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The Role of DNA Methylation and Methyl Binding Domain Protein 2 in the Regulation of Human Embryonic and Fetal Beta Type Globin Genes

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THE ROLE OF DNA METHYLATION AND METHYL DOMAIN BINDING PROTEIN 2 IN THE REGULATION OF HUMAN EMBRYONIC AND FETAL BETA TYPE GLOBIN GENES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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Dedication

This work is dedicated to my loving family who supported me throughout this endeavor: Keira, Bailey, Jackson, Martini, and Guinness.

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Abbreviations

α	alpha
AcH3	acetylation of lysine residues on histone H3
AcH4	acetylation of lysine residues on histone H4
BAC	bacterial artificial chromosome
β	beta
bp	base pairs
°C	degrees Celsius
cDNA	DNA complementary to mRNA
ChIP	chromatin immunoprecipitation
CpG	cytosine-guanine dinucleotide
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DNMT3	DNA methyltransferase 3
dpc	days post coitus
DR	direct repeat
DRED	direct-repeat erythroid definitive
EDTA	ethylenediaminetetraacetic acid
EKLF	erythroid Krüppel like factor
ϵ	epsilon
FITC	fluorescein isothiocyanate
γ	gamma
Hb	hemoglobin
HbF	fetal hemoglobin
HDAC1	histone deacetylase 1
HDAC2	histone deacetylase 2
HPFH	hereditary persistence of fetal hemoglobin
HS	DNase-I hypersensitive site
IgG	immunoglobulin G
IL-4	interleukin-4
kb	kilobase
KLF	Krüppel like factor
LCR	locus control region
LiCl	lithium chloride
mRNA	messenger ribonucleic acid
MBD1	methyl-CpG binding domain protein 1
MBD2	methyl-CpG binding domain protein 2
MBD3	methyl-CpG binding domain protein 3

MBD4	methyl-CpG binding domain protein 4
MCBP	methyl-CpG binding proteins
MeCP1	methyl-CpG binding protein complex 1
MeCP2	methyl-CpG binding protein 2
MEL	mouse erythroleukemia
MENT	mature erythrocyte nuclear termination stage-specific protein
mg	milligram
mL	milliliter
MTA1	metastasis-associated 1
MTA2	metastasis-associated 2
NaCl	sodium chloride
NuRD	Nucleosome Remodeling and Deacetylase complex
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RbAp46	retinoblastoma-associated protein 46 kD
RbAp48	retinoblastoma-associated protein 48 kD
RNA	ribonucleic acid
RPA	ribonuclease protection assay
RPM	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SWI/SNF	switch/sucrose non-fermenting
TE	tris-EDTA
Th1	T-helper lymphocyte, subset 1
Th2	T-helper lymphocyte, subset 2
tRNA	transfer ribonucleic acid
μg	microgram
μL	microliter
YAC	yeast artificial chromosome

Abstract

THE ROLE OF DNA METHYLATION AND METHYL BINDING DOMAIN PROTEIN 2 IN THE REGULATION OF HUMAN EMBRYONIC AND FETAL BETA TYPE GLOBIN GENES

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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The genes of the human β-globin locus are located on chromosome 11 in the order of their expression during development: 5' ε, γ, β 3'. During development, silencing of the 5' gene occurs with activation of the immediate 3' gene. This process occurs twice and is termed hemoglobin switching. The exact mechanism(s) of this process have not been fully described. Herein, we describe a role for DNA methylation and methyl binding domain protein 2 in the transcriptional regulation of the human embryonic and fetal beta type globin genes. Adult mice containing the entire human β-globin locus as a yeast artificial chromosome (βYAC) express very low levels of the fetal γ-globin gene. However, treatment of adult βYAC transgenic mice with the DNA methyltransferase inhibitor, 5-azacytidine, induces a >10-fold increase γ-globin mRNA levels. In addition,

βYAC transgenic mice null for methyl binding domain protein 2 (MBD2) express a similar level of γ-globin mRNA. DNA methylation and MBD2 appear to induce γ-globin expression via the same pathway(s), as treatment of MBD2 null βYAC transgenic mice do not show an additive boost in γ-globin expression. MBD2 does not bind to the γ-globin promoter region *in vivo* indicating MBD2 mediated transcriptional silencing does not occur by recruitment of transcriptional repression complexes to the γ-globin gene promoter. Additionally, these transgenic mice contain only the 5' portion of the β-globin locus through the ε-globin, and do not express the ε-globin genes as adults. However, treatment with 5-azacytidine or loss of MBD2 induces expression of the ε-globin gene in adult transgenic mice. A similar induction of ε-globin is seen in βYAC transgenic mice under the same conditions. The level of expression of the ε-globin gene is much lower than the γ-globin gene, indicating the powerful effect of the *cis* elements mediating transcriptional repression of the ε-globin gene. These studies indicate DNA methylation and MBD2 contribute to the transcriptional repression of the human embryonic and fetal β-type globin genes. Additionally, MBD2 has been identified as a potential target for the therapeutic induction of fetal hemoglobin for the treatment of hemoglobinopathies.

Chapter 1: Introduction

The Human β-globin Locus

The human β-globin locus is located on chromosome 11 and the genes of the locus encode the β chain of hemoglobin. The genes are positioned in the order of their expression during development: 5' ε, γ, δ, and β 3' (Figure 1 and 2). Two switches in globin gene expression occur during development. The first occurs during the switch from embryonic (primitive) to definitive hematopoiesis at 5 weeks post-conception. At this time, γ-globin replaces ε-globin as the major β-chain subunit. This time also marks a change in the site of hematopoiesis from the yolk sac to the fetal liver. At the time of birth, the second switch takes place whereby β-globin replaces γ-globin. By birth, the bone marrow has become the primary site of hematopoiesis.

High level globin gene expression at all stages of development is regulated at least in part by the locus control region (LCR). The LCR acts as an enhancer, resides 6-20 kb upstream of the ε-globin gene, and consists of five DNase I hypersensitive sites (HS1-5) (Stamatoyannopoulos and Grosveld, 2001). In addition, it confers copy-number

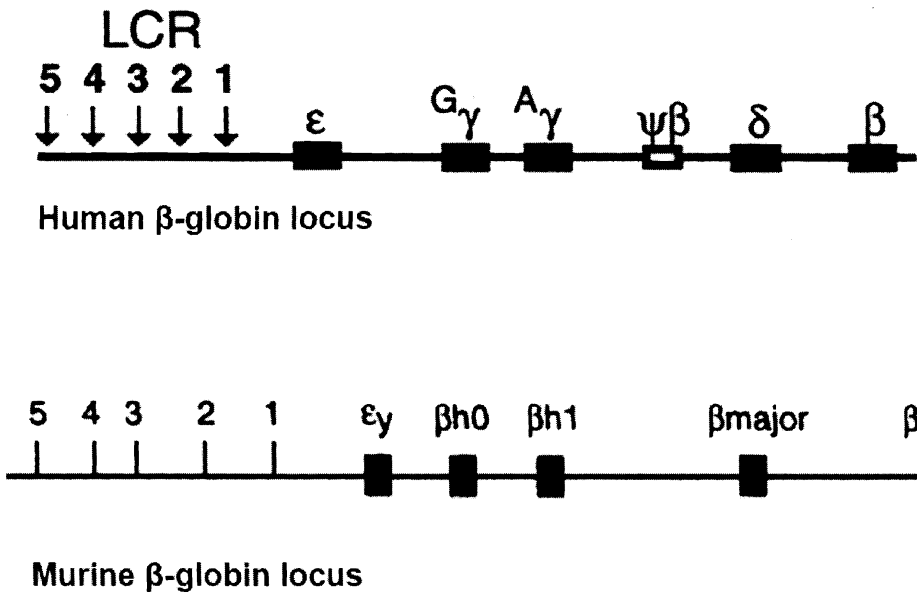

Figure 1: The human and murine β-globin loci. The top picture depicts the human β-globin locus while the bottom depicts the murine β-globin locus. Numbers and arrows indicate the position of DNase I hypersensititve sites within the locus control region (LCR). The black boxes are the positions of the genes within each locus.

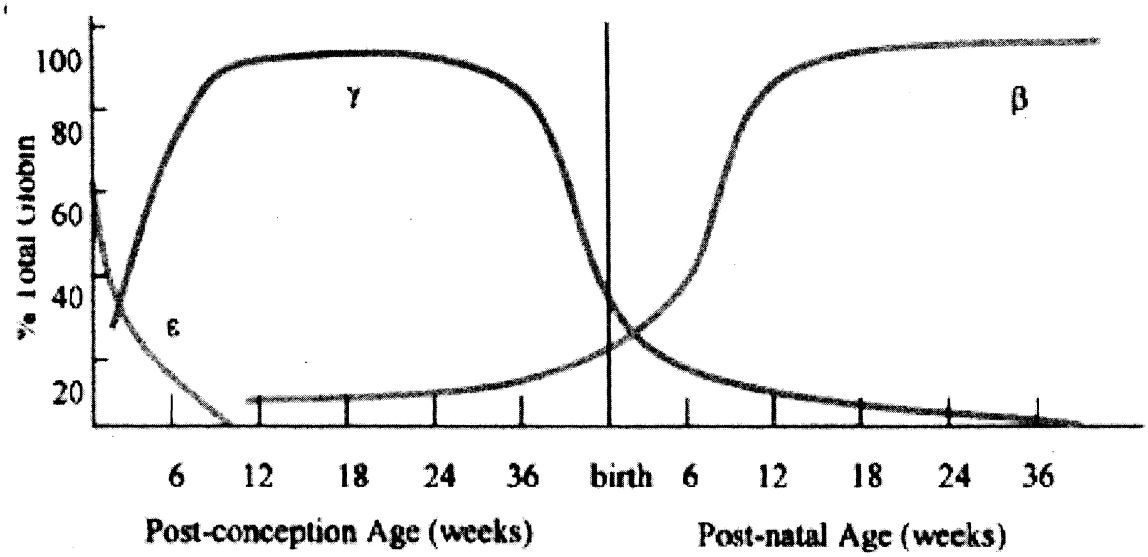

Figure 2: β-type globin chain switching during development. This graph depicts the switch in expression of β-type globin genes during development (Harju et al., 2002).

dependent position-independent expression of a linked gene in transgenic mice (Fraser and Grosveld, 1998; Grosveld et al., 1987).

The mouse has been a useful model system for the study of globin gene regulation. In mice, the first switch in globin gene expression takes place at day 10.5 post-conception, when erythropoiesis shifts from the yolk sac to the fetal liver (Chada et al., 1986; Farace et al., 1984). The mouse yolk sac contains erythroblasts of primitive hematopoiesis and expresses the ϵy (orthologous to the human ϵ) and βh1 (orthologous to the human γ) globin genes. Unlike its orthologue, βh1 is only expressed in the cells of primitive hematopoiesis. The fetal liver is the site of definitive hematopoiesis and contains erythroblasts expressing the adult globin genes β-major and β-minor. Unlike the human locus, the genes of the mouse β-globin locus are not expressed in the order of their position on the chromosome with βh1 expression coming first, followed by ϵy expression (Kingsley et al., 2005). However, mouse β-type globin genes undergo a similar switching program as is seen during human development (Figure 3). Interestingly, mice lack a fetal β-type globin gene as neither the ϵy gene nor the βh1 gene is expressed in the fetal liver. Nevertheless, due to evolutionary conservation, the mouse allows for the study of human globin gene switching. Transgenic mice containing 70 to 248 kb constructs of the entire human β-globin locus as ligated cosmids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and phage artificial chromosomes (PACs) show correct temporal expression patterns of the human genes during mouse development (Gaensler et al., 1993; Imam et al., 2000; Peterson et al., 1993a; Peterson et al., 1993b; Peterson et al., 1997; Peterson et al., 1998; Porcu et al., 1997; Strouboulis et al., 1992) (Figure 3). These

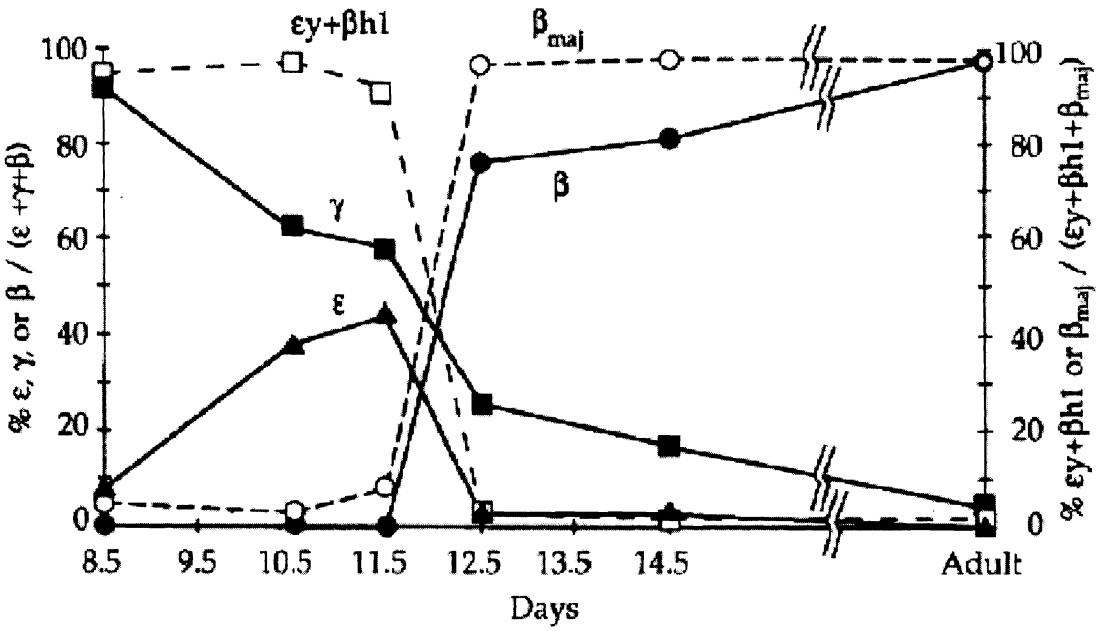

Figure 3: Endogenous and human β-type globin switching in whole locus transgenic mice. This graph depicts the changes in β-type globin gene expression during development. The y-axis on the left half depicts the level of human β-globin expression in transgenic mice containing the entire human β-globin locus as a yeast artificial chromosome during development. The y-axis on the right depicts the level of endogenous mouse β-type globin expression during development (Gaensler et al., 1993).

transgenic mice express the fetal γ-globin gene in the fetal liver, but it is not expressed as highly as the human β-globin gene. The ε-globin gene is expressed solely in the yolk sac during primitive erythropoiesis. Expression of the γ-globin gene begins in the yolk sac during primitive erythropoiesis and continues into the definitive cells of the fetal liver, finally switching off around day 17. Expression of the β-globin gene begins at day 12 in the fetal liver and is the only gene expressed in the adult. The ability of the mouse to correctly regulate human β-globin transgenes has had a profound impact upon the study of globin gene regulation. Through mutation, truncation, and rearrangement, insight has been gained on the impact of *cis* regulatory elements within the β-globin locus. In addition, the effects of gene overexpression or gene knock-out on globin gene regulation have been studied.

Globin Gene Regulation

The globin genes each consist of three exons and two introns. Tissue specific developmental regulation is achieved through the interaction of transcription factors and regulatory elements flanking each gene and elements located at a distance from the genes. Transgenic mice derived from two different human ε-globin constructs express the gene during primitive erythropoiesis, but fail to express upon definitive erythropoiesis (Raich et al., 1990; Shih et al., 1990). Thus, all elements necessary for developmentally correct expression and silencing of ε-globin gene silencing are located within the constructs used in these studies, a mechanism termed autonomous silencing. The mechanism for γ-globin

gene silencing differs slightly, as it is regulated partially by autonomous silencing and partially by competition with other globin genes for upstream enhancer activity. When attached to the LCR in the context of a transgenic mouse, γ-globin mRNA is highly expressed during the embryonic and fetal stages of development and is silenced in adult mice. However, detectable levels of γ-globin mRNA are seen in adult mice (Behringer et al., 1990; Enver et al., 1989; Enver et al., 1990). When linked to a β-globin construct, the γ-globin gene becomes completely silenced in adult transgenic mice (Behringer et al., 1990; Enver et al., 1990). Thus the γ-globin gene is not completely silenced autonomously and is dependent upon competition with other downstream globin genes for complete silencing.

Specific *cis* elements have been investigated for their role in the autonomous silencing of the ε-globin gene. The first element discovered was located between -177 and -392 bp relative to the mRNA initiation site using a transfection assay (Cao et al., 1989). Deletion of a region between -182 and -467, relative to the transcription start site, in transgenic mice corroborated this finding (Raich et al., 1992). The region includes three binding sites important for silencing: a GATA site at -208, a YY1 site at -269, and a CACCC motif at -379 (Peters et al., 1993; Raich et al., 1995). Interestingly, the GATA site seems to act as a silencing motif. Disruption of the GATA site at -208 leads to ε-globin expression in adult transgenic mice (Raich et al., 1995). Furthermore, overexpression of GATA-1 in K562 cells and transgenic mice leads to ε-globin suppression (Ikonomi et al., 2000; Li et al., 1997). Mutations of the YY1 site and CACCC element lead to low levels of ε-globin in adult transgenic mice (Raich et al.,

1995). The -182 to -467 silencer was studied using a 2.5kb micro-LCR and a 3.7 kb EcoRI fragment of the ϵ-globin gene (Raich et al., 1992). Different results were seen upon deleting a fragment from -179 to -304 in the context of a yeast artificial chromosome (Liu et al., 1997). The deletion included the YY1 and GATA binding sites previously defined as silencing elements. Transgenic mice harboring the YAC deletion construct failed to express ϵ-globin during primitive erythropoiesis and displayed reduced γ-globin expression at the same time (Liu et al., 1997). These data indicate the region may act as an activator during development. This work shows the importance of using the complete globin locus to study the autonomous silencing of the ϵ-globin gene, and, in addition, that other silencing elements may be present. In fact, two inverted direct repeats of a short motif analogous to DR-1 binding sites for non-steroid nuclear hormone receptors located in the region of the CCAAT box have been found to act as ϵ-globin silencers (Filipe et al., 1999; Tanimoto et al., 2000). Mutation of the 5′ DR and both DRs in the context of YAC transgenic mice leads to ϵ-globin expression at levels of ~1 and 4.5% relative to yolk sac expression levels, respectively (Tanimoto et al., 2000). This is almost four times more induction than was seen when comparing the -182 to -467 deletion mice to wild type mice at 11 dpc (4.5% vs. 1.4%) (Raich et al., 1990; Raich et al., 1992). Characterization of MEL cell nuclear extracts identified a novel complex, DRED (direct repeat erythroid definitive), that binds to the inverted DRs only during definitive erythropoiesis (Tanimoto et al., 2000). Further characterization of the complex showed it to contain the nuclear orphan receptors TR2 and TR4 as a heterodimer (Tanabe et al., 2002). The autonomous silencing of the human ϵ-globin gene has been mapped to

four regulatory regions. Deletions or point mutations in these regions lead to adult expression in the context of transgenic mice. The regions include, relative to the cap site, -3000, between -2000 and -460, -460 to -180, and the proximal promoter (Li et al., 1998a; Li et al., 1998b; Raich et al., 1995; Wada-Kiyama et al., 1992). However, none of these elements is able to restore expression to levels seen in the yolk sac and, in addition, no studies have been undertaken where multiple silencing elements have been eliminated. This suggests that additional silencing elements are or there are other mechanisms of silencing. In addition, factors specific for primitive erythropoiesis may be required to achieve high levels of ϵ-globin expression in the adult.

Unlike the ϵ-globin gene, the γ-globin gene is regulated by a combination of autonomous silencing and competition. In transgenic mice made from a construct containing portions of the LCR, both the γ-globin gene and the β-globin gene show correct expression profiles during development (Behringer et al., 1990; Enver et al., 1990). In contrast, mice containing the LCR and only the γ-globin gene show incorrect developmental regulation of the γ-globin gene, since the γ-globin gene is expressed in adult mice (Behringer et al., 1990; Enver et al., 1989). These data indicate that the presence of the β-globin gene is needed to silence the γ-globin gene during development, presumably by competing for LCR-mediated enhancer activity. However, another study using a different LCR-γ-globin gene construct showed the correct developmental silencing of the γ-globin gene in the absence of other competing globin genes, illuminating an autonomous silencing mechanism (Dillon and Grosveld, 1991). These differences may be accounted for by the different constructs used in each study. Despite

these contradictory studies, it is generally accepted that both autonomous silencing and competition each contribute to some degree to γ-silencing during development. Sequences within the promoter play a crucial role in γ-globin gene regulation. This point is illustrated when looking at non-deletional mutants of hereditary persistence of fetal hemoglobin (HPFH). Individuals with HPFH express increased levels of fetal hemoglobin as adults due to either genetic deletions or point mutations. Many of the described point mutations of HPFH are due to single base pair substitutions in the γ-globin promoter region (Table 1). Thus, the promoter region of the γ-globin gene contains important regulatory elements for maintaining γ-globin gene suppression. The two identical γ-globin gene promoters each contain a canonical TATA box, a duplicated CAAT box within a 27 bp segment, and a single CACCC box. Located between the CAAT box and TATA box is a G-rich sequence possessing a binding activity called stage selector protein (SSP) (307, 308). Mutation of the SSP binding site results in down-regulation of the γ-globin gene only when competing for the β-globin gene during the second switch (Ristaldi et al., 2001). There are 2 CpG dinucleotides within the SSP binding site, the stage selector element (SSE), at -55 and -50 relative to the transcription start site. When these sites are methylated, Sp1 binds more readily than SSP. These data indicate that SSP may bind the SSE as an activator while Sp1 may mediate repression of the γ-globin gene when the SSE is methylated, as is seen in adult erythroid cells (Humphries et al., 1985). However, mice that contain the human β-globin locus but lack functional Sp1 still express the γ-globin gene, indicating that it is not functionally important *in vivo* or that another factor can compensate for its loss (Marin et al., 1997).

Type and Ethnic Group	Mutation	HbF%
Normal		0.5-1
Japanese	$^{G}\gamma$-114 C to T	11-14
Australian	$^{G}\gamma$-114 C to G	8.6
Black/Sardinian	$^{G}\gamma$-175 T to C	17-30
Tunician	$^{G}\gamma$-200 +C	18-49
Black	$^{G}\gamma$-202 C to G	15-25
Georgian	$^{A}\gamma$-114 C to T	3-6.5
Black	$^{A}\gamma$-114 to -102 deleted	30-32
Greek	$^{A}\gamma$-114 G to A	10-20
Cretan	$^{A}\gamma$-158 C to T	2.9-5.1
Black	$^{A}\gamma$-175 T to C	36-41
Brazilian	$^{A}\gamma$-195 C to G	4.5-7
Chinese/Italian	$^{A}\gamma$-196 C to T	14-21
British	$^{A}\gamma$-198 T to C	3.5-10
Georgian	$^{A}\gamma$-202 C to T	1.6-3.9

Table 1: List of non-deletional mutations of hereditary persistence of fetal hemoglobin (Stamatoyannopoulos and Grosveld, 2001).

Mutation of the CACCC and TATA boxes in transgenic mice and transfected cells interferes with competition and leads to an increase in β-globin transcription at the expense of the γ-globin gene (Sargent et al., 1999; Sargent and Lloyd, 2001). The CACCC box is the binding site for the zinc finger Krüppel like proteins. The erythroid specific EKLF is crucial for β-globin activation and mice null for EKLF are embryonic lethal due to severe anemia (Nuez et al., 1995; Perkins et al., 1995). EKLF is specific for β-globin gene expression, despite the fact that both the γ- and ε-globin genes possess CACCC boxes. Therefore, it was postulated that there exists a CACCC-binding factor that specifically enhances ε- and/or γ-globin expression during development. However, given the large number of known KLFs, no factor specific γ-globin expression has emerged (Zhang et al., 2005). However, KLF2 has emerged as an activator of the ε-globin gene, but not the γ-globin gene, indicating that another KLF or redundancy plays a role in regulation of the γ-globin by the CACCC box (Basu et al., 2005). Similar to the ε-globin gene, DRED mediated transcriptional repression at DR elements of the γ-globin gene has been described (Omori et al., 2005). In addition, a silencer element has been mapped using transgenic mice to -378 to -730 relative to the transcription start site (Stamatoyannopoulos et al., 1993). Cis elements located within the promoters of both the human ε- and γ-globin genes contribute to the transcriptional regulation of the genes during development.

Mechanisms for γ-globin transcriptional repression have also been postulated to occur at sites distant from the γ-globin gene region. Increased expression of fetal hemoglobin in the absence of any known deletional or non-deletional HPFH mutation has

been described. Mapping studies have linked regions of chromosome 6, 8, and X as potential modifiers of γ-globin expression (Chang et al., 1995; Garner et al., 2004; Thein and Craig, 1998; Wyszynski et al., 2004). Potential candidate genes in these regions include Hbs11, GGH, DEPDC2, PIK3C2A, GPM6B, TSPYL1, and ZHX2. Thus, regions outside of the γ-globin gene may contribute to the transcriptional silencing of the γ-globin gene during development.

DNA Methylation

Epigenetic phenomena are biological processes that cause heritable changes in gene expression without affecting the sequence of the gene itself. DNA methylation is the prototypic epigenetic modification involving the addition of a methyl group to the carbon 5 position of the cytosine ring in a CpG dinucleotide by a DNA methyltransferase (DNMT). Cytosine is the only base known to be methylated in eukaryotic DNA. However, methylated cytosine is highly unstable and mutagenic. The number of CpG dinucleotides is estimated to be 20% of what would be expected based on the base composition of DNA owing to the spontaneous deamination of methylated cytosine to thymine (Bird, 1980). Regions of the genome containing the expected frequency of CpG dinucleotides have been termed "CpG islands." They are defined as regions that range from 200 bases to several kilobases in length, with a G + C content greater than 50%, and having a ratio of CpG to GpC greater than 0.6 (Gardiner-Garden and Frommer, 1987).

These CpG islands are believed to represent regions of the genome that have remained unmethylated and thus spared from the deamination process.

The DNA methyltransferase enzymes catalyze the addition of a methyl group to the CpG dinucleotide. DNA methyltransferase 1 (DNMT1) is considered the maintenance methyltransferase as it preferentially methylates hemi-methylated DNA during S-phase (Leonhardt et al., 1992). DNMT3a and 3b preferentially methylates unmethylated DNA and are considered to be "*de novo*" methyltransferases (Yokochi and Robertson, 2002). The importance of DNA methylation is clearly evident in the embryonic lethal phenotype of DNMT1 or DNMT3 knock-out mice (Li et al., 1992; Okano et al., 1999).

Often there exists an inverse correlation between the level of DNA methylation and transcriptional activity: hypermethylated regions of DNA are transcriptionally silent and hypomethylated regions are active. The exact mechanism of DNA methylation induced transcriptional silencing has not been completely discerned, but mechanisms have been proposed (Singal and Ginder, 1999). One mechanism involves direct inhibition of factor binding. AP-2, cMyc/Mym, the cyclic AMP-dependent activator CREB, E2F, and NF-κB have been shown to have impaired binding upon DNA methylation. On the contrary, many transcription factor binding sites lack CpGs and some factors (Sp1 and CTF) can bind regardless of methylation status (Tate and Bird, 1993).

A second mechanism involves the binding of transcriptional repressors directly to methylated DNA. The discovery of a family of proteins, the methyl-CpG binding proteins (MCBP), that specifically bind to methylated DNA has led to the theory that these proteins act as the mediators of transcriptional repression by DNA methylation (Chen et

al., 2003; Hutchins et al., 2002; Lin and Nelson, 2003; Magdinier and Wolffe, 2001; Sarraf and Stancheva, 2004; Zucker et al., 1983). There are currently six well characterized MCBPs, five of which possess a distinct domain, the Methyl-CpG Binding Domain (MBD) that is necessary and sufficient for binding of the proteins to methylated DNA: MeCP2, MBD1, MBD2, MBD3, and MBD4 (Nan et al., 1993). The sixth protein, Kaiso, binds to methylated DNA via its zinc-finger domains and lacks a true MBD (Daniel et al., 2002).

MBD2 binds more avidly to methylated DNA *in vitro* as CpG density increases, however it has been shown to bind to as few as three methylated CpGs *in vitro* (Fraga et al., 2003). MBD2 was identified in the purification of the MeCP-1 complex, the first methyl-CpG specific binding activity to be described (Feng and Zhang, 2001). This protein complex consists of MBD2 and the Nucleosome Remodeling and Deacetylation (NuRD) complex: Mi-2, MTA1, MTA2, MBD3, HDAC1, HDAC2, RbAp46, RbAp48. Targeted knock-out of the MBD2 gene in mice generates a mild phenotype consisting of a decrease in maternal-nurturing of pups (Hendrich et al., 2001). Loss of MBD2 is also associated with dysregulation of the IL-4 gene in Th1 and Th2 lymphocytes (Hutchins et al., 2002). In addition, lack of MBD2 appears to prevent intestinal tumorigenesis in a mouse model (Sansom et al., 2003). However, there are no other genes known to be primarily regulated by MBD2 in non-cancerous cells.

MeCP2 is able to bind to a single symmetrically methylated CpG (Meehan et al., 1992). MeCP2 contains a transcriptional repression domain (TRD) and has been shown to interact with the Sin3a transcriptional repression complex (Nan et al., 1997). However,

this interaction is unstable, but a stable interaction between MeCP2 and Brahma, a component of the SWI/SNF nucleosome remodeling complex, has been reported (Harikrishnan et al., 2005; Klose and Bird, 2004). In addition, MeCP2 recruits histone methyltransfersase activity to the H19 gene (Fuks et al., 2003). MeCP2, unlike MBD2, shows sequence specificity as it preferentially binds to CpGs with adjacent A/T tracks (Klose et al., 2005). Mice null for MeCP2 have a severe phenotype similar to Rett Syndrome, a human neurodevelopmental disorder leading to loss of voluntary movement (Guy et al., 2001). Specifically, the Bdnf and Dlx5 genes have been shown to be regulated by MeCP2 *in vivo* (Chen et al., 2003; Horike et al., 2005; Martinowich et al., 2003).

MBD1 has been identified as a critical component of an S-phase specific complex that propagates the DNA methylation signal into dimethylation of lysine 9 of histone H3 during DNA replication (Sarraf and Stancheva, 2004). In addition, mice lacking functional MBD1 have deficits in hippocampal function (Zhao et al., 2003). MBD3 is a core component of the NuRD complex and has been shown to associate with MBD2, Ikaros, GATA1, and FOG (Hong et al., 2005; Kim et al., 1999; Rodriguez et al., 2005; Zhang et al., 1999). MBD4 functions as a mismatch-specific T/U DNA glycosylase, recognizing the product of spontaneous deamination of a methyl-CpG dinucleotide and excising it (Hendrich et al., 1999). The Kaiso factor contains a transcriptional repressor domain in addition to a zinc finger domain that confers sequence specificity, but lacks an MBD (Prokhortchouk et al., 2001). Kaiso has been reported to interact with the N-Cor complex to mediate DNA methylation dependent repression of the MTA2 gene (Yoon et

al., 2003). The zinc fingers of Kaiso can bind methylated DNA or the Kaiso binding site (Daniel et al., 2002). No Kaiso knock-out mice have been generated, but the Kaiso gene has been shown to be essential for Xenopus development (Ruzov et al., 2004).

MCBPs appear to be most crucial to neural development as genetic knock-out of MBD2, MeCP2, and MBD1 all display a neural deficit phenotype. Knock-out of these proteins is not lethal, unlike the DNMT knock-outs, suggesting each MCBP regulates only a sub-set of genes or that MCBPs can compensate for each other. MBD2 has been shown to bind to MeCP2 sites after knock-down of MeCP2, however, MeCP2 was unable to bind to MBD2 sites after knock-down of MBD2 (Klose et al., 2005). Thus, MeCP2 has more sequence specificity for binding methylated CpGs than MBD2. This may account for the more severe phenotype of MeCP2 null mice versus MBD2 null mice. Interestingly, mice null for MBD3 are embryonic lethal (Hendrich et al., 2001). MBD3 is a core component of the NuRD complex. The NuRD complex is a transcriptional repression complex shown to interact with many proteins including MBD2. Thus, loss of a key transcriptional repression complex is more severe than loss of a single MCBP. In the absence of a MCBP, complexes such as NuRD are still able to interact with other MCBPs. Therefore, the only genes dysregulated will be those that possess sequence specific binding sites. On the other hand, loss of a transcriptional repression complex, for example NuRD in the absence of MBD3, cannot be compensated for by another MCBP. This may account for the phenotypic differences between MCBP knock-out mice and MBD3 knock-out mice.

