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Histone Occupancy of PRMT8 5' UTR in Human Dermal Fibroblasts during the Acquisition of iRC Phenotype

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Abstract

Previous research has found that altering the culture conditions of human dermal fibroblasts to low oxygen and adding the growth factor FGF2 changes the chromatin regulation of the cells creating cells that express an iRC phenotype (Page et al., 2009). One of the chromatin modification enzymes believed to regulate this change is PRMT8, which is highly upregulated in these cells (Runge Sarah, unpublished). PRMT8 is a chromatin modification enzyme which catalyzes arginine methylation reactions (Sayegh et al., 2007). The goal of this project was to determine the histone occupancy of PRMT8 5’ untranslated region in order to identify changes associated with the upregulation. The histone occupancy was determined using Chromatin Immunoprecipitation. Two key histone modifications that play a role in the activation and repression of a gene H4ac and H3K9me2, respectively, were studied (Collas et al., 2009). It was predicted that H4ac, a mark of activation, would be found in the PRMT8 5' UTR of iRC cells, while H3K9me2, a repressive mark, would be found in the 5' UTR of the control cells. Histone mark H4ac, was found to occupy several regions of the 5' UTR of the iRC cells, however it was found over more of the 5' UTR in the control cells and cells grown in hypoxic conditions. H3K9me2 was found to occupy most regions of the 5' UTR in control cells and cells grown in hypoxic conditions as well as iRC cells.

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Introduction

Personalized medicine has transformed our way of thinking about disease and stands to change how medicine is prescribed. Achievements of this field are positioned to be limitless: for example, prosthetics, and even scar tissue, could one day be replaced with functional muscle. However, advancements in personalized medicine are halted by lack of knowledge about how induced pluripotent stem cells (iPSC) acquire regeneration competence. It has now been shown that culture conditions may induce a stem cell-like phenotype and a variety of biological questions not approachable in previous systems can now be answered. The Dominko lab has developed a novel model system that takes advantage of dermal fibroblast plasticity and allows us to study the acquisition of regeneration competence in a cultured environment free of recombinant stem cell genes.

By lowering oxygen concentration and supplementing with fibroblast growth factor FGF2, the Dominko lab has transformed human dermal fibroblasts into more plastic, regeneration-competent cells, which we now term induced regeneration competent (iRC) cells. Previous publications and preliminary research indicates the potential that arginine methylation and protein arginine methyltransferases (PRMTs) have in contributing to tissue regeneration. Unpublished preliminary data from the Dominko lab demonstrates an over 13 fold increase in the arginine methyltransferase PRMT8. Because of this, we hypothesize the promoter region of PRMT8 undergoes changes in histone occupancy in iRC cells compared to dermal fibroblasts from which they were derived.

Background

There is currently a large demand for an autologous cell source that can be readily produced for cell therapy purposes. Ongoing research points to human dermal fibroblasts as a possible way to satisfy this demand. Fibroblasts, highly studied and ubiquitously present in mammalian organisms, provide a good model for researching cell plasticity. *In vivo*, they are able to regenerate complex tissues in salamanders and embryonic mammalian tissues. *In vitro*, they can be induced to demonstrate pluripotency. Once cells become pluripotent, they can form any one of the three germ layers (mesoderm, ectoderm, and endoderm) regardless of where they were derived from. This can allow for many possible cell therapy treatments.

iRC Cells

Standard laboratory practice is to culture cells at atmospheric oxygen (~20%) at 37°C with 5% CO_2. However, physiological oxygen levels have been experimentally determined to be considerably lower and vary significantly between tissue types. Oxygen levels in the brain range from 0.5 to 7% while in the eye levels range from 1 to 5% (Ivanovic, 2009). Previous research has shown lowering oxygen levels to hypoxic *in vitro* conditions (2 to 5% O_2), or normoxic conditions for *in vivo* studies, and supplementing with the growth factor FGF2, allows human dermal fibroblasts to display characteristics similar to human embryonic stem cells (hESC), specifically expression of certain stem cell-specific genes, and increased proliferation compared to primary cells. Because fibroblasts under these conditions show significant increase in cell proliferation compared to the cells from which they were derived, they have been termed extended life span (ELS) cells. ELS cells have an increased rate of proliferation 2 fold higher than control cells and show expression of specific stem cell associated genes, namely Lin28 and Rex1 (Page et al., 2009). Current research has also demonstrated ELS cells lead to significant

decrease in collagen when introduced to a mouse skeletal wound (Page et al., 2011). Changes to this model have been made by changing O_2 levels to 2%. Because of this, current terminology for these cells is induced regeneration competent or iRC cells. Culture conditions of these cells are detailed in Figure 1.

