systematic investigation of the hypothesis stated above was organized into four objectives.

**Objective 1:** Characterize the PRMT8 variant expressed in iRC cells and assess its role in cellular lifespan

Post-translational modifications of proteins diversify the proteome both structurally and functionally. In particular, arginine methylation can influence biological processes such as transcriptional permissiveness, cellular differentiation, and telomere length and stability (148,190,207,230-233). iRC cells here are used as a tool that uncouples the mechanisms of increased cellular lifespan from tumorigenesis to identify molecules that regulate the switch between normal and neoplastic cell phenotypes. Molecular analysis demonstrated significant upregulation of PRMT8 in iRC cells compared to control cells at both the transcript and protein levels. Furthermore, iRC cells express a novel variant of PRMT8, which in knockdown experiments was shown to be required for viability of both human dermal fibroblasts and grade IV glioblastomas. The work presented here not only demonstrates that PRMT8 is a critical chromatin modification enzyme involved in cellular proliferation, but also validates the use of the iRC culture system as a tool to identify biomarkers of a pre-cancerous biological state.

**Objective 2:** Evaluate PRMT8 participation in cell cycle progression and its sufficiency to increase cellular proliferation

It is well-established that most PRMT family members demonstrate elevated expression in proliferating and cancerous tissues (234-236), but whether elevated PRMT levels are sufficient to cause increased proliferation is unknown. Here we evaluate the participation of PRMT8 in cell cycle progression. In this study hydrogen peroxide is used to induce premature senescence in human dermal fibroblasts to gain insight into the relationship between cellular senescence and PRMT8 expression. As cells senesce, PRMT8 expression decreases in a hydrogen peroxide concentration-dependent manner,
suggesting regulation of PRMT8 expression by cell cycle progression. However, PRMT8 overexpression in human dermal fibroblasts did not increase cellular lifespan or alter expression of key cell cycle regulatory genes, indicating that overexpression of PRMT8 alone is not sufficient to induce increased cellular lifespan.

**Objective 3: Investigate PRMT8 promoter occupancy and establish PRMT8 binding partners**

Increased transcriptional expression of PRMT8 is induced by iRC culture conditions, suggesting that changes in culture conditions cause modification of histone occupancy at the PRMT8 promoter to allow for its increased transcription. We show here that reduced oxygen tension is the primary component of the iRC culture system that regulates PRMT8 expression. Sequence analysis of the PRMT8 promoter by bioinformatics reveals the presence of several hypoxia response elements, a DNA motif known to regulate transcription through binding of hypoxia inducible transcription factors. Chromatin immunoprecipitation was employed to explore how PRMT8 promoter occupancy changes with culture conditions. We demonstrate bivalent occupancy of the PRMT8 promoter regardless of culture conditions, implicating the poised activity of this promoter to maintain readiness for transcription, a key feature of biologically critical genes. The mechanism by which PRMT8 participates in cellular lifespan is also explored here using co-immunoprecipitation and mass spectrometry, which identify specific high molecular weight isoforms of FGF2 as PRMT8 binding partners, specifically under standard culture conditions. PRMT8 binding with FGF2 may provide mechanistic insight as to how PRMT8 helps regulate cellular proliferation and identifies the first binding partner of endogenous PRMT8.

**Objective 4: Examine PRMT8 relevance as a cancer biomarker**

Cancer is one of the most prevalent diseases, accounting for 25% of all deaths in the United States (237). As such, medicine has shifted from reactive to proactive. Colonoscopies alone have reduced morality from colorectal cancer by 53% (238). As medical technology advances, preventative screens are becoming less invasive and more widespread as research reveals biomarkers that can be used to identify
cancer-related changes. Here we propose the use of iRC cells as a tool to identify molecules that participate in early cancer-related changes, before cells are cancerous and examine the relevance of PRMT8 as a cancer biomarker. Various publically available databases are used here to gain insight into the expression and function of PRMT8 in cancer development and progression in light of our own data. Such databases demonstrate amplified PRMT8 expression in various cancers and correlate PRMT8 expression to patient survival – either positively or negatively contingent upon the type of cancer. Our own data shows expression of a novel PRMT8 variant in a tumorigenic glioblastoma cell line, further underscoring the importance of understanding PRMT variant expression and function. Taken together, these data suggest that PRMT8 is a viable candidate for further study as a prognostic cancer biomarker, specifically for brain cancer.
CHAPTER 2

Novel protein arginine methyltransferase 8 isoform is essential for cell proliferation
ABSTRACT

Identification of molecular mechanisms that regulate cellular replicative lifespan is needed to better understand the transition between a normal and a neoplastic cell phenotype. We have previously reported that low oxygen-mediated activity of FGF2 leads to an increase in cellular lifespan and acquisition of regeneration competence in human dermal fibroblasts (iRC cells). Though cells display a more plastic developmental phenotype, they remain non-tumorigenic when injected into SCID mice (55,62) allowing for investigation of mechanisms that regulate increased cellular lifespan in a non-tumorigenic system. Analysis of chromatin modification enzymes by qRT-PCR revealed the strongest candidate, a 13.3-fold upregulation of the arginine methyltransferase PRMT8 in iRC cells. Increased protein expression was confirmed in both iRC and human embryonic stem cells (hESCs) – the first demonstration of endogenous human PRMT8 expression outside the brain. Furthermore, iRC cells express a novel PRMT8 mRNA variant. Using siRNA-mediated knockdown we demonstrate that this novel variant was required for proliferation of human dermal fibroblasts (hDFs) and grade IV glioblastomas. PRMT8 upregulation in a non-tumorigenic system may offer a potential diagnostic biomarker and a therapeutic target for cells in pre-cancerous and cancerous states.

INTRODUCTION

Finite cellular proliferative lifespan and onset of irreversible growth arrest, termed “senescence”, has long been recognized in differentiated eukaryotic cells (239). Molecular mechanisms that regulate this terminal arrest of the cell cycle, however, can be deregulated, leading to uncontrolled cellular proliferation in cancer cells or continuous self-renewal in pluripotent stem cells; both cell types becoming neoplastic in parallel. Six biological capabilities have been detailed during the evolution of healthy cells to a neoplastic state. Of the six canonical hallmarks of cancer (resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, and inducing angiogenesis), four are associated with increased cellular lifespan (240,241). Investigating
processes that control lifespan enables us to progress toward the discovery of mechanisms that control the switch between normal cell division and neoplastic proliferation.

Methylation is one of the most widely studied and diverse post-translational modifications (PTMs). Methyl groups can be added to the side chains of various amino acids, such as proline, lysine, histidine, and arginine (233). In particular, arginine methylation can influence biological processes such as transcriptional permissiveness, cellular differentiation, and telomere length and stability (148,190,207,230-233). Many biological processes regulated by arginine methylation are well-described, but limited knowledge exists about how protein arginine methyltransferases (PRMTs) themselves are regulated. However, aberrant expression of PRMT family members has been associated with cardiovascular and pulmonary diseases, as well as various types of cancers including lung, bladder, colon, and breast cancers (160,162,163,168).

PRMT8 was first identified because of sequence similarity with PRMT1 (116), and phylogenetic analysis revealed it to be a paralogue of PRMT1 in vertebrates (224,242). PRMT1 is ubiquitously expressed and is found in both nuclei and cytoplasm (117,185,186). Although members of the PRMT family are all highly homologous, PRMT8 and PRMT1 are most similar with 83% sequence identity, differing only at the N-terminus, where PRMT8 contains 76 additional amino acids (116-118). Northern blot analysis demonstrated that full-length PRMT8 transcript expression was found largely in human brain tissue (116,117,226). However, analysis of PRMT8 in zebrafish found ubiquitous expression during embryonic development, whereas expression only became restricted to brain tissue after neural development (224).

PRMT8 has three described isoforms with unique N-termini translated from differing in-frame start codons. Early characterization of full-length PRMT8 (isoform 1) revealed a glycine residue at the N-terminus modified by a myristoylation motif (116,117). Myristoylation is the addition of a hydrophobic moiety that results in sequestration of modified proteins to the plasma membrane (116,117). However, overexpressed PRMT8 translated from the second (isoform 2) and third (isoform 3) in-frame start codons displays nuclear localization (118). In mice, endogenous PRMT8 localizes to nuclei (118).
Previous studies of PRMT8 utilized overexpression of the full-length isoform, which guided the consensus that the endogenous isoform is the full-length product and that expression is restricted to brain tissue. If, as current literature suggests, PRMT8 is one of the truncated nuclear isoforms and is expressed more widely than initially reported, it challenges the existing paradigm and it suggests that PRMT8, like other PRMT family members, may have a role in critical cellular processes through chromatin modification or regulation of protein-protein interactions. However, no publications to date have assessed expression or function of PRMT8 outside the brain in human cells.

We have developed a unique, reversible cell phenotype from primary human dermal fibroblasts (hDFs), termed induced regeneration competent (iRC) cells. iRC cells are derived by exogenous addition of human fibroblast growth factor FGF2 and culture in reduced oxygen concentration (2%) (Fig. 2.1). Reduction in oxygen concentration has been shown to increase cellular lifespan and to regulate epigenetic changes (56,57). iRC cells display increased proliferative lifespan and increased time to cellular senescence while lacking the propensity to form tumors when injected into SCID mice, a capability that is characteristic of immortalized and pluripotent cells (55,62). This unique phenotype allows us to study molecular changes that lead to increased cellular lifespan without cancerous permanent self-renewal.

Figure 2.1 Derivation of the iRC phenotype.

Supplementation with the growth factor FGF2 under reduced oxygen over 7 day culture period leads to increased plasticity of adult human fibroblasts characterized by a pro-regenerative, non-tumorigenic phenotype.
RESULTS

*PRMT8 is expressed in human dermal fibroblasts* – In an effort to understand molecular mechanisms associated with increased lifespan in iRC cells, known epigenetic modulators were examined as potential candidates. To identify possible target genes, the expression of 84 chromatin modification enzymes were analyzed by an qRT-PCR array in fibroblasts grown under control and iRC culture conditions (n=1). Expression was normalized to the housekeeping gene that showed the least divergent expression between experimental groups, the ribosomal protein RPL13A (data not shown). Fold change was calculated by comparing change in cycle threshold (ΔCt) values between treatment groups (ΔΔCt). Figure 2.2A shows the top five most up- and down-regulated genes, per the array, in iRC cells compared to fibroblasts grown in the absence of exogenous FGF2 and at ambient oxygen. Of the 84 genes examined, the most considerable expression change was observed in protein arginine methyltransferase 8 (*PRMT8*), represented by a 13.3-fold transcriptional increase in iRC cells compared to control cells. Expression levels

![Figure 2.2](image)

**Figure 2.2** Effect of culture conditions on expression of chromatin modification enzymes.

Target genes were analyzed using a qRT-PCR Chromatin Modification Enzyme Array (SA Biosciences) (n=1). (A) Fold change in expression of the top 5 most up- and down-regulated chromatin modifiers as normalized to the housekeeping gene RPL13A. (B) Fold change in expression in members of the PRMT family normalized to the housekeeping gene RPL13A.
of all other arginine methyltransferases remained relatively unchanged between culture conditions (Fig. 2.2B). Expression of the most recently-described PRMT family member, PRMT9, was not assessed.

As the array was used to identify potential targets for further study, PRMT8 expression was examined using RT-PCR and Western blotting. Primers were designed to recognize the region of PRMT8 analyzed in the chromatin modification enzyme array. Careful consideration was given during primer design due to high homology between PRMT8 and others within the PRMT family, especially PRMT1 (116-118,224,242). Upregulation of PRMT8 transcript in iRC cells was validated by RT-PCR (Fig. 2.3A) using mouse brain cDNA as a positive control. Curiously, PRMT8 was also expressed in human embryonic stem cells (hESCs). To determine if upregulation of PRMT8 transcript is accompanied by upregulation of

![Figure 2.3 Effect of culture conditions on PRMT8 expression.](image)

(A) PRMT8 transcript expression in control human dermal fibroblasts (CRL-2352), iRC cells, and hESCs compared to expression in mouse brain by RT-PCR. Actin was used as a loading control. (B) PRMT8 protein expression in control human dermal fibroblasts (CRL-2352), iRC cells, and hESCs compared to expression of purified GST-tagged PRMT8. Actin was used as a loading control. 10µg of total protein were loaded in each lane except for GST-PRMT8 lanes (0.25µg and 0.5µg, respectively). Antibody dilutions are as follows: PRMT8 – 1:200, actin – 1:5000, HRP anti-Rb – 1:10,000. (C) Densitometric representation of protein levels normalized to actin from three separate experiments. *All treatments were significantly different from each other: control compared to iRC, p=0.0002; control compared to hESCs, p =0.002; iRC compared to hESCs, p =0.02. (D) PRMT8 transcript expression in various control human dermal fibroblast lines (CRL-2352; CRL-2097; CT-1005) compared to the same lines grown under iRC conditions by RT-PCR. Actin was used as a loading control.
PRMT8 protein expression, Western blot analysis was performed (Fig. 2.3B). The immunogenic protein was detected at the expected 45kD in both iRC and hESC cells. The control PRMT8 protein migrated at 71kD, due to its 26kD GST tag. Figure 2.3B is a representative blot; densitometry for three replicates can be seen in Figure 2.3C. All samples were normalized to actin and analyzed using a one-tailed T-test.

As iRC cells are derived from primary hDFs, any sample is subject to individual idiosyncrasies in gene expression. Because of this, PRMT8 expression was analyzed in other primary human dermal fibroblast lines to ensure PRMT8 upregulation is a result of iRC culture conditions and not an artifact of the individual from whom the fibroblasts were derived. Though previous and subsequent work was carried out using CRL-2352s (human adult dermal fibroblasts), other cells, specifically CRL-2097s (human foreskin fibroblasts) and CT-1005s (adult female panniculectomy fibroblasts), also demonstrated upregulation of PRMT8 by RT-PCR when grown under iRC culture conditions (Fig. 2.3D). The significance of low expression in young human tissue (CRL-2097s) compared to high expression in mature human tissue (CRL-2352s and CRL-1005s) is not clear, and requires further investigation. However, even in young tissue expression appears to be inducible by iRC conditions, supporting the idea that regulation of PRMT8 is not dependent on organismal age.

Since the published consensus opinion relegates expression of PRMT8 strictly to brain tissue (117,226,233), the transcript detected in iRC cells was sequenced in order to confirm the identity of the transcript as PRMT8. PRMT8 was amplified from iRC cells using RT-PCR, the band was excised from the gel, and the fragment was cloned into pLVX using T4 ligase. Positive transformants were sent for sequencing, and the identity of the transcript was verified as that of PRMT8 (Fig. 2.4).

*Human dermal fibroblasts express a novel PRMT8 variant* – Aberrant PRMT expression plays a role in various disease states, and certain PRMT protein variants are used as prognostic markers for lung and bladder cancers (160,162,163,168). As such, it is critical to understand variant and isoform expression and function of this family of enzymes for development and improvement of diagnostic and therapeutic tools. Previously, through genomic sequencing, a second mRNA variant for PRMT8 was identified (NM_001256536) – one transcribed from an alternate 5’ exon at site 3.38 (Fig. 2.5A). Between mRNA
variants 1 and 2, only exon 1 differs, while exons 2 through 10 are identical. Figure 2.5B illustrates primer locations for both mRNA variants used for sequence determination by 5’ Rapid Amplification of cDNA Ends (RACE) and RT-PCR. Also shown are the four PRMT8 protein isoforms along with their experimentally determined subcellular localizations. To date, only mRNA variant 1 (NM_019854) has been studied, along with the three protein isoforms translated from that variant (116-118). Isoform 1 harbors a myristoylated residue, conferring plasma membrane localization, while isoforms 2 and 3 are truncated at the N-terminus and lack the myristoylation motif, resulting in nuclear localization (118). 5’ RACE was used to reveal which PRMT8 mRNA variants are expressed in both hESCs and iRC cells (Fig. 2.6A-B). 5’ RACE with hESC cDNA produced a band that, when sequenced, was identical to exon 1 of PRMT8 mRNA variant 1, beginning with the 172nd nucleotide. 5’ RACE with iRC cell cDNA produced a band that, when sequenced, was identical to exon 1 of PRMT8 mRNA variant 1, beginning with the 172nd nucleotide.

Figure 2.4 PRMT8 transcript sequence.

(A) Graphic representation of PRMT8 and amplicon location. Boxes represent the 10 exons of PRMT8 where the dashed line in exon 1 represents the lengths of the alternative 5’ exons that differentiate variant 1 from variant 2. The grey line that spans a portion of exons 8-10 represents the amplicon that was sequenced. (B) PRMT8 cDNA from iRC cells was cloned into pLVX at SmaI (CCCGGG) site, and sequenced. The grey line in the sequencing data represents the grey amplicon in (A).
Figure 2.5 Graphic representation of PRMT8 transcript variants, protein isoforms, and localization differences.

(A) Genomic alignment of PRMT8 transcript variants 1 and 2. Numbers represent million base pairs, dashed lines represent introns, and solid vertical lines represent exons. Locations of shRNA sequences are shown with arrows. The red arrows show the location of shRNA #1, which falls at the end of exon 4. The orange arrow shows the location of shRNA #2, which falls in the middle of exon 6. And the yellow arrow shows the location of shRNA #3, which falls in the middle of exon 9. (B) Both PRMT8 mRNA variants are expressed from the PRMT8 gene on chromosome 12. The start of the open reading frame for variant 1 is marked by a green arrow and “v1 ATG.” mRNA variant 1 has 3 alternative translation start sites, responsible for protein isoforms 1-3. mRNA variant 2 is transcribed from an alternative 5` exon and is responsible for translation of isoform 4. Isoform 1 harbors an N-terminal myristoylation motif, represented by the red coil, conferring plasma membrane localization. Isoforms 2 and 3 are truncated at the N-terminus and display nuclear localization. Isoform 4 is a novel variant that has not been explored experimentally. Conserved PRMT core regions are represented in grey, methyltransferase domains are represented in black, and the conserved THW loop is represented in blue. The unique portion of the protein sequence for isoform 4 is represented in orange.
Ch. 2 – Novel PRMT8 isoform is essential for cell proliferation

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sequenced, was identical to exon 1 of PRMT8 mRNA variant 2, beginning with the 1st nucleotide (Fig. 2.6C). In the National Center for Biotechnology Information (NCBI) database, the sequence for PRMT8 variant 2 was predicted by a combination of genomic DNA and transcript sequences. Our cDNA sequence for PRMT8 variant 2 has been curated by NCBI and can be found via the accession number KR014345.