DNA Methylation and Globin Gene Regulation

DNA methylation was first hypothesized to play a role in gene regulation over 30 years ago (Holliday and Pugh, 1975; Riggs, 1975; Scarano, 1971). The first correlation between transcriptional activity and DNA methylation was seen in chicken adult red blood cells (McGhee and Ginder, 1979). Subsequently, similar results were seen in other species (Atweh et al., 2003; Mavilio et al., 1983; Shen and Maniatis, 1980; van der Ploeg and Flavell, 1980). These studies came to the same conclusion that actively transcribed genes were in general hypomethylated around the promoter whereas silent genes were hypermethylated. The globin genes were the first genes shown to be regulated in part by DNA methylation and were among the first genes shown to be up-regulated by the DNMT inhibitor 5-azacytidine, a cytosine analogue that incorporates into DNA and covalently binds to DNMTs leading to their inhibition. 5-azacytidine was first shown to increase fetal hemoglobin (HbF, $\gamma_2\alpha_2$) in anemic baboons (DeSimone et al., 1982). Similar induction of silenced embryonic and fetal globin genes by 5-azacytidine has been reported in chickens, MEL cells, MEL cells containing human chromosome 11, and mice harboring a YAC transgene (Atweh et al., 2003; Ginder et al., 1984; Ley et al., 1984; Pace et al., 1994; Zucker et al., 1983). Taken together, these results indicate DNA methylation plays a role in globin gene silencing during development. In addition, methylation of the avian embryonic ρ-globin gene inversely correlates with its expression during development (Singal et al., 1997).

Interestingly, co-administration of 5-azacytidine with the short chain fatty acid, butyrate, was shown to synergistically increase expression of fetal hemoglobin in baboons and embryonic ρ-globin in chickens versus 5-azacytidine treatment alone (Constantoulakis et al., 1989; Ginder et al., 1984). A similar effect, though less pronounced, was seen in mice harboring a βYAC transgene (Pace et al., 1994). Short chain fatty acids (SCFA), among other functions, inhibit histone deacetylases (HDACs) (Swank and Stamatoyannopoulos, 1998). The synergistic increase in globin gene expression upon treatment with 5-azacytidine and SCFAs is likely due to the co-localization of HDACs with methyl binding complexes. A methyl CpG binding complex has been shown to bind the ρ-globin promoter and proximal transcribed region (Singal et al., 2002). Mass spectrometry analysis of the biochemically purified complex showed it to contain both MBD2 and HDAC2 (Kransdorf and Ginder, 2004). MBD2 was further shown to bind the ρ-globin promoter and proximal transcribed region *in vivo* by chromatin immunoprecipitation assay (ChIP). Finally, this complex differs in biochemical make-up from MeCP1 and shows a preference for the ρ-globin proximal transcribed region versus the CG11 construct used to purify MeCP1. This is evidence that sequence specific binding of MCBPs may in part be determined by the biochemical make-up of the complex it interacts with in a cell and tissue specific manner. Taken together, these results indicate a complex interaction between DNA methylation, histone acetylation, and histone methylation may be at work to regulate globin gene expression.

Induction of Silenced Globin Genes

Short chain fatty acids alone have shown mixed results in the ability to induce globin gene expression. Both sodium butyrate and alpha amino butyric acid were unable to induce expression of silenced globin genes in anemic chickens or βYAC transgenic mice (Ginder et al., 1984; Pace et al., 1994). However both sodium butyrate and proprionate were able to induce mouse εy expression in MEL cells (Little et al., 1995). Increased levels of γ-globin mRNA have been seen in patients receiving SCFA treatment and in children with metabolic disorders leading to increased levels of SCFAs in the serum (Collins et al., 1994; Collins et al., 1995; Little et al., 1995; Olivieri et al., 1997; Perrine et al., 1993; Sher et al., 1995). Treatment of βYAC transgenic mice and baboons with various short chain fatty acid derivates (SCFAD) induced γ-globin mRNA expression (Pace et al., 2002). It has yet to be determined whether the SCFADs used in the study can produce the same synergistic effect on globin expression as seen when combining sodium butyrate or alpha amino butyric acid with 5-azacytidine in chickens and transgenic mice. SCFA impact other cellular functions in addition to acting as inhibitors of histone deacetylation. For example, butyrate has been shown to arrest cell growth in G1 phase, induce protein synthesis, and change cell morphology (Borenfreund et al., 1980; Kruh, 1982; Rastl and Swetly, 1978). Induction of the γ-globin gene has been achieved in the absence of DNA methylation and HDAC inhibition. Specifically, γ-globin mRNA can be induced via manipulation of cell signaling pathways including the p38 MAP kinase pathway, the soluble guanylate cyclase-cGMP dependent kinase pathway, and the l-arginine/nitric oxide/cGMP pathway (Bhanu et al., 2004; Haynes, Jr.

et al., 2004; Ikuta et al., 2001; Pace et al., 2003). In addition, induction of γ-globin has been seen with compounds that do not directly affect epigenetic modifications or cell signaling pathways (Bianchi et al., 1999; Bianchi et al., 2001; Lampronti et al., 2003). Taken together, the regulation of the globin locus involves *cis* elements, *trans* factors, and epigenetic modifications. Understanding how all regulatory elements interact is crucial to the establishment of proper treatment regimens for patients afflicted with hemoglobinopathies.

Significance

Hemoglobinopathies are a group of pathologies characterized by a deficency in the production of the hemoglobin molecule. Most hemoglobinopathies can be categorized into one of two groups. The first group is able to produce structurally normal hemoglobin, but erythropoiesis is ineffective (i.e., the thalassemias). The β-thalassemias are a group of disorders characterized by impaired β-chain synthesis leading to ineffective erythropoiesis. Homozygous patients often die in their teens or early twenties due to the severity of the anemia. The second group produces structurally abnormal hemoglobin due to aberrant amino acid substitutions (i.e., sickle cell disease). Sickle cell disease (SCD) affects 1 out of every 600 African Americans and is due to a single mutation in the amino acid sequence (glutamic acid to valine at position 6 of the β-chain subunit). The mutation allows the sickle hemoglobin to polymerize and distort the shape of the erythrocyte. As a result, cells lose flexibility and are prone to block the

microvasculature leading to painful crises. The course of the disease varies from patient to patient depending on various parameters. However, the life of a sickle cell patient is often shortened and full of pain. Patients with hemoglobinopathies and a genetic mutation leading to hereditary persistence of fetal hemoglobin often show less severe to no signs of the disorder. In β-thalassemia, the expression of the γ-chain can compensate for loss of β- and δ-chain synthesis. The Cooperative Study of Sickle Cell Disease, a large multicenter study of the history of SCD, showed an inverse correlation between levels of fetal hemoglobin and painful crises and early death (Platt et al., 1991; Platt et al., 1994). Fetal hemoglobin was also shown to have a sparing effect on polymerization of deoxyhemoglobin S, the cause of membrane deformability in SCD (Poillon et al., 1993). In addition, the β- and α-embryonic genes, ε- and ζ-globin, have been shown to inhibit the polymerization process *in vitro* and *in vivo* (He and Russell, 2002; He and Russell, 2004). Expression of both these genes was also able to reverse a mouse model of α- and β-thalassemia (Russell and Liebhaber, 1998). The induction of silent globin genes is the primary treatment option for patients with hemoglobinopathies. Many clinical trials are currently ongoing in an effort to find the best compound(s) to treat these patients (Atweh et al., 2003). However, the currently prescribed treatments are either highly toxic or ineffective in a subset of patients. Therefore, dissecting the exact mechanism(s) of globin gene silencing will lead to safer and more effective treatment options for patients. Included in this are compounds that more specifically target the globin gene silencing process and possibly gene specific therapy.

The impact of this study is not solely limited to the realm of hemoglobinopathies. Epigenetic modifications have been implicated in many disease processes. Specifically, abnormalities in DNA methylation are associated with mental retardation, disorders of imprinting, and cancer. Expansion and methylation of a CGG repeat in the *FMR1* 5' untranslated region leads to gene silencing in Fragile X syndrome (Oostra and Willemsen, 2002). DNA methylation plays a crucial role in genomic imprinting. Dysregulation of the imprinted genes is associated with Angelman syndrome, Prader-Willi Syndrome, and Beckwith-Wiedemann Syndrome (Egger et al., 2004). DNA methylation also plays a role in the development of many cancers. In fact, epigenetic silencing has been recognized as a third pathway satisfying Knudson's hypothesis that two hits are necessary for the silencing of tumor suppressor genes (Jones and Laird, 1999). For example, the gene encoding MLH1 is often methylated and silenced in patients with sporadic colorectal cancers with microsatellite instability phenotype (Kane et al., 1997). Thus, discerning the role of epigenetics in globin gene silencing may have a direct impact on other disorders where epigentics play a critical role in pathogenesis.

CHAPTER 2: Generation and Analysis of Globin Gene Regulation in Transgenic Knock-out βYAC/MBD2-/- Mice

Introduction

Methylation of the 5' position of cytosine in the CpG dinucleotide in vertebrates is associated with transcriptional repression. A group of proteins, the methyl-CpG binding proteins (MCBP), specifically recognizes and binds to DNA sequences containing methylated cytosines. Most of these proteins belong to a subgroup based on the common features of a characteristic domain, the Methyl-CpG Binding Domain (MBD), which is necessary and sufficient for binding of the proteins to methylated-DNA (Nan et al., 1993). The MBD protein family consists of MeCP2, MBD1, MBD2, MBD3, and MBD4. MBD2 and MeCP2 have been found to bind methylated-DNA as large multi-protein complexes (Feng and Zhang, 2001; Jones et al., 1998; Nan et al., 1998). MBD4 differs from the other MBD proteins in that its primary function is as a DNA repair enzyme. DNA methylation mediated transcriptional repression is thought to be due to the recruitment of MCBPs and repressive protein complexes to methylated DNA. These complexes are thought to mediate transcriptional repression by recruiting histone deacetylases and transcriptional repressor proteins to methylated-DNA (Chen et al., 2003; Hutchins et al., 2002; Lin and Nelson, 2003; Magdinier and Wolffe, 2001; Sarraf and

Stancheva, 2004). MBD2 is part of the methyl-CpG binding protein complex 1 (MeCP1) which contains Mi-2, MTA1, MTA2, MBD3, HDAC1, HDAC2, RbAp46, and RbAp48 (Feng and Zhang, 2001). Early studies suggested an interaction between MeCP2 and the SIN3A transcriptional repression complex. However, recent evidence indicates this interaction may not be stable (Klose and Bird, 2004). Brahma, a component of the SWI/SNF nucleosome remodeling complex, does form a stable complex with MeCP2 (Harikrishnan et al., 2005). The Kaiso factor represents another type of methyl CpG binding protein that contains a transcriptional repressor domain as well as a zinc finger domain that confers sequence specificity, but lacks an MBD (Prokhortchouk et al., 2001). The zinc fingers of Kaiso can bind methylated CGCGs or the Kaiso Binding Site TCCTGCNA (Daniel et al., 2002).

Targeted deletions of methyl CpG binding proteins have yielded insight into the functions of these proteins *in vivo*. Mice null for MBD1 have deficits in adult neurogenesis and hippocampal function (Zhao et al., 2003). Mice lacking functional MeCP2 display a phenotype similar to Rett Syndrome, a human neurodevelopmental disorder leading to loss of voluntary movements (Guy et al., 2001). Loss of MBD2 has a milder phenotype with the chief manifestation being decreased maternal nurturing of pups (Hendrich et al., 2001). Loss of MBD2 specifically interferes with the regulation of the IL-4 gene in mouse Th1 and Th2 lymphocytes indicating that MBD2 can contribute to the regulation of genes in a tissue specific manner (Hutchins et al., 2002). However, no other genes have been reported to be primarily regulated by MBD2 in non-cancerous cells. Unlike the embryonic lethal phenotype of DNA methyltransferase (DNMT) knock-

out mice, loss of MBDs is not catastrophic, indicating MBDs may compensate for each or that only a small number of genes required for normal cell function are regulated by any specific MBD (Li et al., 1992; Okano et al., 1999). Interestingly, Kaiso, a methyl-CpG binding protein not in the family of MBD containing genes, has been shown to be essential for Xenopus development (Ruzov et al., 2004).

The genes of the human β-globin locus are expressed sequentially during development in the order they appear on chromosome 11: 5' ε-, $^{G}\gamma$-, $^{A}\gamma$-, and β-3'. The individual genes have been shown to be regulated by a complex interplay between *cis* elements, *trans* factors, competition for an upstream enhancer, and epigenetics (Stamatoyannopoulos and Grosveld, 2001). Despite a tremendous amount of progress in this field, the exact mechanism(s) of globin gene silencing is still not understood. The initial observation in vertebrates that DNA methylation is inversely related to gene expression was noted in the globin locus (McGhee and Ginder, 1979). Furthermore, the globin genes were the first group of genes for which the DNA methyltransferase inhibitor, 5-azacytidine, was shown to activate silenced embryonic and fetal genes *in vivo* in both animal models and in a therapeutic setting (Charache et al., 1983; DeSimone et al., 1982; Ginder et al., 1984; Ley et al., 1982). Previously, we have shown a role for DNA methylation and MBD2 in the developmental regulation of the chicken embryonic ρ-globin gene (Burns et al., 1988; Ginder et al., 1984; Singal et al., 1997; Singal et al., 2002). The ρ-globin gene becomes highly methylated at the same time the gene becomes silent. Furthermore, the ρ-globin gene is enriched for MBD2 in adult erythrocytes when the gene is silent, but not when it is actively transcribed. Finally, a protein complex

containing MBD2 purified from primary chicken erythrocytes binds to a methylated ρ-globin gene 5' sequence and differs in biochemical make-up from MeCP1 (Kransdorf and Ginder, 2004). These findings are evidence that MCBPs may form unique tissue restricted complexes that are targeted to specific sets of genes. Taken together, this evidence suggests MBD2 may be involved in the process of globin gene silencing by DNA methylation.

To determine the role of MBD2 in mammalian globin gene silencing, mice containing the entire human globin locus as a yeast artificial chromosome (βYAC) were bred with mice containing a targeted deletion of MBD2. Adult βYAC/MBD2-/- mice inappropriately express the γ-globin gene at a level commensurate to mice treated with 5-azacytidine, while adult βYAC mice express the γ-globin gene at much lower level. This is, to our knowledge, the first evidence of a specific MCBP regulating γ-globin expression. In addition, loss of MBD2 delays the silencing of the γ-globin gene during embryonic development. Loss of MBD2 slightly decreases DNA methylation levels around the γ-globin promoter, and inhibiting DNA methylation does not greatly enhance the expression of the γ-globin gene, indicating the induction seen in knockout mice is not largely dependent on DNA methylation or that the additional effect of 5-azacytidine is mediated by a different MCBP.

Methods

Generation of βYAC/MBD2-/- Mice

MBD2-/- mice (a generous gift from Dr. Adrian Bird) were mated with βYAC mice (a generous gift from Dr. Karin Gaensler). The subsequent βYAC/MBD2+/- mice were bred with MBD2-/- mice to generate βYAC/MBD2-/- mice. To maintain the line, βYAC/MBD2-/- were bred with MBD2-/- mice. All progeny were screened for the presence of the transgene and absence of MBD2 using PCR of DNA from tail snips. Primers used to screen mice are located in Table 2. Two lines of βYAC mice were used to generate transgenic knock-out mice, the A20.1 and A85.68 lines. Both contain a single copy of the βYAC transgene in the C57 Bl/6 background (A20.1) or the FVB/N background (A85.68). The MBD2-/- mice are in the BALB/C background. Through multiple rounds of breeding to establish and maintain lines, the final transgenic knock-out mice were in a heterogeneous genetic background.

Generation of βYAC/MeCP2-/- Mice

Female MeCP2+/- mice (Jackson Laboratories) were bred with A20.1 βYAC transgenic mice. The MeCP2 gene is located on the X-chromosome, therefore only one round of breeding was necessary to generate βYAC/MeCP2-/y mice. All mice were screened for the presence of the transgene and absence of MeCP2 using PCR from tail snip DNA (Table 2).

Red Cell Indices

Peripheral blood was extracted from the inferior vena cava of three non-anemic mice of each genotype. The blood was collected in a citrate coated lavender top tube. The

blood was analyzed on a Coulter Hematology Analyzer (Beckman Coulter). Reticulocytes were counted manually after staining with methylene blue for 15 minutes at 37° C. 1000 cells were counted per analysis.

Timed Matings

For MBD2 wild type embryos, βYAC males were bred with non-transgenic females. For MBD2-/- embryos, βYAC/MBD2-/- males were bred with non-transgenic MBD2-/- females. The presence of a vaginal plug was designated day 0.5. Fetal livers were dissected from day 14.5 and 16.5 embryos.

Treatments

Mice were treated for two days with intraperitoneal injection of 1-acetyl-2-phenylhydrazine (10 mg/mL, Sigma) at a dose of 0.4 mg/10g. On the third day, mice were treated with 5-azacytidine (0.5 mg/mL, Sigma) at a dose of 2 mg/kg for five days via intraperitoneal injection.

RNA Isolation

RNA was extracted from fetal livers or peripheral blood using Trizol (Invitrogen). Fetal livers were homogenized in 200 μL of Trizol in a 1.5 mL tube. The volume was then increased to 1 mL with Trizol. Peripheral blood was added directly to 1 mL of Trizol. 200 μL of $CHCl_3$ was added to the tube and shaken vigorously for 20 seconds and allowed to sit at room temperature for 3 minutes. The tube was spun at

Primer	Sequence
Beta 488 NC	GAGGGGAAAAGGTCTTCTACTTGG
Beta 161 BC	CCCTACGCTGACCTCATAAATGC
MBD2-17390-F	CCTCAGCTGGCAAGATACCT
MBD2-17507-R	GGGGGTCATTCCGGAGTCT
bGeo-3084-F	GGTCAGGTCATGGATGAGCAGA
bGeo-3286-R	CGCGGATCATCGGTCAGACGATT
MeCP2 Common	GGTAAAGACCCATGTGACCC
MeCP2 K/O	TCCACCTAGCCTGCCTGTAC
MeCP2 WT	GGCTTGCCACATGACAA

Table 2: List of primers used to screen transgenic and knock-out βYAC0020mice.

12000 x G for 15 minutes at 4° C. The upper aqueous phase was collected and precipitated with 83% volume of isopropanol at room temperature for 10 minutes followed by centrifugation at 12000x*g* for 10 minutes at 4°C. The resulting RNA pellet was washed with 70% ethanol/30% DEPC-treated water and air dried. The pellet was resuspended in DEPC-water containing 1 μL of SuperRNasIN (Ambion) per 40 μL of DEPC-water. Fetal liver RNA was further purified by LiCl precipitation. The RNA concentration was determined using a UV spectrophotometer.

RNase Protection Assay

Single stranded, radiolabeled cRNA probes were synthesized using SP6 polymerase and α-^{32}P UTP (MP Biomedicals) with various templates. The mouse α-globin probe was derived from a 309 bp fragment at the 3' end of the gene cloned into pSP6 vector (Curtin et al., 1989). Probes for γ-globin analysis were from a 1.7 kb fragment of the $^{A}\gamma$-globin gene protecting two bands (205, 144bp) when cut with EcoRI or one band when cut with NcoI (188bp). The fragment was cloned into the pGEM-3zf(-) plasmid (Promega). To discriminate between $^{G}\gamma$- and $^{A}\gamma$-globin mRNA, a probe identical to one used previously was generated by PCR (Morley et al., 1991). The PCR product was cloned into the pGEM-EZ plasmid (Promega). The forward primer contained a *HindIII* site to make a linear template protecting a 215 bp fragment for $^{A}\gamma$-globin mRNA and a 135 bp fragment for $^{G}\gamma$-globin gene. For the detection of γ-globin, 500 ng RNA was analyzed per RPA. For mouse α-globin analysis, 10 ng of RNA was used per analysis. RNA was hybridized overnight at 42°C with 1x10^{6} cpm of probe in 30 μL of

hybridization buffer containing 80% formamide. After hybridization, the γ-globin and α-globin samples were combined for RNase digestion so both samples could be run on the same gel. The samples were digested with RNase digestion buffer (300mM NaCl, 10mM Tris-Cl, pH 7.4, 5mM EDTA, pH 7.5) containing RNase A (Roche, 20 μg per sample) and RNase T1 (150 U per sample) at 37°C for 30 minutes. The enzymes were inactivated by treating with Proteinase K (60 μg per sample) and SDS (10 μL of 20% stock) at 37°C for 30 minutes. The RNA was extracted with phenol and chloroform and ethanol precipitated. The samples analyzed on a 6% denaturing polyacrylmide gel containing 7 M urea. Band intensity was determined using a Phosphor-Imager (Molecular Dynamics). RNA level was determined relative to the amount per copy of mouse α-globin.

Bisulfite Conversion

Mice were treated with 1-acetyl-2-phenylhydrazine for two days and harvested on the fifth or seventh day. Genomic DNA was isolated from spleens using the Zymo Research genomic isolation kit (Zymo Research). The DNA was bisulfite converted using the Zymo Research EZ methylation kit. Genomic DNA (~ 1 μg) was diluted to 45 μL in water and heated at 95° C for 5 minutes. The DNA was further denatured by adding 5 μL M-dilution buffer and incubated at 37° C for 15 minutes followed by the addition of 100 μL of bisulfite conversion solution. The samples were incubated at 50° C overnight. The bisulfite converted DNA was purified on a spin-column and eluted in water. Bisulfite converted DNA was amplified using nested PCR with primers for either $^{G}\gamma$- or $^{A}\gamma$-globin promoter region. Primers were designed so that the last nucleotide in the primer was a

thymidine in the bisulfite converted and cytosine in normal genomic DNA to prevent the amplification of non-converted DNA. PCR products were cloned, screened, and sequenced. Primers used for bisulfite specific PCR are listed in Table 3.

Chromatin Immunoprecipitation

Mice were treated for two days with 1-acetyl-2-phenylhydrazine. On the fifth day, spleens were harvested and gently brushed into single cell suspension in ice cold RPMI containing 2% FBS, 5 mM Butyrate, PMSF, aprotinin, leupeptin, and pepstatin. Butyrate and protease inhibitors were included in all steps until TE washes. Cells were then passed through a 70 μM nylon filter to remove debris. Cells were spun and washed with ice cold PBS containing 2% FBS (PBS/FBS). Cells were then resuspended in room temperature PBS/FBS. Formaldehyde was added drop-wise to a final concentration of 0.4% and cells crosslinked for 10 minutes at room temperature. The reaction was terminated by adding glycine to 0.125M and incubating at room temperature for 5 minutes. Cells were washed twice with PBS/FBS. Cells were resuspended in SDS/Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) at a concentration of 25-30 mg spleen/200 μL and incubated on ice for 10 minutes. Chromatin (~700 μL with 200 μL glass beads) was sonicated by six 20 second pulses at 27.5% on a sonicator fitted with a 4 mm tip to achieve 200-1000bp (mean 600bp) DNA. Cellular debris was removed by centrifugation at 12000 rpm for 10 minutes at 4°C. The sonicated chromatin was diluted ten-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). Diluted chromatin was pre-cleared with 80 μL

Primer	Sequence
5-A-START-1	GGAGTTATAGATAAGAAGGTC
5-G-START-2	GGAGTTATAGATAAGAAGGTG
G-START-2	GGTATTTTTTATGGTGGGAGAAGA
A-START-2	GGTATTTTTTATGATGGGAGAAGG
3-END-2	CCAAAACTATCAAAAAACCTCTAA
3-END-1	CCTTCTTACCATATACCTTAACTTTA

Table 3: Primers used for bisulfite specific PCR of the γ-globin promoter region.

protein A agarose/salmon sperm DNA slurry (Upstate Biotechnology) per 2 mL diluted chromatin by rotating at 4°C for 30 minutes. The diluted chromatin was spun at 2000 rpm at 4°C for 2 minutes and aliquoted into 2 mL (25 mg spleen) samples for immunoprecipiation with anti-acetyl H3 (Upstate Biotechnology), anti-acetyl H4 (Upstate Biotechnology), anti-Pol II (Santa Cruz), rabbit pre-immune serum (Santa Cruz Biotechnology), and anti-Trimethyl H3 K4 (Abcam and Upstate), and normal rabbit IgG (Upstate Biotechnology) and incubated overnight with rotation at 4°C. A 500 μL aliquot was stored at -20°C for use as the input sample. Samples were mixed with 60 μL protein A agarose/salmon sperm and rotated for an additional 2 hours at 4° C. Samples were spun at 2000 rpm for 2 minutes at 4°C. The supernatant was removed and the beads were washed with 1 mL low salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl) by rotating at 4°C for 5 minutes. The beads were spun down at 2000 rpm for 2 minutes and washed again with 1 mL high salt wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl). The washing process was repeated three more times, once with a LiCl buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10mM Tris-HCl, pH 8.1) and twice with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After the final wash, the immune complexes were eluted by adding 250 μL elution buffer (0.1 M $NaHCO_3$, 1% SDS), vortexing briefly, and rotating at room temperature for 20 minutes. The beads were then spun down at 2000 rpm for 2 minutes at room temperature and the supernatant collected. The beads were then eluted a second time with 250 μL of elution buffer. The formaldehyde crosslinks were reversed by adding 50 μL 3 M NaCl and 1 μL RNase A

(20 mg/mL) and heating at 65° C overnight. Protein was digested by adding 20 μL Tris pH 6.5, 10 μL 0.5 M EDTA, and 1 μL Proteinase K (20 mg/mL) and incubating at 45° C for 2 hours. The samples were then extracted once with phenol and $CHCl_3$ and once with chloroform followed by ethanol precipitation. DNA pellets were dissolved in water and quantitated using the PicoGreen assay (Molecular Probes, Carlsbad, California). The amount of globin gene present in the immunoprecipitated samples was determined by real time PCR using TaqMan™ probes (Perkin-Elmer Applied Biosystems) on an ABI 7500 or 7900 HT. The levels of enrichment were normalized to the non-specific mouse amylase gene to account for variations during immunoprecipitations. Primer and probe sequences used for real-time PCR analysis are located in Table 4. ChIP analysis performed on fetal livers was identical to the above procedure with the following exceptions. Frozen fetal livers were thawed and gently pipetted into a suspension. Half a fetal liver was used per immunoprecipitation.

RT-Real-Time PCR

RNA (3.7 μg) was first treated with DNase I using the Turbo DNA-Free (Ambion). DNase-treated RNA (1 μg) was reverse transcribed using iScript (Bio-Rad) cycling at 25° C for 5 minutes, 42° C for 30 minutes, and 85° C for 5 minutes. cDNA was analyzed using γ-globin primers (25 ng) and mouse α-globin primers (5 ng) and SYBR green chemistry on an ABI 7500 or 7900HT Sequence Detection System. Levels of γ-globin expression were normalized to endogenous mouse α-globin expression. Primer sequence used for real-time PCR analysis are found in Table 5. Additional analysis was

Primer	Sequence
GammaIn2-F	GGAGATGTTTCAGCACTGTTGCCT
GammaIn2-Probe	6famACAGCAGGGTGTGAGCTGTTTGAAGAtamra
GammaIn2-R	AGCCCAGTTAGTCCTCTGCAGTTT
Amylase-F	AGTTCGTTCTGCTGCTTTCCCTCA
Amylase-Probe	tetTTCTGCTGGGCTCAATATGACCCACAtamra
Amylase-R	TTCCTTGGCAATATCAACCCAGCG
hBetaExn#2-F	TACCCTTGGACCCAGAGGTTCTTT
hBetaExn#2-Probe	vicTCCACTCCTGATGCTGTTATGGGCAAtamra
hBetaExn#2-R	TAAAGGCACCGAGCACTTTCTTGC

Table 4: List of primers used for real-time PCR analysis of ChIP assay DNA in Chapter 2.

Primer	Sequence
MouseBetaH1-F	AGGCAGCTATCACAAGCATCTG
MouseBetaH1Probe	6famGAAACTCTGGGAAGGCTCCTGATTGTTTACCtamra
Mouse Beta H1-R	AACTTGTCAAAGAATCTCTGAGTCCAT
Mouse Ey-F	CAAGCTACATGTGGATCCTGAGAA
Mouse Ey-Probe	6famTCAAACTCTTGGGTAATGTGCTGGTGATTGtamra
Human ϵ-globin-F	GCCTTTGCTAAGCTGAGTGAG
Human ϵ-globin-Probe	6famTCAAGCTCCTGGGTAACGTGATGGTGAtamra
Human ϵ-globin-R	TTGCCAAAGTGAGTAGCCAGAA
Human γ-globin-F	GTGGAAGATGCTGGAGGAGAAA
Human γ-globin Probe	6famAGGCTCCTGGTTGTCTACCCATGGACCtamra
Human γ-globin-R	TGCCATGTGCCTTGACTTTG
Mouse GlycophorinA-F	GCCGAATGACAAAGAAAAGTTCA
Mouse GlycophorinA-P	6famTTGACATCCAATCTCCTGAGGGTGGTGAtamra
Mouse GlycophorinA-R	TCAATAGAACTCAAAGGCACACTGT
Mouse zeta-F	GCGAGCTGCATGCCTACAT
Mouse zeta-Probe	6famTGGATCCGGTCAACTTCAAGCTCCTGTtamra
Mouse zeta-R	GCCATTGTGACCAGCAGACA
Malpha-2 F	AATATGGAGCTGAAGCCCTGG
Malpha-2 R	ACATCAAAGTGAGGGAAGTAGGTCT

Table 5: Primers used for real-time PCR expression analysis in Chapter 2.

performed using Taqman™ probes for detections of glycophorin A, human ϵ-, ϵy-, βh1-, and ζ-globin transcripts (Table 5).

Results

Two lines of mice containing the entire human β-globin locus as a yeast artificial chromosome (βYAC) were used in these studies, A20.1 and A85.68 (Figure 4) (Porcu et al., 1997). βYAC/MBD2-/- mice were generated by breeding βYAC mice with MBD2-/- mice and breeding the subsequent transgenic hemizygotes with MBD2-/- mice. βYAC/MBD2-/- mice were treated with 1-acetyl-2-phenylhydrazine to induce hemolytic anemia for the purpose of increasing the number of reticulocytes for RNA analysis. Peripheral blood was collected on the third day after treatment. For 5-azacytidine treatment, βYAC/MBD2+/+ mice were treated for 2 days with 1-acetyl-2-phenylhydrazine followed by 5 days of intraperitoneal 5-azacytidine treatment. Peripheral blood was collected the day after the final treatment. Globin gene expression was analyzed using the RNase protection assay. As seen in Figure 5, γ-globin mRNA was barely detectable by this assay in adult βYAC/MBD2+/+ mice and βYAC/MBD2+/- mice. However, βYAC/MBD2-/- and βYAC/MBD2+/+ mice treated with 5-azacytidine express the silenced γ-globin gene at similar increased levels. Both A20.1 βYAC/MBD2-/- and βYAC mice treated with 5-azacytidine express the γ-globin gene at ~2% of mouse alpha globin (Figure 6). A slightly higher level of expression is seen in MBD2 null mice derived from A85.68 βYAC transgenic mice. In addition, this effect is seen in MBD2-/-

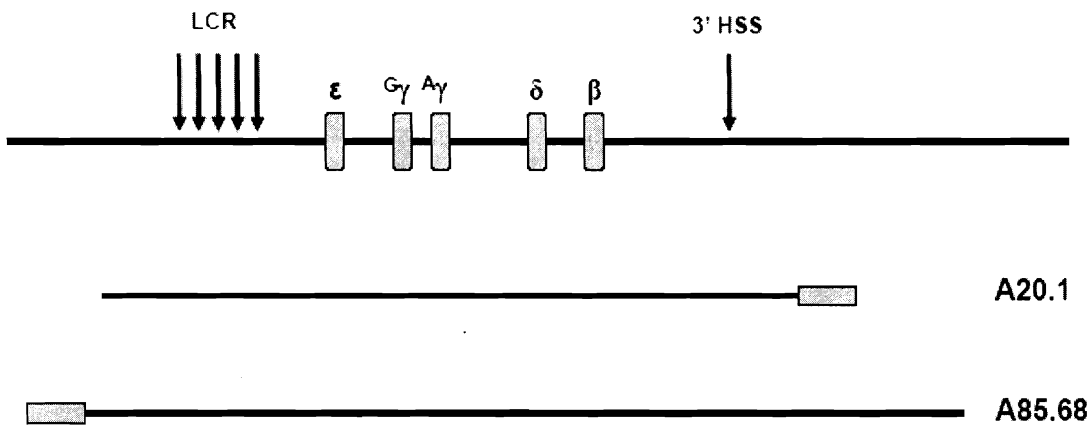

Figure 4: Constructs used to generate βYAC transgenic mice. The top line is a representation of the human β-globin locus. The A20.1 constructs contains ~150kb of sequence while the A85.68 contains >200kb of sequence. The vertical gray bars indicate the position of the genes in the locus. Black arrows indicate DNase I hypersensitive sites. Horizontal gray bars indicate YAC arms. LCR = locus control region. HSS = DNase I hypersensitive site.