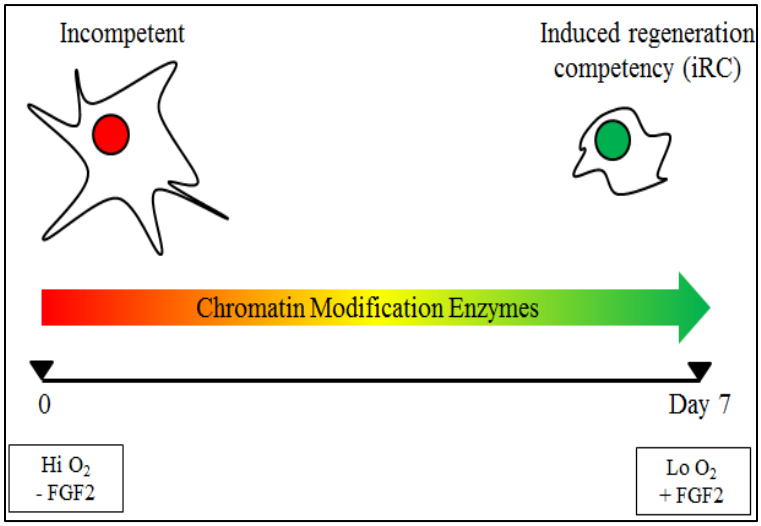


Figure 1: iRC Model System. Human dermal fibroblasts (incompetent cells) are transformed to iRC cells through culture in reduced oxygen and FGF2 supplementation for 7 days.

Human dermal fibroblasts grown in atmospheric oxygen, cells incompetent to regeneration, are induced to become iRC cells with growth over 7 days in 2% oxygen and FGF2 supplementation. The expression of stem cell genes in these cells is hypothesized to be the result of alterations in the epigenetic code, which would allow for transcriptional permissiveness of said genes.

Epigenetics

The change in expression of Lin28 and Rex1 in iRC cells compared to control cells is likely caused by changes in the epigenetic code. Epigenetics is the study of heritable changes in

gene activity that are not caused by changes in DNA sequence (Slatkin, 2009), such as post-translational modifications to proteins that control transcription. Gene expression is often regulated in response to the environment, not only by DNA sequence (Qui, 2006), which correlates to gene expression changes our lab has described due to changes in culture conditions. As a consequence, cellular phenotype is affected by the transcription of certain genes.

Gene expression changes as a result of epigenetics are primarily caused by modifications to DNA and histone associated with the differentially regulated gene. These modifications determine how tightly condensed DNA is around nucleosomes. In order for a gene to be transcribed, the transcriptional machinery has to be able to gain access to that gene. This means that that in order for transcription to happen the chromatin cannot be too tightly packed. The tightly packed, transcriptionally inactive form of chromatin is known as heterochromatin, while "open", or transcriptionally active chromatin, is known as euchromatin (Gan et al., 2006)). Examples of heterochromatin and euchromatin can be seen in Figure 2**.**