Figure 2.6 PRMT8 variant expression.

(A) Sequence for 5′ RACE of hESCs-PRMT8 compared to sequences in the NCBI database. Asterisks represent base pairs mismatched from the NCBI database. Dashed line represents beginning of alignment with database sequences, which begins with the 172nd nucleotide. Solid lines represent exon-exon junctions. (B) Sequence for 5′ RACE of iRC-PRMT8 compared to sequences in the NCBI database. Asterisks represent base pairs mismatched from the NCBI database, and “N” represents sequencing misreads. Solid lines represent exon-exon junctions. (C) Graphic representation of 5′ RACE data from hESCs and iRC cells compared to variant 1 and variant 2. Solid horizontal lines represent mRNA alignments between treatments and boxes represent exons. Numbers delineate base pairs. (D) Variant-specific PRMT8 transcript expression in control cells, iRC cells, and hESCs using RT-PCR. Actin was used as a loading control.
Forward primers were designed to amplify a region on exon 1 of either variant 1 or variant 2 (Fig. 2.5) to develop variant-specific PCR so that RT-PCR could be used to test PRMT8 variant expression in other cell types (Fig. 2.6D). Variant 2 amplification required semi-nested PCR. Sequences for variant-specific primers are presented in Table 2.1. RT-PCR demonstrated that both control fibroblasts and iRC cells express PRMT8 variant 2 transcript, with control cells expressing low levels, while hESCs express PRMT8 variant 1 transcript, validating the 5` RACE data.

**Table 2.1 DNA primer sequences for variant-specific RT-PCR.**

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<th>Primer</th>
<th>Forward (5<code> to 3</code>)</th>
<th>Reverse (5<code> to 3</code>)</th>
<th>Amplicon (bp)</th>
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<td>GCTCATCCCAATTAGCAAGGT</td>
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<td>PRMT8</td>
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<td>GGTCTCGACATTTTGGCATTTGGCTTCAGG</td>
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<td>GGCATAGGAGCTCAGAATAATCTTC</td>
<td>458</td>
</tr>
<tr>
<td>PRMT8 v2</td>
<td>CTGGTTGAATCGGTGCCAGGTGT</td>
<td>GCCATAGGAGCTCAGAATAATCTTC</td>
<td>240</td>
</tr>
<tr>
<td>PRMT8 v2 nested</td>
<td>TGAATGTGTCAGCTGGAATTGGAG</td>
<td>GCCATAGGAGCTCAGAATAATCTTC</td>
<td>235</td>
</tr>
<tr>
<td>GFP</td>
<td>AGCTGACCTGAAGGTTCCATGTG</td>
<td>CTGCTTGTCAGCCATGATAGAC</td>
<td>350</td>
</tr>
<tr>
<td>Actin</td>
<td>TCTGGCCACACACTCTTACAA</td>
<td>CTCTCCCTTAAATGCACAG</td>
<td>392</td>
</tr>
</tbody>
</table>

**PRMT8 variant 2 is critical for proliferation of human dermal fibroblasts** – The iRC phenotype is, in part, characterized by increased cellular lifespan. Because of this, we next examined whether there is a causal link between increased lifespan and upregulation of PRMT8. PRMT8 was knocked down using custom lentiviral particles containing shRNA constructs designed to target both known mRNA variants of PRMT8 – shRNA vector #1 targets PRMT8 within exon 4, shRNA #2 within exon 6, and shRNA #3 within exon 9. To demonstrate knockdown success and specificity, the glioblastoma line U87MG expressing PRMT8 variant 2 was transduced with each shRNA construct separately, including the scramble control, and cells were imaged and then harvested two days post-transduction for analysis by RT-PCR. Microscopy demonstrated that all treatment groups transduced with the scramble control construct, shRNA #1, #2, and #3, express the GFP reporter, indicating successful transduction in all treatments (Fig. 2.7A). RT-PCR demonstrated successful PRMT8 knockdown, as control and scramble control treatments continue to express PRMT8 variant 2 transcript, while each of the three knockdown treatments show no detectable
Figure 2.7 Demonstration of knockdown specificity.

(A) GFP reporter fluorescence of U87MG glioblastomas on day two post-transduction. Scale bars are 200µm. (B) Transcript expression for PRMT8 variant 2 is compared to PRMT1 in all treatment groups using RT-PCR. Actin and GFP were used as loading controls. Cells were harvested two days post-transduction.

Figure 2.8 Effect of PRMT8 on hDF growth and longevity.

(A) Three independent replicates were performed and cumulative population doublings were measured for cells in all treatment groups. Error bars represent standard deviation. The lightest grey line represents control fibroblasts, the medium grey line represents fibroblasts treated with scramble control particles, and the dark grey line represents fibroblasts treated with PRMT8 shRNA. (B) GFP reporter fluorescence on day 17 (left) and day 45 (right). Scale bars are 200µm.
levels (Fig. 2.7B). Of note is the partial knockdown of PRMT8 observed using RT-PCR in the scramble control treatment for unknown reasons. PRMT1 was used to determine knockdown specificity since PRMT8 and PRMT1 are the most homologous members of the PRMT family, sharing over 80% sequence identity (116-118). Based on preliminary data (data not shown), shRNA construct #2 was the most effective and was selected for use in experiments to determine the effect of PRMT8 on cellular lifespan.

Fibroblasts were thawed at passage 7 and transduced at day 0 (Fig. 2.8A). Puromycin selection pressure was applied for 7 days to all treatment groups except control cells, beginning 3 days after transduction. Three replicates were performed and cumulative population doublings (PDs) were measured and averaged. Control cells reached an average of 13.8 cumulative PDs on day 42 post-transduction, and control cells receiving scrambled shRNA (showing a slight PRMT8 knockdown via RT-PCR in glial cells in Fig. 2.7B) reached an average of 9.79 cumulative PDs after selection recovery on day 42 post-transduction. Reduced viability of cells transduced with scrambled shRNA is likely due to cell type-specific effects of transduction on primary cells. It has long been known that primary cells are notoriously difficult

Figure 2.9 Effect of PRMT8 on glioblastoma growth and longevity.

(A) Three independent replicates were performed and cumulative population doublings were measured and averaged for cells in all treatment groups. Error bars represent standard deviation. The light grey line represents control glioblastomas, the medium grey line represents glioblastomas treated with scramble control particles, and the dark grey line represents glioblastomas treated with PRMT8 shRNA. Measurements for cells in the PRMT8 shRNA treatment group were terminated after day 6 due to complete cell loss. (B) GFP reporter fluorescence on day 1 (left) and day 6 (right).
to transduce due to low efficiency and excessive cell death – an effect not observed with immortalized cells (243). Cells transduced with PRMT8 shRNA reached an average of -1.26 cumulative PDs on day 42 post-transduction with a peak of 0.63 cumulative PDs on day 6 post-transduction. Transduction efficiency was monitored by expression of a GFP reporter and GFP fluorescence was imaged weekly throughout the study. Figure 2.8B shows representative images from each treatment on day 17 post-transduction and day 45 post-transduction.

PRMT8 is critical for proliferation of grade IV glioblastomas – PRMT8 was next knocked down in glioblastomas to determine whether PRMT8 expression is required for proliferation of this highly aggressive cancer. The glioblastoma line U87MG was transduced at day 0 and puromycin selection pressure was applied for 3 days to all treatment groups except control cells beginning 3 days after transduction (Fig. 2.9A). Again, three replicates were performed and cumulative PDs were measured and averaged. Control cells reached an average of 4.89 cumulative PDs on day 16 post-transduction. Cells transduced with scramble control shRNA (showing a slight PRMT8 knockdown via RT-PCR in glial cells in Fig. 2.7B) reached an average of 2.83 cumulative PDs on day 16 post-transduction. No data was recorded for PRMT8 shRNA treated cells on day 16 as all cells within the treatment were dead. The experiment was terminated after day 16 due to complete death in the PRMT8 shRNA treatment group. Cells transduced with PRMT8 shRNA reached an average of -3.73 cumulative PDs on day 6 post-transduction. Transduction efficiency was monitored with a GFP reporter and GFP expression in all treatments from day 1 and day 6 can be seen in Figure 2.9B.

DISCUSSION

Small molecule inhibitors of enzymes that catalyze PTMs have been approved by the Food and Drug Administration (FDA) for treatment of human cancers, and arginine methyltransferases are being hailed as the new enzymes to target for personalized cancer therapeutics (244-247). Limited evidence about PRMT regulation prevents understanding of biological consequences of corruption in their regulatory pathways.
However, what is known underscores the significant role this family of enzymes plays in cell viability and in cancer biology (105,110,121,125,157,160,162,163,168,190,248-252).

While it is understandable that PRMT8 specifically has been understudied because of its relatively recent discovery, and because of early reports implicating brain tissue specificity, due to my data showing its importance to fibroblasts, it can no longer be ignored that PRMT8 does in fact have functional relevance outside the brain. An *in vivo* zebrafish study found that PRMT8 is expressed ubiquitously during early development and is critical for embryonic and neural development, as knockdown of *PRMT8* resulted in early developmental defects in all three germ layers and, in many cases, death (224). This was the first evidence that PRMT8 plays a critical role in development before becoming localized specifically to mature brain tissue. Here, we demonstrate PRMT8 expression in hESCs, the first evidence that PRMT8 may also function in human development. Furthermore, PRMT8 expression is demonstrated in human dermal fibroblast-derived cells, clearly indicating human PRMT8 expression outside of the central nervous system (CNS).

Cell culture is routinely performed at atmospheric oxygen levels (between 19% to 20%) even though physiological levels tend to be much lower (ranging from 0.5% to 10%, depending on tissue type) (58-61). Oxygen concentration was reduced in our model system to more closely match the physiological state. Combined with few *in vivo* PRMT8 studies, the fact that physiological oxygen levels are much lower than what is used for standard cell culture methods, and the fact that brain specifically is a hypoxic tissue (58), may be the cause of why PRMT8 has, until now, not been seen widely outside the CNS. It may actually be that iRC culture conditions are not inducing PRMT8 expression but, rather, that standard culture conditions are repressing its expression.

Our demonstration of increased PRMT8 protein expression with reduced oxygen is not the first indication that hypoxic conditions regulate PRMTs. In a study that analyzed PRMTs 1 through 7 in mouse lung tissue, hypoxia was shown to be a regulator of PRMT2 (253). However, it was noted that PRMT8 was not analyzed alongside other PRMT family members in this study due to its assumed specificity to brain,
highlighting the importance of accepting current literature that has shown PRMT8 to be ubiquitously expressed, at least during development (224).

PRMT family members have variant-specific functions in various cancers, which makes them attractive targets for cancer diagnostics and/or therapeutics. For example, specific splice variants of PRMT1 demonstrate distinct activity and substrate specificity, and have been correlated to tumor grade in breast cancer (163,175,176,189). Nevertheless, our current ability to target these molecules is limited by our lack of understanding regarding expression and regulation of specific PRMT variants and the variant-specific effects they have in cancer cell lines and tumors. The mechanism by which a shift from one isoform to another occurs is not known, although this shift is thought to be important for cancer development and progression. Here we identify a novel PRMT8 variant expressed in cells grown under iRC culture conditions, conditions that lead to increased cellular lifespan without the capacity to form tumors when injected into SCID mice (55,62). Increased understanding about the role of PRMTs in cancer-related changes (i.e. bypassing the Hayflick limit) in a non-tumorigenic system will increase our understanding of PRMT regulation while offering molecular tools for development of cancer treatments and diagnostic tests.

Arguably the most interesting phenotype detailed here is that PRMT8 knockdown leads to a loss of cell proliferation in both non-tumorigenic and tumorigenic cell types. Transductions of hDFs were performed under control conditions, with the plan to transfer to iRC conditions following selection. However, cells in knockdown treatments failed to recover following transduction, indicating that the small amount of PRMT8 present in control hDFs is necessary for proliferation, regardless of culture conditions. While other PRMTs have been robustly linked to cell cycle, this is the first evidence of PRMT8 having a functional role in cell proliferation, suggesting that PRMT8 is more similar to other PRMT family members than initially thought. In human lung fibroblasts, PRMTs 1, 4, and 6 are down-regulated as cells senesce and their expression decreases as p21 increases during senescence (254). In osteosarcoma, breast, bladder, and lung cancer lines, PRMT1 knockdown results in G0/G1 arrest, a common hallmark of senescent cells (157,168,255). In mouse embryonic fibroblasts (MEFs), PRMT6 knockdown increases expression of both p53 and p21 (256,257).
Arginine methyltransferases have remained grossly understudied given their critical functional roles and variant-specific functions in cancer biology. The little that is known about this family of enzymes makes them attractive targets for drug development. Further study of PRMT variant expression and regulation will no doubt reveal critical physiological and pathophysiological mechanisms leading to therapeutic developments. We hypothesize that the mechanism by which PRMT8 influences cell proliferation is through regulation of cell cycle. However, work remains to be done to determine exactly which genes and/or proteins are regulated by PRMT8.
CHAPTER 3

PRMT8 expression decreases as cells senesce but overexpression is not sufficient to induce increased proliferation
ABSTRACT

A better understanding of molecules that control normal and aberrant cell cycle progression will provide insight into the nature of ageing, a process that gains significance as average lifespan increases, while also identifying drug targets for diseases associated with uncontrolled cell division, such as cancer. Post-translational modifications have long been known to control rapid switches necessary for cell cycle checkpoints, and increasingly data are indicating the role arginine methylation plays in this process. Many protein arginine methyltransferases (PRMTs) are necessary for cell cycle progression of non-neoplastic and neoplastic cells, as they function to methylate promoter-associated histones of cell cycle-related genes and the cell cycle proteins themselves. While the relationship between most PRMTs and cell cycle progression has been assessed, the role of PRMT8 in this process until this point has not been explored. Here we investigate expression of the arginine methyltransferase PRMT8 during human dermal fibroblast (hDF) senescence. We show for the first time that PRMT8 expression is significantly reduced by premature induction of replicative senescence using sub-cytotoxic hydrogen peroxide (H$_2$O$_2$) treatment. As such, we next investigated the sufficiency of PRMT8 overexpression to increase cell cycle progression. However, PRMT8 overexpression in hDFs causes no significant change in number of population doublings or amount of time spent in culture prior to senescence and does not alter expression of key cell cycle regulatory genes. Taken together, these results suggest that although the maintenance of PRMT8 expression may be critical for cellular proliferation, the ectopic overexpression of PRMT8 alone is not sufficient to increase cellular lifespan.

INTRODUCTION

While phosphorylation and lysine methylation have remained undoubtedly more studied than arginine methylation, it is now evident that arginine methylation plays and equally significant biological role. It is well established that protein arginine methyltransferases (PRMTs) have critical roles in diverse biological processes ranging from gene expression to signal transduction (105,110,121-126,157,160,168,190,248-252). And this breadth of function has recently expanded, as PRMTs have been
acknowledged for their participation in cell cycle regulation as well as cancerous self-renewal (157,168,255,258).

Methylation of arginine residues within histone tails can promote or prevent transcriptional permissiveness either directly or indirectly by influencing the deposition of other histone marks, specifically methylation of lysine residues (125,259). A variety of histone modifications have been associated with specific PRMTs and are summarized in Table 3.1 (105,148,158,159,208,209,213,219,230,252,260-270).

Table 3.1 Histone modifications by arginine methylation.

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</tr>
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<td>PRMT6</td>
<td>H3R2me2a</td>
<td>Repressive – counteracts H3K4me3 activation</td>
</tr>
<tr>
<td>PRMT6</td>
<td>H3R42me2a</td>
<td>Active</td>
</tr>
<tr>
<td>PRMT6</td>
<td>H4R3me2a</td>
<td>Active</td>
</tr>
<tr>
<td>PRMT6</td>
<td>H3R2me2a</td>
<td>Repressive</td>
</tr>
<tr>
<td>PRMT6</td>
<td>H4R29me2a</td>
<td>Repressive</td>
</tr>
<tr>
<td>PRMT7</td>
<td>H4R3me2s</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

One of the ways in which PRMTs contribute to cell cycle progression is by influencing the transcription of cell cycle-related genes via methylation of promoter-associated histones (106,266,271-274). For example, PRMT4, also known as coactivator-associated arginine methyltransferase 1 (CARM1), asymmetrically dimethylates histone 3 on arginine 17 (H3R17me2a), which is enriched in the promoter region of E2F transcription factor 1 (E2F1), a protein essential for cell cycle control and tumor suppressor activation (150,275). CARM1 also confers the H3R26me2a mark associated with the promoter of Cyclin
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E1, a protein required for the G1/S transition (266). PRMTs also influence cell cycle progression by directly modifying proteins that participate in cell cycle. The double strand break repair protein MRE11 is a substrate of PRMT1, and PRMT1 is necessary for normal cell cycle progression, as knockdown leads to cell cycle checkpoint defects in response to DNA damage (276). In agreement with such findings, PRMTs demonstrate similar effects at the organismal level with increased expression in proliferating tissues. An in vivo rat study demonstrated increased expression and activity of PRMT1 and PRMT5 following a partial hepatectomy, indicating a correlation between tissue proliferation and PRMT expression and activity (277).

While many studies have helped define the role PRMTs play in cell cycle progression and proliferation, none have examined PRMT8 expression changes as cell cycle progresses. It has previously been shown that as cells reach replicative senescence, and as cell cycle slows, expression of PRMTs 1, 4, 5, and 6 decreases, indicating a correlation between PRMT expression and cell cycle progression (254). In light of these publications and our own data demonstrating PRMT8 necessity for cellular proliferation (found in chapter 2 of this dissertation), we chose to investigate the connection between PRMT8 expression and cell cycle progression. For this, we chose human dermal fibroblasts (hDFs) due to their regular use as a model for cellular proliferation and aging. Typically, hDF populations demonstrate a sigmoidal growth curve, rapidly dividing before reaching a period of irreversible growth arrest, a process defined as replicative senescence. Replicative senescence can also be achieved prematurely via addition of the oxidative stressor hydrogen peroxide (H2O2). Senescent phenotypes are commonly defined by morphological cellular changes that can be measured with senescence-associated β-galactosidase (SA-β-gal) activity, increased expression of the cyclin-dependent kinase (CDK) inhibitors p16 and p21, and increased activity of checkpoint inhibitors p53 and Rb (278).