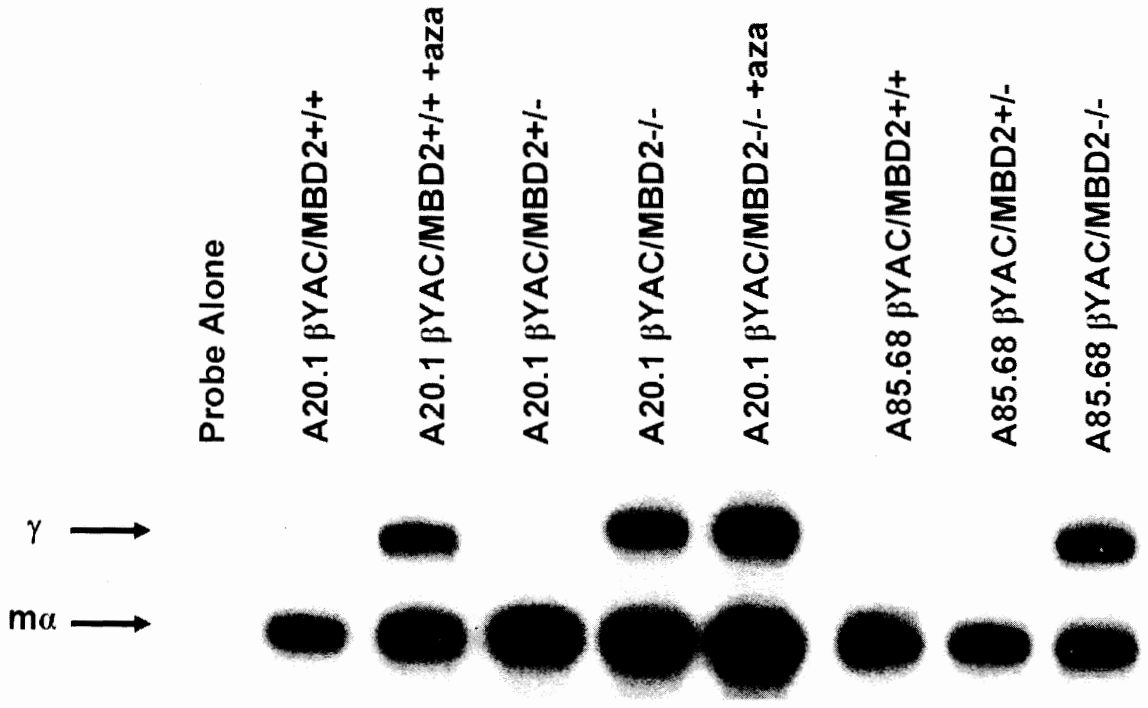

Figure 5: RPA analysis of γ-globin expression in wild type and MBD2 null βYAC transgenic mice. Both lines of βYAC transgenic mice express nominal levels of γ-globin mRNA in the MBD2+/+ and MBD2+/- background. Treatment with 5-azacytidine or loss of functional MBD2 leads to a pronounced increased in γ-globin expression in both lines. Treatment of a 20.1 βYAC/MBD2-/- mouse with 5-azacytidine leads to slight additive boost in γ-globin expression. The endogenous mouse α-globin gene was used as a loading control and was hybridized using 50-fold less RNA. The samples were hybridized separately and combined for RNase digestion.

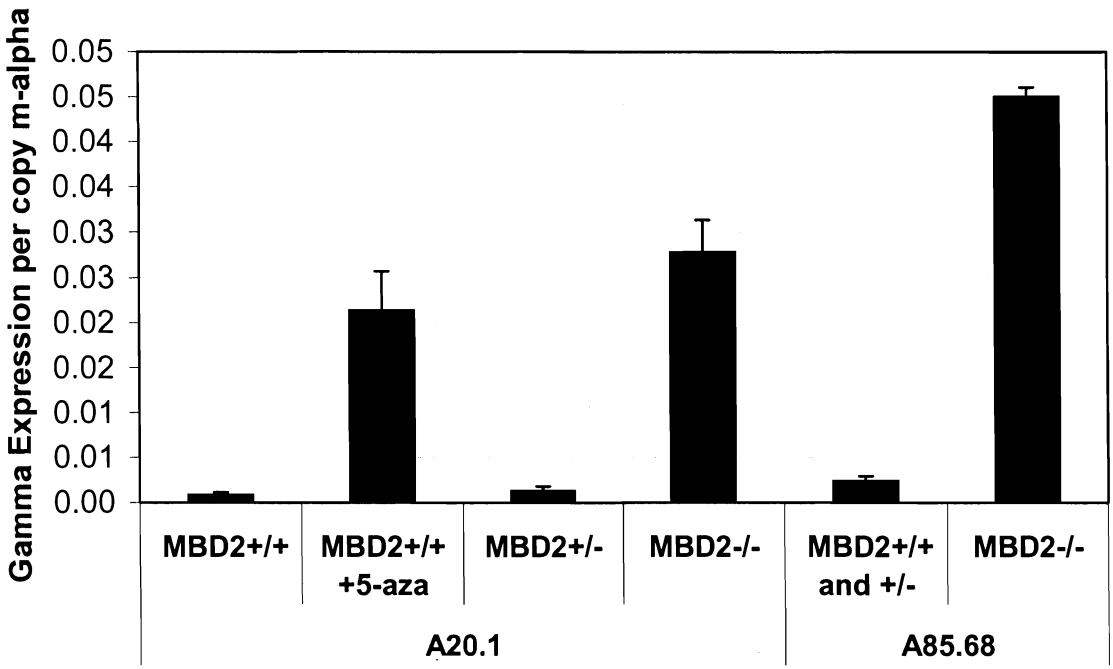

Figure 6: Quantitative analysis of γ-globin expression in wild type and MBD2 null βYAC transgenic mice. Bands from RPA were quantitated using a phosphor-imager. The level of expression of the γ-globin gene was calculated relative to the per copy expression of the mouse α-globin gene. Shown above are the results from the A20.1 line of βYAC transgenic mice and the A85.68 line of βYAC transgenic mice. Either treatment with 5-azacytidine or loss of MBD2 results in expression of the γ-globin gene to ~2% of mouse α-globin expression. The results shown are the average of three RPAs from at least two different mice. For the A85.68 mice, the MBD2+/+ and +/- is the average value from one mouse of each genotype.

mice not made anemic with 1-acetyl-2-phenylhydrazine (Figure 7). No endogenous mouse embryonic globin transcripts were detectable by RNase protection assay (Figure 8). Very low levels of endogenous mouse globin genes are detected by real-time PCR, however, they show large error bars indicating inconsistent low level expression that does not differ between genotype or treatment (Figure 8). The effect on human ϵ-globin expression is discussed in Chapter 3. However, we have observed that a human epsilon transgene containing a full LCR and 4 kb of downstream flanking sequence but no γ- or β-globin gene is activated in the MBD2-/- background. Thus, MBD2 is necessary for maintaining near complete γ-globin silencing in adult mice and loss of MBD2 results in markedly increased γ-globin expression but not mouse or human embryonic β-type globin genes. This is likely due to the ability of the fetal gene but not the embryonic genes to partially compete with adult β-globin gene for the LCR. In addition, the level of induction after treatment with 5-azacytidine is additive, not synergistic, indicating MBD2 and 5-azacytidine likely work through the same mechanism via DNA methylation. Alternatively, the additive increase in γ-globin mRNA may be due to the cytoxic effect of the compound or compensation by a different methyl CpG binding protein. The percentage of $^{G}\gamma$- vs. $^{A}\gamma$-globin was determined using an RNase protection assay probe that discriminates between the two genes (Morley et al., 1991). The % $^{A}\gamma$-globin mRNA was 60% for βYAC/MBD2-/- mice and 70% for βYAC/MBD2+/+ mice treated with 5-azacytidine (Figure 9). These values are very similar to the level seen in adult human F-cells and opposite to the $^{A}\gamma$- to $^{G}\gamma$-globin ratio in K562, cells suggesting that the γ-globin mRNA is from cells with a definitive program rather than due to a reactivation of

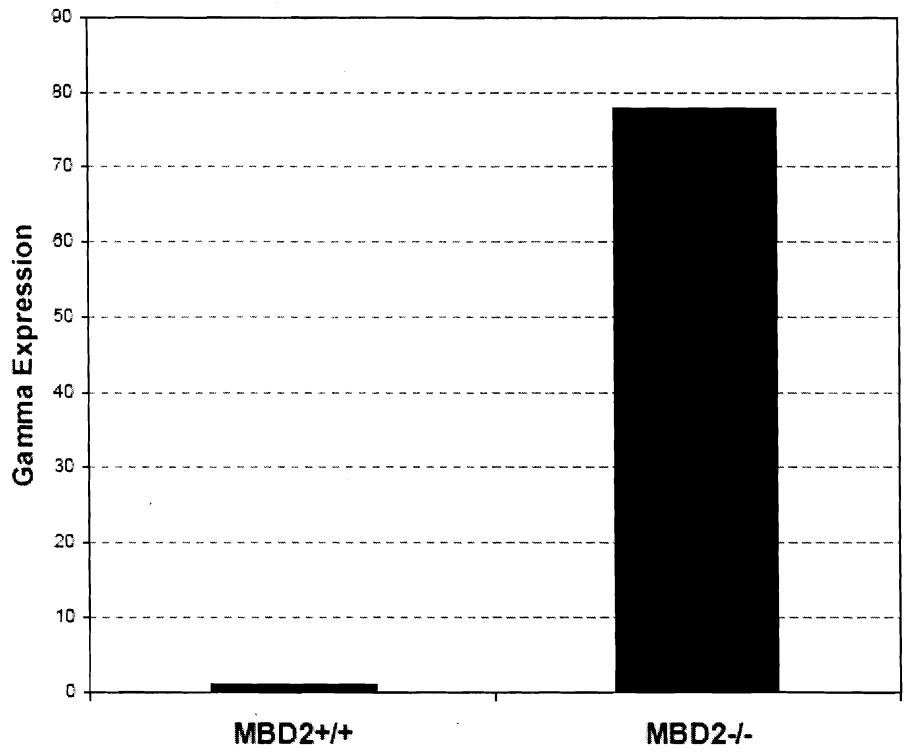

Figure 7: Real-time PCR was performed on peripheral blood taken from non-anemic MBD2+/+ and MBD2-/- A20.1 βYAC transgenic mice. The relative level of γ-globin expression was determined relative to glycophorin A. The results indicate that the γ-globin gene is expressed even when MBD2-/- mice are not treated with 1-acetyl-2-phenyl-hydrazine.

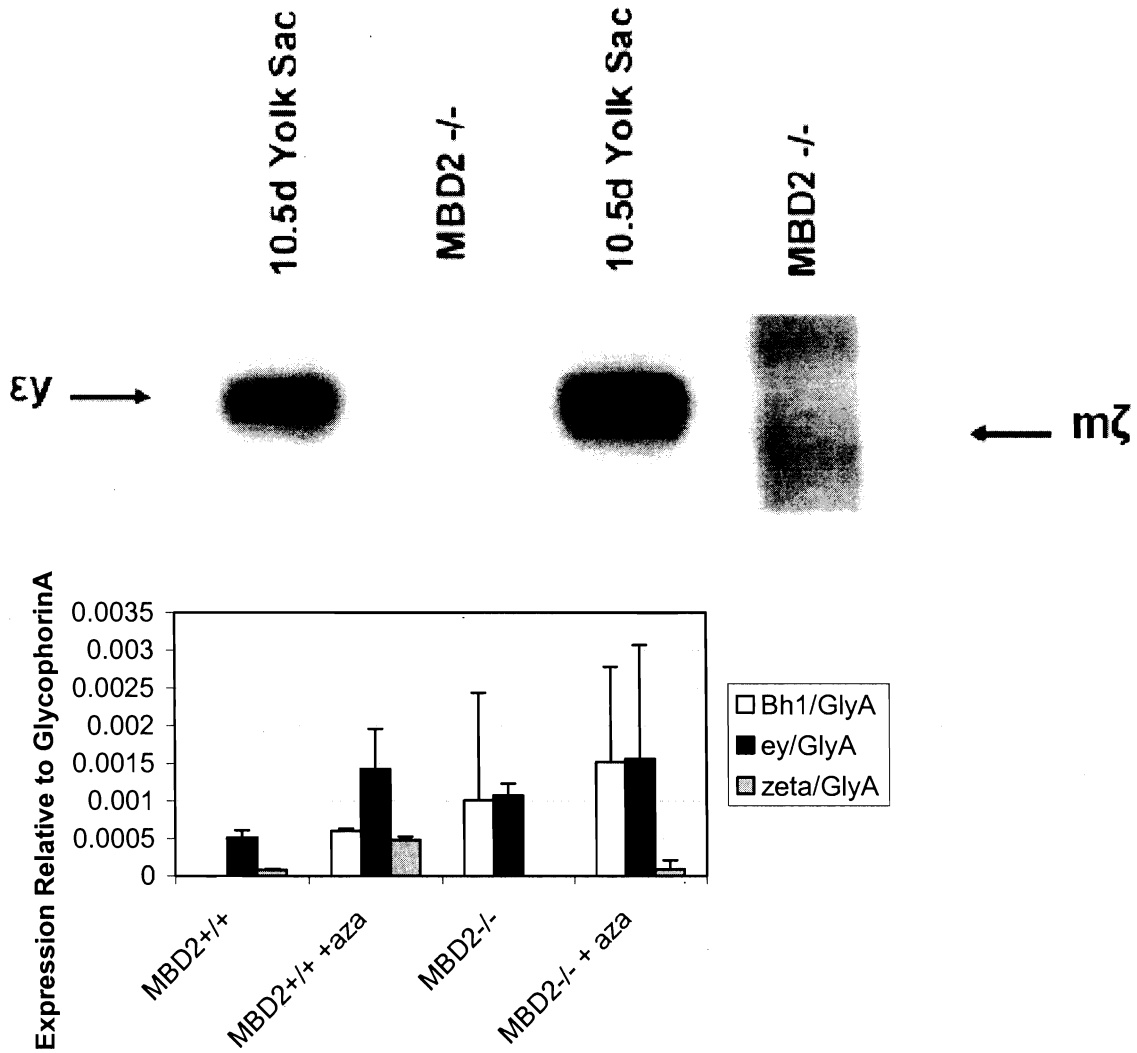

Figure 8: Treatment with 5-azacytidine treatment or loss of MBD2 does not induce expression of any endogenous embryonic globin gene. The top panel shows an RPA of εy- and ζ-globin expression in MBD2+/+ and MBD2-/- peripheral blood. RPA analysis was unable to detect expression of either globin gene. The bottom panel shows real-time PCR analysis of endogenous globin gene expression in MBD2+/+ mice, MBD2+/+ mice treated with 5-azacytidine, MBD2-/- mice, and MBD2-/- mice treated with 5-azacytidine. Low levels of all transcripts are detectable, but no significant increases are observed after 5-azacytidine treatment or after loss of MBD2. In addition, the level of expression is >1000-fold less than that seen in the 10.5 dpc yolk sac.

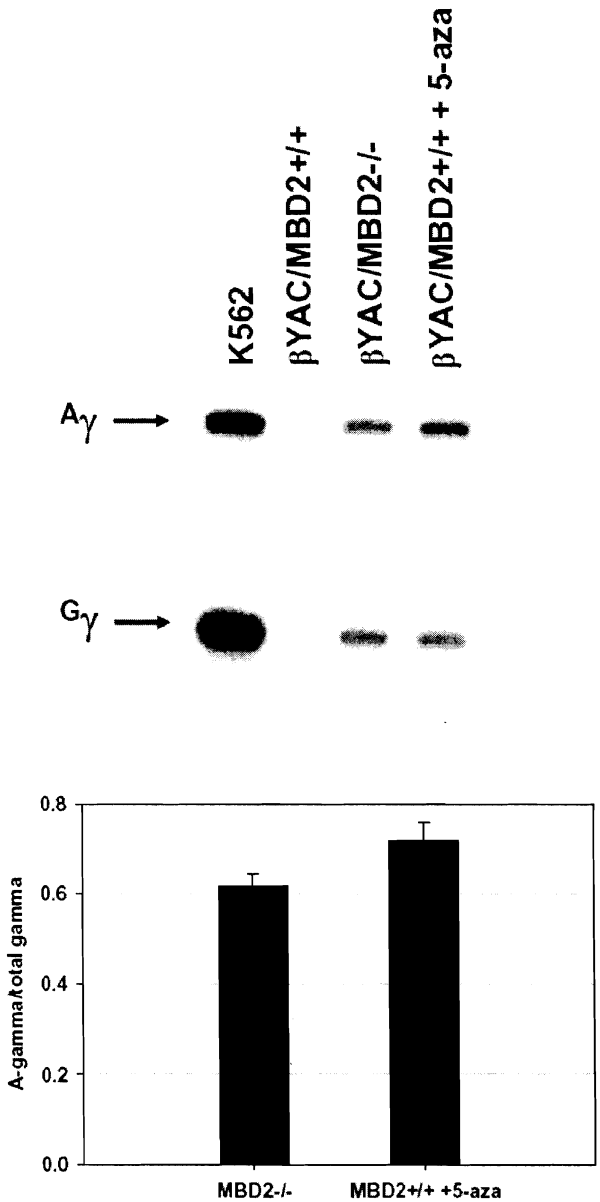

Figure 9: Ratio of $^{A}\gamma$-globin to total γ-globin expression in βYAC transgenic mice treated with 5-azacytidine and βYAC mice null for MBD2. The RPA probe used in the top panel discriminates between the $^{A}\gamma$- and $^{G}\gamma$-globin genes. The ratio of the two genes was determined by phosphor-image analysis and indicates that the level of expression of the $^{A}\gamma$-globin gene is slightly higher in both 5-azacytidine treated mice and MBD2 null mice. K562 cells show a preference for $^{G}\gamma$-globin gene expression.

primitive erythropoiesis. Red cell indices were compared between non-anemic wild-type and knock-out mice. The results show no significant difference except for slight microcytosis in MBD2 null mice (Table 6).

Adult erythroid cells from mice lacking MBD2 inappropriately express the γ-globin gene. We next wanted to determine the impact of MBD2 on globin gene expression during development. Timed matings were performed and embryos harvested on 14.5 and 16.5 days post coitus (dpc). At 14.5 dpc, embryos lacking MBD2 expressed 2-fold more γ-globin than wild type embryos (Figure 10). This difference increased to 3.5-fold in 16.5dpc fetal livers (Figure 10). The effect seen in adult knock-out mice appears to be at least partly due to an inability to fully silence the γ-globin gene during the switch to definitive erythropoiesis. Therefore, the effect is specific for the γ-globin and not the embryonic globin genes in the context of βYAC transgenic mice.

Lack of MBD2 results in expression of the γ-globin gene in adult βYAC transgenic mice. MCBPs bind to methylated DNA and recruit factors that lead to transcriptional repression. In addition, MBD2 has been shown to colocalize with DNA methyl transferase 1 (DNMT1) (Tatematsu et al., 2000). We therefore postulated that if MBD2 binds at or near the γ-globin gene promoter the effect seen in adult βYAC/MBD2-/- mice may be accompanied by a loss of DNA methylation around the γ-globin promoter. Bisulfite sequencing was performed to determine the methylation status in βYAC/MBD2+/+ mice, βYAC/MBD2+/+ mice treated with 5-azacytidine, and βYAC/MBD2-/- mice. A 70bp region containing four CpGs was analyzed around the γ-

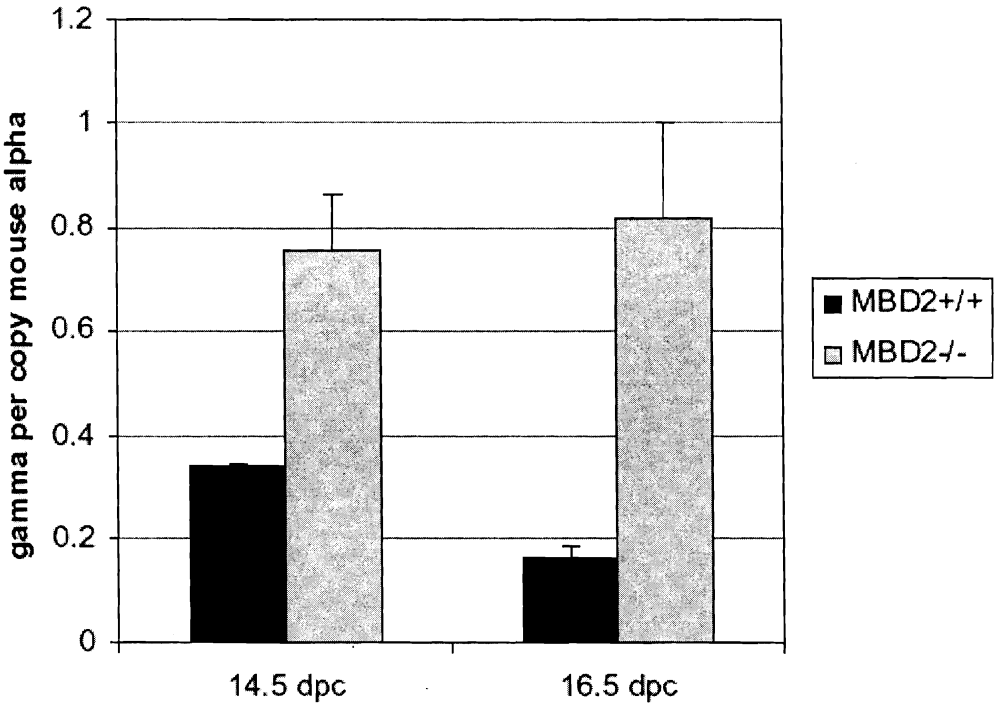

Figure 10: Expression of the γ-globin gene during development in MBD2+/+ and MBD2-/- βYAC transgenic mice. The level of expression of the γ-globin gene was determined during development in MBD2+/+ and MBD2-/- βYAC transgenic mice by RPA. During development, loss of MBD2 results in increased expression of the γ-globin gene in 14.5 and 16.5 dpc fetal liver erythroid cells.

	RBC	Hb	Hct	MCV	MCH	HCHC	Retic
MBD2+/+	7.6±1.1	13.2±1.2	37.2±5.1	49.3±1.5	17.5±0.9	35.8±1.8	6.2±0.9
MBD2-/-	6.7±1.6	11.7±2.7	30.3±8.1	44.6±0.3	17.2±0.4	38.6±1.2	5.9±1.5
P-Value	0.42	0.41	0.28	0.01	0.64	0.08	0.73

Table 6: Red blood cell indices were determined on three mice of each genotype. P-values were calculated by student t-test.

globin promoter. As shown in Figure 11, wild type adult erythroblasts show a high level of methylation (75-100%) at each CpG tested in the γ-globin gene. Furthermore, no individual clone (represented as a row in Figure 8) was either completely or ≥75% unmethylated indicating all cells are highly methylated around the γ-globin promoter. Treatment with 5-azacytidine led to a decrease in methylation at all sites (40-60% methylated) with 4 clones having no methylation at any site tested. This result is similar to results seen in MEL cells containing human chromosome 11 and primary erythroid cells of patients treated with 5-azacytidine (Humphries et al., 1985; Ley et al., 1984). Most MBD2-/- clones show an intermediate level of DNA methylation at each site (60-90% methylated). In addition 1 clone is fully demethylated while another is 75% demethylated. DNA methylation levels are only modestly decreased in the absence of MBD2 compared animals treated with 5-azacytidine. This may be the result of increased transcription of the gene preventing DNA methylation by DNMT rather than MBD2 recruiting DNMT activity to the γ-globin promoter. Nevertheless, γ-globin mRNA levels can be increased in the absence of significant loss of DNA methylation around the promoter and proximal transcribed regions. This suggests 5-azacytidine and MBD2 exert their effect directly at the γ-globin promoter region.

To determine whether loss of MBD2 alters post-translational histone modification around the γ-globin gene, chromatin immunoprecipitation (ChIP) assays were performed. Both βYAC/MBD2+/+ and βYAC/MBD2-/- mice were treated with 1-acetyl-2-phenylhydrazine to promote conversion of the spleen to a primarily erythroid organ

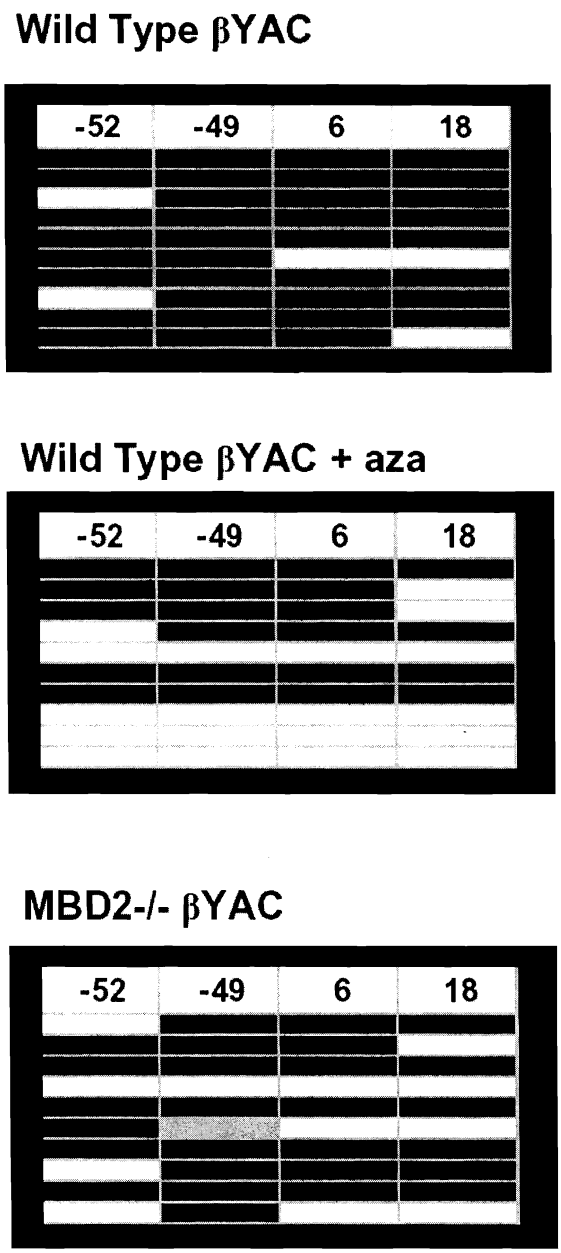

Figure 11: Bisulfite analysis of MBD2+/+ and MBD2-/- βYAC transgenic mice. Genomic DNA from the spleens of anemic mice was treated with bisulfite. The γ-globin gene promoter region was amplified by PCR, cloned, and sequenced. The numbers above each figure indicate the position of CpG relative to the transcription start site. The horizontal row indicates one sequenced clone with white bars indicating an umethylated CpG, a black bar indicating a methylated CpG, and a gray bar indicated an undetermined CpG.

(Spivak et al., 1973). Splenic erythroblasts were used for chromatin immunoprecipitation with antibodies directed against acetylated histone H3 (AcH3), acetylated histone H4 (AcH4) and trimethylated lysine 4 of histone H3 (TriMeK4), all of which are associated with active transcription. Given that the level of γ-globin mRNA is ~2% of mouse α-globin mRNA in βYAC/MBD2-/- mice, we postulated that any effect on histone modifications might be too small to determine. For this reason, the amount of enrichment was normalized to the level seen in the inactive endogenous amylase gene control. Immunoprecipitations were performed on two mice of each genotype. Shown in Figure 12 is the average gamma globin to amylase gene ratio for wild type and knockout mice. The γ-globin promoter region shows enrichment for AcH4, Pol II, and TriMeK4 in knock-out mice and no enrichment for these modifications in wild-type mice. Additionally there is little change in the level of AcH3 in either genotype. Additionally, the 5' region of the γ-globin gene coding region is enriched ~4-fold over baseline for TriMeK4 in MBD2-/- mice while MBD2+/+ mice show baseline levels. Similar levels of enrichment are seen when normalizing the gamma globin value to the active human β-globin gene. The level of enrichment at the highly expressed β-globin gene is > 20-fold higher than enrichment levels at the γ-globin gene in MBD2 null mice (Figure 13). A similar relationship between level of mRNA expression and active histone modification enrichment is seen in mice containing a mutant human βYAC transgene that expresses the human β-globin gene at 3% of the normal transgene (Fang et al., 2005). The enrichment of TriMeK4 in MBD2-/- mice may be due to the recruitment of histone

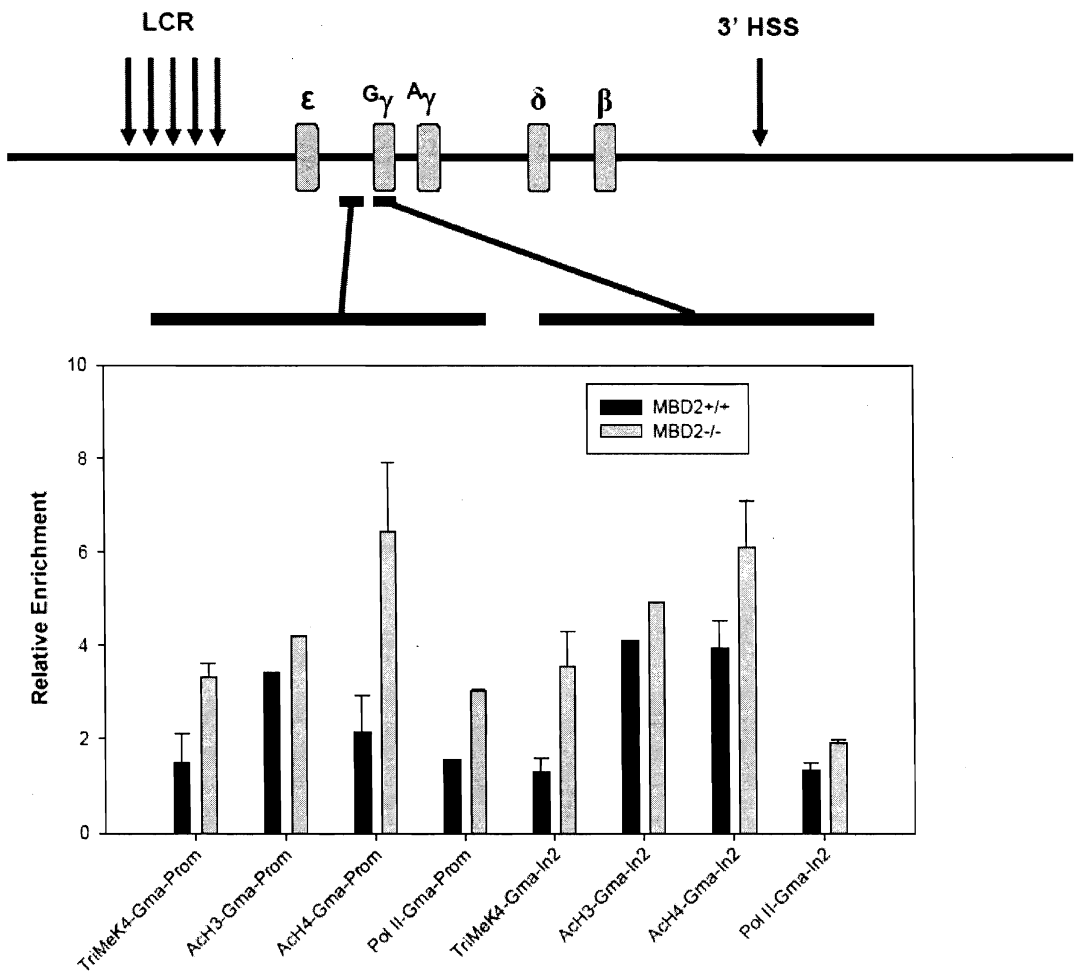

Figure 12: Analysis of post-translational histone modifications at the γ-globin gene promoter and coding regions in wild type and MBD2 null βYAC transgenic mice. ChIP assays were performed on splenocytes from anemic mice using antibodies against TriMeK4, AcH3, AcH4, and Pol II. Enrichment was determined relative to the inactive amylase gene by real-time PCR. Non-specific background was accounted for by dividing each value by the IgG ratio. Small horizontal black bars below the map of the human β-globin locus indicate the positions of PCR amplification.