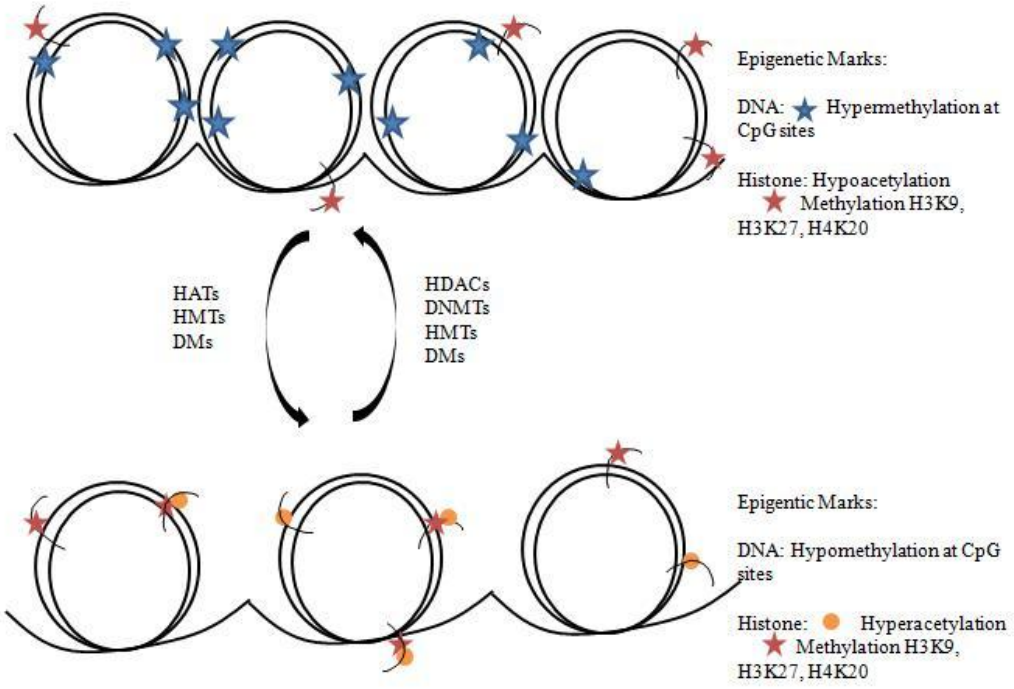


Figure 2: Heterochromatin vs. Euchromatin. Chromatin modification enzymes are responsible for mediating the changes in epigenetic marks seen on heterochromatin and euchromatin.

Two main types of post-translational modifications occur to effect transcription. The first is post-translational modifications to DNA, which occur when methyl groups are either added or removed from the linear dinucleotide cytosine guanine, or CpG, sites in DNA (Collas et al., 2009). The addition of a methyl group generally causes transcriptional inactivity by causing tighter bonds between DNA strands and histones, while the removal of a methyl group removes bonds that restrict DNA from being transcribed. The second type of post-translational regulation of transcription, histone modification, occurs when different groups (most commonly acetyl groups and methyl groups) attach to histone N-terminals, changing association with DNA that is wrapped around them (Gan et al., 2006). There are several histone modifications but the two most common types are acetylation and methylation. The acetylation of histones often confers activity while the methylation of certain histone residues can either cause activation or repression. Table 1 shows the type of activity generally associated with specific residues (Collas et al., 2009).

	H3K4	**H3K9**	**H3K27**	**H3K36**	**H3K79**	**H4**
monomethylated	active	repressed	repressed		active	
dimethylated	bivalent	repressed				
trimethylated	repressed	repressed	repressed	active	active	
acetylated		active				active

Table 1: Transcriptional Activity associated with Histone modifications. Certain histone residues typically associated with transcriptional activity or repression.

Chromatin modification enzymes, such as methytransferases acetyltransferases, deacetylases and demethylases, are known to mediate changes between activity states of specific residues. Methyltransferases work to add methyl groups to either DNA or histones while demethylases remove these methyl groups. Similarly acetyltransferases add acetyl groups to histones and deacetylases remove them (Trievel, 2012). Changes in a cell's environment such as pH levels, oxygen levels or growth factors can often change the chromatin modification enzymes thereby changing the activation and repression of certain genes.

Protein Arginine N-Methyltransferase

Arginine methylation is a common posttranslational modification (Bedford et al., 2011) involved in various processes, such as RNA processing, signal transduction, transcriptional regulation, and DNA repair (Bedford et al., 2005). Arginine methylation occurs posttranslationally when the nitrogen of arginine within polypeptides is modified by a methyl group. Protein Arginine N-Methyltransferases, PRMTs, are chromatin modification enzymes that catalyze methylation reactions on arginine residues.

Currently, there are 11 PRMTs that have been identified (Debats et al., 2009) that typically target proteins harboring glycine/arginine rich (GAR) motifs. Publications have also demonstrated that PRMTs methylate histones and their coactivators (Bedford et al., 2005). Arginine methylation of core histone tails regulates chromatin function (Bedford et al., 2011). Methylated arginines have been shown to regulate cellular processes by blocking some protein interactions and promoting others (Bedford et al., 2005). This can be done one of three ways, through monomethylarginines (MMA), asymmetric dimethylarginines (aDMA) and symmetric dimethylarginines (sDMA).