Due to the role PRMTs play in cell cycle progression, an abundance of data exists regarding the effect of PRMT knockdown on cell proliferation, demonstrating the necessity of many of these enzymes for continued proliferation, even in immortal tumorigenic cell lines. Without PRMT5, the breast cancer line MCF7 arrest in G1 (279). And PRMT6 expression is required for proliferation of the osteosarcoma line U2OS, without which cells arrest at the G2 checkpoint (257). Moreover, in many cancer types, such as
breast, prostate, lung, colon, and bladder cancers as well as leukemia, PRMTs are upregulated (162,168,188,189,255,280-287). However, no studies to our knowledge have assessed the effect of PRMT overexpression to determine if upregulation of these enzymes alone is sufficient for increased cellular proliferation. We have previously shown hDFs require PRMT8 for proliferation (found in chapter 2 of this dissertation) and therefore hypothesize that PRMT8 overexpression will lead to increased stimulation of the cell cycle, leading to increased cellular proliferation.

RESULTS

\textit{H}_2\textit{O}_2\textit{ induction of senescence causes decreased PRMT8 expression} – As cells senescence, cell cycle slows. We therefore chose to examine the relationship between PRMT8 expression and cell cycle by mimicking replicative senescence using oxidative stress. Oxidative stress is a well-documented inducer of premature senescence in fibroblasts (288-292). Therefore, we examined the interplay between PRMT8 expression and senescence in hDFs through treatment of CRL-2352s with sub-cytotoxic doses of H\textsubscript{2}O\textsubscript{2} in two independent experiments. hDFs were treated with various concentrations of H\textsubscript{2}O\textsubscript{2}, ranging from 200\textmu M to 800\textmu M. Concentrations of 200\textmu M, 400\textmu M, 600\textmu M, and 800\textmu M H\textsubscript{2}O\textsubscript{2} were all sufficient to induce senescence, confirmed by morphology compared to cells grown without H\textsubscript{2}O\textsubscript{2} and SA-\beta-gal activity (Fig. 3.1A). Senescence was verified molecularly by qRT-PCR using \textit{p21} as a marker, as levels of \textit{p21} are known to increase in an H\textsubscript{2}O\textsubscript{2} concentration-dependent manner (n=2) (Fig. 3.1B). Expression of \textit{PRMT8} was then assessed using qRT-PCR, which demonstrated down-regulation in a H\textsubscript{2}O\textsubscript{2} concentration-dependent manner, except for treatment with 600\textmu M (n=2) (Fig. 3.1B).

\textit{PRMT8 overexpression is not sufficient to increase cellular lifespan} – PRMTs play a clear role in maintenance of cellular proliferation, but, to our knowledge, no one has assessed the sufficiency of PRMTs for induction of increased cellular proliferation. To demonstrate lentiviral transduction success, initially the transduction process was tested in Chinese hamster ovary (CHO-K1) cells. They were transduced with lentiviral particles containing a construct for the open reading frame (ORF) of \textit{PRMT8} mRNA variant 2 (\textit{PRMT8v2}) tagged with a RFP reporter and driven by a cytomegalovirus (CMV) promoter. Cells were
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imaged then harvested six days post-transduction for analysis by RT-PCR and Western blot. Microscopy demonstrated RFP reporter expression in transduced cells, indicating successful transduction (Fig. 3.2A). RT-PCR demonstrated successful PRMT8 overexpression, as transduced cells express increased levels of PRMT8 compared to untransduced cells to levels observed in mouse brain (Fig. 3.2B). PRMT8 expression is also elevated at the protein level in transduced CHO-K1 cells compared to untransduced control cells, as demonstrated by two of three antibodies specific for PRMT8 using Western blot (Fig. 3.2C). Differences in

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**Figure 3.1 Down regulation of PRMT8 expression in H2O2-induced premature senescent fibroblasts.**

(A) Representative images of SA-β-gal activity in hDFs treated with various concentrations of H2O2 (B) Cells in each treatment were harvested after two treatments with H2O2 and levels of PRMT8 were quantified using qRT-PCR. p21 was used as a control senescence marker. All samples were normalized to actin. Fold change was determined using the ΔΔCt method. Error bars represent standard deviation (n=2).

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Figure 3.2 Demonstration of PRMT8 overexpression in CHO-K1s.

(A) RFP reporter fluorescence in CHO-K1s on day six post-transduction. (B) Transcript expression for PRMT8 in control CHO-K1s and CHO-K1s overexpressing PRMT8 using RT-PCR. Mouse brain was used as a positive control. Actin was used as loading control. Cells were harvested six days post-transduction. (C) PRMT8 protein expression in control CHO-K1s and CHO-K1s overexpressing PRMT8 using Western blot. Mouse brain was used as a positive control. Actin was used as a loading control. Cells were harvested six days post-transduction.

protein size between control and OE cells compared to the positive control (mouse brain) are attributed to species differences. Successful transduction in fibroblasts was next verified in the hDF line CRL-2352 using the same PRMT8v2 ORF construct-containing particles. While microscopy indicates integration of the RFP reporter in some cells, the transduction efficiency appears low based on reporter expression alone (Fig. 3.3A). For this reason, additional primer sets were designed and included in RT-PCR analysis for hDF transduction efficiency; primer sequences for overexpression validation can be found in Table 3.2.
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Primer sets that amplify the RFP reporter and the CMV promoter as well as the nucleotide stretch between the end of the CMV promoter and the start of the PRMT8 v2 ORF demonstrate successful PRMT8 transduction and overexpression in hDFs (Fig. 3.3B).

To determine the effect of PRMT8 overexpression on hDF cellular proliferation, fibroblasts were thawed at passage 7 and transduced at day 0, having undergone 5.34 population doublings (PDs) since passage 6. Cell supplier documentation indicates that this line, under standard culture conditions, can reach 33 PDs before senescence. Two independent replicates of cells were transduced then grown until senescence, and population doubling results were measured and averaged (Fig. 3.4A). Cultures of the first replicate were terminated on day 162 post-transduction due to “negative” PDs in both groups indicative of

Table 3.2 RT-PCR primer sequences for PRMT8 overexpression validation.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT8</td>
<td>GACTACGTCCACGCCCTGGTCACCTATTTTATT</td>
<td>GGTCTCGCACATTTTGGCATTTGGCTTCATGG</td>
<td>205</td>
</tr>
<tr>
<td>CMV</td>
<td>GGCTGACCACCGCCTTTGAGGCTTCTCACTACGCTC</td>
<td>CACGTCCTACCCCTTGGCTTGCCTGCTTGTTCATG</td>
<td>448</td>
</tr>
<tr>
<td>CMV-PRMT8</td>
<td>CGCAAATGGGCGCTAGCCGCTGCTGCTTCTCCAACGCAC</td>
<td>GGCATAGGAAGTCTAGAAAATATGCTAGAGGGTTCGCG</td>
<td>373</td>
</tr>
<tr>
<td>RFP</td>
<td>CCAGAACGGCTGCTGACTCATCTACAACGTCTCTTCAATAATGCTACACGACGATCTTTAT</td>
<td>CCGTACCTACCCCTTGGCTTGCCTGCTTGTTCATG</td>
<td>356</td>
</tr>
<tr>
<td>Actin</td>
<td>TCTTGCCACACCCCTTCTTGCACGACGATCTTTAT</td>
<td>CTTCTCCTTAAATGTCACACGACGATCTTTAT</td>
<td>392</td>
</tr>
</tbody>
</table>

Figure 3.3 Demonstration of PRMT8 overexpression in human dermal fibroblasts.

(A) RFP reporter fluorescence in CRL-2352 hDFs on day eleven post-transduction. (B) Transcript expression for PRMT8 in control hDFs and hDFs overexpressing PRMT8 using RT-PCR. Mouse brain was used as a positive control. Actin was used as loading control. Cells were harvested eleven days post-transduction.
Ch. 3 – PRMT8 expression decreases as cells senesce but overexpression is not sufficient to induce increased proliferation

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**Figure 3.4 Effect of PRMT8 overexpression on growth and longevity.**

(A) Cumulative population doublings were measured for hDFs in control (blue line) and overexpression (red line) groups. Two independent replicates were averaged. Cells from replicate one were grown for 162 days and cells from replicate two were grown for 109 days. Error bars represent standard deviation. (B) RFP reporter fluorescence of representative images on day 11 (left) and day 129 (right). Scale bars are 200µm. Representative images from replicate one are shown. (C) RT-PCR was used to determine maintenance of PRMT8 overexpression. PRMT8 as well as RFP and the CMV promoter driving PRMT8 overexpression were assessed throughout the study. Actin was used as a loading control. Representative images from replicate one are shown.
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cell death. For the first replicate, control cells reach an average of 39.6 cumulative PDs after 162 days in culture and PRMT8 overexpression cells reached an average of 41.2 PDs after 162 days in culture, since passage 6. Cultures of the second replicate were terminated on day 109 post-transduction with control cells reaching an average of 36.3 cumulative PDs, and PRMT8 overexpression cells reaching an equivalent average of 36.5 cumulative PDs.

**Figure 3.4B** shows representative microscopy from an early time point (day 11 post-transduction) and a later time point (day 129 post-transduction) from both cultures for morphological comparison. By day 129 post-transduction, some cells in both treatment groups have adopted a large, flat, highly vacuolated morphology typical of senescent cells. As RFP reporter expression was weak, maintenance of *PRMT8v2* construct expression was not monitored by microscopy, but rather by RT-PCR. Aliquots from each treatment were collected every third passage throughout the study and analyzed using RT-PCR. Transduced

![Graph](image_url)

**Figure 3.5 Effect of PRMT8 overexpression on cell cycle-associated gene expression.**

Cell cycle gene expression was quantified using qRT-PCR in hDFs overexpressing PRMT8. All samples were normalized to control matched time points as well as *actin*. Fold change was determined using the ΔΔCt method. Error bars represent standard deviation. All time points except day 126 post-transduction depict two biological replicates.
cells out to day 129 post-transduction maintain elevated expression of PRMT8, CMV, CMV-PRMT8, and RFP by RT-PCR compared to control cells, showing that the lack of difference of control and CMV-PRMT8 cells is not due to transgene loss (Fig. 3.4B). Interestingly, endogenous levels of PRMT8 in control cells appear to decrease over time with aging.

**PRMT8 overexpression does not influence cell cycle gene expression** – We showed previously that cells with increased proliferative capacity demonstrate upregulation of PRMT8 and that PRMT8 is necessary for cellular proliferation (found in chapter 2 of this dissertation). Here, we show that PRMT8 expression decreases as cell cycle slows and cells senesce. To determine if PRMT8 overexpression is sufficient to induce changes in cell cycle gene expression, key cell cycle-related genes were analyzed in control hDFs and hDFs overexpressing PRMT8. The CDK inhibitors p16 and p21, as well as the tumor suppressor p53 were analyzed by qRT-PCR in samples taken at every third passage throughout the study (Fig. 3.5). Fold change was calculated by comparing change in cycle threshold (∆Ct) values between treatment groups (∆∆Ct). As fold change from 1 to -1 represents no change, p16, p21, and p53 show no significant change between control cells and cells overexpressing PRMT8 when grown until senescence. As overexpression samples are normalized to age matched control samples, any changes in cell cycle gene expression expected due to senescence are masked by normalization. Primer sequences for cell cycle-associated genes can be found in Table 3.3.

**Table 3.3 qRT-PCR primer sequences for cell cycle-associated genes.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>CACTGTTCTTGTACCCTTGTGCC</td>
<td>TTCCTCTTTGGAGAAGATCAGCCG</td>
<td>148</td>
</tr>
<tr>
<td>p53</td>
<td>GTGTGGTGTGCCCTGTCTGGGAG</td>
<td>GCTCTCGGAACATCTCGAAGCG</td>
<td>213</td>
</tr>
<tr>
<td>p16</td>
<td>GAGCACTCAGCCCTAAGC</td>
<td>AGTGACTCAAGAGAAGCCAG</td>
<td>139</td>
</tr>
<tr>
<td>Actin</td>
<td>AGAGCTACGAGCTGCCTGAC</td>
<td>GGATGCCACAGGACTCCA</td>
<td>111</td>
</tr>
</tbody>
</table>
DISCUSSION

Previous reports have detailed the importance of PRMT family members, specifically PRMTs 1, 4 (CARM1), 5, and 6, in cell cycle progression and senescence in fibroblasts (254). The necessary participation of PRMTs in critical cellular processes highlights the importance of understanding the physiological impact of all members of this enzymatic family. Since it’s discovery in 2005, PRMT8 has remained largely understudied due to early reports claiming plasma membrane localization and brain specific tissue distribution for this protein (116,117,226). However, more recent findings detail the importance of PRMT8 for critical cellular processes such as zebrafish developmental progression (224).

As fibroblasts replicatively senesce, levels of PRMTs 1, 4 (CARM1), 5, and 6 decrease as senescence markers increase, indicating an interplay between cellular proliferation and expression of these specific PRMTs (254). Moreover, H$_2$O$_2$-induced senescence causes global decreases in levels of asymmetric arginine methylation, deposited by type I arginine methyltransferases (PRMTs 1, 2, 3, 4 (CARM1), 6, and 8), while levels of global symmetric arginine methylation, deposited by type II arginine methyltransferases (PRMTs 5, 7, and 9), increased in a H$_2$O$_2$ concentration-dependent manner (254). The same observation regarding global arginine methylation deposition has also been described in endothelial cells treated with the more robust oxidants peroxynitrite and glycated collagen (293). We report here that PRMT8 levels also decrease in a concentration-dependent manner during H$_2$O$_2$-induced senescence in hDFs, indicating a potential relationship between PRMT8 expression and cellular proliferation. As PRMT8 is a type I arginine methyltransferase, this data alludes to the possible contribution of PRMT8 to loss of asymmetric arginine methylation as cells senesce.

Knockdown of PRMTs in many cell lines is sufficient to prevent maintenance of cellular proliferation, even in cell lines where cell cycle progression is especially robust, such as in cancers (257,279). We recently demonstrated that knockdown of PRMT8 in the hDF line CRL-2352 as well as the glioblastoma line U87MG leads to a loss of cellular proliferation (found in chapter 2 of this dissertation). However, we show here that overexpression of PRMT8 alone in hDFs is not sufficient to induce increased cellular proliferation, amount of time spent in culture, or expression of key cell cycle-related genes. This
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data pays homage to the inalienable fact that evolution has no doubt developed in such a way that allows for prevention of cell proliferation more readily than acquisition of increased proliferation as a checkpoint for neoplastic transformation. However, it is possible that PRMT8 overexpression causes some phenotypic change not observed by the limited assays performed here; extension of phenotypic characterization would be necessary to establish the significance of exogenous PRMT8 upregulation.
CHAPTER 4

PRMT8 binds high molecular weight fibroblast growth factor 2 in human dermal fibroblasts and demonstrates bivalent promoter occupancy
ABSTRACT

Oxygen tension has demonstrated roles in regulation of gene expression (294). DNA motifs that allow for this regulation via oxygen tension, hypoxia response elements (HREs), regulate gene expression via binding of hypoxia inducible factors (HIFs) to mediate the cellular response to reduced oxygen. And while protein arginine methyltransferases (PRMTs) are regulators of many critical biological processes, surprisingly little is known regarding the regulation of these key enzymes. We demonstrate here that PRMT8 expression is regulated by reduced oxygen in human dermal fibroblasts (hDFs), and analyze transcriptional permissiveness allowing for PRMT8 upregulation by examining the relationship between HREs within the PRMT8 promoter and histone occupancy. Mapping of HREs within the PRMT8 promoter demonstrates no difference between the number of HREs in PRMT8 and a gene known to not be regulated by oxygen, histone deacetylase 3 (HDAC3). However, using dimethylated histone 3 lysine 9 (H3K9me2) as a representative repressive mark and acetylated histone 4 (H4ac) as a representative active mark, we show a bivalent occupancy of the PRMT8 promoter and 5' untranslated region (UTR) regardless of culture conditions. We have previously shown that PRMT8 is necessary for cellular proliferation (found in chapter 2 of this dissertation). The potential mechanism by which this occurs is investigated here. Using co-immunoprecipitation and mass spectrometry, we show that PRMT8 binds specific high molecular weight (HMW) isoforms of the fibroblast growth factor FGF2. Taken together these data indicate that the PRMT8 promoter maintains bivalent histone occupancy regardless of culture conditions to participate in maintenance of cellular plasticity observed in hDFs, potentially through interaction with HMW FGF2, to regulate cellular proliferation.

INTRODUCTION

Many biological processes regulated by arginine methylation are well described (121-126), but little is known regarding regulation of protein arginine methyltransferases (PRMTs) themselves. We have previously shown that expression of the arginine methyltransferase PRMT8 is regulated by culture conditions in human dermal fibroblasts (hDFs), specifically via reduction in oxygen concentration and via
supplementation with basic fibroblast growth factor (FGF2) (found in chapter 2 of this dissertation), conditions that lead to increased plasticity and a pro-regenerative phenotype referred to as induced regeneration competence (iRC) (55,62,295). Until now, however, we have yet to explore the cause of transcriptional permissiveness of PRMT8 by changes in culture conditions.

*In vitro* environmental cues such as FGF2 addition and reduction in oxygen concentration have been shown to influence cellular phenotype in other systems. FGF2 is a well studied growth factor with roles in limb development, wound healing, tumor growth, cell survival, and mitogenesis (296). FGF2 has five protein isoforms translated from a single mRNA. The low molecular weight (LMW) 18kD isoform is a critical component of stem cell cultures; it is a mitogen required for maintenance of pluripotency (53,54). The functions of the four high molecular weight (HMW) 34kD, 24kD, 22.5kD, and 22kD isoforms are not fully understood, although it is known that they participate in intracrine signaling and regulation of cell growth and behavior (296). While HMW FGF2 translocates to the nucleus, LMW mostly remains cytoplasmic and acts through receptor tyrosine kinase (RTK) signaling pathways. In iRC cells, the addition of LMW FGF2 to reduced oxygen conditions induces expression of specific stem cell-associated genes (*OCT4*, *SOX2*, *NANOG*, *LIN28*, and *REX1*) by day 7 (55).