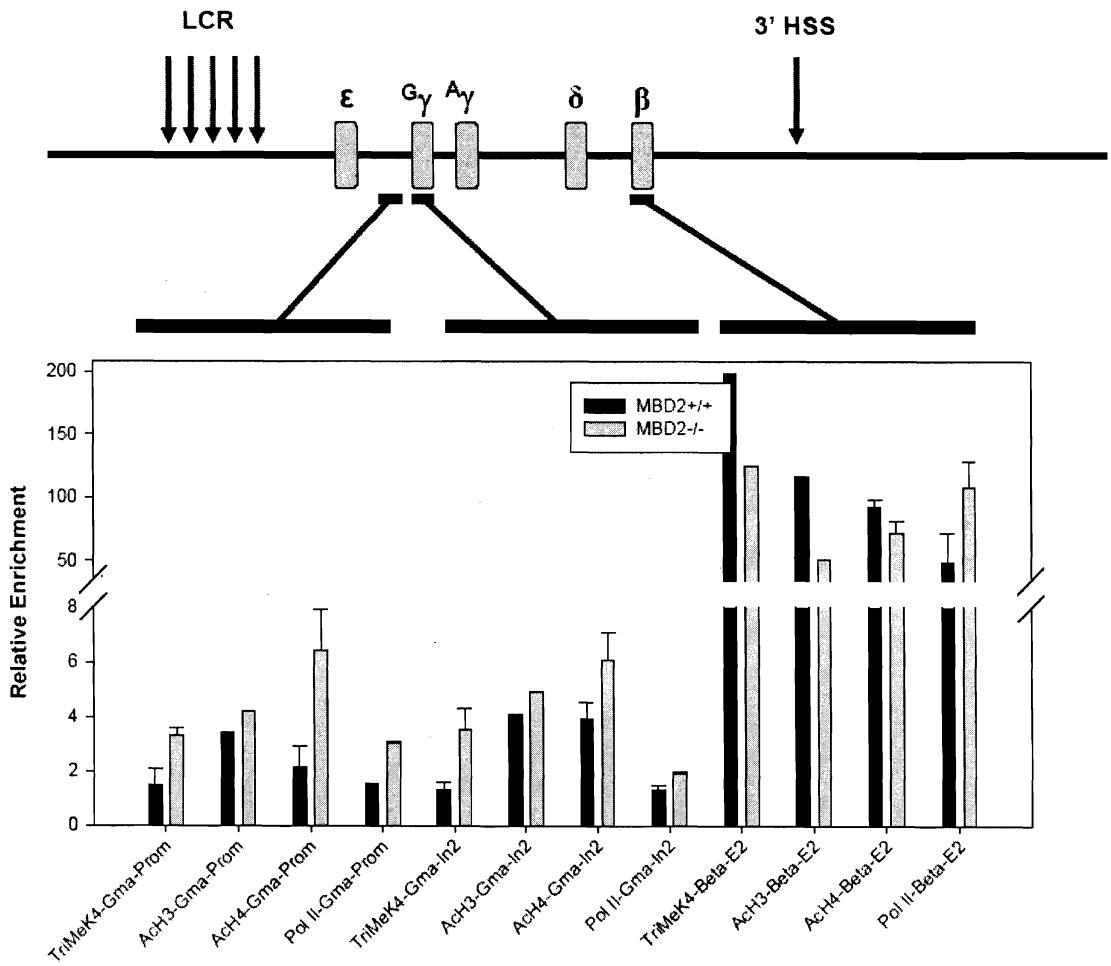

Figure 13: Analysis of post-translational histone modifications at the β-globin gene in wild type and MBD2 null βYAC transgenic mice. ChIP assays were performed on splenocytes from anemic mice using antibodies against TriMeK4, AcH3, AcH4, and Pol II. Enrichment was determined relative to the inactive amylase gene by real-time PCR. The level of enrichment at the β-globin gene is >20-fold higher than at the γ-globin gene.

methyltransferases to the γ-globin gene by RNA polymerase II in the absence of MBD2 (Ng et al., 2003). As shown in Figure 6 and 10, the γ-globin gene is expressed ~10-fold higher in the wild-type 14.5 dpc fetal liver than in adult MBD2 null erythroid cells. As seen in Figure 14, the level of

enrichment for TriMeK4 is ~10-fold higher as well. This data indicates that the level of transcription is proportional to TriMeK4 enrichment and that the increase in γ-globin mRNA is due to increased transcription of the gene. In addition, there is no significant difference between the level of AcH3 enrichment in wild-type versus MBD2 knock-out mice at the γ-globin gene (Figure 12). Once again, the level of enrichment of this modification is much higher at the β-globin gene (Figure 13). Loss of MBD2 leads to an increase in TriMeK4, AcH4, and Pol II levels at the γ-globin gene promoter and increased TriMeK4 at the coding region but does not induce a similar increase in AcH3 levels.

Treatment of βYAC/MBD2+/+ mice with 5-azacytidine leads to a 10-15 fold induction of the γ-globin gene versus mice treated with 1-acetyl-2-phenylhydrazine only (Figure 12) which is similar to the level of activation seen in βYAC/MBD2-/- mice and in a different βYAC line treated with 5-azacytidine (Pace et al., 1994). To determine whether 5-azacytidine and MBD2 both induce γ-globin mRNA via the same mechanism through DNA methylation, βYAC/MBD2-/- mice were treated with 5-azacytidine. Blood was collected prior to 5-azacytidine treatment and again five days after treatment and analyzed by both RNase protection assay and quantitative real-time PCR (Figures 5 and 15, respectively). 5-azacytidine only induces the γ-globin by 2.5-fold (25-fold over wild-

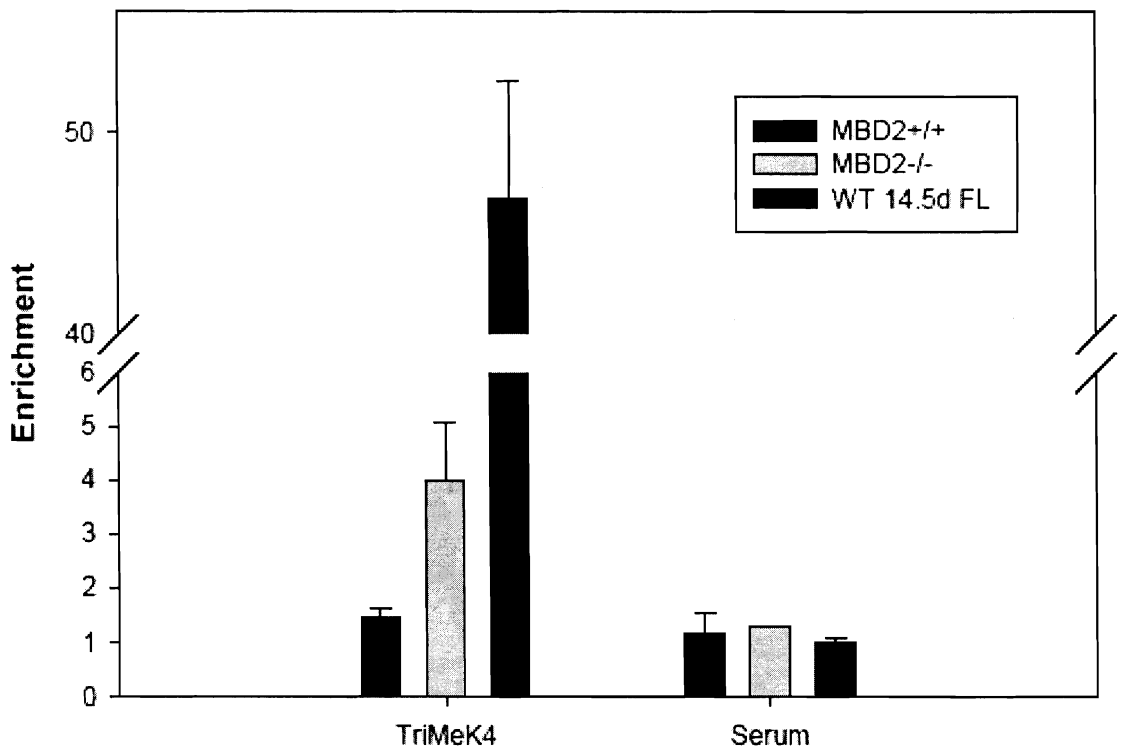

Figure 14: ChIP assay was performed on 14.5 dpc wild-type βYAC fetal livers. Chromatin was immunoprecipitated with anti-TriMeK4. The data is graphed relative to enrichments levels seen in adult MBD2+/+ and MBD2-/- βYAC transgenic mice.

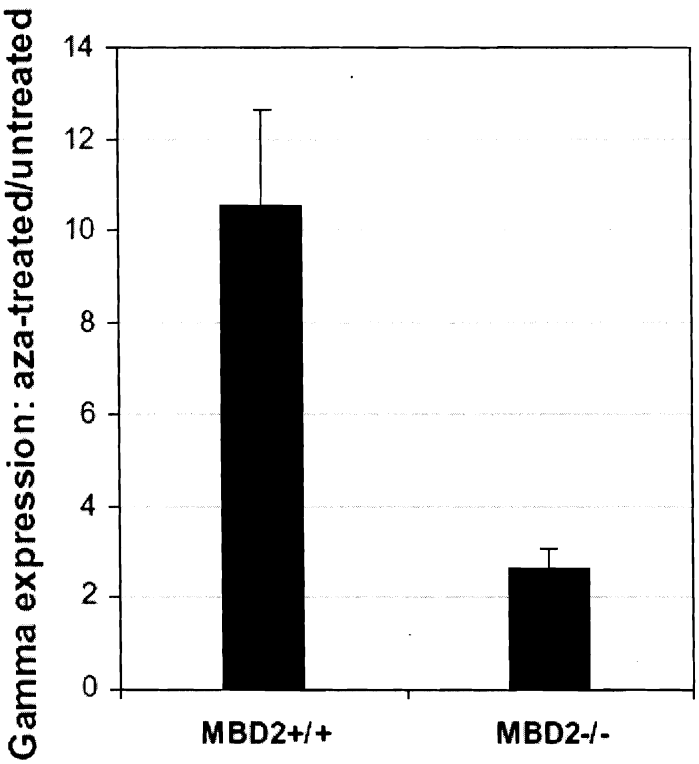

Figure 15: Analysis of 5-azacytidine mediated induction of the γ-globin gene in MBD2+/+ and MBD2-/- βYAC transgenic mice. Induction of the γ-globin gene by 5-azacytidne was determined relative to pre-treatment expression levels by real-time PCR. Treatment with 5-azacytinde results in a >10-fold induction in MBD2+/+ mice while only a 2.5-fold induction is seen in MBD2-/- mice.

type) in MBD2-/- mice. The lack of a synergistic effect (>1000-fold over wild-type) of MBD2 deficiency upon 5-azacytidine treatment strongly suggests that both are largely working through the same pathway, which is mediated by DNA methylation. This induction, while present, is sub-additive. However, the 2.5-fold induction may be due to the cytoxic effect of the drug or compensation by another methyl CpG binding protein. Furthermore, the activation of multiple pathways by 5-azacytidine is a possibility that cannot be ruled out. These alternate pathways may be the cause of the sub-additive induction.

To determine the specificity of the MBD2 mediated γ-globin gene repression, transgenic knock-out mice were made for a different MCBP, MeCP2. MeCP2 knock-out mice have a more severe phenotype than MBD2 knock-out mice characterized by uncoordinated gait, hind-limb collapsing, breathing irregularities, rapid weight loss, and death by day 54 (Guy et al., 2001). These symptoms are similar to the human neurological disorder Rett Syndrome, a disorder associated with MeCP2 loss or mutation. Transgenic knock-outs can be generated in one round of breeding by mating a male transgenic mouse with a hemizygous female. Multiple transgenic hemizygous females were obtained, however only one transgenic knock-out mouse was generated. Transgenic mice hemizygous for MeCP2 were made anemic and peripheral blood analyzed by RNase protection assay. As shown in Figure 16, these adult mice do not express the human γ-globin gene. Given that MeCP2 is X-linked, half of the blood cells should be null for MeCP2. These results indicate that loss of MeCP2 is not sufficient to induce expression of the γ-globin gene. However, these results do not take into account the fact that other

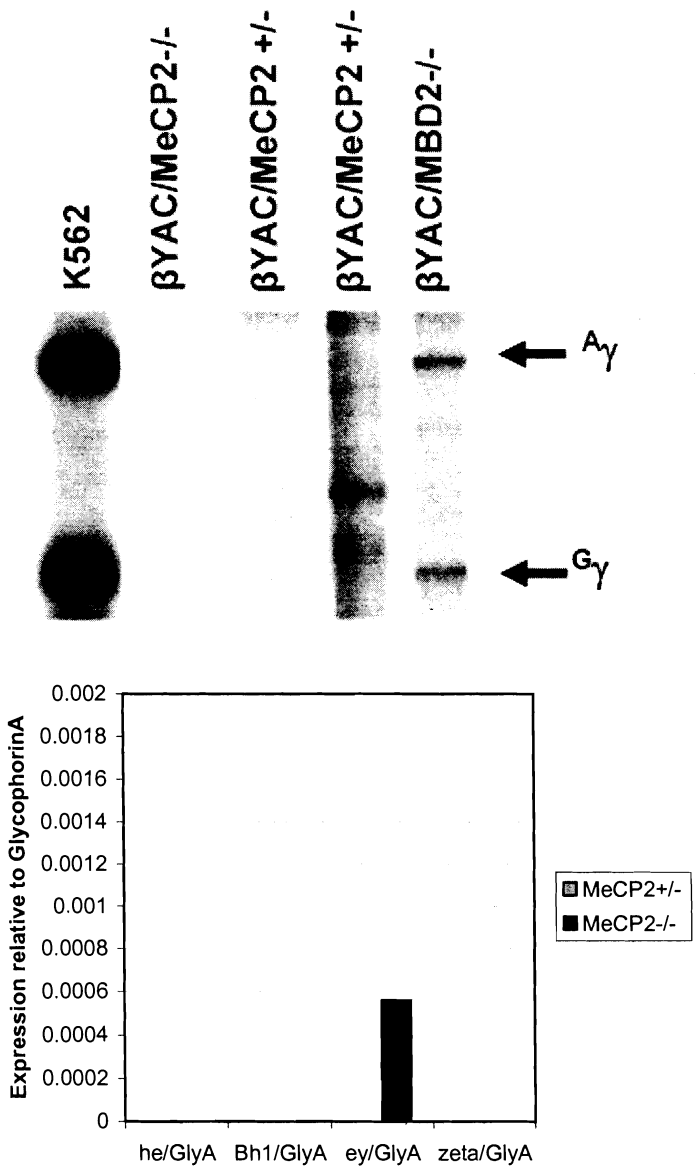

Figure 16: Expression of globin genes in MeCP2y/- and MeCP2+/- βYAC transgenic mice. The top panel shows an RPA for γ-globin expression in MeCP2y/- and MeCP2+/- transgenic mice. The bottom panel shows a real-time PCR analysis of expression of globin genes in MeCP2y/- and MeCP2+/- βYAC transgenic mice. The level of expression of the εy globin gene is >1000-fold less than 10.5 dpc yolk sac expression levels.

factors expressed from other cells (ie cytokines) may be responsible for the induction of the γ-globin gene in MBD2 null mice. One mouse transgenic for the βYAC and null for MeCP2 was generated. Peripheral blood was collected from this mouse without 1-acetyl-2-phenylhydrazine treatment due to the frail condition of the mouse. RNA from peripheral blood was analyzed by RNase protection assay and real-time PCR. No γ-globin RNA is expressed in this mouse (Figure 16). These results indicate that the effect seen due to loss of MBD2 is specific, because loss of MeCP2 does not generate the same phenotype.

Discussion

MBD2 is needed for complete suppression of the γ-globin gene in βYAC transgenic mice. Loss of the protein leads to a level of expression from a single copy of the γ-globin gene commensurate with βYAC mice treated with 5-azacytidine, ~2% of the endogenous mouse α-globin RNA per gene copy. In addition, developmental silencing of the transgene is delayed in the absence of MBD2 as these mice express the γ-globin gene at higher levels in 14.5 and 16.5dpc fetal livers. DNA methylation levels are modestly reduced while TriMeK4 levels are modestly increased around the γ-globin gene in βYAC/MBD2-/- mice. Treatment of βYAC/MBD2-/- mice with 5-azacytidine leads to only a 2.5 fold induction of γ-globin mRNA relative to βYAC mice treated with 5-azacytidine.

The phenotype seen in MBD2 null βYAC transgenic mice appears to be due to increased transcription. The level of enrichment of TriMeK4 is proportional to

transcriptional activity. As shown, there is an increase in TriMeK4 enrichment around the γ-globin gene in MBD2 null transgenic mice. Thus, it seems unlikely that MBD2 loss leads to a stabilization of the γ-globin mRNA or another non-transcriptional mechanism. Cytotoxic compounds such as 5-azacytidine, hydroxyurea, and araC have been proposed to increase fetal hemoglobin by inducing stress erythropoiesis (Stamatoyannopoulos, 2005). In stress erythropoiesis, early differentiating erythroid cells that express the γ-globin gene are accelerated through the differentiation pathway before they can fully silence the γ-globin gene. Cytotoxic drugs result in increased erythropoiesis leading to increased production of γ-globin mRNA. Thus, we investigated whether MBD2 mediates a stress erythropoiesis effect to induce γ-globin mRNA expression. However, the red cell indices, including reticulocyte count, are not different between the two genotypes. Additionally, 1-acetyl-2-phenylhydrazine induced hemolytic anemia fails to increase γ-globin mRNA levels in MBD2-/- mice. It therefore seems unlikely that MBD2 is mediating a stress erythropoiesis effect to increase γ-globin expression. However, it does not eliminate the possibility that MBD2 is somehow altering erythroid differentiation in a way that results in increased γ-globin expression. In addition, the effect is very specific as loss of MeCP2 does not create the same phenotype nor are any endogenous mouse globin genes appreciably up-regulated in the absence of MBD2. Thus, it appears MBD2 is mediating a specific transcriptional up-regulation of the γ-globin gene.

Treatment of MBD2-/- mice with 5-azacytidine does not produce a synergistic increase in γ-globin expression. However, there is a sub-additive increase in γ-globin mRNA levels. There are four possible reasons to account for this effect. First, loss of

MBD2 and 5-azacytidine are working along the same pathway via DNA methylation to repress γ-globin transcription in adult βYAC transgenic mice. Given this, treatment with 5-azacytidine in the MBD2 null background should not cause a further increase if both are working via the mechanism. This idea is supported by the similar level of γ-globin mRNA after treatment with 5-azacytidine or in the absence of MBD2. Second, cytotoxic drugs such as hydroxyurea and AraC have been shown to increase γ-globin mRNA levels in both humans and baboons (Stamatoyannopoulos, 2005). Neither of these compounds affects DNA methylation, but they are hypothesized to exert their effect by stimulating stress erythropoiesis. The 2.5-fold induction seen may be the result of stress erythropoiesis due to the cytotoxic nature of 5-azacytidine. Third, drug treatment can lead to the activation or inactivation of many pathways. Treatment of 5-azacytidine in the MBD2 null background may be impacting a pathway outside of DNA methylation to create the 2.5-fold increase. Fourth, another MCBP may compensate for MBD2 loss. The loss of DNA methylation via 5-azacytidine treatment alleviates its contribution to γ-globin repression and creates a 2.5-fold induction in expression. However, most of the known MCBPs can be eliminated as contributors. MeCP2 loss appeared to have no effect of γ-globin mRNA levels. Kaiso binds to a specific site not found in the γ-globin gene. MBD3 does not bind to methylated DNA and MBD4 is a repair enzyme. As a result, MBD1 is the only other known MCBP that could contribute to γ-globin gene repression.

Most MCBPs are thought to inhibit transcription by binding to promoters and recruiting transcriptional repression complexes. We have shown here only minor changes in DNA methylation status and post translational histone modifications around the γ-

globin gene in βYAC/MBD2-/- mice. This is not completely surprising as the γ-globin promoter does not possess a CpG island, a region of 200bp or more that have a CpG:GpC ratio of 0.6 (Antequera and Bird, 1993; Cross and Bird, 1995). In addition, there are no significant changes in global DNA methylation levels in MBD2-/- mice (Hendrich et al., 2001). Thus, MBD2 is not recruiting DNMT-activity to the γ-globin gene. The modest decrease in DNA methylation levels seen in at the γ-globin promoter region is likely due to increased transcription of the gene preventing DNMT-activity. While MBD2 has been shown to bind to as few as 2 CpG *in vitro*, this has not been shown *in vivo* (Kransdorf and Ginder, 2004). Thus it appears unlikely MBD2 binds directly to the γ-globin promoter to mediate transcriptional repression of the γ-globin gene for three reasons. First, the low CpG density is not an ideal binding site for MBD2. Second, MCBPs mediate their repressive effects through recruitment of transcriptional repression complexes that include HDACs. If MBD2 were recruited to the γ-globin gene there should be an increase in both AcH3 and AcH4 in its absence. Instead, there is only enrichment for AcH4 at the promoter with no increase in AcH3 and no increase in AcH3 or AcH4 within the coding region. Third, work in cell hybrids shows the γ-promoter region is unmethylated in the absence of transcription (Enver et al., 1988). The data here shows that γ-globin expression stays high during development in the absence of MBD2, indicating MBD2 is necessary for silencing during development. If the promoter is still unmethylated at this time, MBD2 cannot be mediating its effect directly at the gene. Loss of MBD2 or 5-azacytidine treatment leads to increased expression of the γ-globin gene in βYAC transgenic mice. Both appear to be working via the same mechanism that likely does not

involve the γ-globin promoter. MBD2 may be mediating its effect at a different location in the locus. The NuRD complex has recently been shown to be recruited to HS2 of the LCR in K562 cells (Mahajan et al., 2005). However, MBD2 was not shown to bind there, but MBD2 may be mediating its effect through a CpG dense region in the locus. The β-globin locus does not possess a true CpG island, but it does contain pockets of increased CpG density. MBD2 binding at these sites may contribute to γ-globin gene silencing. In addition, MBD2 may be regulating a gene in *trans* that when expressed increases γ-globin gene expression.

Many compounds currently available to treat patients with hemoglobinopathies are toxic and, in addition, the response to the agents is variable. Here we show that loss of MBD2 leads to expression of the normally silent γ-globin gene in adult βYAC transgenic mice. Given the mild phenotype of MBD2 null mice, MBD2 may represent an attractive potential target for the treatment of β-thalassemia or sickle cell anemia conditions in which increased γ-globin gene expression has an ameliorating effect.

Chapter 3: The Role of DNA Methylation and MBD2 on Human ϵ-globin Regulation

Introduction:

The genes of the human β-globin locus are located on chromosome 11 in the order of their expression during development: 5' ϵ, γ, and β 3'. During development, two switches occurs whereby the 5' globin gene (ϵ or γ) becomes silent and the gene directly 3' becomes transcriptionally active. The exact mechanism of this process is not yet fully understood, however it has been show to involve many interactions between *cis* elements, erythroid specific *trans* factors, ubiquitous *trans* factors, and epigenetics. Transgenic mice with the human β-type globin genes have provided much insight into the mechanism(s) of globin gene switching. Mice containing the entire β-globin locus as a yeast artificial chromosome (βYAC) transgene show correct developmental expression and silencing of human globin genes (Gaensler et al., 1993; Peterson et al., 1993a; Peterson et al., 1993b; Zucker et al., 1983). In addition, mice transgenic with smaller globin gene constructs show similar developmental regulation (Stamatoyannopoulos and Grosveld, 2001). These transgenic studies have brought about the concept of competitive and autonomous silencing of globin genes. High level expression of globin genes is mediated by an enhancer located 5' of the human ϵ-globin gene termed the locus control

region (LCR). In the competition model, two globin genes compete the enhancer activity of the LCR with one gene being highly expressed at the expense of the other. In the absence of another globin gene, the less competitive gene would still be expressed. In the case of autonomous silencing, the gene would not be expressed even in the absence of other globin genes. The fetal γ-globin gene has been shown to be regulated by a combination of autonomous silencing and competition. This means γ-globin transgenic mice in the absence of other globin genes decrease expression of the transgene during development, but cannot completely silence the gene without another globin gene present (Behringer et al., 1990; Dillon and Grosveld, 1991; Enver et al., 1989; Enver et al., 1990; Zucker et al., 1983). In the case of the ϵ-globin transgenic mice, the gene is silenced in the absence of other globin genes and is thus autonomously silenced (Raich et al., 1990; Shih et al., 1990).

The autonomous silencing of the ϵ-globin gene has been shown to be mediated by many different factors binding to different locations around the gene. Both GATA-1 and YY1 have been shown to bind to different regions of the ϵ-globin gene and mediate transcriptional repression (Li et al., 1997; Raich et al., 1995). In addition, a complex was isolated that bound to two inverted direct repeats of a short motif analogous to DR-1 binding sites for non-steroid nuclear hormone receptors in the region of the CCAAT box (Tanimoto et al., 2000). Mutation of these repeats leads to expression of the ϵ-globin gene in adult βYAC transgenic mice (Filipe et al., 1999; Tanimoto et al., 2000). A complex binding to this site, termed DRED, was found to contain the nuclear orphan receptors

TR2 and TR4 (Tanabe et al., 2002). Thus, it appears many factors contribute to the autonomous silencing of the ϵ-globin gene during development in transgenic mice.

Globin genes have also been shown to be regulated by DNA methylation. The first correlation between transcriptional status and DNA methylation in vertebrates was seen in the avian globin locus (McGhee and Ginder, 1979). Further studies showed a similar correlation in other species and model systems (Atweh et al., 2003; Mavilio et al., 1983; Shen and Maniatis, 1980; van der Ploeg and Flavell, 1980). The compound 5-azacytidine inhibits the enzyme DNA methyltransferase and leads to a decrease in DNA methylation levels. Treatment with 5-azacytidine has been shown to induce the expression of silenced embryonic and fetal β-type globin genes in many different model systems and in patient studies (Charache et al., 1983; DeSimone et al., 1982; Ginder et al., 1984; Ley et al., 1982; Ley et al., 1984; Pace et al., 1994; Zucker et al., 1983). A family of proteins has been identified that bind to methylated DNA and recruits transcriptional repression complexes, which include histone deacetylases (Feng and Zhang, 2001; Jones et al., 1998; Nan et al., 1993; Nan et al., 1998; Zucker et al., 1983). One member of this family is the methyl binding domain protein 2, MBD2. MBD2 has been shown to bind to a methylated ρ-globin construct as a large complex containing histone deacetylase 1 (Singal et al., 2002). In addition, short chain fatty acids, compounds known to inhibit histone deactylases among other functions, have been shown to induce the expression of embryonic and fetal β-type globin genes (Dempsey et al., 2003; Gabbianelli et al., 2000; Little et al., 1995; Marianna et al., 2001; Pace et al., 2002; Skarpidi et al., 2003; Stamatoyannopoulos and Grosveld, 2001; Zucker et al., 1983).

Thus, in addition to factors mediating autonomous and competitive silencing, epigenetic modifications contribute to globin gene silencing.

Herein we describe a role for both DNA methylation and MBD2 in mediating human ϵ-globin silencing in adult transgenic mice. Transgenic mice containing continuous sequence from 5' of HS5 of the LCR through various lengths 3' of the ϵ-globin polyA site correctly silence the transgene during development. This silencing is correlated with low levels of DNA methylation in the embryonic yolk sac and high levels in adult erythrocytes. Adult transgenic mice treated with 5-azacytidine express the normally silenced transgene. Furthermore, adult transgenic mice null for MBD2 also express the transgene. However, βYAC transgenic mice treated with 5-azacytidine or null for MBD2 express very low levels of the ϵ-globin gene. These results indicate that competition may be necessary to silence the ϵ-globin gene during 5-azacytidine treatment or in the absence of MBD2.

Methods

Generation of Transgenic Mice

Transgenic mice were generated using two different constructs derived from the cosmid cosLCRϵ (a generous gift from Frank Grosveld) that contain the entire sequence from 5' of HS5 of the LCR through 12 kb 3' of the human ϵ-globin gene polyA site. A 28 kb construct was generated by digesting cosLCRϵ with NotI and BstBI to release a fragment containing 4 kb of the 3' sequence. The constructs were run on a low-melting 0.6% agarose gel and gel purified using the Bio 101 Gene Clean Spin Kit (Qbiogene).

Constructs were then injected into fertilized eggs and implanted into pseudopregnant mothers to generate transgenic mice. Founders were determined by performing PCR on tail snipped DNA. Primers used to screen LCRϵ mice are shown in Table 7. Three lines were established for each construct.

Copy Number Determination

Genomic DNA was isolated from tails of positive mice. The DNA was digested with EcoRI and transferred to a nylon membrane. The blot was probed with a 600 bp fragment binding to the 3' end of the 3.7 kb portion of the human ϵ-globin gene. The intensity of this band was compared to EcoRI digested negative mouse genomic DNA containing 2, 5, 10, 20, and 40 copies of the LCRϵ cosmid.

Expression Profile of Mice

The developmental regulation of the transgene in mice was determined by analyzing expression of the transgene at different developmental stages. Timed matings were performed as described in Chapter 2. RNA was extracted from 10.5 dpc yolk sacs, 14.5 dpc fetal livers, and peripheral blood from adult anemic mice. RNase protection assays were used to analyze human ϵ-globin mRNA expression. RPA probes protecting either exon 1 or exon 3 of the human ϵ-globin gene were used for analysis.

Generation of 28kb LCRϵ/MBD2-/- Mice

28kb LCRϵ mice from lines 1 and 2 were used to generate transgenic knock-out mice. As performed in Chapter 2, transgenic mice were bred with knock-out mice to generate hemizygous transgenic mice. These mice were bred with MBD2-/- mice to generate 28 kb LCRϵ/MBD2-/- mice. Mice were screened for the presence of the transgene and absence of MBD2 by PCR of DNA from tail snips. Primers used to screen these mice are in Table 2 and 6.

Treatment of Mice

Mice were treated for two days with intraperitoneal injection of 1-acetyl-2-phenylhydrazine (10 mg/mL, Sigma) at a dose of 0.4 mg/10g. On the third day, mice were treated with 5-azacytidine (0.5 mg/mL, Sigma) at a dose of 2 mg/kg for five days via intraperitoneal injection.

Bisulfite Conversion

EcoRI digested genomic DNA (1 μg) from adult anemic spleens and 10.5 dpc yolk sacs was diluted to 18 μL in water, boiled for 5 minutes and further denatured by treating with 2 μL fresh 2N NaOH at 37°C for 15 minutes. Sodium bisulfite solution was prepared by dissolving 1.9 g sodium bisulfite in 2 mL water and 400 μL 2N NaOH and dissolving 100 mg hydroquinone in 2 mL water. After mixing the solution, 1 mL of the hydroquinone solution was added to the sodium bisulfite solution. The solution pH was adjusted (if necessary) to pH 5.0. After denaturing the DNA, 130 μL of the bisulfite solution and 2 μg tRNA was added and the solution incubated in the dark for 4 hours at

Primer	Sequence
E11	CTCCCCTTCACCGAATCTCATCC
HBE1202	CTTAGCGCTTCCAGCTCTACTCCACTG

Table 7: Primers used to screen LCRϵ transgenic mice.

55° C. After incubation, add 5X volume of Buffer PB (Qiagen) and 30 μL 3M sodium acetate (pH 5.2) was added to the sample and the solution applied to a spin column containing 20 μL sodium acetate. Columns were spun at 10000 xg for 1 minute and the flow-through discarded. Column was washed with 700 μL Buffer PE and centrifuged again. The sample was eluted by adding 40 μL Elution Buffer, incubating at room temperature for 5 minutes, and centrifuging. The sample was desolfonated by addition of 4.4 μL 3M NaOH and incubated at 37° C for 15 minutes. The sample was precipitated by addition of 28 μL 5 M ammonium acetate (pH 7.0), 2 μL pellet pain, 2 μg tRNA, and 180 μL ethanol. Sample was then centrifuged for 10 minutes at 10500 rpm at 4° C. The final pellet was resuspended in 100 μL Elution Buffer.

Single Nucleotide Primer Extension

The DNA methylation status at a single CpGs was determined using single nucleotide primer extension (SNuPE). Primer extension was performed using nested PCR product from bisulfite converted DNA as a template. Primers were designed to 1 nucleotide upstream of the CpG of interest. Primer extension was performed using α-^{32}P dCTP or TTP (MP Biomedicals) in separate reactions. Primer extension was performed by heating at 95° C for 1 minute, annealing for 2 minutes, and extension at 72° C for 1 minute. Only methylated CpGs will incorporate the dCTP while only unmethylated template will incorporate TTP. The reaction was terminated by adding RPA loading buffer. The products were analyzed on a 15% polyacrylamide gel after heating at 90° C for 5 minutes. The gel was exposed to X-Ray film and bands were quantitated using a

phosphor-imager. The percentage of methylation at each site was determined by the following formula: intensity of dCTP band / (intensity of dCTP band + intensity of TTP band). The primers used for bisulfite specific PCR and SNuPE assay are listed in Table 8.

Chromatin Immunoprecipitation

Performed as described in Chapter 2.

Real-time PCR

Real-time PCR was performed as described in Chapter 2. The list of primers and probes used are listed in Table 5.

Results

Two constructs were used to generate transgenic mice. Both constructs contained the entire sequence from 5' of HS5 through 12 kb (36kb) and 4 kb (28kb) 3' of the human ϵ-globin polyA site (Figure 17). Three independent transgenic lines of 36kb and 28kb LCRϵ were established. Only 36kb LCRϵ line 1 and 28kb LCRϵ lines 1 and 2 were used in this work as 28kb LCRϵ line three appeared to have the transgene inserted in the Y-chromosome and similar results were seen with 36kb line 1 and both 28kb lines used. Previous work with similar human ϵ-globin transgenic mice showed the transgene is highly expressed in the primitive erythroblasts of the 10.5 dpc yolk sac and appreciably silenced in the definitive cells of the 14.5 dpc fetal liver (Raich et al., 1990; Shih et al., 1990). In addition, unlike transgenic mice containing various γ-globin constructs, these

Primer	Sequence
Bis_Prim2_19417	GGAGAATAGGGGGTTAGAAT
Bis_Prim1_19667	CCCTTCATTCCCATACATTAAA
PE_19573	GTTGAGGAGAAGGTTGT
PE_19446	GGAGAATAGGGGGTTAGAATTT
PE_19499	GAGAGGTAGTAGTATATATTTGTTTT

Table 8: List of primers used for bisulfite specific PCR and SNuPE assay at the ϵ-globin promoter region.