The 11 PRMTs have been further subdivided into two groups, type I and type II. Type I PRMTs lead to the production of aDMA, using MMA as an intermediate. Like Type I, Type II uses MMA as an intermediate but these PRMTs lead to the production of sDMA (Bedford et al., 2005).

Protein Arginine N-Methyltransferase 8

A lesser studied PRMT is PRMT8, discovered in 2005 through sequence homology to PRMT1 (Lee et al., 2005). Currently very little is known about the activity of PRMT8. PRMT8 is a 394aa long protein coded by a gene on chromosome 12. It is grouped with the Type I PRMTs, PRMT1, 2, 3, 4 and 6, as it produces aDMA. It is closely related to its type I group mate PRMT1, sharing an over 80% identity. PRMT8 differs from PRMT1 by a distinct 76aa long N-terminal region which harbors a myriostoylation motif (Lee et al., 2005). Published results of this myriostoylation motif indicate its plasma membrane association resulting in expression mainly in the brain. Tissue specificity is unique to PRMT8, as PRMT family members are generally ubiquitously expressed enzymes (Lee et al., 2005). Brain specific expression has been reported to suggest PRMT8 plays a role in neuronal differentiation (Lee et al., 2005). Interestingly, unpublished data from our lab demonstrates PRMT8 presence in both iRC cells and human embryonic stem cells (hESC). This may indicate a role for PRMT8 in development and cellular plasticity.

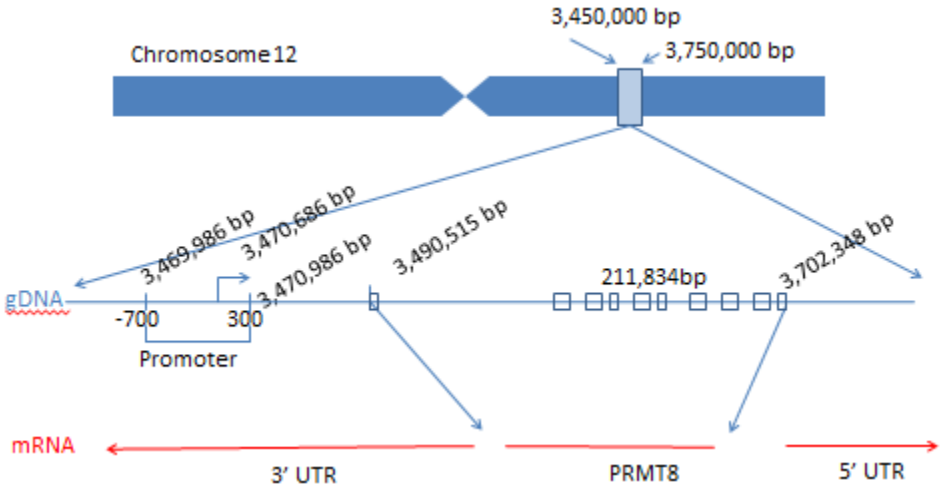


Figure 3: PRMT8 Gene. The PRMT8 gene is located on Chromosome 12. Blue rectangles indicate exons, arrow indicates transcription start site (TSS) and relative distances from the TSS are indicated by numbers of nucleotides upstream or downstream

Histone Occupancy of PRMT8

The current research aims to identify the histone occupancy of the 5' UTR of PRMT8 in regards to histone residues H4ac, a mark of activation and H3K9me2, a mark of repression. Unpublished research has shown a 13.8 fold increase in PRMT8 expression in human dermal fibroblasts cultured in low oxygen (2%) and supplemented with FGF2, also known as iRC cells, when compared to cells cultured in 19% oxygen without the addition of FGF2, or control cells. An increase in PRMT8 expression is also seen in cells cultured in 2% oxygen alone, or hypoxic cells. Determining the histone occupancy of PRMT8 will uncover what histone modifications are allowing for the upregulation of PRMT8 in iRC cells and hypoxic cells. This was done by using Chromatin Immunoprecipitation to determine the portions of genomic DNA in PRMT8 that were occupied by H4ac and H3K9me2 and analyzing the results using PCR.

It was hypothesized that iRC culture conditions will increase occupancy of histone residue H4ac and decrease occupancy of H3K9me2 on the PRMT8 5' UTR when compared to

the PRMT8 5’ UTR occupancy in control cells. These predictions are expected to be mirrored in hypoxic cells when compared to control cells.