Reduction in oxygen concentration has been shown to increase cellular lifespan and to regulate epigenetic changes (56,57). Molecular changes induced by oxygen concentration are carried out by hypoxia inducible factors (HIFs), transcription factors that bind to hypoxia response elements (HRE) within promoters of target genes to mediate the cellular response to changes in oxygen concentration (297-302). Only five nucleotides long, the HRE motif is found promiscuously throughout the genome. While we show here enrichment for this sequence within the PRMT8 promoter compared to that which would be expected by uniform probabilistic distribution of HREs throughout the genome, this “enrichment” is no greater than what is present in a gene whose expression is not controlled by hypoxia, histone deacetylase 3 (HDAC3). We therefore assessed histone occupancy of the PRMT8 promoter using representative active and repressive histone marks to gain insight into the regulation of PRMT8 expression.
The main mechanisms of transcriptional regulation are 1) binding of transcription factors that cause activation or repression, 2) influence from enhancers and silencers that sit up or downstream of the gene and, 3) DNA methylation or histone modifications at or near gene promoters (303-306). For either transcription factors or enhancers/silencers to access the promoter of a gene, the DNA must be exposed and free of protein occupation. Chromatin exposure is a highly dynamic regulatory process directed by modification of amino acids within histone tails. Generally, most attention is paid to acetylation (Ac) and methylation (Me), although other modifications also influence transcription (such as phosphorylation, ubiquitination, and sumoylation) (307-312). It is generally accepted that acetylation correlates positively with transcriptional activity, while different methylation sites correlate either positively or negatively with transcriptional activity (Table 4.1) (231,305). Similarly, most attention is paid to methylation of lysine (K) residues within histone tails, although modifications to other residues (such as methylation of arginine residues) also influences transcription (105).

<table>
<thead>
<tr>
<th>Active</th>
<th>Repressive</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4</td>
<td>H3K9</td>
</tr>
<tr>
<td>H3K36</td>
<td>H3K27</td>
</tr>
<tr>
<td>H3K79</td>
<td>H4K40</td>
</tr>
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</table>

We have previously shown that PRMT8 is necessary for cellular proliferation of both hDFs as well as tumorigenic glioblastomas (found in chapter 2 of this dissertation). Here we explore the potential mechanism by which PRMT8 may be influencing cellular lifespan by assessing PRMT8 binding partners. It is well-documented that most PRMT family members preferentially methylate glycine arginine rich (GAR) motifs with arginine-glycine-glycine (RGG) repeats (128,131,313). Currently, no binding partners or substrates of endogenous PRMT8 have been described in any cell type. Studies to identify binding partners and substrates have only done so with full-length, overexpressed PRMT8 (116,227,228), a myristoylated isoform with plasma membrane localization – an isoform we have demonstrated is not the
endogenous isoform expressed in hDFs (found in chapter 2 of this dissertation). Four substrates of full-length overexpressed PRMT8 have been identified: histone H4 (116), Ewing sarcoma (EWS) (227,228), Dishevelled3 (Dvl3) (314), and Nucleosome assembly protein 3 (NPL3) (116). Along with the enzymes themselves, protein isoforms of PRMTs also exhibit substrate specificity. PRMT1, the most structurally homologous PRMT family member to PRMT8, has seven described isoforms that each preferentially methylate different substrates (163). This suggests overexpression studies done with full-length PRMT8 may have erroneously identified PRMT8 interaction partners and substrates, as the endogenous isoform has since been reported to be truncated at the N-terminus, localizing to cell nuclei (118).

RESULTS

PRMT8 is upregulated by culture under reduced oxygen tension – We have previously demonstrated upregulation of PRMT8 in hDFs via iRC culture conditions, specifically with reduced oxygen concentration and FGF2 supplementation (found in chapter 2 of this dissertation). Here we analyze which component of the iRC cell system, oxygen or FGF2, causes PRMT8 upregulation. Western blot analysis suggests PRMT8 upregulation by reduced oxygen when compared to cells grown under control conditions (Fig. 4.1).

HREs are likely not controlling PRMT8 expression – As the sequence for HREs is only five nucleotides in length (298,299), it is present quite promiscuously throughout the genome. Because of this we calculated the frequency at which HREs are probabilistically expected to be present within PRMT8 as
well as the frequency at which they are actually present, allowing us to determine if HREs are enriched in the *PRMT8* gene and predicted promoter. The frequency with which the sequence coding for HREs ([A/G]CGTG) is expected to be present within the genome was determined by multiplying the frequency with which each nucleotide is expected to be present within the genome. However, as the HRE motif contains a CG dinucleotide, and this combination is underrepresented by 79%, the phenomenon known as “CG suppression” must be considered (315). The frequency with which a typical nucleotide is expected to be present is once in every four nucleotides (¼), however the frequency at which the CG dinucleotide is expected to be present is 21 times in every 1600 nucleotides (¼ multiplied by ¼ multiplied by 0.21). Therefore, the frequency HREs are expected to be present within the genome is approximately 21 times out of 51,200 nucleotides. The promoter of *PRMT8* has not been defined experimentally. We therefore analyzed HREs that fall between the start of the predicted *PRMT8* promoter, which falls 700 base pairs upstream and 300 base pairs downstream of the transcription start site, and the start of exon 1 of variant 2. As the genomic region between the start of the predicted promoter and the first exon of *PRMT8* variant 2 is 20,530 nucleotides long, 8.4 HREs would be expected to be present. In reality, we actually observe 30 HREs, 3.6 times more than expected, indicating enrichment of the HRE motif within the region between the start of the predicted *PRMT8* promoter and the first exon of variant 2 relative to a purely probabilistic assumption. However, analysis of a gene whose expression qRT-PCR suggests is not controlled by hypoxia (data not shown), HDAC3, also demonstrates HRE “enrichment.” Using the same calculations, the HDAC3 promoter demonstrates 3.2-fold enrichment of HREs relative to a purely probabilistic assumption. Therefore HREs do not appear to be a regulatory element controlling PRMT8 expression under hypoxic conditions.

*PRMT8 promoter demonstrates bivalent histone occupancy* – Given the contribution of reduced oxygen concentration to PRMT8 expression and the enrichment of HREs in the *PRMT8* promoter, mediators of the hypoxic response were examined. HREs were mapped within the PRMT8 genomic sequence (Fig. 4.2). Primers were designed to tile HREs within the PRMT8 promoter and 5’ UTR (Fig. 4.3A and Table 4.2). Validation of all primer sets was first performed using human genomic DNA from
Ch. 4 – PRMT8 binds HMW FGF2 and demonstrates bivalent promoter occupancy

**Figure 4.2 Hypoxia response elements within genomic PRMT8 sequence.**

PRMT8 mRNA variant 1 (v1) and variant 2 (v2) are aligned with genomic coordinates of chromosome 12. Exons are represented by black vertical bars, introns by dashed horizontal lines, and HREs by blue vertical lines. The promoter is predicted to fall between 700 base pairs upstream and 300 base pairs downstream of the hypothesized transcription start site. The expanded genomic region within the inset highlights HREs that fall between the start of the predicted promoter and the start of exon 1 of variant 2. Bowtie arrows represent primer sets used to tile the promoter region.

whole blood (**Fig. 4.3B**). Any primer set resulting in less than optimal signal (sets 1, 5, 8 and 13) was excluded from chromatin immunoprecipitation (ChIP) analysis.

ChIP was performed using a representative repressive (H3K9me2) and a representative active (H4ac) mark with both control and iRC cells. RT-PCR was performed using validated primers on ChIP samples as well as input controls (**Fig. 4.4A**). All validated primer sets amplified bands of the expected size in input samples from control and iRC cells for both H3K9me2 and H4ac. ChIP of H3K9me2 from control cells demonstrates occupancy at all sites, except those amplified by primer set 2 (**Fig. 4.4B**). ChIP of H4ac from control cells demonstrates occupancy at all sites tested, indicating bivalent occupancy at all amplified sites except those amplified by primer set 2, which only demonstrates occupation by the active
H4ac mark. ChIP of H3K9me2 from iRC cells demonstrates occupancy at all sites analyzed with validated primers. ChIP of H4ac from iRC cells demonstrates occupancy at sites 3’ of and including primer set 6 (sets 6, 7, 9, 10, 11 and 12). This data indicates bivalent occupancy of the 3’ region of the PRMT8 promoter and repressive occupancy by H3K9me2 of the 5’ region of the PRMT8 promoter in iRC cells. For control cells, 5 HREs demonstrate bivalent occupancy and 3 HREs demonstrate active occupancy by H4ac, while for iRC cells, 4 HREs demonstrate bivalent occupancy and 4 HREs demonstrate occupancy by the repressive H3K9me2 mark.
Figure 4.4 ChIP for H4ac and H3K9me2 within the *PRMT8* promoter region.

(A) RT-PCR of ChIP samples from control and iRC cells with H3K9me2 (left) and H4ac (right) antibodies using primer sets mapping HREs of the *PRMT8* promoter region, detailed above. Input levels (control – first row; iRC – third row) are compared to ChIP levels (control – second row; iRC – forth row) between respective samples. (B) Graphical summary of ChIP pulldown in control and iRC cells with H3K9me2 and H4ac antibodies. Genomic DNA is represented by the black horizontal line, HREs by blue vertical lines, the transcription start site by the green arrow, and primer sets by bowtie arrows. Occupation of specific genomic regions by the H4ac mark is delineated by the presence of green circles, occupation by the H3K9me2 mark by red circles, and bivalent occupation by both H4ac and H3K9me2 marks by yellow circles.
**Figure 4.5 PRMT8 binding partners via co-IP.**

PRMT8 was pulled down from control and iRC fibroblasts as well as U87MG glioblastomas. iRC cells without adding antibody were used as a negative control. Western blot was performed for PRMT8 to confirm successful pull-down (A) and for FGF2 (B) and HIF1α (C) to assess binding. Signal in the IP lanes is compared to signal of the respective sample input and supernatant (Sup). W1 is the first wash of the IP.

**PRMT8 binds the 22.5kD and 22kD isoforms of FGF2** – We next examined the ability of PRMT8 to bind key molecules that regulate phenotypic changes resulting from iRC culture conditions, namely HIF1α, the primary regulator of the adaptive response to hypoxia, and FGF2, a mitogen supplemented in iRC cell media. PRMT8 was immunoprecipitated from control cells, iRC cells, and U87MG glioblastoma cells (a cell type shown to require PRMT8 for proliferation (found in chapter 2 of this dissertation)) and samples were blotted for HIF1α or FGF2. HIF1α was not found to be bound by PRMT8 in any cell type tested, but is present in both control and iRC cells, as expression of the 93kD protein is present in the supernatant in both cell types (Fig. 4.5A). HIF1α appears to not be expressed by the glioblastoma cell line tested. The FGF2 antibody detects all 5 protein isoforms, the HMW 34kD, 24kD, 22.5kD, and 22kD as well as the LMW 18kD isoforms. Purified 18kD FGF2 was used as a positive control (Fig. 4.5B). While all isoforms except the 34kD are expressed in control cells, PRMT8 only binds the 22.5kD and 22kD isoforms, as evidenced by presence of these isoforms in the IP lanes. The 24kD and 18kD isoforms...
appear in the input and supernatant, while the 22.5kD and 22kD isoforms appear in the supernatant but not the input of control cells. PRMT8 also binds the 22.5kD and 22kD FGF2 isoforms in iRC cells, also as evidenced by presence of these isoforms in the IP lane. However, iRC cells appear to express neither the 34kD nor the 24kD isoforms in either the input or supernatant samples. The 18kD isoform, as in control cells, can be found in both the input and supernatant, while the 22.5kD and 22kD isoforms are found in the supernatant but not the input of iRC cells. The U87MG glioblastomas appear to not express any FGF2 isoforms. Binding of the 22.5kD FGF2 isoform in control cells was validated using mass spectrometry (data not shown).

DISCUSSION

Our earlier discussed findings that PRMT8 expression changes as a result of culture conditions did not explore which factor(s) of the iRC culture system contribute(s) to PRMT8 upregulation – supplementation with FGF2 or reduction in oxygen concentration. Here we show using Western blot that culture in reduced oxygen concentration is the primary cause of PRMT8 upregulation. For this reason, histone occupancy of HREs within the PRMT8 promoter and 5’ UTR were examined as a possible regulatory mechanism underlying PRMT8 upregulation in iRC cells. Even though hypoxia is the primary cause of PRMT8 upregulation, we chose to analyze histone occupancy of the PRMT8 promoter in iRC cells instead of fibroblasts simply grown under hypoxic conditions due to phenotypic shifts seen specifically under iRC conditions and not with reduced oxygen alone, namely increased expression of specific stem cell-associate genes and wound healing participation in a skeletal muscle defect (55,62).

Although HREs initially appear to be enriched within the PRMT8 promoter region, examination of the HDAC3 promoter, a gene whose expression is suggested to not be controlled by hypoxia, revealed a similar level of HRE “enrichment” relative to purely probabilistic assumption. Thus, HRE motifs are likely not the regulatory element responsible for PRMT8 upregulation under hypoxic conditions. Also, the representative repressive and active histone marks assessed here appear to show no correlation between PRMT8 expression and histone occupancy at HREs. While PRMT8 is strongly expressed in iRC
cells compared to control cells, the assumed activation mark (H4ac) assessed here does not show a stronger association with HREs of PRMT8 in iRC cells compared to HREs of PRMT8 in control cells. However, this analysis makes no assumptions about the occupancy of other histone marks also associated with transcriptional permissiveness or repression, such as those detailed in Table 1. Moreover, a majority of the PRMT8 promoter in both control and iRC cells is represented by occupancy of both the repressive H3K9me2 and the active H4ac marks, indicating that perhaps PRMT8 maintains a poised, bivalent state to maintain transcriptional readiness regardless of culture conditions. Bivalent domains refer to histone marks associated with opposite transcriptional outcomes within a single promoter, with some classically active as well as repressive marks. Bivalence allows for genes to maintain a transitory state poised between activation and repression allowing cells to rapidly change expression levels of certain genes (316). Typically, bivalent promoters have been associated with stem cell differentiation and cellular plasticity, but they have more recently been described in cell types with more limited plasticity (317,318). It is also noteworthy that portions of the genomic region between the promoter and first exon of variant 2 in both control and iRC cells maintains bivalence, while portions of the genomic region between the promoter and first exon of variant 2 in iRC cells displays occupation by a repressive mark even though PRMT8 under these conditions is highly expressed. This could indicate that histone occupancy of portions of the genomic region between the promoter and first exon of variant 2 does not dictate expression, or that other marks not examined here are important.

We have previously shown that PRMT8 is necessary for cellular proliferation in hDFs (found in chapter 2 of this dissertation). To elucidate the potential mechanism of PRMT8 involvement in cellular lifespan we assessed physical interaction between PRMT8 and HIF1α as well as between PRMT8 and FGF2, two essential mediators of the iRC cell phenotype. PRMT1 and PRMT5 have both been shown to be essential components of the HIF signaling pathway (319,320). However, we show here that PRMT8 does not bind HIF1α under either control or iRC culture conditions, indicating that regulation of the iRC phenotype likely does not occur via a PRMT8-HIF1α interaction. Given the propensity of PRMTs to methylate GAR motifs and the presence of eight GAR motifs within the 22.5kD FGF2 isoform and seven
within the 22kD isoform of FGF2, it may be expected that PRMT8 would bind to these isoforms for the prospect of methylation. It was shown previously that methylation of the HMW FGF2 isoforms is necessary for nuclear localization (321), that the 22.5kD and 22kD isoforms contain five dimethylated arginine residues, that PRMT1 methylates HMW FGF2 (though the specific FGF2 isoform methylated is not clear (322)), and that PRMT5 methylates the 22.5kD isoform (323). Here we show interaction between PRMT8 and the 22.5kD and 22kD FGF2 isoforms under both control and iRC conditions, allowing for the possibility that PRMT8 regulates lifespan and/or other cellular processes via interaction with and potential methylation of FGF2. However the significance of PRMT8 binding specific isoforms of FGF2 remains to be explored.

Interestingly, the co-IP data indicates that FGF2 appears to be less abundant in iRC cells compared to control cells, even though FGF2 is a supplemented component of iRC cell media. However, this is in agreement with literature that details the down regulation of HMW FGF2 isoforms by supplementation with the LMW FGF2 isoform via regulation by its antisense RNA Nudix6 (324-327).

Our demonstration that PRMT8 binds specific HMW FGF2 isoforms not only adds to the field a novel binding partner of a key mitogenic protein, but it also reports for the first time a binding partner for endogenous PRMT8. This finding gives potential mechanistic insight into how PRMT8 may help to regulate cell proliferation. Further exploration of the relationship between FGF2 and PRMT8 will help to elucidate how this arginine methyltransferase plays a critical role in proliferation and why only specific HMW FGF2 isoforms are bound.
CHAPTER 5

*PRMT8* variant 2 is a feasible biomarker candidate for glioblastomas
ABSTRACT

Identification of regulators of molecular mechanisms necessary for oncogenic transformation will contribute to our understanding of cancer development as well as aid in discovery of new biomarkers and therapeutic strategies. It is well accepted that epigenetic mechanisms participate in the transition from a normal to a neoplastic cell phenotype. For this reason, emphasis in cancer diagnostics and therapeutics research has been placed on discovering biomarkers and designing drugs that recognize, target, or inhibit epigenetic regulators and modifications. As the capabilities of precision medicine increase and as the number of cancer therapeutics grows, discovery of biomarkers for tracking disease development, progression, and remission is essential. The similarities between iRC cells and cells in a pre-neoplastic state (increased lifespan while lacking tumorigenesis) led us to hypothesize that iRC cells can be used as a tool to identify cancer biomarkers. Here we describe the utility of the arginine methyltransferase PRMT8 as a cancer biomarker by interpreting publically available data in light of our own observations. The Human Protein Atlas (328-332) shows high PRMT8 expression in 72.3% of cancers tested. cBioPortal (333,334) for Cancer Genomics has curated studies comparing PRMT8 expression between tumor and control tissues, which demonstrates amplified expression in a variety of cancers – specifically, PRMT8 demonstrates amplified expression in all glioblastoma studies. Most notably, KM Plotter (335) significantly correlates PRMT8 expression to patient survival in 3 of 4 tested cancer types (breast, ovarian, and gastric). Furthermore, we detail the importance of understanding variant-specific expression of PRMTs in cancer and demonstrate expression of a unique PRMT8 mRNA variant in a tumorigenic glioblastoma cell line (U87MG). Taken together, these data suggest that PRMT8 is a viable candidate for further study as a prognostic cancer biomarker, specifically for brain cancer.