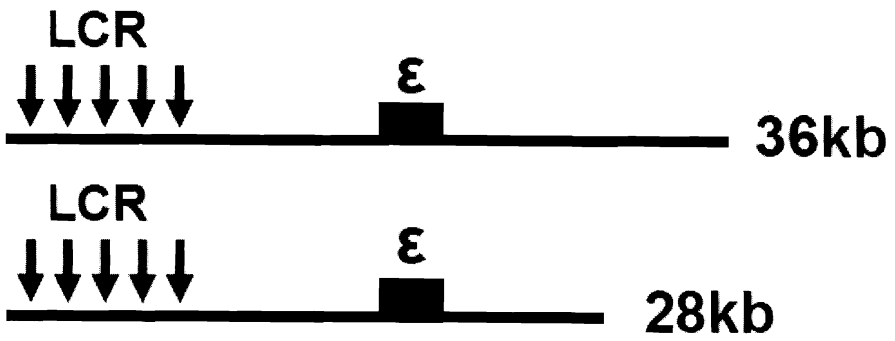

Figure 17: Constructs used to generate LCRϵ transgenic mice. The constructs only differ by the amount of sequence 3' of the ϵ-globin polyA site: 12kb for the 36kb construct and 4kb for the 28kb construct.

mice showed complete autonomous silencing in the absence of any other human β-globin gene. To determine whether the construct used to generate the 28kb and 36kb LCRε mice was autonomously silenced, timed matings were performed to analyze expression of the transgene in 10.5 dpc yolk sacs and 14.5 dpc fetal livers. All timed matings were performed by breeding a transgene positive male with a non-transgenic female. The presence of a vaginal plug was designated 0.5 dpc. Positive embryos were determined by screening placentas for the presence or absence of the transgene by PCR. RNA was isolated from transgene positive yolk sacs and fetal livers from 36kb LCRε line 1 and 28kb LCRε lines 1 and 2 mice. Expression of the transgene was determined by RNase protection assay. As shown in Figure 18, the transgene is expressed in 10.5 dpc yolk sac and becomes silenced by 14.5 dpc. Thus, the 28kb and 36kb LCRε constructs show correct developmental silencing of the transgene, indicating the constructs contain all sequences necessary for autonomous silencing. Furthermore, transgene expression cannot be detected in the peripheral blood of anemic adult mice by RNase protection assay (Figure 19).

The copy number of the transgenic mice was determined using Southern blotting. Genomic DNA was isolated from all lines and digested with EcoRI. A standard curve was generated using the LCRε cosmid at known concentrations mixed with non-transgenic genomic DNA. As shown in Figure 17, line 1 contains ~10 copies of the transgene while line 2 contains ~20 copies of the transgene. The 36kb LCRε line 1 mice contains ~25 copies of the transgene (Figure 20).

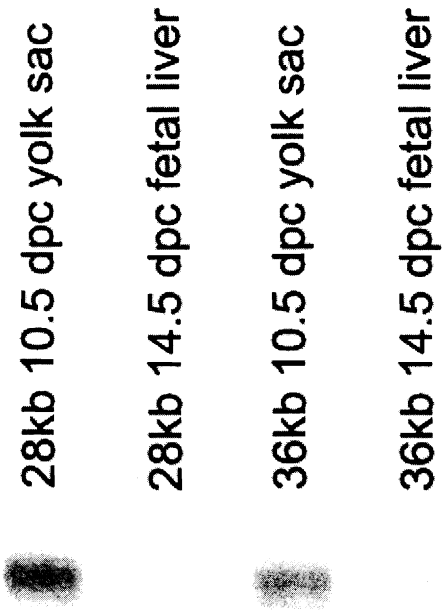

Figure 18: Developmental silencing of the human ϵ-globin gene in LCRϵ transgenic mice. The transgene is expressed in the 10.5 dpc yolk sac erythroblasts and becomes nearly silent in the erythroid cells of the 14.5 dpc fetal liver.

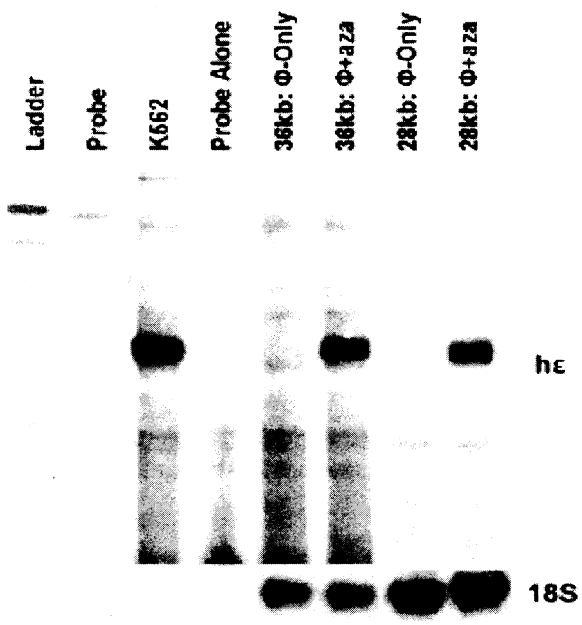

Figure 19: RPA analysis of human ϵ-globin expression in adult LCRϵ transgenic treated with and without 5-azcytidine. No human ϵ-globin mRNA is detected in adult 28kb and 36kb LCRϵ transgenic mice peripheral blood. Treatment of both lines with 5-azacytidine induces expression of the normally silent transgene in adult mice peripheral blood. Φ=1-acetyl-2-phenylhydrazine, aza=5-azacytidine.

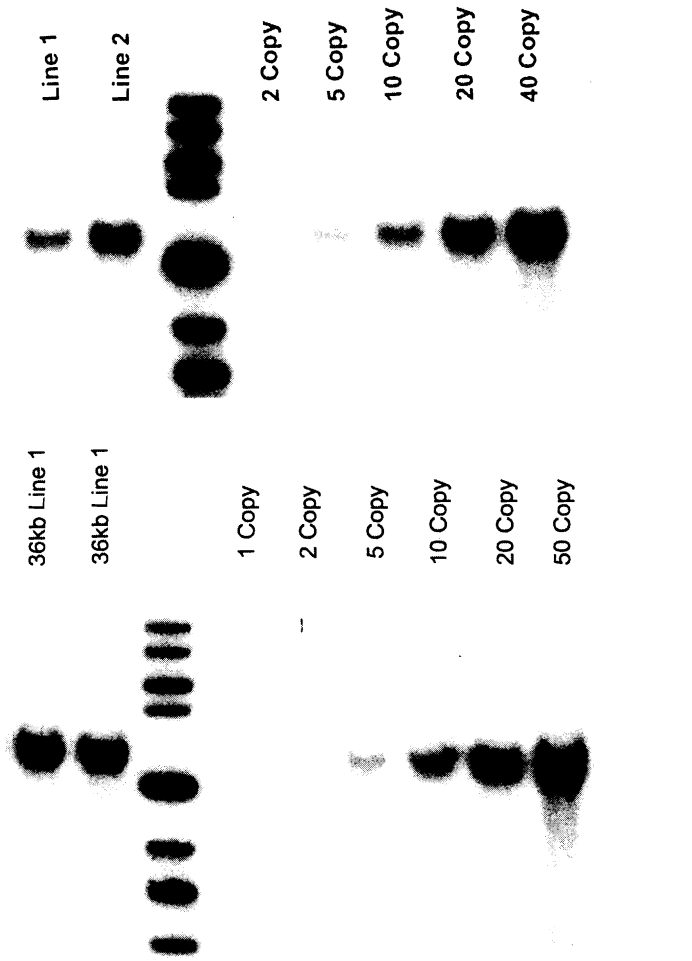

Figure 20: Copy number determination of 28kb and 36kb LCRϵ transgenic mice. Southern blots were performed to determine the copy number using a probe located at the 3' end of human ϵ-globin gene. The copy number was by comparing signal intensity to a standard curve of known amounts of cosmid LCRϵ mixed with transgene negative genomic DNA.

The inverse correlation between DNA methylation and transcriptional activity was first shown in vertebrates in the chicken β-globin locus (McGhee and Ginder, 1979). In addition, a compound that inhibits the enzyme responsible for DNA methylation (DNMT) activates silenced embryonic and fetal globin genes in a variety of model systems, chickens, and patients (Ginder et al., 1984; Ley et al., 1982; Ley et al., 1984; Nienhuis et al., 1985; Pace et al., 1994). We sought to determine whether 5-azacytidine could activate the silenced human embryonic globin gene in the context of 28kb and 36kb LCRε transgenic mice. Adult mice made anemic via 1-acetyl-2-phenylhydrazine treatment fail to express the transgene as determined by RNase protection assay. However, after a five day treatment regime with 5-azacytidine, adult 28kb and 36kb LCRε transgenic mice express the human ε-globin gene (Figure 19 and 21). In the work done by Pace and colleagues, βYAC mice treated with 5-azacytidine express the γ-globin gene, but expression of the human ε-globin gene was not measured. Interestingly, we find that when treated with 5-azacytidine, adult βYAC transgenic mice fail to express the human ε-globin gene as determined by RPA (Figure 22). However, when measured by real-time PCR, low levels are detectable (Figure 23). The 28kb and 36kb LCRε transgenic mice contain all necessary regulatory elements needed for autonomous transgene silencing. However, inhibition of DNA methylation is able to overcome these repressive elements.

Given the ability of 5-azacytidine to overcome the autonomous silencing mechanisms of the human ε-globin gene in 28kb and 36kb LCRε mice, we sought to look at the methylation status of CpGs around the promoter and proximal transcribed region of

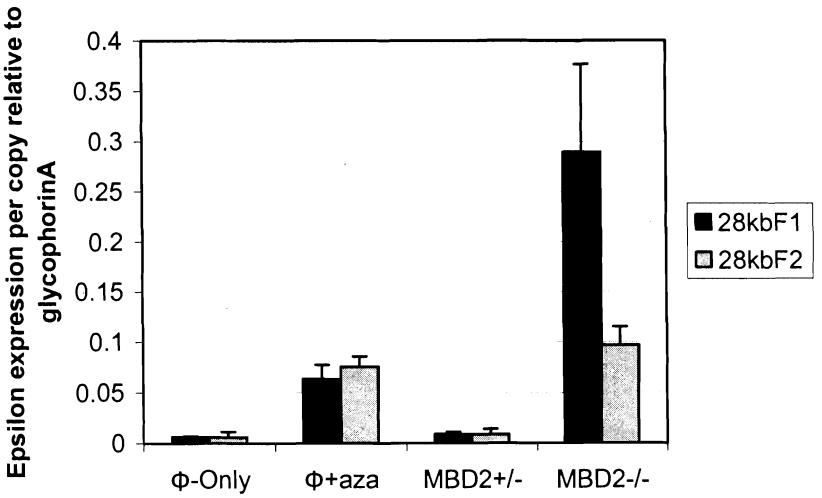

Figure 21: Real-time PCR analysis of human ϵ-globin expression after treatment with 5-azacytidine and loss of MBD2 in LCRϵ transgenic mice. Wild type 28kb LCRϵ lines 1 and 2 mice were treated with 5-azacytidine. Expression of the transgene was determined by real-time PCR using peripheral blood RNA from prior to treatment and after treatment. Peripheral blood RNA was also collected from both lines of mice in the MBD2+/- and MBD2-/- background.

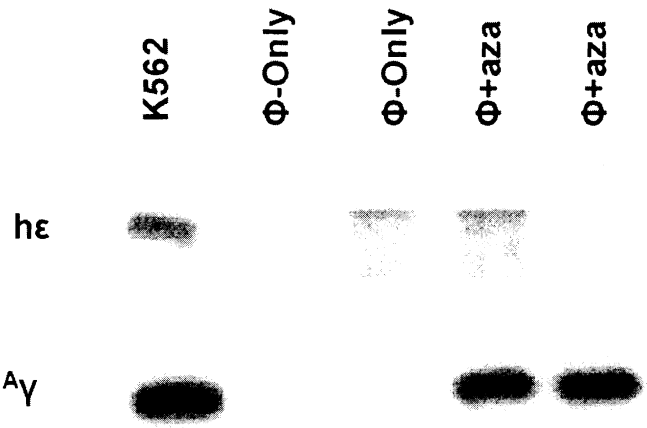

Figure 22: RPA analysis of human ϵ-globin expression in βYAC transgenic mice treated with 5-azacytidine. In contrast, 5-azacytidine is able to induce expression of the γ-globin gene in adult βYAC transgenic mice. RPA was performed on peripheral blood of mice treated with and without 5-azacytidine.

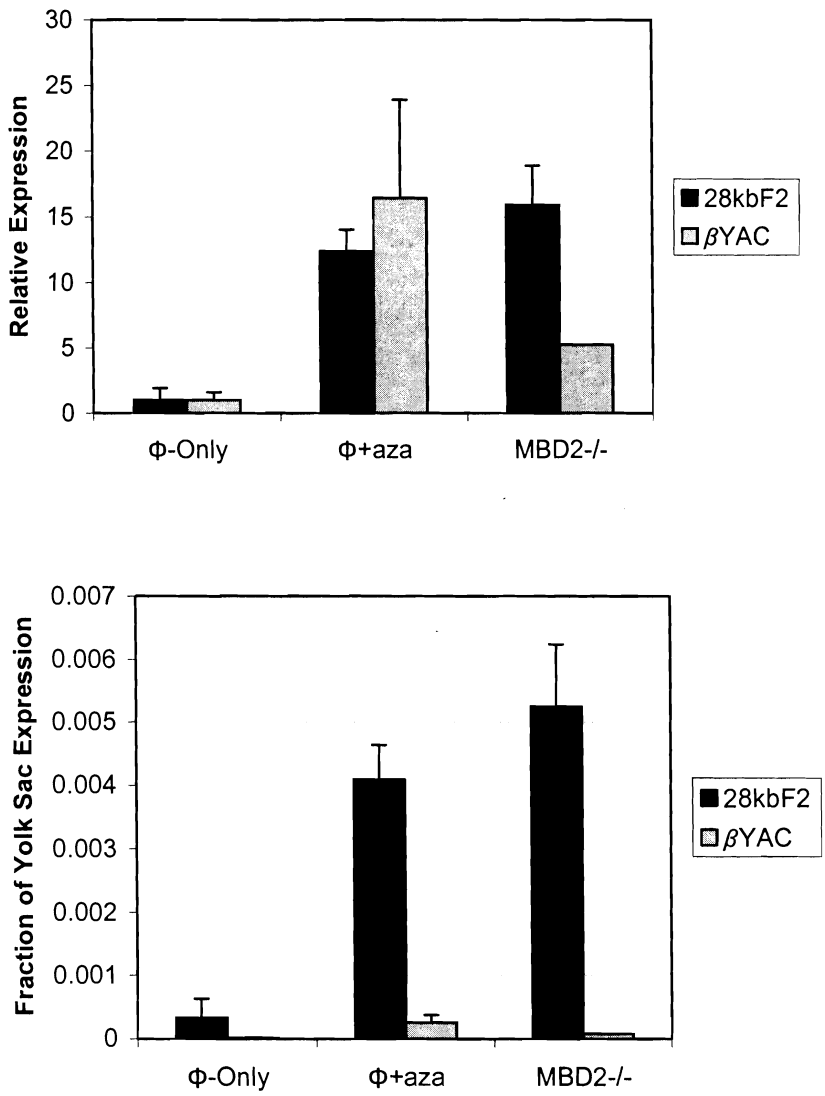

Figure 23: Human ϵ-globin expression in LCRϵ line 2 and βYAC transgenic mice. The top panel shows expression levels relative to 1-acetyl-2-phenylhydrazine treatment only. The bottom panel represents the expression of the transgene relative to yolk sac expression levels.

the transgene. Previous work in our laboratory has shown a correlation between DNA methylation in the promoter and proximal transcribed region of the chicken ρ-globin gene and the silencing of this gene during development (Ginder et al., 1984; Singal et al., 1997; Singal et al., 2002). To examine the methylation status of 3 CpGs around the human ϵ-globin promoter and proximal transcribed region, bisulfite conversion followed by single nucleotide primer extension was performed. The technique allows for the determination of the percentage of methylation at any CpG of interest in the genome. The level of methylation was compared at three different CpGs in 28kb LCRϵ line 2 transgenic mice in 10.5 dpc yolk sac, adult anemic mice, and adult mice treated with 5-azacytidine. The promoter and proximal transcribed region in adult anemic mice is highly methylated (>90%) at all CpG sites tested (Figure 24). This result is in agreement with the idea that high levels of DNA methylation correspond to low levels of transcription. The transgene is highly expressed in the primitive erythroblasts of the 10.5 dpc yolk sac. At this time, the level of CpG methylation is much lower than in adult mice, ~40% at each site tested. The genomic DNA used in this analysis came from total yolk sac tissue which contains epithelial cells in addition to erythroid cells. Epithelial cells do not express globin genes and are presumably highly methylated in the globin locus. In addition, erythroid cells have been found to only account for ~11% of the total yolk sac population (Redmond and Lloyd, 2005). As a result, the level of DNA methylation seen in the 10.5 dpc yolk sac may in fact be much lower than 40%. Adult mice treated with 5-azacytidine show an intermediate level of methylation at each site (~80%). This correlates with expression data. While 5-azacytidine is able to induce expression of the

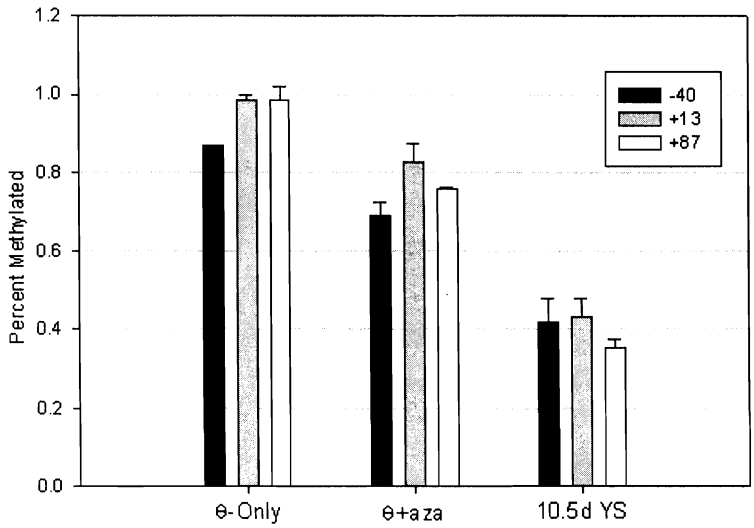

Figure 24: MS-SNuPE analysis of DNA methylation levels around the ϵ-globin promoter and proximal transcribed region. Bisuflite conversion was performed on genomic DNA from the splenocytes of anemic mice or 10.5 dpc yolk sac. After amplification by PCR, the DNA methylation levels were analyzed using MS-SNuPE at three sites around the ϵ-globin promoter and proximal transcribed region in 28kb LCRϵ mice, mice treated with 5-azacytidine and 10.5 dpc yolk sac erythroid cells.

silenced transgene, it is expressed at a much lower level than in the 10.5 dpc yolk sac (Figure 23). The compound 5-azacytidine is highly toxic. While the dose administered to the mice is not fatal, it may be insufficient to produce high enough levels of demethylation to recapitulate the level of expression seen in the yolk sac. In addition, 5-azacytidine is not specific for the β-globin locus and acts as a global inhibitor of DNA methylation. Hypomethylated DNA is associated with genetic instability and mice lacking DNMTs are embryonic lethal presumably due to this instability (Li et al., 1992; Okano et al., 1999). Cells hypomethylated due to 5-azacytidine treatment may undergo cell death due to the instability of genome leaving only moderately demethylated cells for the analysis. Finally, these mice have 20 copies of the transgene and the analysis does not discriminate between individual transgenes. Thus, the relatively low level of demethylation seen after 5-azacytidine treatment may be due to the number of copies of the transgene where only highly demethylated copies are expressing the transgene while methylated copies do not. This kind of variation on a cell-to-cell basis may account for the low level of demethylation seen after 5-azacytidine treatment. Taken together, these results show a positive correlation between the level of unmethylated CpGs around the human ε-globin promoter and proximal transcribed region in 28 kb LCRε line 2 transgenic mice and the level of transgene expression.

Inhibition of DNA methylation leads to expression of the γ-globin gene in βYAC transgenic mice and the human ε-globin gene in 28 kb and 36 kb LCRε transgenic mice. Furthermore, loss of MBD2 leads to expression of the γ-globin gene in βYAC transgenic mice. We sought to determine what impact MBD2 has on maintaining repression of the

human ε-globin gene in 28 kb LCRε transgenic mice. Mice null for MBD2 were bred with mice containing the 28 kb LCRε transgene. The resulting hemizygous mice were bred with MBD2 null mice again to generate transgenic knock-out mice. As was done previously, adult mice were made anemic to increase erythropoiesis. Blood was collected from the tail vein and analyzed by real time PCR. Adult 28kb LCRε transgenic mice null for MBD2 express the transgene at a level ~16-45-fold over baseline (Figure 19). The variation in expression of the transgene between line 1 and line 2 may be due to position effects due to the location of transgene insertion that were not revealed during 5-azacytidine treatment. In addition, the higher copy number of the line 2 mice may be a rate limiting factor in transcription level. Mice containing the βYAC transgene and null for MBD2 express the γ-globin gene and low levels of the human ε-globin gene (Figure 20). When standardizing expression to determine the fold induction due to 5-azacytidine treatment or loss of MBD2, the level of induction is similar in βYAC transgenic mice and 28kb LCRε F2 mice (Figure 23). However, when calculating expression relative to yolk sac expression levels, the human ε-globin gene is expressed 15-60-fold higher in LCRε transgenic mice versus βYAC transgenic mice (Figure 23). This is not surprising as loss of MBD2 leads to no enrichment in TriMeK4, AcH3, AcH4, and Pol II levels at the human ε-globin gene promoter or coding regions (Figure 25). This result supports the notion that competition also plays a role in the silencing of the human ε-globin gene.

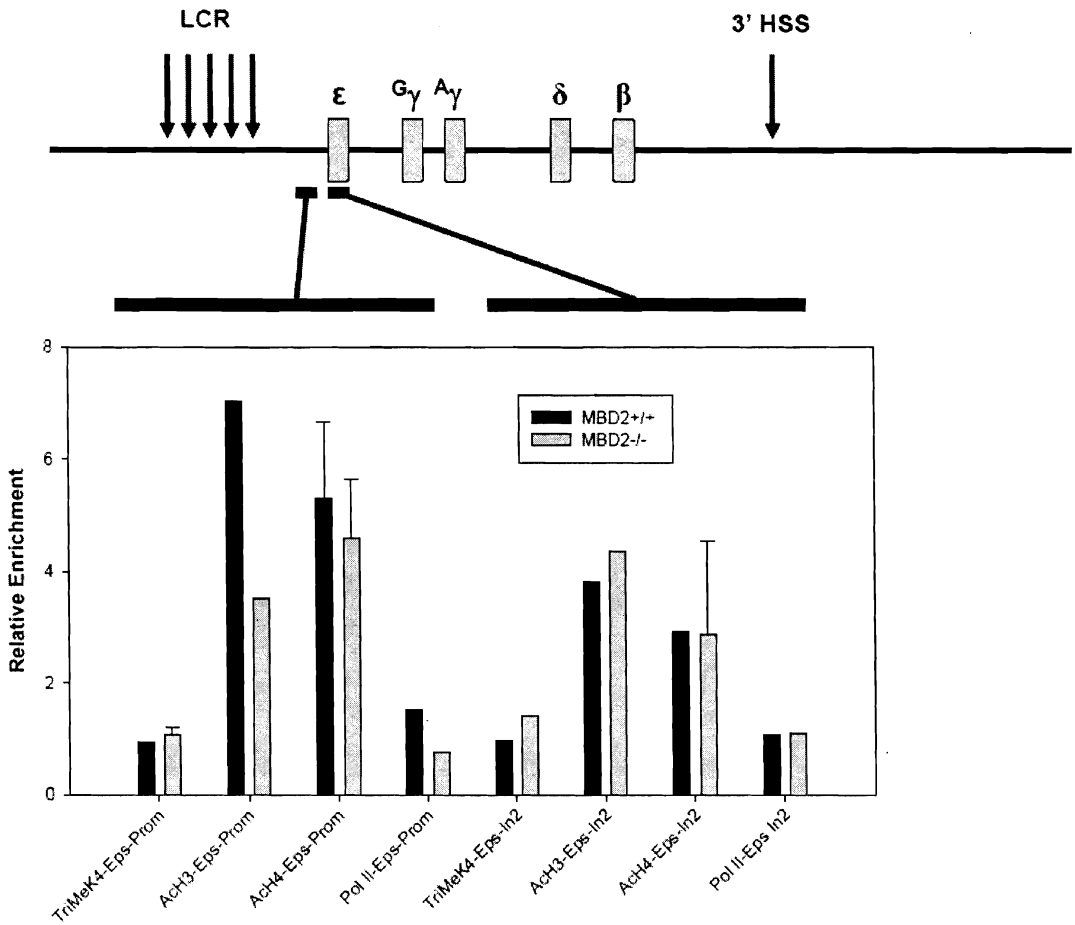

Figure 25: Post-translational histone modification changes around the human ϵ-globin gene promoter and proximal transcribed region in MBD2+/+ and MBD2-/- adult βYAC transgenic mice. Relative enrichment values were determined relative to the endogenous amylase gene to control for loading and IgG values to control for non-specific immunoprecipitation.

Discussion

Transgenic mice harboring constructs containing the entire sequence from 5' HS5 through 4 kb 3' of the human ϵ-globin gene correctly express and silence the gene during development. Similar to other human ϵ-globin transgenic mice, the mice generated here contain all necessary *cis* elements for autonomous silencing (Raich et al., 1990; Shih et al., 1990). Many factors have been shown to bind to the human ϵ-globin promoter to mediate transcriptional repression and activity (Filipe et al., 1999; Li et al., 1998a; Li et al., 1997; Li et al., 1998b; Peters et al., 1993; Raich et al., 1995; Tanabe et al., 2002; Tanimoto et al., 2000; Wada-Kiyama et al., 1992). While studies have shown DNA methylation plays a causative role in the regulation of the chicken embryonic ρ-globin gene, little was known of the role of DNA methylation in human ϵ-globin gene regulation (Ginder et al., 1984; Singal et al., 1997; Singal et al., 2002). Here, we show that inhibition of DNA methylation leads to expression of a silenced human ϵ-globin transgene in adult mice. Furthermore, there is an inverse correlation between the level of DNA methylation around the ϵ-globin promoter and proximal transcribed region and the level of expression of the transgene. Furthermore, loss of the methyl CpG binding protein, MBD2, leads to expression of the 28 kb LCRϵ transgene in adult mice.

Initial studies with human ϵ-globin transgenic mice indicated the gene does not require the presence of an additional human β-type globin gene to become fully silenced during development. This was in contrast to the γ-globin gene which requires the presence of an additional β-type globin gene to become fully silenced in transgenic mice (Behringer et al., 1990; Dillon and Grosveld, 1991; Enver et al., 1989; Enver et al.,

1990). The γ-globin gene is autonomously silenced, but requires competition for the upstream LCR enhancer for complete silencing. Until now, it was thought that competition was not needed for the complete silencing of the ε-globin gene. We show that both 5-azacytidine and loss of MBD2 can induce expression of the silenced human ε-globin transgene in LCRε transgenic mice. Using the same conditions, mice containing the entire β-globin locus yeast artificial chromosome express 15-60-fold less ε-globin RNA. The presence of additional β-type globin genes inhibits expression of the human ε-globin gene when βYAC transgenic mice lack MBD2 or are treated with 5-azacytidine. The other globin genes out-compete the ε-globin gene for upstream LCR enhancer activity.

The data presented here suggest the human ε-globin gene is silenced by three mechanisms. First, and most likely strongest, is the autonomous silencing mediated by *cis* elements. This appears to be the primary mechanism by which the human ε-globin gene is silenced. Disruption of *cis* elements leads to significant ε-globin expression in adult mice (Raich et al., 1992; Raich et al., 1995; Tanimoto et al., 2000). In addition to elements located 5' of the human ε-globin gene, we have shown 3' sequences may be involved. As shown here, 36kb and 28kb LCRε transgenic mice correctly express and silence the transgene during development. However, two lines of transgenic mice containing similar 5' sequence and only 100 bp 3' of the polyA site had an embryonic lethal phenotype (data not shown). The embryos were pale and small in size. We hypothesized the mice were suffering from an α-thalassemia due to overexpression of the ε-globin chain that prevented normal hemoglobin tetramer formation. To test this

hypothesis, a cosmid containing the entire sequence from 5' of HS5 through 100 bp 3' of the ϵ-globin polyA site was proposed to be inserted into a specific chromosomal location in MEL cells. As a negative control, the 28kb construct was to be inserted into the same chromosomal location to prevent any position effects. Expression analysis was to be performed to determine whether lack of 3' sequence resulted in expression of the ϵ-globin gene in adult erythroid cells. However, generation of these cells proved problematic. Using various transfection mechanisms, large cosmids were unable to be effectively transfected. Currently, alternative mechanisms are being proposed to determine whether the 3' silencer exists.

Another layer of silencing is mediated by DNA methylation and MBD2. Loss of MBD2 or treatment with 5-azacytidine induces expression of the human ϵ-globin gene in 36kb and 28kb LCRϵ transgenic mice. However, low levels are also seen in βYAC transgenic mice under the same conditions. This revealed a third layer of silencing, namely competitive mechanism of human ϵ-globin silencing as βYAC mice express 15-60-fold less ϵ-globin mRNA than LCRϵ mice. The presence of other globin genes or additional down-steam sequences may prevent an effective interaction between the LCR enhancer and the ϵ-globin gene in a >15-fold decrease in expression (Figure 26). However, the effect may be due to sequences present on the βYAC transgene rather than competition mediating this effect. Taken together, these results indicate the human ϵ-globin gene is silenced by three mechanisms. We have shown significant expression when removing two of these mechanisms. An interesting study would be to combine

elements of all three mechanisms to determine what fold repression each exerts on the ϵ-globin gene.

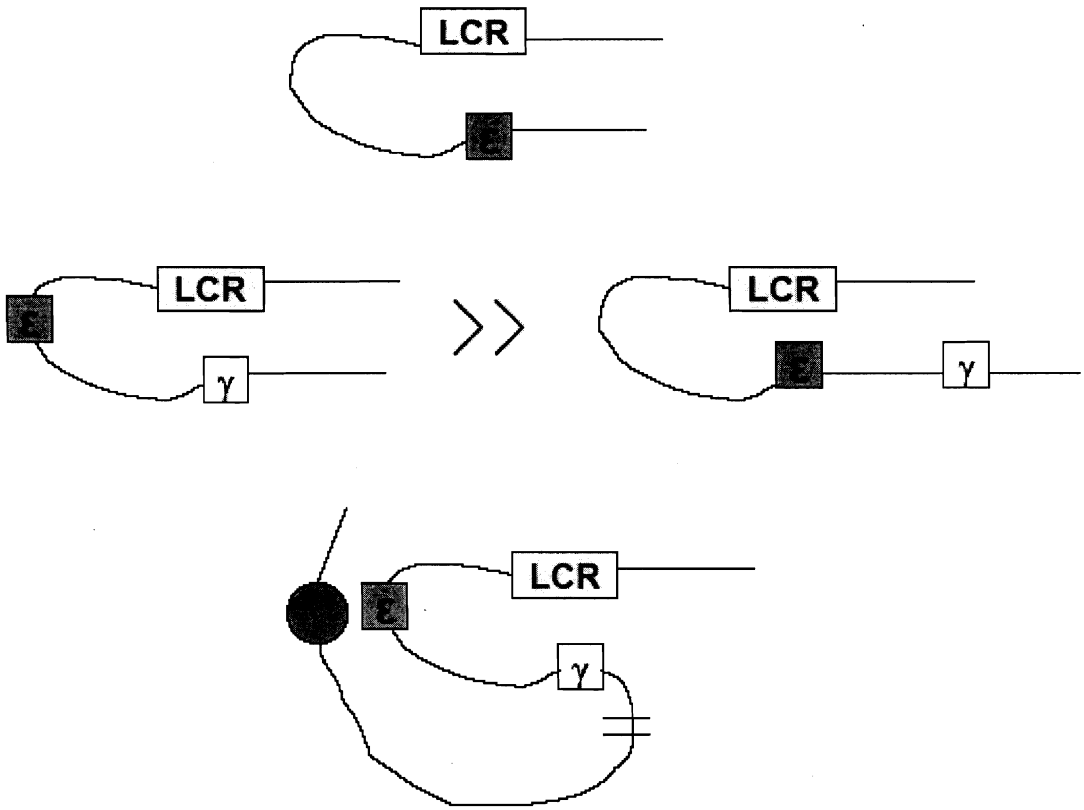

Figure 26: Model of ϵ-globin silencing in adult βYAC transgenic mice by sequences downstream of the ϵ-gene. In the absence of other downstream sequences, loss of MBD2 or treatment with 5-azacytidine allow for an effective interaction between the ϵ-globin gene and the LCR enhancer leading to transcription of the transgene (top). In βYAC transgenic mice, the ϵ-globin gene is expressed at a much lower level to disruption of the enhancer interaction by other globin genes or downstream sequences (middle and bottom).