Materials and Methods

Cell Culture

Primary Adult Human Fibroblasts (CRL-2352) from a 24 year old Caucasian male were obtained from the American Type Culture Collection (ATCC; Manassas, VA) at passage 2. Cells were cultured in medium containing 45% DMEM, 45% Ham's F12 and 10% FCIII. The DMEM was supplemented with 4mM L-glutamine. Cells were cultured in a 37°C incubator with 5% CO_2 and either 2% or 19% oxygen. Cells cultured at 2% oxygen were supplemented with FGF2 at 4ng/mL. Cells were grown in 100mm x 20mm plastic culture dishes. All cultures were processed on Day 7.

Crosslinking

37% Formaldehyde was added directly to culture dishes with fresh medium to a final concentration of 1%. Cells were incubated for 10 minutes at room temperature on a shaker platform. 2.5M glycine to a final concentration of 125mM was then added to stop the reaction and cells were incubated for 10 minutes at room temperature. Medium was discarded and cells were washed twice with ice cold 1x PBS. 1ml of 1x PBS with the addition of protease inhibitor cocktail PIC was then added to the cells. Cells were scraped into tubes and pelleted by centrifugation. The supernatant was removed and pellets were stored at -80°C.

Chromatin Immunoprecipitation

Crosslinked cells were lysed in aliquots of $3x10^6$ cells for 10 minutes at 4°C in 250µL IP/Wash Buffer (10mM Tris Acetate; 1% NP-40; 100mM NaCl; 1mM Sodium Orthovanadate; PIC). Lysate was placed on ice and sonicated 11 times using 20 second pulses at power level 10. 750µL of IP/Wash buffer was added. At this point an aliquot was set aside as an input sample. An aliquot was also taken for samples with and without antibody. 10µg of primary antibody (H4ac- 06598 Milipore or H3K9me2-D5567 Sigma) was added to the plus antibody sample and

both were incubated on ice for 2 hours. A 50:50 mixture of protein A:G beads were then added to both samples and left to incubate at 4°C overnight. Nonspecifically associated DNA and proteins were then removed by washing beads 1 time with wash 1 (.1%SDS; 1% Triton-X; 2mM EDTA; 20mM Tris HCl, pH 8.0; 150mM NaCl), 1 time with wash 2 (.1%SDS; 1% Triton-X; 2mM EDTA; 20mM Tris HCl, pH 8.0; 500mM NaCl), 1 time with wash 3 (.25M LiCl; 1% NP-40; 1% sodium deoxycholate; 1mM EDTA; 10mM Tris HCl, pH 8.0), and 2 times with wash 4 (10mM Tris HCl, pH 8.0; 1mM EDTA). Chromatin was eluted from both samples by incubating with 250µL 1%SDS/.1M $NaHCO_3$ for 15 minutes at room temperature then centrifuging the beads at 2500rpm for 1 minute. The eluted complexes were then transferred to a microfuge tube and the chromatin was eluted a second time as above and the complexes were pooled. 500µL of 1%SDS/.1M $NaHCO_3$ was then added to the input sample. 20µL of 5M NaCl was added to all samples. The formaldehyde crosslink was reversed by incubating at 68°C overnight. 20µL of 1M Tris HCl, pH 6.5; 10µL .5M EDTA; 40µg proteinase K was added and the samples were the incubated at 45°C for 1 hour. DNA was extracted using a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol with 10µg of glycogen as a carrier. DNA was precipitated by incubating samples at -80°C for 1 hour. The DNA was washed using ice-cold 70% ethanol to remove any residual salt. The samples were centrifuged and the supernatant was removed. After the pellet was air dried, it was resuspended in 20µL of TE (20mM Tris HCl, pH 8.0; 1mM EDTA, pH 8.0).