INTRODUCTION

In 1942, the concept of epigenetics was introduced by Conrad Waddington, who famously proposed that cells are like a ball rolling down a valley, with epigenetics guiding cells unidirectionally toward a single developmental fate (336). In 1958, John Gurdon dispelled this theory, demonstrating the intrinsic
plasticity of cells by cloning a frog using somatic cell nuclear transfer (6). The field has since reimagined Waddington’s view as a pinball machine (337) – where cell fate is malleable and can be redirected by moving the proverbial ball back up the hills of the epigenetic landscape, and down a different path, committing those cells to a different developmental lineage. Aptly, maintenance of innate chromatin modification and regulation during dedifferentiation or transdifferentiation is required for prevention of oncogenic transformation.

Modifiers and regulators of epigenetic mechanisms, such as DNA methylation and histone modifications, are highly dysregulated in various cancers. DNA methylation, perhaps the most studied chromatin modification, demonstrates global hypomethylation in cancerous cells and tissues (81-85). Histone modifications are also perturbed in cancer. Global lysine acetylation on both histone (281,338) and non-histone proteins (339) tends to be much higher in malignancies, making acetyltransferase inhibitors attractive therapeutic targets. Along with lysine methylation, arginine methylation and protein arginine methyltransferases (PRMTs) as a family of enzymes tend to be highly dysregulated in various cancers with aberrant expression so far characterized in breast, prostate, lung, colon, and bladder cancers as well as in leukemia (162,168,188,189,255,280-287). Specifically, PRMTs demonstrate amplified expression in tumor tissues compared to tissue-matched controls. Increased PRMT1 expression has been linked to cancerous self-renewal and reduced expression leads to loss of viability of bone, breast, bladder, liver, and lung cancer cell lines (157,161,255,258), highlighting the potential this family of enzymes may have as biomarkers or therapeutic targets.

Not only can expression of PRMTs themselves be used to provide new targets for cancer prevention and treatment, but PRMT substrates and the methylation status deposited by PRMTs can also be tracked for potential therapeutic gain. The methylation status of histone 4 arginine 3 (H4R3) deposited by PRMT1 positively correlates with prostate tumor grade and is currently being used as a biomarker for prostate cancer patients (230,281,340).

While it is clear that aberrant PRMT expression plays a role in various disease states (Table 1), it appears that alternatively spliced PRMT variants do as well, adding additional layers of complexity to
such diseases. Currently, specific PRMT protein isoforms are being used as prognostic markers of lung and bladder cancers (160,162,163,168). PRMT1 has seven distinct isoforms displaying tissue-specific expression with unique substrate specificity and functional effects (163,174-177). Furthermore, certain isoforms are hypothesized to play individual roles in cancers, as expression is elevated in tumor tissue compared to normal tissue and in certain instances has been correlated to cancer progression (163,176,189). For this reason, we explore variant-specific PRMT8 expression in several cancer cell lines.

Table 5.1 Dysregulation of PRMT family members in cancer.

<table>
<thead>
<tr>
<th>PRMT</th>
<th>Cancer type</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT1</td>
<td>Breast, lung, colon, bladder, prostate, leukemia</td>
<td>Invasion, cell proliferation, cell survival, transformation and resistance to DNA damage</td>
<td>(162,163,168,188,189,255,281-284,341,342)</td>
</tr>
<tr>
<td>PRMT2</td>
<td>Breast</td>
<td>Invasion, cell proliferation and ERα status</td>
<td>(181,343)</td>
</tr>
<tr>
<td>PRMT3</td>
<td>Breast</td>
<td>Loss of DAL1 and cell survival</td>
<td>(344-347)</td>
</tr>
<tr>
<td>CARM1</td>
<td>Breast, prostate, colorectal</td>
<td>Cell proliferation</td>
<td>(150,266,285,286,348,349)</td>
</tr>
<tr>
<td>PRMT5</td>
<td>Lung, leukemia, lymphatic, melanoma, gastric, colorectal</td>
<td>Increased activity in tumor cells, invasion, cell proliferation, transformation and resistance to DNA damage</td>
<td>(252,287,350-355)</td>
</tr>
<tr>
<td>PRMT6</td>
<td>Lung, bladder</td>
<td>Cell proliferation</td>
<td>(168)</td>
</tr>
<tr>
<td>PRMT7</td>
<td>Breast</td>
<td>Resistance to DNA damage and metastasis</td>
<td>(166,168,356-358)</td>
</tr>
<tr>
<td>PRMT8</td>
<td>Ovarian, skin, colorectal</td>
<td>Cause of somatic mutations</td>
<td>COSMIC database</td>
</tr>
<tr>
<td>PRMT9</td>
<td>ND</td>
<td>ND</td>
<td>ND=not determined</td>
</tr>
</tbody>
</table>

RESULTS

PRMT8 is expressed in various cancers – To explore the possibility of PRMT8 being used as a cancer biomarker, the cancer proteome, as curated by The Human Protein Atlas (328-332), was assessed for PRMT8 expression. Protein expression was measured using immunohistochemical staining in patient-derived primary cancer tissue samples. The Human Protein Atlas analyzed 216 independent cancer samples from 20 of the most common cancers for 16,613 genes. PRMT8 expression was evaluated using a single primary antibody. Of all human cancers tested, PRMT8 expression was detected in 98.5% of the tissue samples (Fig. 5.1A). And, PRMT8 is highly expressed in 72.3%, expressed at moderate-levels in
23.8%, and at low-levels in 2.4%. **Figure 5.1B** shows PRMT8 expression levels categorized by cancer type. PRMT8 is highly expressed in breast, glandular, cervical, head and neck, prostate, and thyroid.

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**Figure 5.1** Histological PRMT8 expression in cancers.

(A) Overall PRMT8 expression intensity in all cancer types tested represented as percentages of total cancer cases. Dark blue represents high PRMT8 expression (72.3% of cases). Blue represents medium PRMT8 expression (23.8% of cases). Light blue represents low PRMT expression (2.4% of cases). White represents PRMT8 expression at undetectable levels (1.5% of cases). (B) PRMT8 expression intensity in specified cancers represented by number of patients with varying PRMT8 expression. Cancer type is listed on the far left, histological antibody staining correlated to PRMT8 expression level is represented by colored bars, and specific patient numbers with varying PRMT8 expression levels are listed on the right. Dark blue represents high PRMT8 expression, blue represents medium PRMT8 expression, light blue represents low PRMT8 expression, and white represents PRMT8 expression at undetectable levels. Source: http://www.proteinatlas.org/.

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Note: The text above includes a table and a bar chart illustrating the distribution of PRMT8 expression across different cancer types, with varying levels of staining intensity represented by different colors and the number of patients at each expression level indicated.
cancers. PRMT8 is expressed at moderate- to high-levels in colorectal, endometrial, brain, lung, ovarian, pancreatic, skin, testicular, and urothelial cancers. PRMT8 is expressed at low- to high-levels in renal and stomach cancers. And PRMT8 expression ranges from undetectable to high or undetectable to moderate in liver and lymphatic cancers, respectively.

**PRMT8 is differentially expressed in various cancers** – For consideration of PRMT8 as a cancer biomarker, expression levels of PRMT8 or one of its variants must differ between cancer tissue and normal tissue. To determine how PRMT8 expression in various primary cancer tissues compares to tissue-matched controls, The Cancer Genome Atlas (TCGA) was mined for PRMT8 expression using cBio Portal for Cancer Genomics (cBio) (333,334). cBio pools results from 91 genomic cancer studies in a single, publicly available interactive infrastructure for the purpose of validation and discovery research. A cross-cancer query was performed to assess alteration frequencies and mutation data for PRMT8 across all cancer types curated by cBio. Each bar on the graph (Fig. 5.2) represents an independent genome-wide study where PRMT8 expression in the specified cancer tissue is normalized to expression in healthy, patient-matched control tissue. Studies showing no change in PRMT8 expression were excluded from the graph. Each study, and therefore each bar of the graph, focuses on a specific type of cancer and contains mutation data, copy number alteration (CNA) data, or both, as detailed on the x-axis. Of the 49 studies demonstrating altered PRMT8 frequency, 47 contain mutation data and 38 of those studies show PRMT8 to be mutated in specified cancers. Of the 49 studies demonstrating altered PRMT8 frequency, 39 contain CNA data and 12 of those studies show PRMT8 deletion, while 33 of those studies show PRMT8 amplification in specified cancers. Focus here is called to 5 specific types of cancers (breast, lung, ovarian, colorectal, and brain) due to the prevalence of those particular cancer types within the population as well as inclusion of PRMT8 expression for these cancer types in other databases, to be discussed later. **Table 5.2** lists alteration frequency values for all breast, lung, ovarian, colorectal, and brain cancer data sets, presented in Figure 5.2.
Figure 5.2 Aberrant PRMT8 expression in various cancer studies (from previous page).

Histogram of genomic data regarding the frequency of PRMT8 expression changes in various cancer studies. Alteration frequency, plotted on the y-axis, shows the percentage of cases from each study in which PRMT8 demonstrates some type of aberrant expression. Various genomic cancer studies are shown on the x-axis where each bar represents an individual cancer study and each cancer type is assigned a unique color. The x-axis also details which data sets have mutation data or copy number alteration (CNA) data with plus (+) or minus (-) symbols. Types of aberrant PRMT8 expression are represented by different color bars, where green delineates genomic PRMT8 mutations, blue represents genomic PRMT8 deletions, red represents genomic PRMT8 amplifications, and grey represents multiple alterations of genomic PRMT8. Source: http://www.cbioportal.org.

Table 5.2 Aberrant PRMT8 expression statistics for specific cancers grouped by type.

<table>
<thead>
<tr>
<th>Study abbreviation</th>
<th>Study name</th>
<th>Number of altered cases</th>
<th>Total number of cases</th>
<th>Percent of cases altered</th>
</tr>
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<tbody>
<tr>
<td>Breast cancer studies:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast (BCCRC Xenograft)</td>
<td>Breast cancer patient xenografts (British Columbia, Nature 2014)</td>
<td>6</td>
<td>29</td>
<td>20.7%</td>
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<tr>
<td>Breast (TCGA)</td>
<td>Breast Invasive Carcinoma (TCGA, Provisional)</td>
<td>38</td>
<td>962</td>
<td>4.0%</td>
</tr>
<tr>
<td>Breast (TCGA pub)</td>
<td>Breast Invasive Carcinoma (TCGA, Nature 2012)</td>
<td>9</td>
<td>482</td>
<td>1.9%</td>
</tr>
<tr>
<td>Breast (Sanger)</td>
<td>Breast Invasive Carcinoma (Sanger, Nature 2012)</td>
<td>1</td>
<td>100</td>
<td>1.0%</td>
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<tr>
<td>Breast (BCCRC)</td>
<td>Breast Invasive Carcinoma (British Columbia, Nature 2012)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Breast (Broad)</td>
<td>Breast Invasive Carcinoma (Broad, Nature 2012)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Lung cancer studies:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lung squ (TCGA)</td>
<td>Lung Squamous Cell Carcinoma (TCGA, Provisional)</td>
<td>13</td>
<td>178</td>
<td>7.3%</td>
</tr>
<tr>
<td>Lung SC (JHU)</td>
<td>Small Cell Lung Cancer (Johns Hopkins, Nature Genetics 2012)</td>
<td>3</td>
<td>42</td>
<td>7.1%</td>
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<tr>
<td>Lung adeno (TCGA)</td>
<td>Lung Adenocarcinoma (TCGA, Provisional)</td>
<td>10</td>
<td>172</td>
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<tr>
<td>Lung adeno (TCGA pub)</td>
<td>Lung Adenocarcinoma (TCGA, Nature 2014)</td>
<td>12</td>
<td>230</td>
<td>5.2%</td>
</tr>
<tr>
<td>Lung squ (TCGA pub)</td>
<td>Lung Squamous Cell Carcinoma (TCGA, Nature 2012)</td>
<td>4</td>
<td>178</td>
<td>2.2%</td>
</tr>
<tr>
<td>Lung adeno (Broad)</td>
<td>Lung Adenocarcinoma (Broad, Cell 2012)</td>
<td>3</td>
<td>182</td>
<td>1.6%</td>
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<td>Lung adeno (TSP)</td>
<td>Lung Adenocarcinoma (TSP, Nature 2008)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Lung SC (CLCGP)</td>
<td>Small Cell Lung Cancer (CLCGP, Nature Genetics 2012)</td>
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<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Ovarian cancer studies:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian (TCGA)</td>
<td>Ovarian Serous Cystadenocarcinoma (TCGA, Provisional)</td>
<td>39</td>
<td>311</td>
<td>12.5%</td>
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<tr>
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<td>Ovarian Serous Cystadenocarcinoma (TCGA, Nature 2011)</td>
<td>19</td>
<td>316</td>
<td>6.0%</td>
</tr>
<tr>
<td>Ovary SC (MSKCC)</td>
<td>Small Cell Carcinoma of the Ovary (MSKCC, Nature Genetics 2014)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Gastric cancer studies:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal (Genentech)</td>
<td>Colorectal Adenocarcinoma (Genentech, Nature 2012)</td>
<td>3</td>
<td>72</td>
<td>4.2%</td>
</tr>
<tr>
<td>Colorectal (TCGA)</td>
<td>Colorectal Adenocarcinoma (TCGA, Provisional)</td>
<td>9</td>
<td>220</td>
<td>4.1%</td>
</tr>
<tr>
<td>Colorectal (TCGA pub)</td>
<td>Colorectal Adenocarcinoma (TCGA, Nature 2012)</td>
<td>8</td>
<td>212</td>
<td>3.8%</td>
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<td>Colorectal (MSKCC)</td>
<td>Colorectal Adenocarcinoma Triplets (MSKCC, Genome Biology 2014)</td>
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<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Brain cancer studies:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glioma (TCGA)</td>
<td>Brain Lower Grade Glioma (TCGA, Provisional)</td>
<td>20</td>
<td>286</td>
<td>7.0%</td>
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<tr>
<td>GBM (TCGA)</td>
<td>Glioblastoma Multiforme (TCGA, Provisional)</td>
<td>9</td>
<td>273</td>
<td>3.3%</td>
</tr>
<tr>
<td>GBM (TCGA 2008)</td>
<td>Glioblastoma (TCGA, Nature 2008)</td>
<td>2</td>
<td>91</td>
<td>2.2%</td>
</tr>
<tr>
<td>GBM (TCGA 2013)</td>
<td>Glioblastoma (TCGA, Cell 2013)</td>
<td>6</td>
<td>281</td>
<td>2.1%</td>
</tr>
<tr>
<td>MBL (Broad)</td>
<td>Medulloblastoma (Broad, Nature 2012)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>MBL (ICGC)</td>
<td>Medulloblastoma (ICGC, Nature 2012)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>MBL (PCGP)</td>
<td>Medulloblastoma (PCGP, Nature 2012)</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
PRMT8 expression correlates significantly with increased patient survival in some cancers and decreased survival in others – The utility of many biomarkers comes not simply from their ability to define the presence of a disease state, but by defining some metric that can be used to characterize that disease state. A metric frequently assessed in cancer research is patient survival. Correlation of biomarker expression with increased or decreased patient survival can be used to define the efficacy of various treatments, which is becoming increasingly important as more patients develop resistance to currently available pharmaceuticals. The Kaplan-Meier Plot is a general statistical tool that can be used for display and determination of lifespan and survivability under many conditions. An online tool called Kaplan-Meier Plotter (KMplot) makes use of microarray data from 10,188 cancer samples for the purpose of biomarker assessment (335). Currently, KMplot is capable of assessing the effect of 70,632 genes on survival of 4,142 breast, 2,437 lung, 1,648 ovarian, and 1,065 gastric cancer patients using Kaplan-Meier survival plots. Two probe sets for PRMT8 are included in microarray data curated by KMplot. Probe set 1 (Affymetrix 230839_at) was used to test 1,660 breast cancer patients, 849 of which show low PRMT8 expression and 811 of which show high PRMT8 expression (Fig. 5.3). Using probe set 1 to measure PRMT8 expression, patients with higher PRMT8 expression were shown to live significantly longer.

![Kaplan-Meier survival plot of breast cancer patients with varying PRMT8 expression using probe set 1.](image)

Differences in PRMT8 expression in breast cancer patients were determined with Affymetrix PRMT8 probe set 1 using a microarray and survival was plotted over time. The red curve includes all patients with high PRMT8 expression. The black curve includes all patients with low PRMT8 expression. Patients with high PRMT8 expression live significantly longer ($p=0.0082$). Source: http://kmplot.com.
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than patients with low PRMT8 expression (p=0.0082). Probe set 2 (Affymetrix 207772_s_at) was used to measure PRMT8 expression in breast, lung, ovarian, and gastric cancer patients (Fig. 5.4). Of the 3,554
breast cancer patients assessed with this probe set, the 1,770 patients with high PRMT8 expression live significantly longer than the 1,784 patients with low PRMT8 expression \((p=9.6e-08)\). Of the 1,926 lung cancer patients, the 949 patients with high PRMT8 expression showed no significant difference in survival compared to the 977 patients with low PRMT8 expression \((p=0.84)\). Of the 1,305 ovarian cancer patients, the 641 patients with high PRMT8 expression lived significantly longer than the 664 with low PRMT8 expression \((p=0.014)\). And of the 876 gastric cancer patients, the 574 patients with high PRMT8 expression lived significantly less time than the 302 with low PRMT8 expression \((p=1.1e-09)\). Thus, elevated PRMT8 correlates with patient survival in some cancers and higher patient death in others.