Chapter 4: Mechanism of MBD2 Mediated Repression of the γ-globin Gene

Introduction

Adult transgenic mice null for MBD2 express silenced embryonic and fetal globin genes. The level of induction of the γ-globin gene in βYAC/MBD2-/- mice is similar to the level seen in βYAC mice treated with the DNA methyltransferase inhibitor 5-azacytidine. In addition, treatment of βYAC/MBD2-/- with 5-azacytidine does not produce an additive induction of γ-globin RNA relative to treatment of wild type βYAC mice. These results indicate that MBD2 and 5-azacytidine work via the same mechanism to induce globin gene expression. In addition to the work presented here, previous work has shown treatment with 5-azacytidine leads to hypomethylation around the globin gene promoters and a concomitant increase in embryonic or fetal globin transcription (Ginder et al., 1984; Ley et al., 1983; Ley et al., 1984; Zucker et al., 1983). With the discovery of proteins that specifically bind to methylated DNA and recruit transcriptional repression complexes, it seemed a likely hypothesis that these proteins were mediating the repressive effect seen on genes up-regulated upon DNMT inhibition by binding to methylated CpGs around the promoter and inhibiting transcription. However, only a handful of genes have been shown to be bound by MCBPs *in vivo* in non-cancerous cells

(Chen et al., 2003; Hutchins et al., 2002; Klose et al., 2005; Kransdorf and Ginder, 2004; Martinowich et al., 2003). The γ-globin gene has been reported to be regulated by many mechanisms including by multiple signaling pathways and by epigenetic modifications (Bhanu et al., 2004; Bhanu et al., 2005; Pace et al., 2003). Thus, MBD2 mediated transcriptional repression of the ε- and γ-globin genes in adult LCRε and βYAC transgenic may be due to direct recruitment of MBD2 to the ε- or γ-globin promoter, or via a cell signaling pathway or both. Therefore, we sought to determine whether MBD2 binds the ε- or γ-globin promoter *in vivo* and what gene(s) were aberrantly expressed in MBD2 null mice that mediated these effects. To do this, chromatin immunoprecipitation assays were performed to locate where MBD2 was binding to mediate transcriptional repression of the ε- and γ-globin genes. Microarray analysis was performed on MBD2+/+ and MBD2-/- mice to determine what genes are differentially expressed in adult erythroid cells. The data from the microarray was further analyzed using a pathway analysis program to determine what pathways were differentially impacted due to the loss of MBD2.

Methods

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as described in Chapter 2 with the following modifications. Chromatin was crosslinked with 1% formaldehyde for 10 minutes at room temperature. Sonication was performed 8x instead of 6x. Chromatin was washed three times with low salt buffer and one time with medium salt buffer (equal

parts high salt and low salt buffer). Immunoprecipitations were performed with anti-MBD2 (Upstate), anti-MBD2/3 (Upstate), ant-MTA2 (Santa Cruz), goat IgG (Santa Cruz), sheep IgG, and protein G salmon sperm agarose beads. DNA was analyzed using real-time PCR and SYBR Green chemistry. Primers used for real-time PCR analysis are listed in Table 9.

Lower stringency washes were used because DNA binding proteins that are more difficult to ChIP than histones owing to lower abundance. Thus, to account for increased non-specific pull-down, enrichment was determined with the following formula:

$$\frac{\text{Sample-(gene bound/input)/(amylase bound/input)}}{\text{IgG-(gene bound/input)/(amylase bound/input)}}$$

Microarray Analysis

Four wild type mice and four MBD2-/- mice were treated with 1-acetyl-2-phenylhydrazine for two days. Spleens were harvested on the fifth day and RNA isolated using Trizol. The RNA was treated with DNase I using the Turbo DNase kit (Ambion). The RNA was further cleaned and concentrated using the RNeasy MinElute Cleanup kit (Qiagen). The RNA was eluted in DEPC-treated water to which 1 μL of SuperRNasin was added per 40 μL water. RNA purity was judged by the ratio of absorbance at 260 and 280 nm (A_{260} / A_{280}) and 260 and 270 nm (A_{260} / A_{270}). In addition, RNA, cDNA, and cRNA integrity were assessed by running 1 μL of every sample in RNA 6000 Nano or

Primer	Sequence
Ugt8-F-1574	TTAGAGCTCTGAAGGGCATGTGGA
Ugt8ChIP-Probe	6famTGCTCTGCGTCTCCCAAACTCAAGAAtamra
Ugt8-R-1671	TGTGCTAATTCCTCCCGCAACAGA
GammaProm-ChIP-F	CCTTGCCTTGACCAATAGCCTTGA
GammaProm-ChIP-Probe	6famACAAGGCTACTATCACAAGCCTGTGGtamra
GammaProm-ChIP-R	TTCCAGAAGCGAGTGTGTGGAACT
-3.4GATA2 F	TCCATCCAGCAGCTTTAGGAA
-3.4GATA2 R	GGGTTCGAAGCCACTCCAA

Table 9: List of primers used for real-time PCR for ChIP assays in Chapter 4.

Pico LabChips on the 2100 Bioanalyzer (Agilent) depending on the RNA concentration, according to manufacturers protocol. RNA (5 μg) was reverse transcribed to cDNA using a 24-mer oligodeoxythymidylic acid primer with a T7 RNA polymerase promoter sited added the 3' end of the T7-olidgo(dT) using the Superscript cDNA Synthesis Systems (Life Technologies). In vitro transcription was performed using the Enzo BioArray High Yield RNA Transcript Labeling kit (Enzo) to produce biotin-labeled cRNA. For each sample, 20 μg of the cRNA product was fragmented and hybridized for 18-20 h onto an Affymetrix Genechip 430A 2.0 microarray (Affymetrix). Each microarray contains 45,000 probes sets analyzing the expression of over 39,000 different transcripts where one transcript is generally interrogated by more than one probe set. Each probe set contains probes that either perfectly match (PM) the sequence in a segment of the target gene or contain a single mismatched (MM) nucleotide in the middle position of the corresponding perfectly matched probe. The intensity difference of PM probes and MM probes is used to make detection calls which determine whether the transcript of a gene is detected or undetected. Each microarray was washed and stained with streptavidin-phycoerythrin and scanned at a 6 μm resolution with the Agilent G2500A Technologies Gene Array scanner (Agilent Technologies) according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix).

After scanning, the scaling factor, the noise (RawQ) value for a given probe array hybridization, the percentage of probe sets declared "Present" (%P) by the detection call algorithm, the 3'/5' ratios of the signal intensity values for two house-keeping genes

(GAPDH and β-actin), and the presence or absence call for the ribosomal RNAs 18S and 28S were determined for quality control purposes.

For every probe set, normalization, background subtraction, and expression summaries were calculated using three different methods. First the Microarray Suite 5.0 method was used to obtain probe set summaries. Model-based expression indices were calculated by use of a multiplicative model to account for probe affinity effects in calculating probe set expression summaries. Lastly, the multiarray average method was used. This method uses quantile normalization followed by a median polish to remove probe affinity effects when calculating probe set summaries. Only genes significantly changed using all three methods were used for further analysis. All microarray studies were performed by Dr. Cathrine Dumur in the Virginia Commonwealth University Department of Pathology.

Pathway Analysis

To determine whether the differentially expressed genes in the microarray were impacting cell signaling pathway(s), the gene list was analyzed using the Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems). Two sets of gene lists were analyzed using different P-values, <0.005 and <0.01. This program uses the Ingenuity Pathways Knowledge Base (IPKB) to provide insights into observed gene expression changes across biological samples. The input to this application corresponds to a user generated dataset file containing differentially expressed genes. Genes of interest to the user, that are also known to directly (or indirectly) interact with other genes in the IPKB,

are called Focus Genes and are used to identify molecular networks that indicate how these genes may influence each other. IPA dynamically computes a large "global" molecular network based on hundreds of thousands of curated direct and indirect physical and functional interactions between orthologous mammalian genes from the published, peer-reviewed content in Ingenuity's Knowledge Base.

CpG Island Determination

The genomic DNA sequence of up-regulated genes from microarray analysis were obtained from the Mouse Genome Informatics web site (http://www.informatics.jax.org/). This sequence was analyzed with the CpG Island Searcher program using default parameters (http://cpgislands.usc.edu/).

Real-Time PCR

Candidate genes from microarray analysis were analyzed using real-time PCR to confirm differences in expression between wild-type and knock-out splenocytes. Candidate gene were chosen based on the level of significance (P-value <0.01) and presence in a hematological pathway. RNA from six of the eight mice analyzed in the microarray was first reverse transcribed using iScript (Bio-Rad). Candidate genes were analyzed using pre-designed Taqman probes sets (Applied Biosystems) or SYBR Green chemistry with pre-designed primer sets (Wang and Seed, 2003). All values were standardized using mouse cyclophilin A as an internal control. Primers used for real-time PCR analysis are located in Table 10.

Primer	Sequence
BAF57-114-F	AAAAGACCATCTTATGCCCCAC
BAF57-114-R	CCTGTAGTTGTTGTAGGCGAG
Ugt8-121-F	ACTCCATATTTCATGCTCCTGTG
Ugt8-121-R	AGGCCGATGCTAGTGTCTTGA
ROG-101-F	GCTCTGAGAGAGGACTTGGGA
ROG-101-R	TGCTTTATGCTTGTGTGACATCT
mCyclophilinA-F	GAGCTGTTTGCAGACAAAGTTC
mCyclophilinA-R	CCCTGGCACATGAATCCTGG
Jund1	ABI Part #: Mm01343193_s1
MAPK3	ABI Part #: Mm01973540_g1

Table 10: Primers used for real-time PCR validation of microarray results and confirmation of MBD2 knock-down.

Results

The promoter region of the γ-globin gene does not contain a CpG island. In fact, no region of the promoter contains more than 4 CpGs within a 70 bp span. Preliminary ChIP results indicated MBD2 was not bound to the promoter region of the γ-globin gene, but was slightly enriched in the 5' region of the GATA2 gene (Figure 27). Taken together, these results led us to believe MBD2 was not mediating its repressive effect by binding to the γ-globin promoter region. Studies in cell culture lines and primary human erythroid cells have shown cell signaling pathways play a role in γ-globin transcriptional regulation (Bhanu et al., 2004; Bhanu et al., 2005; Ikuta et al., 2001; Pace et al., 2003). Thus, we postulated MBD2 may be differentially regulating a factor needed for γ-globin activation directly or by regulating a factor in a signaling pathway necessary for γ-globin transcriptional activation. To determine which gene(s) were involved, microarray analysis was performed. Four wild-type and four MBD2 knock-out mice were made anemic by 1-acetyl-2-phenylhydrazine treatment. Spleens were harvested on the fifth or seventh day and RNA isolated. After amplification, the RNA was hybridized to the Affymetrix 430A2.0 mouse microarray chip. ANOVA analysis on each probe set identified ~25 genes upregulated significantly ($p<0.005$) in MBD2-/- erythroblasts and 266 genes differentially regulated using significant but less stringent analysis ($p<0.01$) (Table 11). Upon initial observation, there was no known erythroid specific factor that was up- or down-regulated in MBD2-/- erythroblasts. However, almost every gene upregulated in MBD2-/- cells contained a CpG island (Table 12) using the default settings of the CpG Island Searcher Program (http://cpgislands.usc.edu/).

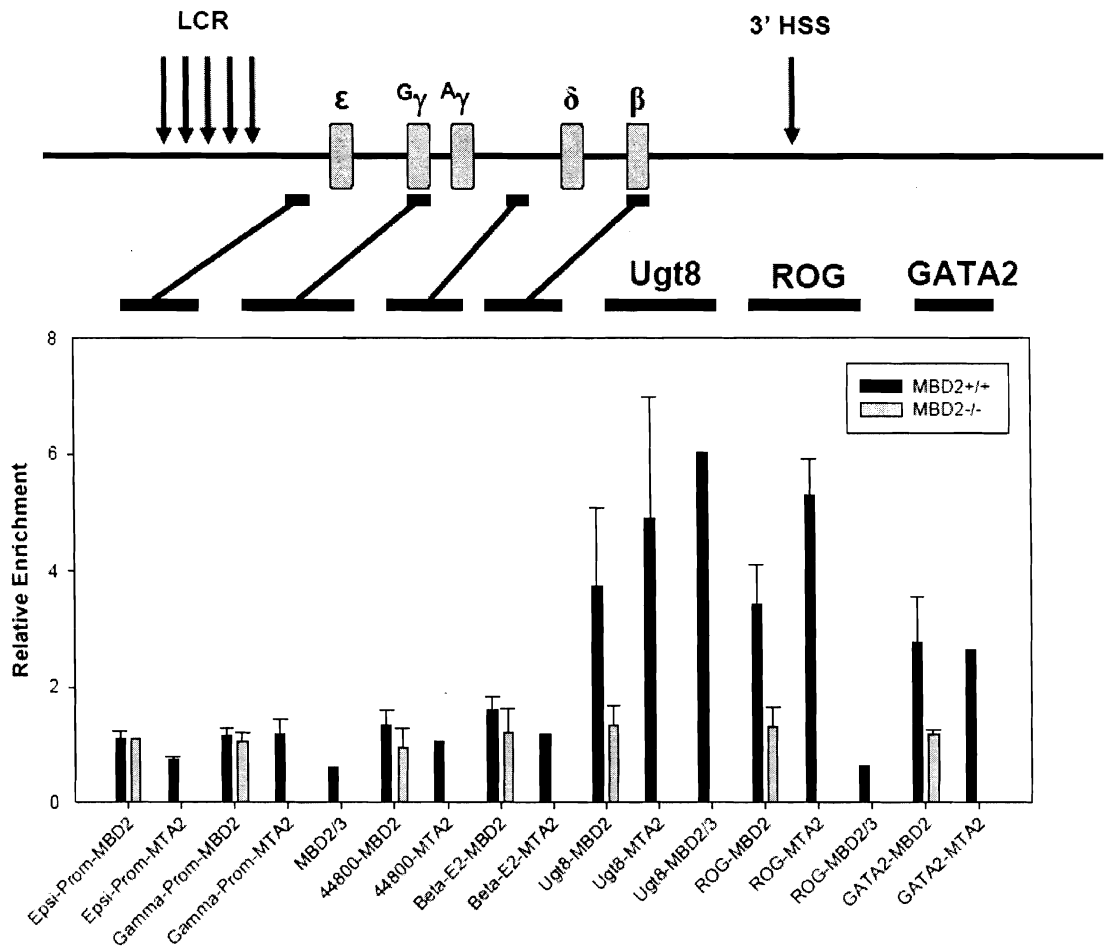

Figure 27: Determining *in vivo* binding of MBD2 using the ChIP assay. ChIP assays were performed using anti-MBD2, ant-MTA2, and sheep and goat IgG antibodies. DNA was analyzed using real-time PCR at the human ϵ- and γ-globin promoters, a weak CpG island between the γ-globin and δ-globin genes, the human β-globin coding region, the Ugt8 gene CpG island, the ROG gene CpG island, and the upstream region of the GATA2 gene. Data was normalized to endogenous amylase levels to account for loading errors and IgG to account for non-specific pull-downs.

Fold difference	Gene
-6.10	methyl-CpG binding domain protein 2
1.97	inositol polyphosphate-5-phosphatase B
-1.56	tripartite motif protein 30-like
-2.67	apolipoprotein B editing complex 3
-6.45	3-phosphoglycerate dehydrogenase
-3.40	RIKEN cDNA 2610034N03 gene
2.53	histocompatibility 28
-4.74	tripartite motif protein 12
-1.34	tyrosyl-tRNA synthetase
1.71	RAB3A interacting protein
-1.47	RAP2B, member of RAS oncogene family
2.19	expressed sequence C79248
5.71	UDP-glucuronosyltransferase 8
1.47	tropomodulin 4
1.69	G kinase anchoring protein 1
-1.37	RIKEN cDNA 2410026K10 gene
1.21	LanC (bacterial lantibiotic synthetase component C)-like 1
2.06	16 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A130032A08 product: unclassifiable, full insert sequence
-1.69	cell division cycle associated 3
-4.42	cDNA sequence BC004690
-4.61	tripartite motif protein 34
-6.54	RIKEN cDNA 2810417H13 gene
1.90	cysteine-rich protein 1 (intestinal)
1.17	hairy/enhancer-of-split related with YRPW motif 2
2.78	solute carrier family 35 (UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter), member D1
2.57	stearoyl-Coenzyme A desaturase 2
2.56	interferon-induced protein 44
1.61	RIKEN cDNA 2610034N03 gene
-1.62	3-hydroxyisobutyryl-Coenzyme A hydrolase
1.14	MAS1 oncogene
1.81	receptor-like tyrosine kinase
-3.47	RIKEN cDNA 6720467C03 gene
1.14	transcriptional regulator, SIN3B (yeast)
1.70	receptor-like tyrosine kinase
1.58	2'-5' oligoadenylate synthetase 1D /// 2'-5' oligoadenylate

	synthetase 1E
1.13	RIKEN cDNA 2010002A20 gene
-1.56	pan hematopoietic expression
-1.29	RIKEN cDNA 2510015F01 gene
2.11	zinc finger, FYVE domain containing 21
2.00	zinc finger, FYVE domain containing 21
-1.71	RIKEN cDNA 2510048K03 gene
-4.57	expressed sequence AI447904
1.15	RIKEN cDNA 2300006M17 gene
-1.31	cDNA sequence BC028528
-2.01	RIKEN cDNA 9130208E07 gene
1.58	histidyl-tRNA synthetase
-1.21	expressed sequence AW060207
-1.67	polymerase (DNA directed), delta 2, regulatory subunit
-1.99	RIKEN cDNA 1810010O14 gene
1.53	suppressor of Ty 16 homolog (S. cerevisiae)
1.17	thioredoxin 2
-6.33	methyl-CpG binding domain protein 2
1.89	transcription factor 7-like 2, T-cell specific, HMG-box
-1.36	deleted in polyposis 1
-2.58	RNA-binding region (RNP1, RRM) containing 2
-1.08	RIKEN cDNA 1700001E16 gene
1.15	signal recognition particle receptor, B subunit
-1.36	glycyl-tRNA synthetase
1.12	solute carrier family 39 (zinc transporter), member 9
-1.65	serine hydroxymethyl transferase 2 (mitochondrial)
1.33	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19 /// RIKEN cDNA 2810457M08 gene
-1.26	3-ketoacyl-CoA thiolase B
-1.74	RIKEN cDNA 1810010O14 gene
-2.68	Diabetic nephropathy-related gene 1 mRNA, partial sequence
1.87	phytanoyl-CoA hydroxylase
2.02	GTP cyclohydrolase 1
-1.22	cell division cycle 26
1.35	5-azacytidine induced gene 2
-1.21	RIKEN cDNA 8430432M10 gene
1.50	cDNA sequence BC002199
-1.18	expressed sequence 2 embryonic lethal
1.41	cardiac lineage protein 1
1.17	RIKEN cDNA 4932432K03 gene
-1.20	hemoglobin alpha, adult chain 1
1.12	RIKEN cDNA 1110028N05 gene

2.14	myelin basic protein
-2.08	kinesin family member 5B
1.71	tripartite motif protein 34
-1.64	membrane-associated protein 17
-1.21	RIKEN cDNA C730025P13 gene
-1.19	expressed sequence AU040320
1.31	thioredoxin 2
-3.02	DNA segment, Chr 7, ERATO Doi 760, expressed
-1.12	coiled-coil-helix-coiled-coil-helix domain containing 5
1.76	RIKEN cDNA 3110007P09 gene
1.51	RIKEN cDNA 1110053F04 gene
-1.29	RIKEN cDNA 2610001E17 gene
-1.89	runt related transcription factor 1
1.41	cDNA sequence BC053917
2.02	ROD1 regulator of differentiation 1 (S. pombe)
-1.79	DNA-damage inducible transcript 3
-1.18	epidermal growth factor-containing fibulin-like extracellular matrix protein 1
1.60	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
-2.26	secreted acidic cysteine rich glycoprotein
1.76	tyrosine kinase, non-receptor, 1
1.52	spermatogenesis associated 13
-1.60	glutathione S-transferase omega 1
-1.39	tripartite motif protein 24
1.12	RIKEN cDNA E030006K04 gene
1.37	ubiquitin carboxyl-terminal esterase L5
-1.18	regulatory factor X-associated ankyrin-containing protein
-1.21	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)
1.29	leucine-rich repeats and immunoglobulin-like domains 1
-1.69	RIKEN cDNA 2310057G13 gene
1.10	cDNA sequence BC019537
-1.16	crumbs homolog 1 (Drosophila)
1.24	casein kinase 1, alpha 1
-1.61	ubiquitin C
1.64	DNA segment, Chr 8, ERATO Doi 354, expressed
1.55	RIKEN cDNA 2610034N03 gene
-1.18	KDEL (Lys-Asp-Glu-Leu) containing 1
-1.44	RIKEN cDNA 2410026K10 gene
-2.92	coproporphyrinogen oxidase
1.19	thioredoxin 2

1.26	zinc finger protein X-linked
-1.37	RIKEN cDNA 1200006F02 gene
-1.31	sphingosine kinase 1
-2.48	RIKEN cDNA 9830147J24 gene
1.37	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
1.44	HIV-1 Rev binding protein
-1.16	Ceruloplasmin
1.39	Rho guanine nucleotide exchange factor (GEF) 12
6.42	microfibrillar-associated protein 1
-1.28	cDNA sequence BC011467
-1.96	tripartite motif protein 34
-1.31	RIKEN cDNA 3110005G23 gene
1.28	2'-5' oligoadenylate synthetase 1E
1.46	SUMO/sentrin specific protease 2
-1.53	membrane-associated protein 17
-1.18	RIKEN cDNA 1110014L17 gene
-1.49	phosphoinositide-3-kinase, class 3
1.44	SUMO/sentrin specific protease 2
-1.32	diptheria toxin resistance protein required for diphthamide biosynthesis (Saccharomyces)-like 1
1.20	protein phosphatase 2a, catalytic subunit, beta isoform
-1.74	deaminase domain containing 1
1.29	interferon alpha responsive gene
-2.01	growth arrest and DNA-damage-inducible 45 alpha
1.22	spectrin beta 2
1.17	rabaptin, RAB GTPase binding effector protein 1
2.03	Clone L2MZB-13.5 immunoglobulin heavy chain variable region mRNA, partial cds
-1.06	DnaJ (Hsp40) homolog, subfamily C, member 4
1.16	phosphate cytidylyltransferase 2, ethanolamine
1.40	TRAF family member-associated Nf-kappa B activator
1.17	protein phosphatase 1, catalytic subunit, gamma isoform
-1.83	RIKEN cDNA 2310009N05 gene
-1.21	F-box only protein 31
-1.65	zinc finger protein 386 (Kruppel-like)
1.79	cDNA sequence BC008163
1.14	casein kinase II, alpha 2, polypeptide
1.64	CCR4 carbon catabolite repression 4-like (S. cerevisiae)
-1.31	phosphomannomutase 1
1.32	adaptor-related protein complex 3, mu 1 subunit
1.29	UDP-glucose pyrophosphorylase 2

-1.80	ubiquitin C
1.55	low density lipoprotein receptor-related protein associated protein 1
1.28	Sjogren syndrome antigen B
1.58	Similar to PCAF acetyltransferase; p300/CBP-associated factor (LOC330129), mRNA
-3.40	guanylate nucleotide binding protein 2
1.25	RIKEN cDNA 6820402O20 gene
1.27	zinc finger, CW-type with coiled-coil domain 3
1.40	eukaryotic translation initiation factor 3, subunit 1 alpha
-1.12	Similar to ubiquitin A-52 residue ribosomal protein fusion product 1 (LOC383341), mRNA
1.41	Janus kinase 1
1.43	Ninein
1.08	RIKEN cDNA 6330512M04 gene
4.32	microfibrillar-associated protein 1
-1.09	nuclear receptor subfamily 2, group C, member 1
-2.40	3-phosphoglycerate dehydrogenase
2.40	microfibrillar-associated protein 1
1.18	growth factor receptor bound protein 2-associated protein 1
1.34	ribulose-5-phosphate-3-epimerase
1.93	cyclin M2
1.20	Rab geranylgeranyl transferase, a subunit
1.63	GTP cyclohydrolase 1
1.68	lipocalin 7
-1.14	Era (G-protein)-like 1 (E. coli)
1.64	carnitine acetyltransferase
-1.18	nurim (nuclear envelope membrane protein)
1.22	RIKEN cDNA 1810017G16 gene
1.47	RIKEN cDNA 2700084L22 gene
1.79	adaptor-related protein complex 3, mu 1 subunit
1.22	RIKEN cDNA 2310020A21 gene
1.31	RIKEN cDNA 2610020H15 gene
-1.07	PYD and CARD domain containing
1.47	DNA segment, Chr 16, ERATO Doi 480, expressed
1.20	pregnancy-specific glycoprotein 23
-2.61	purine-nucleoside phosphorylase
1.34	topoisomerase (DNA) I
-1.25	Clone IMAGE:3983419, mRNA
1.44	expressed sequence C78339
-1.44	nucleotide binding protein 2
1.25	Itchy
1.53	RIKEN cDNA 4833415E20 gene

-1.53	fibulin 5
1.80	peptidase 4
1.53	tripartite motif protein 25
1.31	nudix (nucleoside diphosphate linked moiety X)-type motif 4
-1.50	synaptogyrin 1
-3.13	purine-nucleoside phosphorylase
1.42	niban protein
1.18	ribonucleotide reductase M2
1.38	HIV-1 Rev binding protein
-1.35	scavenger receptor class B, member 2
-1.30	RIKEN cDNA D230019K24 gene
-2.46	lymphocyte antigen 6 complex, locus A
-1.30	eukaryotic translation initiation factor 3, subunit 8
-1.36	Jun proto-oncogene related gene d1
-1.31	palmitoyl-protein thioesterase 2
1.46	procollagen, type V, alpha 1
-1.18	RIKEN cDNA 1110035L05 gene
1.67	aminopeptidase puromycin sensitive
-2.87	CD5 antigen-like
-1.19	RIKEN cDNA 1200014P03 gene
1.57	hypothetical protein A130072J07
1.19	RIKEN cDNA 2310037I24 gene
1.52	vesicle-associated membrane protein 3
1.31	nuclear transport factor 2
1.41	platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit
2.11	repressor of GATA
-1.41	solute carrier family 12, member 2
1.99	glioblastoma amplified sequence
1.30	protein phosphatase 4, regulatory subunit 2
1.11	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3
-1.60	DnaJ (Hsp40) homolog, subfamily D, member 1
1.30	UDP-N-acetylglucosamine pyrophosphorylase 1
1.26	protein kinase, lysine deficient 1
1.22	SUMO1/sentrin specific protease 1
1.31	RIKEN cDNA 3300001P08 gene
1.33	zinc finger, CW-type with coiled-coil domain 3
1.22	interferon (alpha and beta) receptor 2
-1.61	pyruvate dehydrogenase kinase, isoenzyme 1
1.12	cellular nucleic acid binding protein 1
1.33	ubiquitin-conjugating enzyme E2, J1
1.15	LIM domain only 7

-1.72	serine (or cysteine) proteinase inhibitor, clade G, member 1
1.26	RIKEN cDNA 2810055E05 gene
1.37	adducin 1 (alpha)

Table 11: List of all genes differentially regulated in MBD2-/- mice from microarray Analysis at $P<0.01$.

Gene	Gene Symbol
UDP-glucuronosyltransferase 8	Ugt8
Kinesin family member 24	Kif24
Solute carrier family 35	Slc35d1
GTP cyclohydrolase 1	Gch1
Regulator of differentiation 1	Rod1
Inositol polyphosphate-5-phosphatase B	Inpp5b
Cysteine-rich protein 1	Crip1
Transcription factor 7-like 2	Tcf7l2
Recptor-like tyrosine kinase	Ryk
RAB3A interacting protein	Ryk
RAB3A interacting protein	Rab3ip
Procollagen, type V, alpha 1	Col5a1
Crystallin, lamda 1	Cryl1
SUMO/sentrin specific protease 2	Senp2
Phosphatidylinositol-4phosphate 5-kinase	Pip5k2c

Table 12: List of genes significantly up-regulated in the absence of MBD2 possessing a CpG island ($P<0.005$).

No gene from the high specificity list ($p<0.005$) appeared to be an erythroid specific factor. Therefore, we analyzed both lists using the Ingenuity Pathway Analysis (IPA) program to discern the impact of MBD2 on known cellular pathways. This program uses the Ingenuity Pathways Knowledge Base (IPKB) to provide insights into observed gene expression changes across biological samples. The input to this application corresponds to a user generated dataset file containing differentially expressed genes. Genes of interest to the user, that are also known to directly (or indirectly) interact with other genes in the IPKB, are called Focus Genes and are used to identify molecular networks that indicate how these genes may influence each other. IPA dynamically computes a large "global" molecular network based on hundreds of thousands of curated direct and indirect physical and functional interactions between orthologous mammalian genes from the published, peer-reviewed content in Ingenuity's Knowledge Base. Analysis of both data sets ($P<0.005$ and 0.01) identified several genes in known hematologic pathways (Figure 28). We analyzed four genes by real-time PCR we thought may represent candidate genes from these pathways: mitogen activated protein kinase 3 (MAPK3), zinc finger and BTB domain containing protein 32 (TZFP), jun proto-oncogene related gene d1 (JunD1), and Swi/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member i (Baf57). As a positive control, the gene having the highest fold difference between knock-out and wild type cells, UDP galactoyltransferase 8A (Ugt8), was also analyzed. As shown in Figure 29, only TZFP and Ugt8 were upregulated in MBD2 null mice. This result indicates the importance of confirming the results of microarray analysis especially

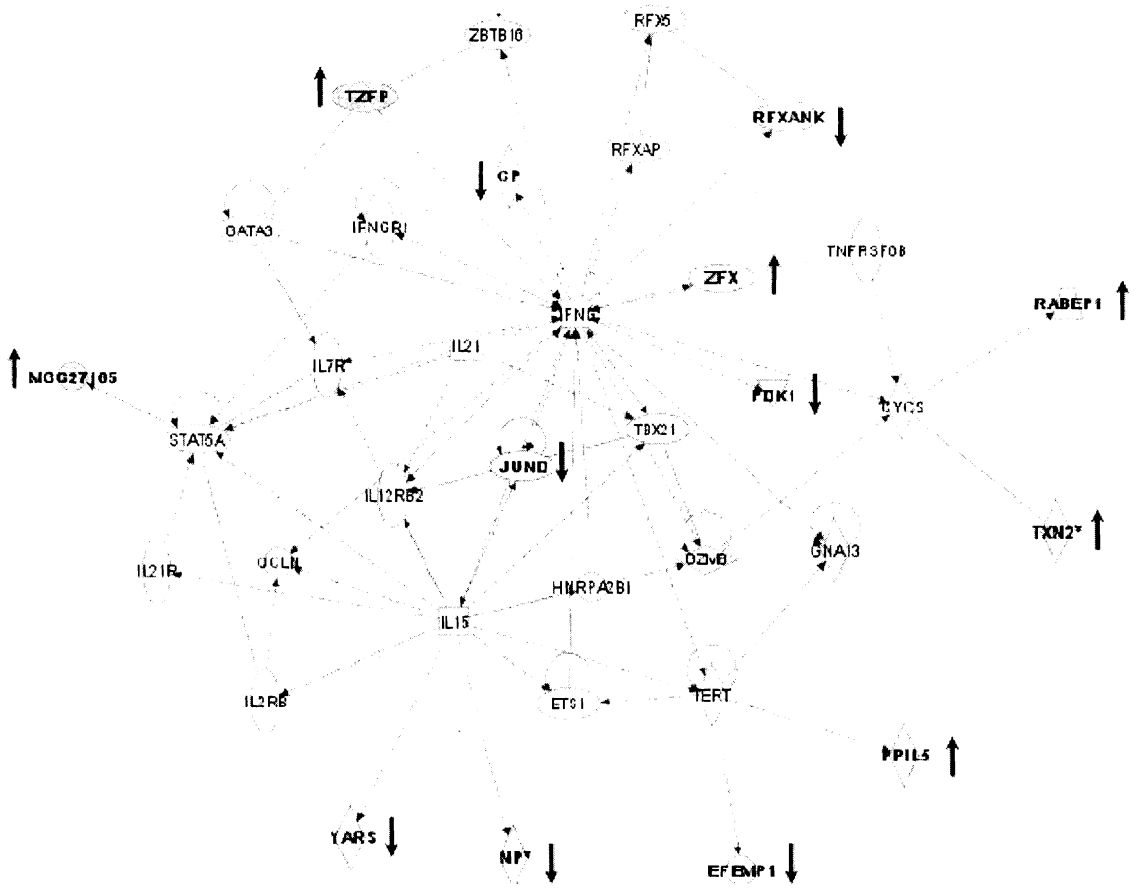

Figure 28: Hematological pathway identified by Ingenuity Pathway Analysis software.

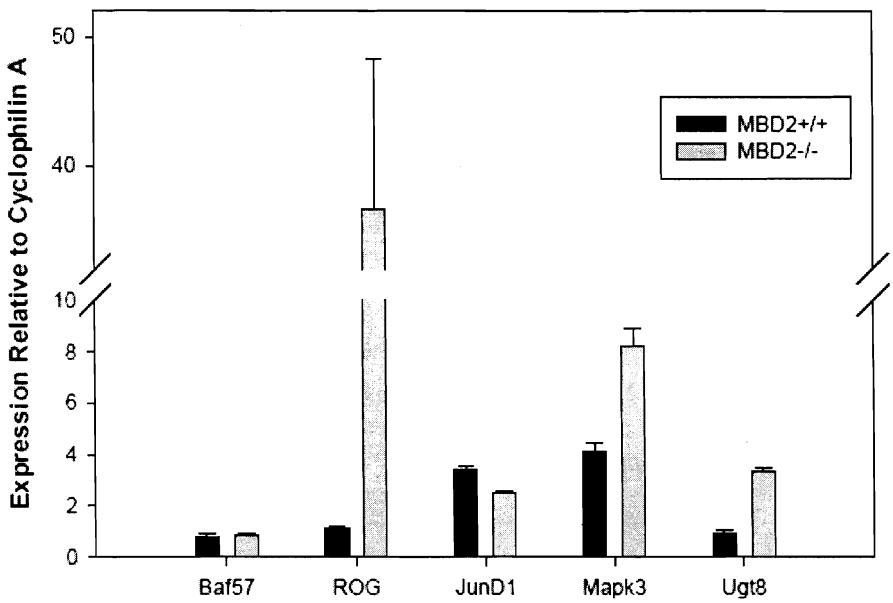

Figure 29: Validation of potential candidate genes by real-time PCR. The results shown are the average of three mice of each phenotype. The RNA used for this validation is the same used for the microarray analysis.

when lowering the P-value for analysis purposes. TZFP was found to be increased >20-fold in MBD2-/- erythroblasts. TZFP is also known as repressor of GATA (ROG), PLZF-like zinc finger protein (PZLP) and Fanconi anemia zinc finger (FAZF). TZFP is a POZ (POZ and Krüppel) protein containing three C_2-H_2 Krüppel-type zinc finger domains and has been shown to heterodimerize with FANC-C and PLZF and is therefore proposed to play a role in Fanconi's anemia and acute promyelocytic leukemia (APL) (Hoatlin et al., 1999). In addition, TZFP has been shown to interact with GATA-2, although the effect of the interaction has not been determined (Tsuzuki and Enver, 2002). GATA-2 has been shown to upregulate γ-globin expression in K562 cells (Ikonomi et al., 2000). Interestingly, a CpG island is located just 3' of the TZFP gene and just upstream of the MLL2 gene. Taken together, these results implicate TZFP as a possible candidate gene for the upregulation of the γ-globin gene in MBD2-/- mice.

Preliminary results indicated MBD2 does not bind to the γ-globin promoter region to mediate a repressive effect. In addition, the CpG island content of the locus was analyzed. Only two weak CpG islands were found, but they were not located near any gene within the locus. To determine whether MBD2 was recruited to one of these weak CpG islands, ChIP was performed. No enrichment for MBD2 was seen at the island located between the γ- and δ-globin genes (Figure 27). Furthermore, no enrichment was seen at the ϵ-globin gene promoter region or at the human β-globin transcribed region. The same data seemed to indicate that MBD2 was bound to the GATA-2 upstream region similar to results seen in MEL cells (Rodriguez et al., 2005). However, the level of enrichment seen is not very high. The microarray data provides the opportunity to define

a strong positive control to confirm the initial MBD2 ChIP studies. The genes up-regulated in MBD2-/- erythroblasts are presumably primarily regulated by MBD2. In addition, most genes up-regulated by MBD2 in knock-out splenocytes contains a CpG island (Table 12). Thus far, only one gene has been shown to be up-regulated in the absence of MBD2 in mice and the study was performed in Th1 and Th2 lymphocytes (Hendrich et al., 2001). No studies have been focused on the genes regulated by MBD2 in erythroid cells. As a result, MBD2 recruitment to the Ugt8 CpG island was analyzed by real-time PCR. Ugt8 displayed the highest fold difference between MBD2-/- and MBD2+/+ erythroblasts in microarray analysis and was confirmed to be expressed ~4-fold higher in MBD2-/- cells. ChIP analysis showed MBD2 to be recruited to the Ugt8 CpG island in wild-type but not knock-out splenocytes suggesting MBD2 mediates a transcriptional repressive effect directly at the Ugt8 (Figure 27). This is in contrast to what occurs at the γ-globin gene. Loss of MBD2 leads to increased transcription, but MBD2 does not bind directly to the γ-globin promoter region to mediate the effect. The Ugt8 ChIP data validates the result that MBD2 does not bind to the γ-globin promoter in addition to validating the ability of the microarray to identify genes regulated by MBD2. In addition, MBD2 enrichment is seen at the CpG island located 3' of the TZFP gene. As another control, MBD2 was not enriched at the highly expressed human β-globin gene.

To further confirm the result that MBD2 does not bind to the γ-globin promoter region, ChIP was performed with an ant-MBD2/3 antibody. No enrichment is seen at the γ-globin promoter while enrichment is seen at the CpG islands near the Ugt8 and TZFP gene (Figure 27). Thus neither MBD2 nor MBD3 are binding near the γ-globin promoter.

ChIP was also performed with an anti-MTA2 antibody. MTA2 is part of the NuRD complex which associates with MBD2. MTA2 is not recruited to the γ-globin promoter but is recruited to the Ugt8 and TZFP CpG islands.

Discussion

Loss of MBD2 leads to expression of the normally silent fetal globin gene, γ, in adult βYAC transgenic mice. MBD2 mediated transcriptional repression is thought to occur via MBD2 recruitment to methylated CpGs in the promoter region of a gene. MBD2 may bind as a complex or once bound recruit a protein complex to repress transcription via HDACs and transcriptional repressive proteins. To determine whether MBD2 represses the human γ-globin gene in adult βYAC transgenic mice, ChIP assays were performed to determine whether MBD2 binds to the γ-promoter region *in vivo*. No enrichment was found for MBD2 at the γ-globin promoter region in wild-type or MBD2 null splenocytes using two different antibodies. However, MBD2 is enriched in wild-type, but not knock-out, splenocytes at the GATA2 upstream promoter, the Ugt8 CpG island, and the CpG island 3' of the TZFP gene. This result is not surprising despite the high level of CpG methylation in adult erythroblasts. The γ-globin gene does not contain a CpG island. While MBD2 can bind to as few as 3 CpGs *in vitro,* this has not been shown *in vivo* nor has it been shown whether MBD2 can bind as a complex to a region with low CpG density. MBD2 binding has been shown to increase with increasing CpG density (Kransdorf and Ginder, 2004). The MBD2 containing complex, MeCP1, is able to bind sparsely methylated DNA but it is unstable and its repressive properties can be

overcome by a strong enhancer, similar to the LCR (Boyes and Bird, 1992). Taken together, it is not surprising that MBD2 mediated transcriptional repression of the γ-globin gene is not due to MBD2 recruitment to the γ-globin promoter region.

Studies have shown that mechanisms other than simple *cis* element *trans* factor binding to the γ-promoter exists for γ-globin gene regulation. We postulated that MBD2 mediated γ-globin gene silencing may be due to up-regulation of a factor that binds to the γ-promoter region or a factor involved in a signaling pathway needed for γ-globin activation. As a result, we performed a microarray analysis of erythroblasts from four MBD2+/+ and four MBD2-/- mice. No erythroid specific factors were identified on the initial highly significant ($p<0.005$) gene list generated by microarray analysis. However, after analyzing the gene lists through the Ingenuity Pathway Analysis program, several genes involved in hematologic pathways were identified. One of these genes, TZFP, was found to be >20-fold enriched in MBD2-/- erythroid cells. This protein appears to be a potential candidate for MBD2 mediated γ-repression. This protein has been shown to interact with GATA-2, but its effect on GATA-2 activity has not been determined (Tsuzuki and Enver, 2002). Overexpression of Gata-2 in K562 cells leads to increased expression of the γ-globin gene (Ikonomi et al., 2000). TZFP may interact with Gata-2 to promote γ-globin gene expression, however further experiments must be performed to prove or disprove this hypothesis. Interestingly, the TZFP gene does not contain a CpG island in its 5' promoter region, but does contain a CpG island 3' of the coding sequence, but 5' of the downstream MLL2 gene. We have shown MBD2 is recruited to this island.

Given the large induction of TZFP expression in the absence of MBD2, this CpG island may be mediating a novel 3' repressive effect on TZFP.

The results described here show the importance of validating candidate genes acquired from microarray analysis. Three genes considered to be potential candidates did not show a difference of expression between wild type and MBD2 knock-out erythroblasts: MAPK3, JunD1, and Baf57. These genes were also located in hematologic pathways. Given that these genes were not validated, but TZFP was validated provides further evidence that TZFP may be involved in MBD2 mediated γ-globin repression.

The results of the microarray analysis provided the opportunity to search for potential positive controls for MBD2 ChIP analysis in mouse erythroblasts. Previously, MBD2 had only been shown to be recruited to the IL-4 locus in mouse Th1 and Th2 cells (Hendrich et al., 2001). No previous work has shown *in vivo* occupancy of MBD2 in erythroid cells. Interestingly, the IL-4 gene was not detected in the microarray analysis likely owing the lymphoid-specific factors being necessary for its expression. However, microarray analysis and real-time PCR indicate the gene Ugt8 is up-regulated in MBD2-/- erythroid cells. Furthermore, ChIP experiments show this gene to be enriched at the CpG island region of the Ugt8 gene thus establishing a positive control. This result confirmed that MBD2 does not bind directly to the γ-globin promoter region to mediate transcriptional repression. Thus, it is highly likely MBD2 is negatively regulating a gene in *trans* that when activated results in expression of the normally silent γ-globin gene, for example TZFP.

The mice used in this analysis were bred on a heterologous background. As a result, the results shown here may result in false negatives due to background modifiers. Thus, the gene responsible for the up-regulation of the γ-globin gene may not be found using this technique with these samples. False positives would likely be eliminated by multiple repetitions in different progeny. In addition, the spleens used in this study are highly erythroid in composition, but are not 100% erythroid. It is possible the genes analyzed are from lymphoid cells and that induction of these genes in erythroid cells may have no bearing on γ-globin gene expression. However, we hypothesized the effect seen may be due to secretion of a cytokine or some other factor from a non-erythroid cells to mediate up-regulation of the γ-globin gene. A final factor to consider in the microarray analysis is that erythroid cells are present at all stages of differentiation in the anemic spleen. The gene responsible for the phenotype may only be differentially regulated at a distinct stage of erythroid maturation. If this is the case, the gene will likely be difficult to identify amidst other erythroid cells and lymphoid cells which compromise the anemic spleen. To eliminate these variables, the MBD2-/- and MBD2+/+ mice must be bred into the same genetic background and the microarray repeated. In addition, it may prove necessary to isolate erythroid cells and erythroid cells from specific stages of differentiation from the contaminating lymphoid cells to correctly identify candidate genes. However, overexpression of candidate genes in MEL cells containing human chromosome 11 or βYAC transgenic mice would allow one to assess the effect of the candidate gene(s) on γ-globin gene expression. These initial microarray studies have

provided a dearth of information, but care must be taken when identifying candidate genes to incorporate all possible modifying variables.

Lastly, MBD2 may be regulating a gene that results in increased levels of γ-globin by a mechanism other than via increased transcription. For example, a protein that stabilizes γ-globin mRNA or increases its export from the nucleus may account for the increased levels of γ-globin mRNA. However, unless these factors are specific for the γ-globin gene, more genes should have been up-regulated in the microarray analysis. Lastly, the increased enrichment of TriMeK4 in MBD2 null βYAC transgenic mice suggest the mechanism is transcriptionally related.

Chapter 5: Loss of MBD2 in other Erythroid Models

Introduction

We have shown that loss of MBD2 leads to expression of the γ-globin gene in two lines of adult βYAC transgenic mice. The lack of a severe phenotype in MBD2-/- mice makes this protein a potential target for therapeutic induction of the γ-globin gene for patients suffering hemoglobinopathies (Hendrich et al., 2001). In addition, MBD2 has been shown to inhibit tumor suppressors in cancer; therefore, compounds inhibiting MBD2 activity may have potential benefits to cancer patients (Magdinier and Wolffe, 2001). However, loss of MBD2 must be shown to induce γ-globin in other systems to show the effect seen in transgenic mice is conserved and thus a viable target to pursue.

A cell culture model would be an ideal system to study this effect. Cell culture has the advantage of generating large amounts of material to analyze in a relatively short period of time. In addition, generation of transgenic cells lines is much more rapid than generation of transgenic mice. For analysis of potential candidate genes generated from microarray studies, a cell culture model has the benefit of allowing relatively rapid analysis of gene overexpression or knock-down using siRNA. To this end, we are using the mouse erythroleukemia (MEL) cell line. MEL cells are virally transformed cells maintained at the proerythroblast stage of differentiation. However, upon chemical treatment, these cells can be terminally differentiated into adult erythroid cells. Upon

induction, these cells express high levels of adult globin transcripts. These cells provide a cell culture model with which to study the endogenous mouse globin genes in adult definitive cells. In addition, these cells have been used to study embryonic and fetal regulatory elements (Wandersee et al., 1996). MEL cells have also been created that contain the entire human chromosome 11 by using cell fusion (Forrester et al., 1990). These cells mimic adult erythroid cells in that they predominantly express the β-globin gene. MEL cells containing human chromosome 11 express the γ-globin upon treatment with 5-azacytidine (Ley et al., 1984). Thus, MEL cells containing human chromosome 11 (N-MEL) behave similarly to βYAC transgenic mice and human patients.

Another model system employed is the differentiation of human hematopoietic stems cells (HSC) down the erythroid pathway. Human HSCs express high levels of the glycoprotein CD34. As these cells differentiate they lose expression the CD34 surface marker. CD34+ cells are capable of initiating long-term hematopoiesis both *in vitro* and *in vivo*. CD34+ cells can be found in bone marrow, mobilized peripheral blood, cord blood, and the fetal liver. When grown in the right mixture of chemicals and cytokines, these cells can differentiate into cells of any hematopoietic lineage. Numerous studies have demonstrated methods for differentiation of CD34+ cells down the erythroid pathway (Bhanu et al., 2004; Fibach et al., 2003; Migliaccio et al., 2002). This system has the benefit of using human cells. However, these cells are not immortal and selection of transfected constructs is difficult to accomplish. Thus, to insert foreign DNA sequences these cells must be infected with viruses carrying the DNA of interest. Lentiviruses have been used to successfully transduce human CD34+ cells (Gimeno et al., 2004).

The goal of these model systems is to first recapitulate the effect seen in βYAC transgenic mice in a cell culture system. The reason for this is two-fold. First, demonstrating the phenotype in multiple systems suggests the effect is evolutionarily conserved and not unique to the βYAC model. As a result, MBD2 becomes a potential target for manipulating HbF levels in patients with hemoglobinopathies. Second, the cell culture system is much more conducive to validating potential candidate genes generated from microarray analysis. Validating candidate genes through gene over-expression and siRNA knock-down is much more efficient than generating transgenic and knock-out mice. Thus, additional model systems are needed for proof of principle as well as further dissecting the exact mechanism of MBD2 mediated γ-globin transcriptional repression.

Methods

Culture and analysis of N-MEL cells

N-MEL cells were a generous gift from Dr. M.A. Bender (Fred Hutchinson Cancer Research Center, Seattle, Washington) (Forrester et al., 1990). Cells were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum and grown at 37° C and 5% CO_2. The human chromosome 11 does not have a selectable marker and the cell eventually loses the chromosome with culture. In order to keep a high percentage of N-MEL cells, the cells were periodically purified using an antibody directed against a cell surface marker expressed from the human chromosome, UME7. 1-2 x 10^8 cells were spun down and resuspended in 500 μL of UME7 anti-serum. The cells were then incubated at room temperature for 15 minutes. The cells were washed in 15 mL

PBS/2% FBS/1 mM EDTA and spun at 1000 x G. The cells were resuspended in PBS/2% FBS/1 mM EDTA to 1-2 x10^8 cells/mL. FITC conjugated goat anti-mouse antibody (MP Biomedicals) was added to 3 μg/mL. The cells were allowed to incubate for 15 minutes at room temperature. The cells were washed again with 15 mL PBS/2%FBS/1mM EDTA and resuspended to 1-2 x 10^8 cell/mL in a 5 mL Falcon Polystyrene tube. Anti-FITC antibody (Stem Cell) was added at 100 μL per 1 mL of cells and allowed to react for 15 minutes at room temperature. Magnetic nanoparticles (Stem Cell) were added at 50 μL per mL of cells and allowed to react for 10 minutes. After incubation, the volume was increased to 2.5 mL with PBS/2%FBS/1 mM EDTA and placed in an EasySep Magnet (Stem Cell) for 5 minutes. The supernatant was then decanted and the tube removed from the magnet. The cells were resuspended in 2.5 mL of PBS/2%FBS/ 1 mM EDTA and placed back in the magnet for 5 minutes. This process was repeated for a total of three magnetic purifications. After the final wash, the cells were resuspended in normal medium and allowed to grow.

After purification, N-MEL cells were tested for their ability to express the human globin genes. Cells were treated for 3 days in the presence of 2% DMSO or not treated. RNA was isolated using Trizol, DNase I treated, and reverse transcribed as described earlier. Expression of human γ-globin and β-globin transcripts was determined using real-time PCR.

ChIP Assay with N-MEL cells

The ChIP assay was performed essentially as described in Chapter 2 with the following changes. Each IP was performed with ~6 x 10^6 cells.

Treatment of N-MEL cells with 5-azacytidine

N-MEL cells were plated at a density of 3 x 10^6 cells per 10mL medium. Cells were treated with 2.5 μM 5-azacytidine (Sigma) for 2 days. Every 24 hours the cells were spun down and replaced with fresh medium and 5-azacytidine. On the third day, the cells were treated with 5-azacytidine at a concentration of 9.82 μM. The cells were harvested 48 hours later. Another set of cells was treated 24 hours after dilution with 2% DMSO. Another set of cells was treated with 2.5 μM 5-azacytidine for 24 hours, the cells spun down and resuspended in fresh medium containing 2% DMSO. Finally, a group of cells was left untreated.

RNA was isolated from cells using Trizol as described earlier. The RNA was DNaseI treated and reverse-transcribed. Analysis of γ-globin gene expression was determined using real-time PCR.

Cloning a mouse MBD2 shRNA

The sequence for mouse MBD2 mRNA (GeneBank ascension NM_010773) was analyzed using three different web-based siRNA design programs: Ambion (www.ambion.com), Qiagen (www.qiagen.com), and IDT DNA Technologies (www.idtdna.com). Three siRNAs present in all three analyses were used. To generate a shRNA expressing vectors, the siRNAs from the analyses were fitted with a loop

sequence and compatible ends for cloning into the EcoRI and XbaI site of the pSuperior vector. The pSuperior vector was cut with EcoRI and XbaI at 37° C for 2 hours followed by heat inactivation of the enzymes at 65° C for 20 minutes. The cut plasmid was gel purified on a 1% agarose gel using the Qiagen gel extraction kit (Qiagen). Oligos corresponding to the three siRNAs of interest were ordered as oligos from IDT DNA Technologies. The oligos were ordered as top and bottom strand and left and right half. The top and bottom of each half were phosphorylated with T4 polynucleotide kinase at 37° C for 40 minutes. The enzyme was inactivated by heating at 65° C for 15 minutes. The oligos were annealed by boiling for 5 minutes followed by gradual cooling to room temperature to make a dsDNA at 5 pmol/μL. The ligation was performed in two steps. First, 1 μL of the left half dsDNA was added to 36 ng of cut and purified pSuperior. The fragments were ligated with T4 ligase in a volume of 20 μL at room temperature for 3 hours. Afterwards, 1 μL of pellet paint (Novagen) was added and the DNA ethanol precipitated at -20° C for 30 minutes. The DNA was spun and the pellet washed in 70% ethanol. The pellet was resuspended in 20.5 μL water. The second ligation was performed by adding 1 μL of the right dsDNA and T4 ligase in a volume of 25 μL at room temperature for 2 hours. Alpha-Select Bronze Efficiency cells (Bioline) were transformed with 5 μL of ligation product. Colonies were screened for clones containing the insert. Positive clones were then sequenced to confirm the presence and sequence of the insert. The list of oligos used for cloning is located in Table 13. The cloned siRNAs were labeled as pSuperior30, 45, and 95.

Oligo Name	Sequence
mM2-45-Top1	GATCCTGAACAACCACGTCAGCTT
mM2-45-Bot1	CTCTTGAAAAGCTGACGTGGTTGTTCAG
mM2-45-T2-XhoI	TTCAAGAGAAAGCTGACGTGGTTGTTCATTTTTTC
mM2-45-B2-XhoI	TCGAGAAAAAATGAACAACCACGTCAGCTTT
mM2-95-Top-1	GATCCAGGTAGCACTTACGTGAAA
mM2-95-Bot1	CTCTTGAATTTCACGTAAGTGCTACCTG
mM2-95-T2-XhoI	TTCAAGAGATTTCACGTAAGTGCTACCTTTTTTTC
mM2-95-B2-XhoI	TCGAGAAAAAAAGGTAGCACTTACGTGAAAT
mM2-30-Top1	GATCCGGGTAAACCAGACCTGAAC
mM2-30-Bot1	CTCTTGAAGTTCAGGTCTGGTTTACCCG
mM2-30-T2-XhoI	TTCAAGAGAGTTCAGGTCTGGTTTACCCTTTTTTC
mM2-30-B2-XhoI	TCGAGAAAAAAGGGTAAACCASGACCTGAACT

Table 13: Oligos used to generate MBD2 shRNA constructs.

Transfection of shRNA constructs into N-MEL Cells

Each siRNA vector (4.5 μg) was diluted to 250 μL with Opti-Mem (Invitrogen) and lightly vortexed. Lipofectamine 2000 (10 μL) (Invitrogen) was diluted to 250 μL in Opti-Mem, vortexed lightly, and incubated at room temperature for 5 minutes. The DNA mixture and Lipofectamine mixture were combined, vortexed gently and incubated at room temperature for 20 minutes. This mixture was then added to 2 mL of magnetically purified N-MEL cells and grown overnight at 37° C in 5% CO_2. After 24 hours, the cells were split and diluted 1:10 (final volume 5 mL) in DMEM and grown for 24 hours at 37° C in 5% CO_2. 48 hours after transfection, G418 (Invitrogen) was added to the medium at 1 mg/mL to select for transfected cells. After selection, a pool of transfected cells was generated. From this pool, single cell colonies were grown. These single cell clones were screened for expression of MBD2 by real-time PCR and Western Blot. The list of primers used for real-time PCR to assay for transcript level is located in Table 14.

Western Blot

Nuclear extracts were prepared by the Dignam method (Dignam et al., 1983). Protein samples were run on 10% Tris-glycine SDS-PAGE ReadyGels (Bio-Rad) in Running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS). The gel was equilibrated in Transfer buffer (25 mM Tris-HCl pH 8.0, 192 mM glycine, 20% methanol) and then transferred to activated PVDF overnight at 30V. The next day the membrane was washed twice for 10 minutes with 1x PBS and then blocked for 1 hour at room temperature with rocking in 10 mL MPBST (5% (w/v) non-fat milk, 1x PBS,

Primer	Sequence
mMBD2-1757-F-73	TTTATAATAGCAAGCAGGGCCCTTCCGGTG
mMBD2-1757-R-215	GGGAAACTTATTTGGTACTCCTGATCTGT
mMBD2-1061-F-49	CAAATTCACGAACCACCCGAGCAA
mMBD2-1061-R-157	TGATGCGCTAAGTCCTTGTAGCCT
mMBD2-938-F-15	TTGACTTCAGGACCGGCAAGATGA
mMBD2-938-R-129	GGCAATGTTGTGTTCAGGTCTGGT

Table 13: Primers used to analyzed transcript levels in N-MEL cells transfected with MBD2 shRNA vector.

0.05% Tween-20). The blocking solution was removed and the primary antibody, anti-MBD2 (Upstate) was incubated with the membrane at 3 μg/mL for 2 hours at room temperature with rocking in MPBST. This solution was removed and the membrane washed three times with 10 mL PBS-T for 5 minutes. The secondary antibody was then incubated with the membrane for 1 hour at room temperature with rocking in MPBST. This solution was removed and the membrane was washed three times with 10 mL PBS-T for 5 minutes and one time with 10 mL PBS-T plus 0.1% Triton-X 100. Bands were visualized with ECL Plus (Amersham Pharmacia) and exposed to film. The blot was stripped by incubating in 100 mM 2-mercaptoethanol, 2%SDS, and 62.5 mM Tris-HCl ph 6.7 at 50° C for 30 minutes. The blot was washed in PBST and re-probed as above using an anti-βactin antibody (Sigma).

Isolation and Differentiation of human CD34+ Cells

CD34+ cells were isolated from two sources: cord blood and aphaeresis pack. Aphaeresis packs were obtained from the VCU Bone Marrow Transplant Lab. The pack was thawed at 37° C until it became slushy, at which time thawing was completed at room temperature. Cord blood was obtained from the VCU department of Obstetrics and used within 24 hours of collection. The cells were diluted with an equal volume of PBS/2% FBS. 30 mL of cells was loaded onto 15 mL of Ficoll Paque Plus (Stem Cell) and spun at 400 x G for 30 minutes with no brake. The middle mononuclear layer was extracted, diluted to 40 mL with PBS/2%FBS/1 mM EDTA and filter through a 70 μM nylon filter (BD Biosciences). The cells were counted, washed twice in PBS/2%FBS/1

mM EDTA, and diluted to 4 x 10^8 cell/mL in a 5 mL Falcon Polystyrene tube. Human CD34+ positive selection cocktail (Stem Cell) was added at 100 μL per mL of cells and allowed to incubate for 15 minutes at room temperature. Magnetic nanoparticles were then added at 50 μL per mL of cells and allowed to react at room temperature for 10 minutes. The cells were then magnetically purified as described above except the cells were washed 4 times. The cells were resuspended at ~1 x 10^6 cells per mL in amplification medium: IMDM, 20% FBS, 10 ng/mL Stem Cell Factor (R&D Systems), 1 U/mL EPO (Amgen), 1 mg/mL IL-3 (R&D Systems), 10^{-6} M dexamethasone (Sigma), and 10^{-6} M estradiol (Sigma). Cells were grown for two weeks and maintained at 1 x 10^6 cells per mL. After two weeks, cells were spun down and washed 1X with IMDM medium and resuspended in differentiation medium: IMDM, 20% FBS, 1 U/mL EPO, and 10 ng/mL insulin (Sigma). The cells were allowed to differentiation for 5 days. After differentiation, cells were spun down and RNA isolated using Trizol. The presence of globin gene transcripts was determined using RNase protection assay.

Results

The N-MEL cell line does not possess a selectable marker. As a result, the cell is prone to losing the human chromosome 11 with prolonged culture. We first had to ensure we could maintain the cell line in culture, purify it, and determine if it was expressing the β-globin gene. Cells were successfully grown-up in culture and MEL cells containing chromosome 11 were enriched using an anti-serum against a chromosome 11 marker and magnetic beads. After purification, cells were grown in culture and differentiated down

the erythoid pathway with DMSO. After three days of differentiation, RNA was isolated and analyzed by RT-PCR. As shown in Figure 30, the N-MEL cells were actively expressing the human β-globin gene. A sub-set of purified cells were amplified in culture to generate enough cells for ChIP assay. To ensure we could successfully use the N-MEL cell line for ChIP assays, we performed a ChIP assay for TriMeK4 at the human β-globin locus. As shown in Figure 31, the human β-globin gene is highly enriched for TriMeK4, indicating successful application of the N-MEL cell line. In addition, the γ-globin gene is not enriched in these cells, consistent with low levels of expression (Figure 31).

Activation of the γ-globin gene has been shown in MEL cells containing human chromosome 11 (Ley et al., 1984). However, it is not known whether the N-MEL cells used here express the γ-globin gene after 5-azacytidine treatment. N-MEL cells were treated with 5-azacytidine and showed a ~10-fold induction in γ-globin expression as determined by real-time PCR (Figure 32). This level of induction is similar to the level seen after treating βYAC transgenic mice with 5-azacytidine or when MBD2 has been genetically deleted in βYAC transgenic mice. However, treatment for 24 hours prior to DMSO induction was unable to induce expression, but was shown to induce expression in a different MEL human chromosome 11-containing cell line (Ley et al., 1984). However, the amount of time the cells were allowed to induce and the inducing agent differed in these studies and may account for the variations in expression. The N-MEL cells here were harvested four days after 5-azacytidine treatment, which may be enough time for the cells to recover from the demethylating effects of the compound.

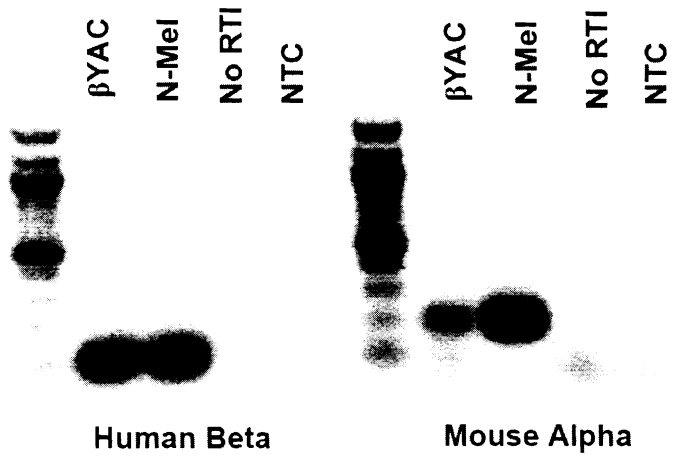

Figure 30: Expression of the human β-globin gene in N-MEL cells after magnetic purification. N-MEL cells were purified magnetically using an anti-serum against a chromosome 11 marker and magnetic beads. The purified cells were differentiation down the erythroid pathway with DMSO and RNA analyzed using RT-PCR. Shown above are the results of the RT-PCR analysis.

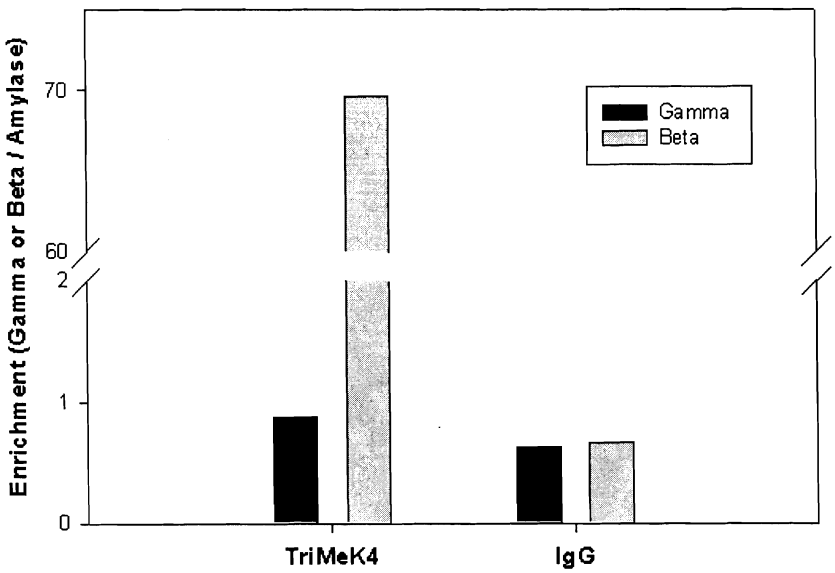

Figure 31: ChIP assay with magnetically purified N-MEL cells at the human β-globin gene. After magnetic purification, N-MEL cells were amplified and subjected to ChIP assay with an anti-body directed against TriMeK4 and IgG. DNA was analyzed using real-time PCR at the human β-globin gene and the endogenous amylase gene.

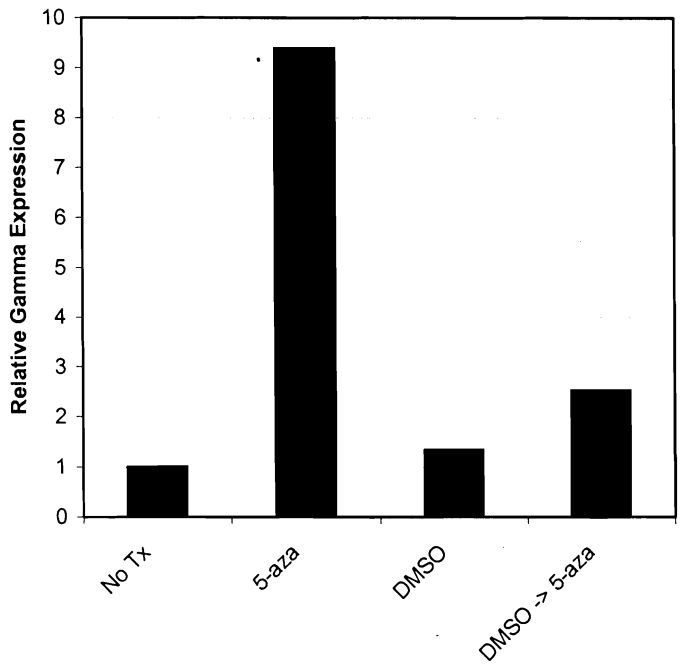

Figure 32: γ-globin expression in N-MEL cells after 5-azacytidine treatment. N-MEL cells were treated with 5-azacytidine either with or without DMSO. Expression of the γ-globin gene was performed by real-time PCR relative to the GlycophorinA gene.

After establishing we could enrich for N-MEL cells after prolonged culture, we sought to transfect these cells with a vector expressing a siRNA against mouse MBD2. Three web-based design programs were used to identify potential siRNA sequences. Three regions were found in all three programs and siRNA constructs were generated for these regions and cloned into the pSuperior vector. N-MEL cells enriched by magnetic purification were transfected with all three constructs and grown in medium containing a selection agent. After isolating a pool of transfected cells, single cell clones were isolated and screened for MBD2 expression levels by Western blot and real-time PCR. As shown in Figure 33, multiple clones had >90% knock-down of MBD2, while others showed minimal knock-down. Clone 45, which showed the greatest level of MBD2 knock-down, was selected for further studies.