**PRMT8 variant 2 is expressed in tumorigenic glioblastoma** – While data from KMplot is useful for determining if PRMT8 is an efficacious biomarker for specific types of cancer, the effect of variant-specific expression on survival cannot be determined from these datasets, as array data is reported in sets and cannot be retrieved from individual probes. To gain insight into the variant-specific expression and function PRMT8 may have in various cancers, we tested individual cell lines for specific PRMT8 variants using RT-PCR. **Table 5.3** lists the various cancer lines tested, the tissue from which they were derived, associated disease type, and tumorigenicity. This preliminary assay reveals that the osteosarcoma U-2OS line, the glioblastoma lines A172 and U87MG, the teratocarcinoma line CRL-2073, as well as three cell lines derived from mammary glands, MCF-10A, SK-BR-3, and MDA-MB-231, all express PRMT8, although at varying levels (Fig. 5.5). The colorectal adenocarcinoma line Caco-2 does not express

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>Disease</th>
<th>Tumorigenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-2OS</td>
<td>Bone</td>
<td>Osteosarcoma</td>
<td>No</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Colon</td>
<td>Adenocarcinoma</td>
<td>Yes</td>
</tr>
<tr>
<td>A172</td>
<td>Brain</td>
<td>Glioblastoma</td>
<td>No</td>
</tr>
<tr>
<td>U87MG</td>
<td>Brain</td>
<td>Glioblastoma; astrocytoma</td>
<td>Yes</td>
</tr>
<tr>
<td>CRL-2073</td>
<td>Embryo, placenta</td>
<td>Teratocarcinoma</td>
<td>Yes</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>Mammary gland</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>Mammary gland</td>
<td>Adenocarcinoma</td>
<td>Yes</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Mammary gland</td>
<td>Adenocarcinoma</td>
<td>Yes</td>
</tr>
</tbody>
</table>
PRMT8. Only the glioblastoma line U87MG expresses PRMT8v2. The osteoscarcoma line U-2OS and the teratocarcinoma line CRL-2073 both express PRMT8v1, and the glioblastoma line A172 along with all three mammary gland lines expresses neither variant 1 nor 2. Here, hESCs were used as a PRMT8 positive control, as well as a positive control for PRMT8v1 expression. U87MGs in Figure 5.5B were used as a positive control for PRMT8v2.

**Figure 5.5 Variant-specific PRMT8 expression in various cancer cell lines.**

(A) PRMT8 expression was measured by RT-PCR in cancers of the bone, colon, and brain along with teratomas. hESCs were used as a positive control for PRMT8 expression. Actin was used as a loading control. (B) PRMT8 expression was measured by RT-PCR in breast cancer cell lines. hESCs and U87MGs were used as positive controls for PRMT8 expression. Actin was used as a loading control.

**DISCUSSION**

This report is not the first to identify the correlation of increased PRMT expression with an oncogenic disease state. So far, all PRMT family members except PRMT9 show overexpression in various cancers, including breast, lung, colorectal, bladder, prostate, lymphatic, skin, and ovarian cancers as well as leukemia (150,162,163,166,168,181,188,189,252,255,266,281-287,341-358). Specifically, PRMT1 expression is amplified in breast cancer tissues and is correlated with patient age, tumor grade, and menopausal status (168,189). In lung and bladder cancers, PRMTs 1 and 6 demonstrate elevated expression and regulate cancer cell growth, as knockdown leads to decreased proliferation in both types of cancer cell lines (168). Here, we provide the first compiled evidence that PRMT8 demonstrates amplified expression in many of the same cancers as other PRMT family members.
PRMT8 expression in any tissue, healthy or diseased, has remained largely uncharacterized due to long-held theories that PRMT8 is exclusive to brain tissue and localizes to the plasma membrane (116). Because of this consensus, PRMT8 hasn’t been scrutinized by the same rigorous experimentation to determine expression and function as other PRMT family members. However, with the rise in popularity of microarrays and bioinformatics, large datasets are becoming increasingly available and accessible, allowing for explorative data analysis of even previously ignored genes.

It is slowly becoming a more accepted theory in the field that PRMT8, like other PRMTs, is ubiquitously expressed during development before tissue specific expression in mature organisms occurs (i.e. brain tissue) – an in vivo zebrafish study demonstrated that PRMT8 is expressed during early development and is critical for normal development of all three germ layers (224). For this reason, PRMT8 in particular is an especially attractive therapeutic target. If aberrant PRMT8 expression is confirmed outside of brain tissue in a mature organism, it can be targeted without concern for off-target effects, so long as the therapeutic agent lacks the ability to cross the blood-brain barrier.

PRMT8 expression is highly altered in breast cancer – in one breast cancer study curated by cBio PRMT8 was amplified in 20.7% of cases. PRMT8 expression is also significantly correlated to breast cancer patient survival as measured using two separate Affymetrix chips by the online tool KMplot. Although much information can be gained by exploring such datasets, limitations prevent understanding how genes that have variant-specific effects may affect disease progression. For these reasons, we examined variant-specific PRMT8 expression in breast cancer cell lines – two tumorigenic lines, SK-BR-3 and MDA-MB-231, and one non-tumorigenic line, MCF-10A, for comparison. However, results show no correlation between PRMT8 variant expression and tumorigenicity. Nevertheless, the significant correlation between PRMT8 expression and breast cancer patient survival implores analysis of other breast cancer cell lines and primary breast tumors for variant-specific expression, especially since only adenocarcinoma derived lines were analyzed here. Other breast cancer tumor types, such as sarcomas, may demonstrate a tumorigenic correlation to variant-specific expression.
While PRMT8 expression correlates with patient survival for some types of cancers, namely breast, ovarian, and gastric, expression does not have the same effect on survival for all cancer types. High PRMT8 expression correlates with a significantly better prognosis for breast and ovarian cancer patients but also correlates with a significantly worse prognosis for gastric cancer patients. This may implicate an unexplored tissue specific role for PRMT8 or could be the effect of a variant-specific function, also seen with PRMT1, potentially indicating that the relationship between PRMT8 expression and these specific cancers may be more intricate than current data allows us to assess. PRMT1 variant 1 expression has a strong correlation with poor patient prognosis in breast cancer, while variants 2 and 3 show no clinical or pathological correlation (343). Strong variant 1 expression is also correlated to relapse and death of colon cancer patients, for which strong variant 2 expression also demonstrates a correlation, albeit at lower probability than with high variant 1 expression (162). Also, PRMT1 variant 1 expression increases significantly throughout colon cancer progression, implicating utility for this variant as a cancer biomarker.

Interestingly, the cBio data presented here demonstrates amplified PRMT8 expression in all glioblastoma studies, but not in other types of brain cancer. We therefore examined PRMT8 variant expression in two glioblastoma cell lines. Our data show PRMT8 variant 2 expression in the tumorigenic U87MG line but not in the non-tumorigenic A172 line. Taken together, these data indicate the feasibility of PRMT8 variant 2 as a glioblastoma cancer biomarker. However, further testing of a multitude of other cell lines and primary samples is required to conclude that PRMT8 variant 2 can be used to distinguish between healthy and diseased tissue.

As cancer becomes more prevalent in an aging population, it becomes important to identify molecules that can be used as therapeutics and for diagnostic screens. And as cancers progress within a patient, drug resistance may develop, necessitating the discovery of biomarkers that can be used to track disease progression and treatment efficacy. Currently, clinical development for cancer pharmaceuticals targeting epigenetics marks and molecules are mainly focused on therapeutics that target histone deacetylases (HDACs), however the benefits of drug development for the other classes of acetyl and
methyl writers, readers, and erasers (histone acetyl transferases, protein methyl transferases, lysine demethylases, bromodomain-containing proteins, and methyl-lysine readers) should undoubtedly be exploited (Table 5.4) (247,359-361). In total, the Food and Drug Administration (FDA) has approved four pharmaceuticals with epigenetic targets designed to treat cancer; two DNA methyltransferase (DNMT) inhibitors, Azacitidine and Decitabine, and two histone deacetylases (HDAC) inhibitors, Vorinostat and Romidepsin (247,359-361). However, many more are currently undergoing clinical trials and will likely reach market soon to add to the number of available drugs.

**Table 5.4 Targets of epigenetic cancer therapeutics.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Pharmaceutical inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT</td>
<td>Azacitidine, Decitabine, Vidaza, FCDR, Zebularine, S110, Procaine, Procainamide, Hydralazine, EGCG, RG108</td>
</tr>
<tr>
<td>SIRT</td>
<td>SEN196, Sirtinol, EX-527, Splitomicin, Cambinol, Salermide, Tenovin-1, Tenovin-6</td>
</tr>
<tr>
<td>HMT</td>
<td>Chaetocin, DZNep, BIX-0294, AMI-1, AMI-5, Allantodapsone, RM-65</td>
</tr>
<tr>
<td>HAT</td>
<td>Pargyline, Phenelzine, Tranylcyromine</td>
</tr>
<tr>
<td>HDAC</td>
<td>Vorinostat, Romidepsin, Panobinostat, Belinostat, Entinostat, Mocetinostat, Resminostat, Givinostat, SB939, CUDC-101, PCI-24781, 4SC-202, AR-42, CG200745, ACY-1215, RG2833, EVP-0334, NaB, Phenylacetate, Phenylbutirate, VPA, TSA, MGCD-0103, (1H) Pyrroles</td>
</tr>
</tbody>
</table>
CHAPTER 6

Thesis conclusions
The work contained in this thesis builds on previous data demonstrating that simple manipulation of *in vitro* culture conditions activates complex biological pathways leading to induced expression of specific stem-cell associated genes, longer cellular lifespan both temporally and in number of population doublings, and participation in a regenerative response at a skeletomuscular wound site (55,62). Collectively, these are referred to as the induced regeneration competent (iRC) phenotype, and cells grown under reduced oxygen with FGF2 supplementation are referred to as iRC cells. While iRC cells display some characteristics of pluripotent cells, such as stem cell gene expression, they lack others, such as tumorigenicity. iRC cell implantation in SCID mice lacks the tumor forming capacity exhibited by pluripotent cells. These data indicate that while some level of cellular reprogramming occurs, full reprogramming to the pluripotent state is not achieved.

The work presented in this thesis experimentally and theoretically extends the knowledge of iRC cells by suggesting their phenotype mimics cells in a pre-cancerous state. The ability of iRC cells to live longer in culture than control counterparts, and their inability to form tumors is a previously unexplored phenotype of this unique model system that has potential for identification of molecules that participate in the first stages of cancer related changes before cells become cancerous. Here, we identify a novel variant of an enzyme contributing to increased cellular lifespan of the iRC phenotype, offer a potential mechanism by which this occurs via a novel binding partner, and illustrate the utility of the iRC system for identifying pre-cancer and cancer biomarkers for the possibility of prognostic and therapeutic intervention.

**Chapter 2**

In chapter two we explore chromatin modification enzymes as a possible cause of the phenotypic shift in cellular lifespan observed in cells grown under iRC culture conditions. Target genes were identified using qRT-PCR, which revealed the highest 13.3-fold upregulation of the protein arginine methyltransferase *PRMT8*. Increased expression of PRMT8 in iRC cells was confirmed using Western blot, and upregulation of *PRMT8* by iRC culture conditions was validated in other primary dermal
fibroblast cell lines. Implication of other PRMTs in critical cellular processes, such as signal transduction, cell cycle progression, and protein expression (148,190,207,230-233), led to the hypothesis that upregulation of PRMT8 in iRC cells is necessary for the increased lifespan phenotype. While the biological functions of most PRMTs have been described, PRMT8 itself has remained largely unstudied due to initial reports erroneously concluding that PRMT8 localizes to plasma membrane and is only expressed in brain tissue (116,117,226). Aberrant expression of almost all PRMT family members has been reported in a variety of different cancers and, interestingly, specific PRMT variants have implicated roles in cancer progression through unique localization patterns as well as binding partners and substrates (160,162,163,168). For this reason, we next explored PRMT8 variant expression in iRC cells. PRMT8 has two mRNA variants, however only the first variant has been explored experimentally. Using 5’ Rapid Amplification of cDNA Ends and variant-specific PCR we demonstrate that human dermal fibroblasts as well as iRC cells express the novel PRMT8 variant 2, previously only described through genomic sequencing. To test the hypothesis that PRMT8 expression is necessary for the increased proliferative phenotype induced by iRC culture conditions, PRMT8 was knocked down using lentiviral transduction. PRMT8 knockdown in human dermal fibroblasts led to a loss of proliferation before iRC culture conditions could be employed, indicating that PRMT8 is essential for proliferation of even cells grown under control conditions. We went on to explore the effect of PRMT8 on proliferation of immortal, tumorigenic grade IV glioblastomas. Even in this highly aggressive cancer we demonstrated that PRMT8 is essential for proliferation. The major conclusions of this work were that PRMT8 expression is upregulated by iRC culture conditions, that human dermal fibroblasts as well as iRC cells express a novel mRNA variant of PRMT8, and that PRMT8 is essential for cellular proliferation of both primary human dermal fibroblasts as well as highly aggressive glioblastomas. Therefore, PRMT8 is a novel therapeutic target.
Chapter 3

In chapter three we examine the potential role that PRMT8 plays in the cell cycle and determine the sufficiency of PRMT8 overexpression for inducing increased cellular proliferation. Many members of the PRMT family have known roles in cell cycle regulation (266,276). To determine how cell cycle progression influences PRMT8 expression, sub-cytotoxic levels of hydrogen peroxide were added to cells to mimic replicative senescence. The data showed that as cells senesce, PRMT8 expression decreases in a hydrogen peroxide concentration-dependent manner as assessed by qRT-PCR, indicating a correlation between PRMT8 expression and cell cycle progression. Because of this, we hypothesized that PRMT8 overexpression would lead to increased stimulation of the cell cycle, leading to increased cellular proliferation. Given the critical role PRMT family members play in maintenance of cell cycle regulation, many studies have assessed the function of PRMTs in cellular proliferation using knockdown. While these studies demonstrate that expression of many PRMTs are critical for cellular viability and proliferation (257,279), none to our knowledge have explored if PRMT overexpression is sufficient to induce increased cellular proliferation. Using lentiviral particles, we overexpressed PRMT8 in human dermal fibroblasts. However, we found no significant difference in the number of population doublings or amount of time spent in culture prior to senescence between human dermal fibroblasts grown under control conditions and those overexpressing PRMT8, indicating that PRMT8 overexpression alone is not sufficient to induce increased cellular proliferation. Confirming these conclusions are qRT-PCR data demonstrating that PRMT8 overexpression does not influence the expression of key cell cycle genes, specifically expression of p21, p53, or p16. Therefore, while biological processes controlled by cell cycle progression regulate PRMT8 expression, overexpression of PRMT8 alone is not sufficient to influence the cell cycle or increase cellular proliferation.

Chapter 4

In chapter four we explore the cause of PRMT8 upregulation by culture conditions by assessing histone occupancy of the PRMT8 promoter and try to determine a mechanism by which PRMT8
influences cellular proliferation through binding partner identification. iRC culture conditions are defined by growth under reduced oxygen with fibroblast growth factor FGF2 supplementation. Of these culture conditions, it was determined that PRMT8 expression is primarily upregulated by reduced oxygen. As such, DNA motifs that regulate expression changes by oxygen tension, hypoxia response elements (297-302), were examined within the PRMT8 promoter as potential regions where changes in histone occupancy may regulate PRMT8 expression. Sequence analysis revealed 3.6-fold enrichment of hypoxia response elements within the PRMT8 promoter. For these reasons we hypothesized that classically repressive histone marks would be associated with hypoxia response elements within the PRMT8 promoter in cells grown under control conditions, while classically active histone marks would be associated with hypoxia response elements within the PRMT8 promoter in cells grown under iRC conditions. Using dimethylated histone 3 lysine 9 as a representative repressive mark, and acetylated histone 4 as a representative active mark (231,305), we found that the PRMT8 promoter demonstrates bivalent occupancy of both repressive and active marks regardless of culture conditions. This groups PRMT8 with other essential genes whose promoters maintain bivalent histone occupancy to remain in a state of poised activation for ease of transcriptional access (316-318).

While this data provides some insight into the regulation of PRMT8, we remained curious about the regulation of cellular proliferation by PRMT8. To gain insight into the mechanism by which PRMT8 influences cellular proliferation we assessed PRMT8 binding to key iRC-related proteins using co-immunoprecipitation and mass spectrometry. As the two main components of the iRC culture system are reduced oxygen and FGF2 supplementation, we assessed the relationship between the two primary molecules that regulate these biological processes: hypoxia inducible factor 1α (HIF1α) and FGF2. While PRMT8 does not bind HIF1α under control or iRC conditions, PRMT8 does bind specific isoforms of FGF2 under both control and iRC conditions. FGF2 has five isoforms, one low molecular weight 18kD isoform and four high molecular weight 34kD, 24kD, 22.5kD, and 22kD isoforms. The low molecular weight isoform is required for maintenance of stem cell pluripotency (53,54) and is the isoform
supplemented in iRC cell media. The high molecular weight isoforms are not as well studied, but are known to participate in intracrine signaling and cell growth and behavior (296). The high molecular weight 22.5kD and 22kD isoforms of FGF2 were bound by PRMT8 in both control and iRC cells, as demonstrated by co-immunoprecipitation. Mass spectrometry validated binding of the 22.5kD FGF2 isoform to PRMT8 in control cells.