Another model system we wished to employ for the study of MBD2 mediated transcriptional repression of the γ-globin gene is the human CD34+ differentiation model. In this system, CD34+ cells isolated from human aphaeresis packs are forced down the erythroid pathway using a cocktail of cytokines favoring this pathway. A goal we set for this system was for γ-globin expression to be very low at the end of culture (<2% of human β-globin). To compare expression levels, adult and cord blood CD34+ cells were used. After isolation and differentiation of CD34+ cells, RNA was isolated and analyzed by RPA. As shown in Figure 34, erythroid differentiated CD34+ cells isolated from adults express high levels of β-globin mRNA and low levels of γ-globin mRNA. Thus, this culture system expresses low enough levels of γ-globin that loss of MBD2 should

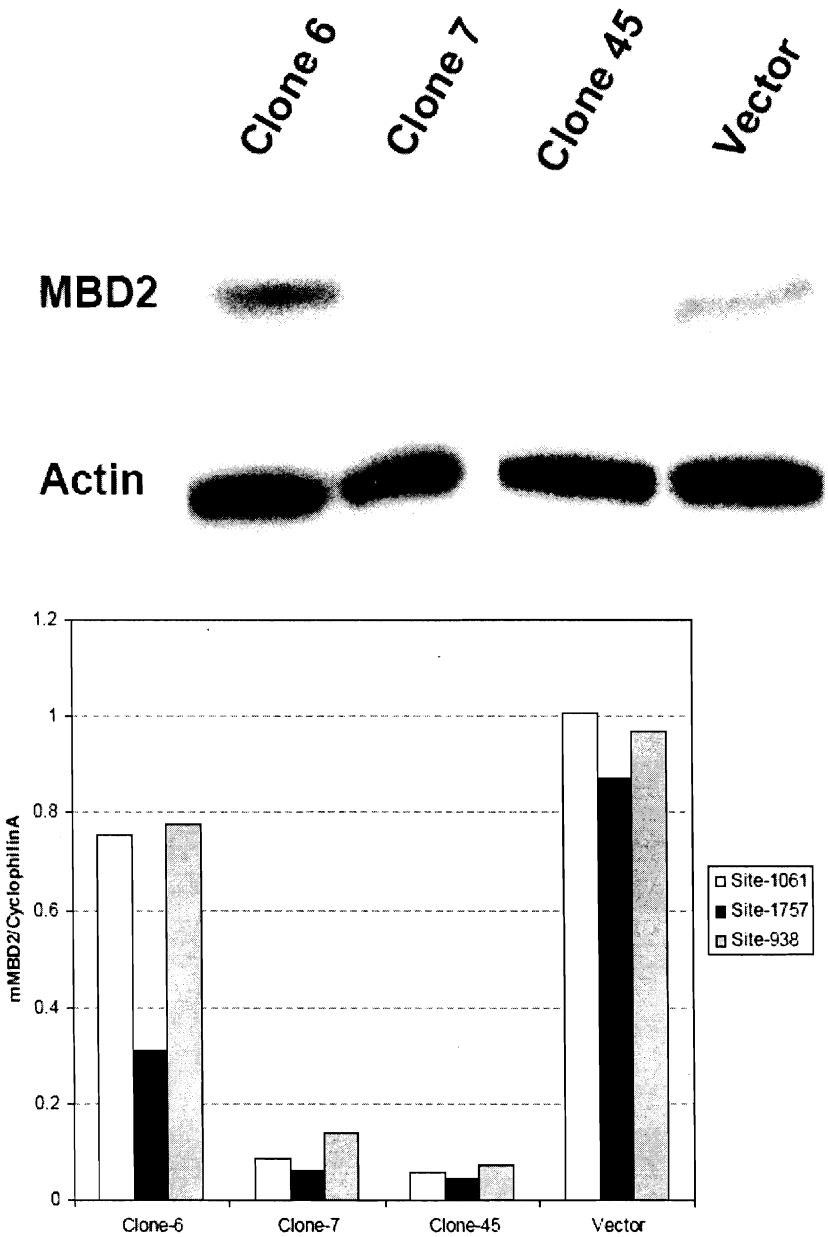

Figure 33: Western blot (Top) and real-time PCR (Bottom) analysis of N-MEL clones contain a MBD2 shRNA vector. After transfection and selection, N-MEL cells contain a MBD2 shRNA vector were grown as single cell clones. Individual clones were screened for expression of MBD2 by Western blot and real-time PCR.

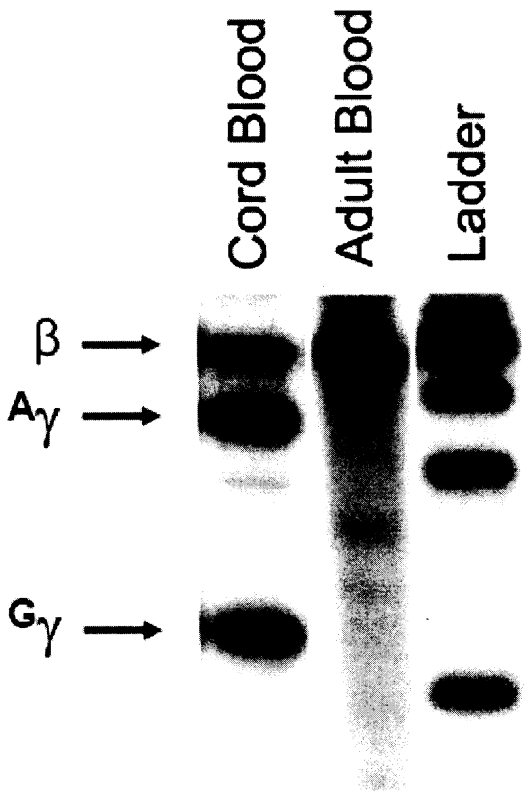

Figure 34: RPA analysis of γ-globin expression levels in erythroid differentiated CD34+ cells obtained from adult and cord blood. CD34+ cells were isolated and differentiated down the erythroid pathway. After differentiation, levels of γ- and β-globin mRNA were determined by RPA.

produce a measurable increase in expression. As expected, CD34+ cells isolated from cord blood express high levels of γ-globin after erythroid differentiation.

Discussion

Here we have shown the characterization of two model systems with which to study MBD2 mediated transcriptional repression of the γ-globin gene. The first system is a mouse erythroleukemia cell line containing human chromosome 11. The chromosome has no selectable marker and the cells have a tendency to expel the foreign DNA after prolonged passage. We first needed to ensure these cells could be purified and maintained in culture. Using a magnetic purification system, N-MEL cells were specifically enriched and were shown to express high levels of the human β-globin gene. Next, we wanted to determine whether N-MEL cells could be ChIPed using our protocol and whether they behaved similar to adult βYAC erythroid cells. The results of the ChIP assay demonstrated that the β-globin gene region was highly enriched while the γ-globin gene was not enriched for histone H3 trimethylated at lysine 4. This result was consistent with the results seen in the wild type βYAC transgenic mice as well as with the levels of expression of these two genes in N-MEL cells. Additionally, N-MEL cells were treated with 5-azacytidine to determine whether or not the γ-globin gene could be induced. Consistent with the previous data in another MEL cell line containing human chromosome 11, 5-azacytidine led to a ~10-fold induction the of the γ-globin gene, a level of induction similar to βYAC mice treated with 5-azacytidine (Ley et al., 1984). However, pre-treatment with 5-azacytidine followed by DMSO induction was unable to

induce expression. However, these cells were harvested four days following 5-azacytidine treatment. This may have been enough time for effects of demethylation to be reversed, or 24 hours of treatment may not be long enough to induce γ-globin expression in N-MEL cells.

Taken together, these results indicate that N-MEL cells may be a good model system in which to study MBD2 mediated repression of the γ-globin gene. These cells appear to behave very similarly to adult βYAC erythroid cells. As a result, N-MEL cells were transfected with a construct generating a siRNA against murine MBD2. Analysis of these cells identified multiples sub-lines with >90% knock-down of MBD2 mRNA and protein levels. Due to the long selection time following siRNA transfection, the pool likely has lost chromosome 11 in many cells. As a result, the cells were grown and magnetically purified. Hemizygous MBD2 βYAC transgenic mice do not show any significant increase in γ-globin expression relative to wild-type mice. Thus, we postulated that it would take nearly 100% knock-down of MBD2 to mirror the phenotype seen in MBD2 null transgenic mice. These cells have a dramatic knock-down of MBD2 and should express the γ-globin gene if the effect is cell autonomous.

Since N-MEL cells appear to accurately mimic adult βYAC erythroid cells, this system will be very useful for testing candidate γ-globin regulators determined from microarray analysis. We have shown that these cells can be transfected and can knock-down gene expression using siRNA. As a result, these cells can be used to test candidate genes. For example, adult MBD2-/- erythroid cells express a high level of TZFP. An expression plasmid can be used to over-express this gene in N-MEL cells to determine

whether it induces γ-globin expression. The converse also applies to genes down-regulated in the absence of MBD2; siRNA vectors to these genes can be generated and used to knock-down the gene to determine whether the γ-globin gene is up-regulated. Overall, the N-MEL system has the potential to be a very valuable and powerful system with which to study MBD2-mediated transcriptional repression of the γ-globin gene.

The other model system explored here was the erythroid differentiation of human CD34+ cells. We have shown successful differentiation of adult and cord blood derived CD34+ cells down the erythroid pathway. In addition, we needed to ensure the level of γ-globin expression in adult derived erythroid cells was low. Given the high level of transcription of the β-globin gene, there may be a level of γ-globin expression that cannot be exceeded without first down-regulating β-globin expression. The main goal of this model system is to determine whether MBD2 knock-down results in increased γ-globin expression in a human model system. If this can be shown, the potential exists that MBD2 may be a target for pharmacological induction of HbF. Thus, we needed to determine the level of γ-globin expression in adult erythroid cells. There was no detectable γ-globin expression in adult erythroid differentiated CD34+ as determined by RNase protection assay. This result indicates that if MBD2 is playing a similar role in human cells, knock-down of the gene may lead to a detectable increase in γ-globin expression. Now that γ-globin levels have been shown to be barely detectable, the next step is to transduce CD34+ with an MBD2 siRNA followed by differentiation and globin expression analysis.

Chapter 6: Summary and Future Directions

The genes of the human β-globin locus undergo a switch in expression during development. During this sequential process, the 5' gene in the cluster is silenced while the immediate 3' gene(s) becomes active. Thus, the genes are expressed in the order in which they lie on chromosome 11: 5' ε, γ, and β 3'. The silencing and activation of the β-type globin genes involves conserved mechanisms in addition to unique mechanisms for each gene. For example, each gene requires the LCR for high level expression. On the other hand, the β-globin gene requires the transcription factor EKLF while the ε- and γ-globin do not require it for expression (Nuez et al., 1995; Perkins et al., 1995). The exact mechanism for globin gene switching is not fully understood, but it has been shown to involve many interdependent processes including *cis* elements, *trans* factors including tissue specific factors, developmental specific factors and ubiquitous factors, and epigenetics. Our lab has a particular interest in the latter mechanism, epigenetics.

Epigenetic mechanisms result in heritable changes in gene transcription that do not alter DNA sequence. A prototypical epigenetic modification is the addition of a methyl group to the 5' position of cytosine in the context of the CpG dinucleotide. Increased levels of DNA methylation are associated with decreased transcription, a

correlation first noted in the β-globin locus (McGhee and Ginder, 1979). Since that time, DNA methylation has been shown to play a part in β-type globin gene regulation in many different model systems and inhibition of DNA methylation by the compound 5-azacytidine has been shown to activate silenced globin genes in model systems and patients with hemoglobinopathies (Atweh et al., 2003; Charache et al., 1983; DeSimone et al., 1982; Ginder et al., 1984; Ley et al., 1982; Ley et al., 1983; Ley et al., 1984; Pace et al., 1994; Zucker et al., 1983). The mechanism by which DNA methylation mediates transcriptional silencing was fostered by the discovery of a family of proteins that specifically bind to methylated DNA. Furthermore, the proteins were shown to bind as large complexes containing transcriptional repressive proteins. One such protein, MBD2 was shown to bind to a methylated ρ-globin construct as part of a large complex (Singal et al., 1997; Singal et al., 2002). This complex was further purified from primary erythrocytes and shown to contain MBD2. Finally, MBD2 was shown to bind to the ρ-globin proximal transcribed CpG islands *in vivo* (Kransdorf and Ginder, 2004).

We sought to determine the impact MBD2 on the regulation of the human embryonic and fetal globin genes. Mice transgenic for different human β-type globin constructs were utilized as a model system. Mice containing the entire β-globin locus as a yeast artificial chromosome were the model system used to study regulation of the γ-globin gene. Similar to results published with a different line of βYAC transgenic mice, we observed activation of the γ-globin gene in adult mice upon treatment with the compound 5-azacytidine (Pace et al., 1994). DNA methylation and MBD2 have been shown to, in part, mediate the silencing of the avian embryonic ρ-globin gene (Ginder et

al., 1984; Singal et al., 1997; Singal et al., 2002). βYAC transgenic mice were bred with mice null for MBD2 to generate transgenic knock-out mice. Adult βYAC transgenic mice lacking functional MBD2 express mRNA from the normally quiescent γ-globin gene. Loss of MBD2 leads to a 10-20 fold induction of γ-globin mRNA in two different βYAC transgenic mouse lines. This effect appears to be very specific as βYAC mice hemizygous and homozygous for MeCP2, another MCBP, do not show the same effect. Furthermore, the endogenous embryonic mouse globin transcripts are not increased in the absence of MBD2. The impact of MBD2 loss on γ-globin expression is not only seen in adult mice, but also during development. Embryos null for MBD2 at 14.5dpc and 16.5dpc express the γ-globin gene at higher levels than wild-type embryos. MBD2 is needed to maintain γ-globin gene repression in adult mice and is necessary for developmental silencing. The increased expression of the γ-globin gene in adult mice is due to expression of the gene during definitive erythropoiesis rather than a reactivation of embryonic erythropoiesis as the level of $^{A}\gamma/^{A}\gamma+^{G}\gamma$ is consistent with the ratio seen in adult human F-cells.

It is hypothesized that epigenetic modifications beget other epigenetic modifications to impart transcriptional silencing. For example, it has been shown that loss of a histone methyltransferase leads to decreased levels of DNA methylation (Tamaru and Selker, 2001). To determine precisely how MBD2 mediated a repressive effect on γ-globin transcription, we first looked for changes in DNA methylation around the γ-globin promoter. In adult mice, the γ-globin promoter and proximal transcribed region are highly methylated while treatment with 5-azacytidine leads to ~50% decrease in DNA

methylation levels and a concomitant increase in γ-globin transcription. If MBD2 binding to the γ-globin promoter region results in subsequent recruitment of DNMT, DNA methylation levels should be significantly decreased around the γ-globin promoter in the absence of MBD2. However, DNA methylation levels around the γ-globin promoter are only modestly decreased to ~75% of wild-type levels in MBD2 null mice. Thus it seems unlikely MBD2 is recruiting DNMT to the γ-globin promoter region and more likely that instead the modest decrease in DNA methylation around the γ-globin promoter is due to increased transcription of the gene. We next determined what impact loss of MBD2 has on post-translational histone modifications around the γ-globin gene. Not surprisingly, histone H3 trimethylated on lysine 4 was enriched ~4-fold in knock-out mice versus wild-type mice. This modification is associated with active transcription, indicating the increase in γ-globin expression is due to increased transcription of the gene. On the other hand, no significant difference was seen in the levels of acetylated histones H3 and H4 between wild-type and knock-out mice. These data indicate that MBD2 is not recruiting HDACs to the γ-globin gene region due to the lack of increase in enrichment of acetylated histones H3 and H4 in MBD2 null mice. Adult MBD2 null βYAC transgenic mice and βYAC mice treated with 5-azacytidine express the γ-globin gene at similar levels. To determine whether MBD2 and 5-azacytidine function in the same pathway(s), MBD2 null and wild-type βYAC transgenic mice were treated with 5-azacytidine to compare levels of induction of the γ-globin gene relative to pre-treatment levels. The nominal additive induction of the γ-globin gene in MBD2 null mice treated with 5-

azacytidine suggests that both MBD2 and 5-azacytidine are working chiefly along the same pathway(s).

We next explored the role of DNA methylation and MBD2 on the regulation of the embryonic ϵ-globin gene. To study human ϵ-globin regulation, transgenic mice containing the entire sequence from 5' of HS5 through 4 and 12kb 3' of the ϵ-globin polyA site were generated. Similar to results with other human ϵ-globin gene transgenic mice, these mice show correct developmental silencing of the transgene during development: high expression in the 10.5 dpc yolk sac and low to no expression in the 14.5 dpc fetal liver and adult erythroid cells. Three lines were used in the studies: two 28kb lines and one 36kb line. Adult mice from all three lines effectively express the normally silenced transgene upon a 5-day treatment course with 5-azacytidine. 28kb LCRϵ transgenic mice show high levels of DNA methylation around the promoter and proximal transcribed region in adult erythroblasts (>90%) and low levels of DNA methylation in 10.5dpc yolk sacs (<40%). The level in the yolk sac is likely to be even lower than calculated, due to contaminating epithelial cells. Treatment with 5-azacytidine reduces the level of DNA methylation ~20%. This modest decrease in DNA methylation is likely a copy number effect as not all copies of the transgene may be equally demethylated. In contrast to the results in human ϵ-globin construct mice, in the context of mice containing the entire β-globin locus as a yeast artificial chromosome transgene, the ϵ-globin gene is expressed at a much lower level upon 5-azacytidine treatment. Similar to the results seen with 5-azacytidine, adult 28kb LCRϵ lines 1 and 2 express the transgene when they are null for MBD2. Once again, βYAC mice do not express high

levels of the ε-globin gene in the MBD2 null background. βYAC mice lacking MBD2 express the γ-globin gene at a level commensurate with wild-type mice treated with 5-azacytidine. One line of 28kb LCRε transgenic mice expresses the ε-globin gene at higher levels in the MBD2-/- background than upon treatment with 5-azacytidine. This result seems to indicate a possible position effect of the transgene rendering them more sensitive to changes in epigenetic status than βYAC transgenic mice. The ε-globin gene has been reported to be silenced by a process termed autonomous silencing whereby the promoter and 5' flanking region of the gene contain all elements necessary for correct developmental silencing in the absence of other β-type globin genes competing for enhancer activity (Raich et al., 1990; Shih et al., 1990). On the other hand, the γ-globin gene has been shown to be silenced by a combination of autonomous silencing and competition with the β-globin gene for the enhancer activity of the LCR (Behringer et al., 1990; Dillon and Grosveld, 1991; Enver et al., 1989; Enver et al., 1990). These results indicate that the ε-globin gene can be activated upon 5-azacytidine treatment or in the MBD2-/- background, but it remains highly repressed under the same conditions when other globin genes are present. Therefore, it appears that a combination of 5' cis sequence mediated autonomous silencing, LCR competition, and DNA methylation contribute to maintain the 10^4 level of silencing of the ε-globin gene in adult erythroid cells.

To determine the exact mechanism of DNA methylation and MBD2-mediated repression of the ε- and γ-globin genes, ChIP assays were performed to determine whether MBD2 was bound to the promoter region of either gene. MBD2 does not associate with the γ-globin promoter in either MBD2+/+ or MBD2-/- mice. However,

MBD2 does associate with the Ugt8 CpG island, the upstream GATA2 promoter, and the CpG island 3' of the TZFP gene in MBD2+/+ but not MBD2-/- mice indicating the specificity of the antibody and validity of the assay. These results indicate that MBD2 mediated transcriptional silencing of the γ-globin gene is not due to the direct binding of MBD2 to the promoter region of the γ-globin gene. MBD2 is likely repressing a gene *in trans* that when expressed induces transcription of the γ-globin gene. In an effort to determine what gene(s) MBD2 may be regulating, a microarray analysis was performed on splenocytes from MBD2 wild-type and knock-out mice. The initial analysis of up-regulated genes failed to identify an erythroid specific transcription factor. However, after analyzing the data set using a pathway analysis program, a group of genes from known hematopoietic pathways were identified. Validation of potential candidate genes revealed TZFP as a candidate. The expression of this gene is increased ~30-fold in MBD2 null erythroid cells. In addition, TZFP interacts with GATA2, a protein shown to increase transcription of the γ-globin gene upon over expression (Ikonomi et al., 2000; Tsuzuki and Enver, 2002). While the nature of this interaction is not known, TZFP is currently a lead candidate for future analysis.

Through the course of these studies, it was revealed that MBD2 is necessary for silencing the γ-globin gene in adult βYAC transgenic mice and during development. The effect of MBD2 on γ-globin is very specific, as no other embryonic globin genes are up-regulated in its absence nor was loss of another MCBP able to recapitulate this phenotype. Absence of MBD2 is associated with modest changes in DNA methylation and histone acetylation around the γ-globin gene, but the increased level of transcription

is associated with an increase in trimethylated histone H3 lysine 4. MBD2 is not mediating its repressive effect at the γ-globin promoter region as MBD2 did not ChIP to the γ-globin promoter region. The phenotype observed is likely due to MBD2 mediated repression of a γ-globin activator(s) of which TZFP has been identified as a potential candidate. Taken together, we believe MBD2 is repressing a gene that when activated either directly or indirectly activates γ- and ε-globin transcription (Figure 35). Finally, inhibition of DNA methylation and loss of MBD2 revealed a competitive silencing mechanism for maintaining ε-globin transcriptional repression. In the absence of other globin genes, loss of MBD2 and 5-azacytidine each led to expression of the ε-globin gene in adult mice. However, these same conditions fail to induce significant expression of the ε-globin when other β-type globin genes are present on the transgene.

In order to fully understand how MBD2 mediates γ-globin transcriptional repression more work must be performed. One obvious place to begin is to determine whether the effect seen is unique to the mouse model utilized. N-MEL cells provide an opportunity to determine whether the effect can be seen in a cell culture system. N-MEL cells are a hybrid cell line in which normal mouse erythroleukemia cells contain a copy of human chromosome 11. These cells have been shown to express the β-type globin gene in a pattern similar to that seen in adult βYAC transgenic mice: high levels of β-globin and low levels of γ-globin mRNA (Forrester et al., 1990). N-MELs, and a similar cell line containing human chromosome 11 were shown to increase expression of the γ-globin gene upon treatment with 5-azacytidine (Ley et al., 1984). Thus, this system in essence mimics adult βYAC erythropoiesis. Since cells in culture can be transfected with

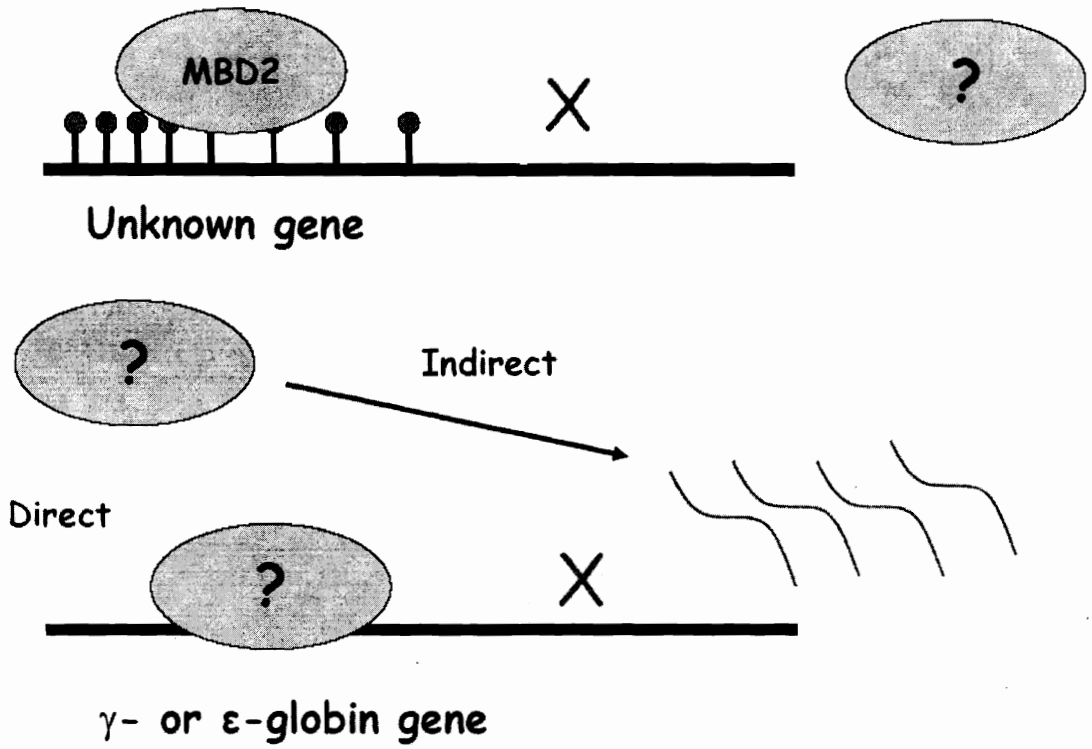

Figure 35: Model for MBD2 mediated transcriptional repression of the human γ- and ε-globin genes. In adult mice, MBD2 silences and unknown gene (?). In the absence of MBD2 or upon treatment with 5-azacytidine, this gene becomes expressed and either directly or indirectly activates transcription of the human γ- or ε-globin genes.

constructs producing shRNA to downregulate the target genes, the phenotype can potentially be reproduced in another system, adding more credibility to the results presented here. Further proof can be provided using erythroid differentiation of human CD34+ cells. CD34+ cells can be cultured and induced down the erythroid pathway to express globin genes (Bhanu et al., 2004; Migliaccio et al., 2002). We have shown that CD34+ cells obtained from adult and cord blood can be differentiated into erythroid cells. In addition, CD34+ cells from cord blood express high levels of γ-globin mRNA while those derived from adult blood show <2% γ-globin mRNA. As a result, this system has the potential to study the effect of MBD2 loss in human primary cells. CD34+ cells have shown the ability to be transduced by lentiviral vectors encoding shRNAs (Hong et al., 2005). In addition, a human siRNA for MBD2 has been described (Lin and Nelson, 2003). Transducing adult CD34+ cells with a lentiviral vector containing an MBD2 shRNA followed by erythroid differentiation would be a valuable approach to obtain data to strengthen the argument that MBD2 mediates γ-globin transcriptional repression in a variety of model systems. If these data can be obtained, the potential then exists for MBD2 to be a target protein for therapeutic induction of fetal hemoglobin.

MBD2 appears to be necessary, at least in part, for the developmental silencing of the γ-globin gene. The potential exists that the effect seen in adult βYAC transgenic mice lacking MBD2 is due in some way to the absence of the protein during development. In this case, induction of γ-globin by inactivation of MBD2 in adult erythroid cells may not be occur if MBD2 is present during development. The N-MEL system and CD34+ culture systems described above would help counter this argument as these cells have

always possessed functional MBD2. However, to test this *in vivo*, a conditional MBD2 knock-out must be produced. To perform this, the MBD2 gene must be surrounded by loxP sites so that upon activation of Cre recombinase, the gene will be removed. The conditional knock-out can be performed in a variety of ways. Placing a Cre-transgene under the control of the β-globin gene promoter would allow removal of the gene at an earlier time point, however the β-globin gene begins to be activated around the time primitive erythropoiesis ends and thus does not allow for adult excision of the gene. Placing the Cre-gene under the control of the Tet-repressor would allow for chemical induction of cre expression upon treatment with tetracycline. This system would allow for analysis of transgenic mice that possess MBD2 during development and have only lost the gene as adults. This mouse model would allow one to assess the importance of MBD2 expression during development on γ-globin activation in adult mice via MBD2 elimination.

One theory for the activation of globin genes upon treatment with 5-azacytidine is that the cytotoxic nature of the drug accelerates the differentiation of an erythroid progenitor. Adult erythroid cells early in the differentiation pathway have been shown to express the γ-globin gene (Stamatoyannopoulos, 2005). Along this line, MBD2 loss may lead to an increase in the number of erythroid progenitors or may favor erythroid differentiation of hematopoietic stem cells. The increased number of erythroid cells may overwhelm the bone marrow leading to a situation similar to the cytotoxic theory where early progenitors are forced to differentiate at an accelerated rate. In this case, the increased number of erythroid cells would cause some early differentiating cells to

differentiate at an accelerated rate and thus still produce γ-globin mRNA. To test this theory, colony assays can be performed to determine whether bone marrow cells from MBD2 null mice produce more erythroid colonies than wild-type bone marrow cells. In addition, it is not yet known whether the effect of MBD2 upon γ-globin expression in adult mice is pancelluar or heterocellular. To determine this effect, an anti-fetal hemoglobin antibody can be used on peripheral blood cells. The cells can either be scored under a microscope or via flow cytometry to determine whether all cells or a subset of cells express the γ-globin gene.

While the initial microarray studies on MBD2+/+ and MBD2-/- mice have been very informative, the analysis should be repeated to account for possible confounding elements. First, the mice used in the study were from a mixed genetic background. As a result, potential modifier genes may not have been detected. To account for this problem, the MBD2-/- mice can be bred into a homogeneous genetic background and the results compared to the pilot study to identify the effects of potential modifier genes. In the initial studies, erythroid cells were not purified out of the cell population. While 1-acetyl-2-phenylhydrazine treatment results in conversion of the spleen to a >80% erythroid organ, lymphocytes are still present (Spivak et al., 1973). We initially wanted to include lymphocytes in the instance that loss of MBD2 led to cytokine release from non-erythroid cells that induced erythroid cells to express the γ-globin gene. The potential exists that these cells are preventing the detection of a gene involved in γ-globin induction or are expressing a gene at a high level that may be interpreted as a γ-globin activating gene. Thus, the microarray analysis should be repeated after purifying erythroid cells using an

erythroid specific marker such as Ter-119. Finally, the gene(s) responsible for γ-globin expression may only be expressed during a certain phase of erythroid differentiation. Lodish and colleagues have described a method for isolating murine erythroid cells at different stages of erythropoiesis based on expression of the cell surface markers CD71 and Ter-119 (Socolovsky et al., 2001). Microarray analysis can be repeated on erythroid cells from different stages of differentiation to discern whether the factor(s) responsible for γ-globin expression are expressed transiently during differentiation. Furthermore, candidate genes from the pilot microarray study, as well as the additional studies, need to be validated in a cell culture model (N-MEL or CD34+) or in a mouse model. If a candidate gene is expressed highly in MBD2 null cells, the cDNA should be over expressed in N-MEL cells to determine whether it is able to induce expression of the γ-globin gene. A more ideal system, though more time-consuming and laborious, would be to over-express the gene in βYAC transgenic mice to determine whether it can recapitulate the effect seen in MBD2 null mice. If a candidate gene is under-expressed in MBD2 null cells, siRNA should be performed in N-MEL or transgenic mice to determine whether knock-down of the gene is able to induce expression of the γ-globin gene. While the initial microarray study was very informative, more characterization must be performed to ensure the validity of the results.

Loss of MBD2 and DNA methylation each revealed that a competitive silencing mechanism exists for the human ε-globin gene. It should be noted that the βYAC transgene contains much more additional sequence both 5' and 3' relative to the 28kb LCRε construct used to generate transgenic mice. Thus, the possibility exists that other

sequences within the βYAC are responsible for the observed effect on competition. To test this, multiple transgenic mice lines can be made. First, a line continuing the 3' sequence through either γ-globin gene can be generated. This transgene will only differ at the 3' end of the gene and would reveal whether the presence of the γ-globin promoter can suppress expression of the ε-globin gene during 5-azacytidine treatment or in the absence of MBD2. It may be that the γ-globin promoter is insufficient to maintain ε-globin repression, thus a construct continuing 3' through the β-globin gene can be generated and assayed for its ability to prevent ε-globin expression. These constructs also contain sequence that could be confounding to the results. Thus, either the γ- or β-globin promoter and gene could be added immediately 3' to the 28kb LCRε construct and used to generate transgenic mice. This construct would show that all that is needed to suppress the ε-globin gene is an additional β-type globin gene and strengthen the argument that a competitive silencing mechanism exists for the ε-globin gene.

The results presented here and proposed above serve to positively impact one group of patients directly and another group indirectly. One current therapeutic approach for the treatment of hemoglobinopathies is through the pharmacological induction of fetal hemoglobin. While promising results have been obtained treating patients with 5-azacytidine, hydroxyurea, and short chain fatty acids, many problems still exist. These compounds are highly toxic and not all patients respond to the therapy. In addition, most of these agents have to be administered intravenously. As a result, a market for better pharmacological inducers of fetal hemoglobin exists. The work described here has the potential to lead to a new class of therapeutics, those that target MBD2. We have shown

that in the absence of MBD2, the γ-globin gene is activated in adult transgenic mice. In addition, mice lacking functional MBD2 display only a mild phenotype (Hendrich et al., 2001). Thus, targeted disruption of MBD2 through binding site interaction or co-repressor interaction may be less toxic than the current line of agents. At the very least, it has shown an additional mechanism for γ-globin silencing that may be beneficial at a later date. Epigenetic regulation of transcription is not limited to the β-globin locus. Epigenetic modifications play a role in regulating tumor suppressor genes as well as imprinting genes whose dysregulation can lead to disease. Thus, understanding the mechanism of how MBD2 mediates repression in a tissue specific manner may be beneficial to the study of other pathologies where epigenetics play a crucial role in pathogenesis.

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Vita

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