Chapter 5

In chapter five we assessed the feasibility of using PRMT8 as a prognostic cancer biomarker by mining publically available data and interpreting our own RT-PCR observations. Modifiers and regulators of epigenetic mechanisms are highly dysregulated in cancers (81-85,281,338,339). Furthermore, the aberrant expression of PRMTs has so far been described in breast, prostate, lung, colon, and bladder cancers as well as in leukemia (162,168,188,189,255,280-287). Currently, specific PRMT protein isoforms are being used as prognostic markers of lung and bladder cancers (160,162,163,168). Using publically available databases, we found that PRMT8, like other PRMT family members, demonstrates increased expression in a variety of different cancers. The Human Protein Atlas (328-332) uses immunohistochemistry to assess expression of proteins in various cancer samples. This database detects PRMT8 expression in 98.5% of all cancers tested, 72.3% of which highly express PRMT8. The cancers that most highly express PRMT8 according to The Human Protein Atlas are breast, glandular, cervical, head and neck, prostate, and thyroid. We next assessed if the PRMT8 gene is mutated or is differentially expressed between tumorigenic tissue and tissue-matched controls using the cBio Portal for Cancer Genomics (333,334), a database that curates genomic data from 91 separate cancer studies. Thirty-eight of 47 studies containing mutation data show PRMT8 to be mutated in various cancers and 33 of 39 studies containing copy number alteration data show PRMT8 to be amplified in various cancers. Of the cancers in which PRMT8 is mutated, it tends to be most mutated in lung cancer, and of the cancers in which PRMT8 is amplified, it tends to be most amplified in breast, ovarian, uterine, and brain cancers. While many molecules demonstrate amplification in neoplastic tissue, the utility of a cancer biomarker comes when
correlation can define a metric of that disease state, such as patient survival. The tool Kaplan-Meier Plotter (335) uses microarray data to assess patient survival in specific cancers relative to high or low expression levels of a particular gene plotted using Kaplan-Meier survival plots. Regarding *PRMT8* expression, breast and ovarian cancer patients with high expression lived significantly longer than patients with low expression. However, gastric cancer patients with low *PRMT8* expression lived significantly longer than patients with high expression. While these data significantly correlate *PRMT8* expression to patient survival they also allude to a tissue- or disease-specific role for *PRMT8* expression. Other PRMT family members have variant-specific functions in cancers, and specific isoforms are currently used as prognostic biomarkers (163,174-177,189). Mining of publically available data sources allows us to make conclusions regarding the expression and function of *PRMT8* mRNA variant 1 in cancer, but no available database currently curates data for *PRMT8* mRNA variant 2. For this reason we carried out variant-specific RT-PCR for *PRMT8* on various cancer cell lines, revealing *PRMT8* variant 2 expression in a tumorigenic glioblastoma line. In line with other data implicating specific *PRMT* variants as prognostic indicators of disease states, we conclude that *PRMT8* variant 2 is a feasible biomarker candidate for glioblastomas.

Taken together, the data presented in this thesis add to knowledge about the critical role PRMTs play in various biological processes, such as regulation by environmental cues, participation in cellular proliferation, regulation by cell cycle progression, binding to essential mitogenic proteins, and amplification and variant-specific expression in neoplastic tissue. Participation in all of these biological processes is novel regarding functional roles for PRMT8. This thesis also demonstrates the first experimental description of *PRMT8* mRNA variant 2, the first demonstration of PRMT8 expression in human embryonic stem cells, and the first binding partners of endogenous PRMT8, the 22.5kD and 22kD isoforms of FGF2.

**Figure 6.1** adds the findings from this dissertation to the original iRC cell model presented in chapter 2.
Human dermal fibroblasts (hDFs) cultured under atmospheric oxygen tension (19%) without the addition of fibroblast growth factor (FGF2) express low levels of the arginine methyltransferase PRMT8. Culture under reduced oxygen tension (2%) with FGF2 supplementation, conditions referred to as induced regeneration competent (iRC) culture conditions, cause a shift in expression of chromatin modification enzymes. Levels of a novel PRMT8 variant (PRMT8 v2) and levels of PRMT8 protein increase under iRC culture. When PRMT8 expression is knocked down in hDFs, cellular proliferation is lost. Under both control and iRC culture conditions PRMT8 binds specific high molecular weight (HMW) isoforms of FGF2. However, binding is reduced under iRC conditions likely due to expression of the antisense FGF2 molecule Nudix6.

Figure 6.1. Graphic summary of thesis conclusions.
CHAPTER 7
Future directions
The work presented here has questioned the paradigm that the arginine methyltransferase PRMT8 is solely expressed in brain tissue at the plasma membrane, and has demonstrated expression of a novel \textit{PRMT8} mRNA variant, identified a novel role for PRMT8 in cell proliferation, and identified the first binding partner of endogenous PRMT8, the fibroblast growth factor FGF2. While this work has advanced the field regarding knowledge of PRMT8 expression and function, tangential studies described here could be carried out to continue the work presented in this thesis.

**Investigate involvement of PRMT8 in the cell cycle**

Chapter two demonstrated the requirement of PRMT8 for maintenance of cellular proliferation, but was unable to investigate the involvement of PRMT8 in the cell cycle using knockdown due to immediate cell death. The lack of biological material as a result of the experimental phenotype prevented any further examination of the relationship PRMT8 may have with key cell cycle-associated genes. Future experiments could make use of this knowledge by performing transductions with an inducible construct for knockdown. Alternatively, an inducible overexpression construct could be transduced first, followed by transduction with a knockdown construct. Following PRMT8 knockdown, the overexpression could be induced to determine if restoration of PRMT8 expression rescues the phenotype. As loss of proliferation is the primary phenotype observed upon PRMT8 knockdown, cell cycle genes directly involved in this biological process should be examined first, such as p53, p21, p16, retinoblastoma, Cyclin D, Cyclin E, and E2F. While PRMT8 itself may not be directly binding any of these proteins, expression changes in PRMT8 may indirectly affect expression of such genes, allowing for the loss of cellular proliferation observed upon PRMT8 knockdown using mutation studies that prevent binding of PRMT8 to FGF2.

**Examine regulation of FGF2 by PRMT8 arginine methylation**

Chapter four identifies the first endogenous PRMT8 binding partners as specific high molecular weight isoforms of FGF2, namely the 22.5kD and 22kD isoforms. Previous reports have demonstrated that methylation affects the subcellular localization of high molecular weight FGF2 isoforms (362) and
that specific arginine residues of FGF2 are methylated by PRMT1 (322,362,363) and PRMT5 (323), but to date no publications have detailed the relationship between PRMT8 and FGF2 and its functional consequence. It remains unexplored if the binding of PRMT8 to FGF2 serves an enzymatic purpose. Functional aspects of PRMT8-FGF2 binding could also be examined, specifically in light of the iRC phenotype by preparing mutants that no longer bind. The necessity of PRMT8 for cellular proliferation as well as the critical functions of FGF2 implicate potential for interesting interplay between these molecules, specifically regarding certain elements of the iRC phenotype such as increased lifespan. Given the participation of FGF2 in mitogenesis, it would be an interesting extension of this thesis to determine the necessity of PRMT8 interaction with or methylation of FGF2 for the increased lifespan phenotype of iRC cells.

**Explore the consequence of PRMT8 binding with other molecules identified via mass spectrometry**

Mass spectrometry was used to validate FGF2 binding to PRMT8 in chapter four. This analysis provides a foundation for future projects related to the iRC phenotype through identification of a variety of other interesting PRMT8 binding partners (*Table 7.1*). For example, in control cells, but not iRC cells the protein PGBM (basement membrane-specific heparan sulfate proteoglycan core protein) binds PRMT8. PGBM is a core protein to which three heparan sulfate chains are attached. Heparan sulfate proteoglycans are components of the extra-cellular matrix that interact with ligands, such as FGF2, to mediate biological processes (364). Given the known role of heparan sulfate proteoglycans in FGF2 receptor binding, dimerization, and internalization (365), the interaction between PRMT8 and PGBM is of interest for study with the iRC system. Another protein of interest is bound to PRMT8 in iRC cells, but not control cells – the protein 14-3-3 gamma. Of interest to the iRC cell system, 14-3-3 participates in biological processes such as cell survival through interaction with BAD (Bcl-2-associated death promoter) (366), cell cycle progression through interaction with the cyclin-dependent kinase inhibitor p27/KiP1 (367), and apoptosis inhibition through interaction with PRAS40 (proline-rich AKT1 substrate 1) (368). 14-3-3
Table 7.1 Mass spectrometric analysis of PRMT8 immunoprecipitated from control and iRC cells.

**Proteins bound to PRMT8 in control cells, but not iRC cells**

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein name</th>
<th>Gene symbol</th>
<th>Number of peptides</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P08195-2</td>
<td>Isoform 2 of 4F2 cell-surface antigen heavy chain</td>
<td>4F2</td>
<td>2</td>
<td>4.9</td>
</tr>
<tr>
<td>Q15758</td>
<td>Neutral amino acid transporter B(0)</td>
<td>AAAT</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>Q9UG63-2</td>
<td>Isoform 2 of ATP-binding cassette sub-family F member 2</td>
<td>ABCF2</td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td>Q8IVF2-3</td>
<td>Isoform 3 of Protein AHNAK2</td>
<td>AHNAK2</td>
<td>17</td>
<td>4.3</td>
</tr>
<tr>
<td>Q9NW6B-2</td>
<td>Isoform 2 of Arginine and glutamate-rich protein 1</td>
<td>ARGL1</td>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>Q9NYF8-2</td>
<td>Isoform 2 of Bcl-2-associated transcription factor 1</td>
<td>BCLF1</td>
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<tr>
<td>Q9NS6-2</td>
<td>Isoform B of Bromodomain and WD repeat-containing protein 1</td>
<td>BRWD1</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>P54299-4</td>
<td>Isoform 4 of Voltage-dependent calcium channel subunit alpha-2/delta-1 CA2D1</td>
<td>3</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>P21127-2</td>
<td>Isoform SV1 of Cyclin-dependent kinase 11B</td>
<td>CD11B</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>P39035-1</td>
<td>Collagen alpha-1(XV) chain</td>
<td>COFA1</td>
<td>3</td>
<td>2.2</td>
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<tr>
<td>P21127-2</td>
<td>Isoform SV1 of Cyclin-dependent kinase 11B</td>
<td>CD11B</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>P39035-1</td>
<td>Collagen alpha-1(XV) chain</td>
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<td>3</td>
<td>2.2</td>
</tr>
<tr>
<td>Q01780-2</td>
<td>Isoform 2 of Exosome component 10</td>
<td>EXOSX</td>
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<td>P09038-1</td>
<td>22.5kD isoform of Fibroblast growth factor 2</td>
<td>FGF2</td>
<td>2</td>
<td>8.1</td>
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<tr>
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**Proteins bound to PRMT8 in iRC cells, but not control cells**

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<tr>
<th>Accession number</th>
<th>Protein Name</th>
<th>Gene symbol</th>
<th>Number of peptides</th>
<th>Sequence coverage (%)</th>
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<td>NEST</td>
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<td>P54727-2</td>
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also interacts with TERT to prevent apoptosis (211). Insight into the relationship between PRMT8 and 14-3-3 gamma within the iRC system would add to the topics covered in this thesis with a possible mechanism by which PRMT8 influences cellular proliferation.

Figure 7.1 is a Venn diagram depicting the number of genes identified via mass spectrometry for each sample.

Figure 7.1. Number of genes under control and iRC conditions that bind PRMT8.

Three samples were analyzed using mass spectrometry: two treatment samples, 19-, also known as control cells and 2+, also know as iRC cells, and one control sample, 2+ cells without antibody added during the IP procedure. Numbers represented in the Venn diagram indicate the number of genes bound to PRMT8 identified using mass spectrometry.
Characterize the utility of *PRMT8* as a glioblastoma biomarker

Chapter five details the expression of PRMT8 in various cancers and concludes that *PRMT8* variant 2 has potential as a glioblastoma biomarker. However, use of this variant for diagnostic purposes would require rigorous testing with a much larger sample pool. Online tissue repositories, such as BioServe, could be used to acquire primary glioblastoma tissue samples as well as tissue-matched controls. These samples could then be tested for the presence and abundance of *PRMT8* variant 2 to determine if glioblastoma-associated tissue has higher expression than healthy tissue. A strong correlation of PRMT8 variant 2 expression with diseased tissue would greatly increase commercial interest of *PRMT8* as a glioblastoma biomarker.
CHAPTER 8

Materials and methods
Chapter 2

Cell culture – The adult human fibroblast line CRL-2352 was obtained from American Tissue Culture Collection (ATCC; Manassas, VA) at passage 2. The foreskin fibroblast line CRL-2097 was obtained from ATCC. The adult human fibroblast line CT-1005 was obtained from a panniculectomy at UMass Medical (Worcester, MA) through their tissue distribution program. Cells were cultured in medium consisting of DMEM: Ham’s F12 (50:50; MediaTech) with 10% Fetal Clone III (HyClone). The DMEM (without L-Gln or phenol red) was supplemented with 4mM fresh L-Gln (MediaTech, Manassas, VA) prior to use. Cultures were carried out in a 37°C incubator in a humidified environment of 5% CO₂ and either 19% or 2% O₂ depending on experimental requirement. All cultures were processed for analyses on day 7. When used, media was supplemented with human recombinant FGF2 (PeproTech) at 4ng/mL. Human embryonic stem cells - hESCs (W09; WiCell, Madison, WI) were cultured on mytomycin C-treated mouse embryonic fibroblasts seeded onto 0.1% gelatin coated six-well plates using 80% Knockout™ DMEM (Invitrogen), 20% Knockout™ serum replacement supplemented with 2.0mM L-Gln, 0.055mM 2-mercaptoethanol, and 4.0ng/mL FGF2, as recommended by the supplier. Glioblastomas (U87MG; ATCC) were cultured in medium consisting of DMEM: Ham’s F12 (50:50; MediaTech) with 10% Fetal Clone III (HyClone).

RT-PCR - RNA was prepared by Trizol (Invitrogen, Inc.) according to the manufacturer’s instructions and quantified by spectrophotometry (NanoDrop 2000). One microgram of total RNA was used to perform first strand cDNA synthesis using qScript™ cDNA SuperMix (Quanta Biosciences™). Mouse brain RNA was a generous gift from RXi Pharmaceuticals. For RT-PCR, 50ng first-strand cDNA was used as a template for each reaction. PCR was performed using 12.5µL GoTaq (Promega) and 0.2mM each of forward and reverse primers. PCR cycling for PRMT8 was performed as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 seconds; annealing at primer-specific annealing temperature for 30 seconds; and extension at 72°C for 1 minute. Final extension was performed at 72°C for 10 minutes and samples were held at 4°C until use. PCR cycling for actin was performed as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of
denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 15 seconds; and extension at 72°C for 15 seconds. Final extension was done at 72°C for 7 minutes and the samples held at 4°C until use. PCR cycling for PRMT1: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 seconds; annealing at primer-specific annealing temperature for 30 seconds; and extension at 72°C for 60 seconds. Final extension was performed at 72°C for 10 minutes and samples were held at 4°C until use. PCR cycling for GFP: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 15 seconds; and extension at 72°C for 30 seconds. Final extension was done at 72°C for 7 minutes and the samples held at 4°C until use. PCR cycling for PRMT8 variant 1: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds; annealing at primer-specific annealing temperature for 30 seconds; and extension at 72°C for 30 seconds. Final extension was done at 72°C for 10 minutes and the samples held at 4°C until use. PCR cycling for PRMT8 variant 2: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 15 seconds; and extension at 72°C for 15 seconds. Final extension was done at 72°C for 10 minutes and the samples held at 4°C until use. PCR products from this primary round of amplification were diluted 1:100 with Tris EDTA and the diluted primary PCR product was used as product for the second round of amplification. The second round of amplification for PRMT8 variant 2 was done as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 15 seconds; and extension at 72°C for 15 seconds. Final extension was done at 72°C for 10 minutes and the samples held at 4°C until use. Amplification products were resolved on 2% agarose gels containing 0.5µg/mL ethidium bromide in 1x TAE buffer and photographed using a BioRad Gel Doc XR System. Primer sequences and product sizes are listed in Table 8.1.
Ch. 8 – Materials and methods

Table 8.1 RT-PCR primer sequences for PRMT8 variant-specific PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
<th>Amplicon (bp)</th>
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<tr>
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<tr>
<td>Actin</td>
<td>TCTGGCACCCACCTTCTACAA</td>
<td>CTTCCTCCTTATACCTAGAC</td>
<td>392</td>
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</table>

qRT-PCR array analysis - RNA was prepared using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions and quantified by spectrophotometry (NanoDrop 2000). Two micrograms of total RNA was used to perform first strand cDNA synthesis using RT² First Strand Kit (SABiosciences) as recommended by the supplier. Relative quantification was determined using a 7500 Real Time PCR system (Applied Biosystems, Bedford, MA) measuring SYBR green fluorescence (RT² SYBR® Green/ROX qPCR Master Mix, SABiosciences). RT² Profiler™ PCR Arrays from SABiosciences for chromatin modifying enzymes containing 84 probes were used to identify genes with altered expression in the presence of FGF2 and when oxygen levels were reduced. Analysis was performed by SABiosciences RT2 Profiler PCR Array Data Analysis Template v3.3. Fold change was calculated based on difference in Ct values.

Cloning – PRMT8 was amplified using RT-PCR described above. The PCR product was resolved on a 2% agarose gel and the 205bp band was excised and cleaned using a NucleoSpin Gel and PCR Clean-up column (Macherey Nagel) according to the manufacturer’s instructions. A Klenow (New England Biolabs) reaction was performed using the entire PCR product. The reaction was incubated at room temperature for 15 minutes then stopped with the addition of 10µM EDTA, followed by a column clean up (NucleoSpin, Macherey Nagel). 70ng from the Klenow reaction were treated with T4 kinase (New England Biolabs). The kinase reaction was incubated at 37°C before cleaning over a column (NucleoSpin, Macherey Nagel). A T4 ligation was performed with 20ng pLVX-puromycin (Clontech Laboratories, Inc.) and PCR product in a 1:1 ratio overnight at 4°C. 10µL of ligated pLVX was then transformed into
chemically competent *E. coli* cells. Transformants were incubated on ice for 30 minutes and heat shocked at 42°C for 45 seconds before 250µL S.O.C. media was added. Transformants were incubated at 37°C for 1 hour with agitation prior to overnight incubation on puromycin-containing agar plates at 37°C. Colonies were picked and plasmids were cultured in 3mL LB broth containing ampicillin overnight with agitation at 37°C. Minipreps were performed on plasmid cultures using a NucleoSpin Plasmid Kit (Macherey Nagel) according to the manufacturer’s instructions. Insertion of the PCR product was confirmed with a double restriction digest using 500ng DNA, 5 units ClaI (New England Biolabs), and 5 units BamHI (New England Biolabs) prior to sequencing (GeneWiz, Cambridge, MA).

**Immunocytochemistry** - Fibroblasts were seeded into 24-well plates (BD Falcon) onto 12-mm round glass coverslips (VWR, West Chester, PA) at 6000 cells per well in medium described above. Cells were cultured as above. After 7 days, the cells were washed with DPBS without Ca/Mg (Mediatech) and fixed in methanol (-20°C) or 2% paraformaldehyde for 15 minutes, washed with DPBS, and permeabilized with 1.5N HCl or 0.1% Triton X-100 in PBS, respectively, for 20 minutes. Cells were washed with phosphate-buffered saline (PBS)/Tween and blocked for 15 minutes at room temperature with PBS containing 5% bovine serum albumin (BSA). Primary antibodies were added in PBS/Tween for 30 minutes at room temperature. Cells were washed four times in PBS/Tween. Alexafluor-568 or 488 labeled appropriate secondary antibodies (4µg/mL, Invitrogen) in PBS/Tween was added for 30 minutes. Cells were washed four times in PBS/Tween. Nuclear counterstaining was added with 0.5µg/mL Hoechst for 10 minutes. Cells were washed four times with PBS and stored in PBS at 4°C until image analysis. Antibodies used were: PRMT8 (Dr. Mori; Novus NBP1-87102). Coverslips were removed and mounted onto glass slides using Prolong Gold (Invitrogen) according to the manufacturer’s recommendation. Fluorescent images were acquired using confocal microscopy.

**5’ Rapid Amplification of cDNA Ends** - 5’ sequences were determined using a 5’ RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen) according to the manufacturer’s instructions. Briefly, cDNA was synthesized using a primer specific to *PRMT8* (5’- CGAGACCTCGATTTCACAG), the sample was purified over a column, and the enzyme terminal deoxynucleotidyl transferase (TdT) was
used to add a series of cytosine residues to the 3’ end of the product. Nested PCR was then performed, the products were run on a 1.5% agarose gel, and bands were excised, purified (Macherey Nagel; Nucleospin Extract II), and sequenced (GeneWiz, Cambridge, MA). Primer sequences for nested amplification are as follows: primary PCR – forward primer provided by Invitrogen (abridged anchor primer); reverse primer 5’ - CTTGGCAGCGAACATGGAAA (hES), 5’ - CACCAGTGAGTTTGAAGTCCTTG (iRC); nested PCR – forward primer provided by Invitrogen (abridged universal amplification primer); reverse primer 5’ - CATCCAGTACCACTTTGTCTC (hES), 5’ - CTGGAAACATAAGCCCTCCAGG (iRC).

**Transduction** – Custom lentiviral particles were designed and produced by GenTarget Inc. (San Diego, CA) to target PRMT8 for knockdown using shRNA. Particles contained shRNA constructs driven by an H1 promoter with a GFP-puromycin reporter tag driven by an RSV promoter. Human dermal fibroblasts were seeded at 1.6x10^4 cells per well of a 12 well plate and incubated at 37°C overnight. Media was removed and 0.4mL serum-free media was added to each treatment well, followed by lentiviral particles to a multiplicity of infection of 50. Cells were incubated at 37°C for 6 hours. Six hours post-transduction, 1mL complete media was added to each well. Cells were imaged every 24 hours for GFP expression and cumulative population doublings were determined via cell counts. Glioblastomas were seeded at 4.0x10^4 cells per well of a 6 well plate and transduced with lentiviral particles to a multiplicity of infection of 50. Transfection efficiency was monitored by expression of GFP on a Zeiss inverted epi-fluorescence microscope (Axiovert 200M) using AxioVision software (AxioVs40 V 4.8.2.0, service pack 4.8.2 SP1). All images were obtained with an AxioCam MRm camera using a 20xLD Plane-Neofluar objective (20x/0.4 Ph2 Korr) using identical settings.

**Protein isolation and Western blotting** - Total protein was isolated from subconfluent cells with cell lysis buffer (200mM Tris-HCl; pH 7.5, 750mM NaCl, 40% glycerol, 0.0626% Triton-X 100, 0.025% Tween-20, 0.1% NP-40), supplemented with compete protease inhibitor cocktail (PIC, Santa Cruz Biotechnology). Lysis was performed using sonication (Misonix XL-2000) on power 3 with 5 pulses performed 3 times. Protein concentration was determined using Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). Protein supernatant and 5x loading dye (10% SDS, 40% glycerol, 1% Bromophenol
Blue, 31.3% 1M Tris-HCl; pH 6.8, 5% 2-β-Dmercaptoethanol) were mixed in a 5:1 ratio and boiled for 5 minutes. Proteins were separated using 12% SDS-PAGE at indicated concentrations of total protein in the lysate and transferred to PVDF membranes (BioRad Laboratories) using Towbin transfer buffer (25mM Tris Base, 192mM glycine, 20% methanol, 0.037% SDS). The membranes were blocked with Tween-20-Tris-buffered saline (TBST: 25mM Tris Base, 137mM NaCl, 2.7mM KCl, 0.2% Tween-20) and 5% dry milk while shaking at room temperature for 60 minutes. Primary antibodies were incubated with the membrane in TBST and 1% dry milk rotating overnight at 4°C: anti-PRMT8 (Novus NBP1-81702; 1:200) and anti-actin (Sigma A-2066; 1:5000). HRP-conjugated secondary antibodies (BioRad 170-5046; 1:5000) were incubated with the membrane in TBST and 1% dry milk rotating at room temperature for 2 hours. Between and after antibody incubations, membranes were washed 4 times for 10 minutes each with TBST. Chemiluminescence signal was developed by luminol (SantaCruz Biotechnolgies) and luminescence detected using a BioRad Gel Doc XR System. Densitometry was used for quantitation of the signal. Obtained values (n=3) were normalized to actin and means compared using one-tailed T-test. Difference between the means was judged at p<0.05.

Chapter 3

Cell culture – Cell culture was performed as described in Chapter 2 materials and methods.

Transduction – Custom lentiviral particles were designed and produced by transOMIC technologies (Huntsville, AL) to overexpress PRMT8 mRNA variant 2. Particles contained the open reading frame of PRMT8 mRNA variant 2 driven by a CMV promoter with a TurboRFP-puromycin reporter tag driven by a PGK promoter. Human dermal fibroblasts were seeded at 1.6x10^4 cells per well of a 12 well plate and incubated at 37°C overnight. Media was removed and 0.5mL serum-free media was added to each treatment well, followed by 0.075mL lentiviral particles. Cells were incubated at 37°C for 6 hours. Six hours post-transduction, 0.5mL complete media was added to each well. Cells were monitored every 24 hours for RFP expression on a Zeiss inverted epi-fluorescence microscope (Axiovert 200M) using AxioVision software (AxioVs40 V 4.8.2.0, service pack 4.8.2 SP1). All images were obtained with an
AxioCam MRm camera using a 10xLD or 20xLD Plan-Neofluar objective (20x/0.4 Ph2 Korr) using identical settings. Cumulative population doublings were determined via cell counts.

**RT-PCR** – RNA and cDNA were prepared and RT-PCR was performed as described in Chapter 2 materials and methods without performance of nested PCR. PCR cycling for CMV, CMV-PRMT8, and RFP were performed as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 15 seconds; annealing at primer-specific annealing temperature for 15 seconds; and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 7 minutes and samples were held at 4°C until use. Primer sequences can be found in Table 8.2.

### Table 8.2 RT-PCR primer sequences to validate PRMT8 overexpression.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’ )</th>
<th>Reverse (5’ to 3’ )</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRMT8</td>
<td>GACTACGTCCACGCCCTGGTCACCTATTTTATT</td>
<td>GGTCCTCGACATTTTTGGCATTGCCTTCATGG</td>
<td>205</td>
</tr>
<tr>
<td>CMV</td>
<td>GGCTGACGGCCCAACGACC</td>
<td>CACGCTACGGCCCAAGTCTCC</td>
<td>448</td>
</tr>
<tr>
<td>CMV-PRMT8</td>
<td>CGCAAATGGGCGGTAGCGGTG</td>
<td>GGCATAGGAGGCTGAAATAATCTCT</td>
<td>373</td>
</tr>
<tr>
<td>RFP</td>
<td>CCAGAACGCTCAGCTCAGCTACACCGTC</td>
<td>CCAGTTTTGCTAGGGAGGTCGC</td>
<td>356</td>
</tr>
<tr>
<td>Actin</td>
<td>TCTGGCAACCACCTTCTACAA</td>
<td>CTTCCTCTAATGTCAACGCACG</td>
<td>392</td>
</tr>
</tbody>
</table>

**Protein isolation and Western blotting** – Protein isolation and Western blotting were performed as described in Chapter 2 materials and methods. Additional PRMT8 antibodies used were from Abcam (ab134774; 1:2500) and Santa Cruz Biotechnology, Inc. (sc-130853; 1:500). Densitometric quantification was done with 2 independent biological replicates.

### Chapter 4

**Cell culture** – Cell culture was performed as described above.

**Chromatin immunoprecipitation** – Thirty seven percent formaldehyde was added directly to culture dishes with fresh medium to a final concentration of 1%. Cells were incubated for 10 minutes at room temperature on a shaker platform. Glycine was added to a final concentration of 125mM and cells were incubated for 10 minutes at room temperature. Medium was discarded and cells were washed twice with ice cold PBS. PBS supplemented with protease inhibitors was added and cells were scraped into tubes and pelleted by centrifugation. Supernatant was discarded and pellets were stored at -80°C. Crosslinked cells
were lysed in aliquots of $3 \times 10^6$ cells for 10 minutes at 4°C with 250µL IP/wash buffer (10mM Tris Acetate; 1% NP-40; 100mM NaCl; 1mM Sodium Orthovanadate; PIC). Lysate was placed on ice and sonicated 11 times using 20-second pulses at power level 10. IP/wash buffer was added to a final volume of 1mL and aliquots were taken (input samples). Ten micrograms of primary antibody (H4ac; Milipore, 06598 or H3K9me2; Sigma, D5567) was added and samples were incubated on ice for 2 hours. Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnologies, Inc., sc-2003) were added and samples were incubated at 4°C overnight. Samples were washed 1 time with buffer 1 (.1%SDS; 1% Triton-X; 2mM EDTA; 20mM Tris HCl, pH 8.0; 150mM NaCl), 1 time with buffer 2 (.1%SDS; 1% Triton-X; 2mM EDTA; 20mM Tris HCl, pH 8.0; 500mM NaCl), 1 time with buffer 3 (.25M LiCl; 1% NP-40; 1% sodium deoxycholate; 1mM EDTA; 10mM Tris HCl, pH 8.0), and 2 times with buffer 4 (10mM Tris HCl, pH 8.0; 1mM EDTA). Chromatin was eluted by incubating with 250µL 1%SDS/.1M NaHCO₃ for 15 minutes at room temperature and samples were centrifuged at 2500rpm for 1 minute. The eluted complexes were transferred to a microfuge tube and chromatin was eluted a second time as above and fractions were pooled. Five hundred microliters of 1%SDS/.1M NaHCO₃ was added to input samples. Twenty microliters of 5M NaCl was added to all samples. Formaldehyde crosslinking was reversed by incubating at 68°C overnight. Twenty microliters 1M Tris HCl, pH 6.5; 10µL 0.5M EDTA; 40µg proteinase K was added and samples were the incubated at 45°C for 1 hour. DNA was extracted using a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol with 10µg of glycogen. DNA was precipitated at -80°C for 1 hour and washed with ice-cold 70% ethanol. Samples were centrifuged and the supernatant was discarded. Pellets were resuspended in 20µL TE (20mM Tris HCl, pH 8.0; 1mM EDTA, pH 8.0).

RT-PCR – Primers were initially tested on human genomic DNA from whole blood (Clontech, Cat # 636401).

$H_2O_2$ treatment – Fibroblasts were seeded in 60mm plates at 1x10^5 cells per plate and cells were incubated at 37°C overnight under iRC culture conditions. Three percent $H_2O_2$ (882mM) was diluted to 200µM, 400µM, 600µM, and 800µM in 5mL media (50:50 DMEM:F-12 with 10% FCIII supplemented with 4ng/mL FGF2) and incubated with cells at 37°C for 2 hours. Cultures were washed 3 times with PBS.
Materials and methods

Cells were allowed to recover for 3 days. H$_2$O$_2$ treatment was repeated and cells were again allowed to recover for 3 days prior to harvesting.

**qRT-PCR** – RNA and cDNA were prepared as described in Chapter 2 materials and methods for RT-PCR. Relative quantification was determined using a 7500 Real Time PCR system (Applied Biosystems, Bedford, MA) measuring SYBR green fluorescence (applied biosystems PowerUp™ SYBR® Green Master Mix, A25741). Forty nanograms template cDNA was used per reaction with primers found in Table 8.3. All reactions were performed in triplicate and Ct values were averaged. All samples were normalized to age-matched actin expression and fold change was calculated using the ΔΔCt method. Statistical analysis was performed using a one-tailed T-test and difference between the means was judged at p<0.05.

**Table 8.3 qRT-PCR primer sequences for cell cycle gene analysis.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>CACTGTCTTGTACCCCTGTTG</td>
<td>TTCCTCTTGGAGAAGATCAG</td>
<td>148</td>
</tr>
<tr>
<td>p53</td>
<td>GTGTGTGTGCTGCTCTGGGAG</td>
<td>GCTCTGGAACATCTCGAAGCG</td>
<td>213</td>
</tr>
<tr>
<td>p16</td>
<td>GAGCACTACGGCTAAGC</td>
<td>AGTGTGACCTCAAGAGC</td>
<td>139</td>
</tr>
<tr>
<td>Actin</td>
<td>AGAGCTACGAGCTGCTGAC</td>
<td>GGATGCCACAGGACTCCA</td>
<td>111</td>
</tr>
</tbody>
</table>

**Immunoprecipitation** – 1mL RIPA Buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail) was added per 3x10^6 cells. All samples were pulled through a 25-gauge syringe to homogenize. A 60µL input sample was taken. 0.3µg PRMT8 antibody (abcam ab134774) was added to appropriate samples. Samples were rotated end-over-end for 2 hours at 4°C. 20µL Santa Cruz A/G PULS-Agarose beads (sc-2003) were added to each sample and samples were rotated end-over-end overnight at 4°C.

**Protein isolation and Western blotting** – Protein isolation and Western blotting were performed as described in Chapter 2 materials and methods. Antibodies used were anti-PRMT8 (abcam ab134774; 1:1000), anti-HIF-1α (abcam ab1; 1:200), and anti-FGF2 (Millipore bFM-2; 1:1000).

**Mass spectrometry** – All IP samples were run on a 4-20% pre-cast PAGE gel until samples were a few mm into the gel. Gel slices were cut into 1x1 mm pieces and placed in 1.5ml eppendorf tubes with
1ml of water for 30 min. The water was removed and 100ul of 250 mM ammonium bicarbonate was added. For reduction 20 ul of a 45 mM solution of 1, 4 dithiothreitol (DTT) was added and the samples were incubated at 50 C for 30 min. The samples were cooled to room temperature and then for alkylation 20 ul of a 100 mM iodoacetamide solution was added and allowed to react for 30 min. The gel slices were washed 2 X with 1 ml water aliquots. The water was removed and 1ml of 50:50 (50mM Ammonium Bicarbonate: Acetonitrile) was placed in each tube and samples were incubated at room temperature for 1hr. The solution was then removed and 200 ul of acetonitrile was added to each tube at which point the gels slices turned opaque white. The acetonitrile was removed and gel slices were further dried in a Speed Vac. Gel slices were rehydrated in 75 ul of 4ng/ul trypsin (Sigma or Promega sequencing grade) in 0.01% ProteaseMAX Surfactant (Promega): 50mM Ammonium Bicarbonate. Additional bicarbonate buffer was added to ensure complete submersion of the gel slices. Samples were incubated at 37C for 21hrs. The supernatant of each sample was then removed and placed in a separate 1.5 ml eppendorf tube. Gel slices were further dehydrated with 100 ul of 80:20 (Acetonitrile: 1% formic acid). The extract was combined with the supernatants of each sample. The samples were then dried down in a Speed Vac. Samples were dissolved in 25 ul of 5% Acetonitrile in 0.1% trifluoroacetic acid prior to injection on LC/MS/MS. A 4.0 µl aliquot was directly injected onto a custom packed 2cm x 100µm C_{18} Magic 5µ particle trap column. Peptides were then eluted and sprayed from a custom packed emitter (75µm x 25cm C_{18} Magic 3µm particle) with a linear gradient from 95% solvent A (0.1% formic acid in water) to 35% solvent B (0.1% formic acid in Acetonitrile) in 90 minutes at a flow rate of 300 nanoliters per minute on a Waters Nano Acquity UPLC system. Data dependent acquisitions were performed on a Q Exactive mass spectrometer (Thermo Scientific) according to an experiment where full MS scans from 300-1750 m/z were acquired at a resolution of 70,000 followed by 10 MS/MS scans acquired under HCD fragmentation at a resolution of 17,500 with an isolation width of 1.6 Da. Raw data files were processed with Proteome Discoverer (version 1.4) prior to searching with Mascot Server (version 2.5) against the Uniprot database. Search parameters utilized were fully tryptic with 2 missed cleavages, parent mass tolerances of 10 ppm and fragment mass tolerances of 0.05 Da. A fixed modification of carbamidomethyl
cysteine and variable modifications of acetyl (protein N-term), pyro glutamic for N-term glutamine, oxidation of methionine and dimethyl arginine were considered. Search results were loaded into the Scaffold Viewer (Proteome Software, Inc.) for assessment of protein identification probabilities and label free quantitation.

Chapter 5

Cell culture – Cell culture was performed as described above. The human bone osteosarcoma cell line U2OS was a gift from the Billiar lab at WPI. The adult human colorectal adenocarcinoma cell line Caco-2 was a gift from the Weathers lab at WPI. The adult human glioblastoma cell lines A172 and U87MG were gifts from the Jain lab at WPI. The human teratocarcinoma line CRL-2073 was obtained from American Tissue Culture Collection (ATCC; Manassas, VA).

RT-PCR – RNA and cDNA were prepared and RT-PCR was performed as described in Chapter 2 materials and methods. Primer sequences can be found in Table 8.1.
CHAPTER 9

References


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