Polymerase Chain Reaction

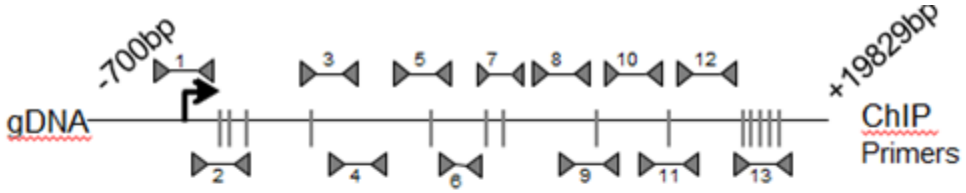
DNA extracted from adult human fibroblasts (ATCC CRL-2352) through Chromatin Immunoprecipitation and quantified using spectrophotometry (NanoDrop 200). 150ng of total DNA from each sample was used. PCR was performed using 12.5 µL GoTaq and 0.2mM each of

forward and reverse primers per reaction. The PRMT8 primers were (1) CAG AAA GCT AGA GAC TGG ATA GTA AAA C (forward) and TGT TGA AGA GAT TAA ACT TAC ACC CAA G (reverse), (2) TGG TGA ATA TTT GTG CAC AGT GTA TAT T (forward) and GGA GAA ACC ACA CAT GGA TAT TTG ATA G (reverse), (3) AAG ATT CTT CCT AAT CTG GAG CAA AAA G (forward) and CAG ATC TCC TGA TTT CCA AAT CTA AAC C (reverse), (4) GAG AGG GTA GAA AAG ATA GAG AGA ACA G (forward) and CGT TTT TCT GAC AAT TCC TAT CTC TTG A (reverse), (5) GAC TAG AAA TGG TAT GAA GGA GAT GAG A (forward) and GGT TGT CAA GGG AAA TTT TGT TAT CTT G (reverse), (6) GAA GAG TTG CAA GAT AAC AAA ATT TCC C (forward) and GAT TGT GAG GTT CAA TTC TAC AGT CAT T (reverse), (7) AGC TGA GCA AAT ATA TAA ATA ACA CCC G (forward) and TAC AAA TGT GGT CAA ATT CCT CTT TGA A (reverse), (8) ATT CAT CAA TTA CCT GAC CAT AGG AAG A (forward) and TAT TGT TGC TGG AAA TTA AGG AAG GAT T (reverse), (9) AAT GAC ATA GGA ACT GAA ACA AAT TGG A (forward) and TCA CATC AAT AAG AAC AA GTC ACT TAG C (reverse), (10) TTT CTG TTC TGA GTT TAC GGT CTA GTA T (forward) and TCT CAG TGA GTA CTA ATT TCT ACA GGT G (reverse), (11) GGT CAC CTT GTC TAA TCT CAT TAC AAT C (forward) and CTC TCT CCA GAA GTC TCA TGT ATC TAT C (reverse), (12) AGA TAG ATA CAT GAG ACT TCT GGA GAG A (forward) and TTC AAT GGT CAG TGG TAT TTT GGT TTA T (reverse), and (13) CTT ATT TCT CTG AGA TGT AGT TTT GGC A (forward) and CCA CAC ACT AAA AAC CCA TAA TGT TCT A (reverse). PCR cycling was done as follows: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 1 minutes; annealing at primer-specific annealing temperature for 1 minute; and extension at 72°C for 1.5 minutes. Final extension was done at 72°C for 10 minutes and the samples held at 4°C until use. Amplification

products were resolved on 1% agarose gels containing .06% ethidium bromide in 1x TAE buffer and images acquired using a Kodak 4000MM Image Station.

Results

Cells were cultured on plastic 10cm plates for 7 days in 2% oxygen in 50/50 DMEM/F12 + 10%FCIII. A second set of cells was cultured in these conditions with the addition of FGF2 at 4ng/mL. Control cells were cultured in 19% oxygen without the addition of FGF2. These cells were then crosslinked and Chromatin Immunoprecipitation (ChIP) was performed. The ChIP results were analyzed using PCR. PCR primers were designed to cover the promoter region of PRMT8 as well as in the 5'UTR as can be seen in Figure 4.



Primer	Start	Stop
1	-498	+467
2	+544	+1543
3	+5191	+6082
4	+5869	+6781
5	+7804	+8761
6	+8725	+9422
7	+9714	+10,667
8	+11,802	+12,456
9	+12,682	+13,626
10	+13,921	+14,903

Primer	Start	Stop
11	+14,938	+15,848
12	+15,820	+16,621
13	+18,273	+19,207

Figure 4: PRMT8 PCR Primers. Primers were designed to span the 5' UTR and promoter region of PRMT8. Primer positions are relative to the transcription start site.

PCR was performed using the PRMT8 primers with genomic DNA from teratocarciomas (ATCC 2073) as a control. The results of this can be seen in Figure 5.

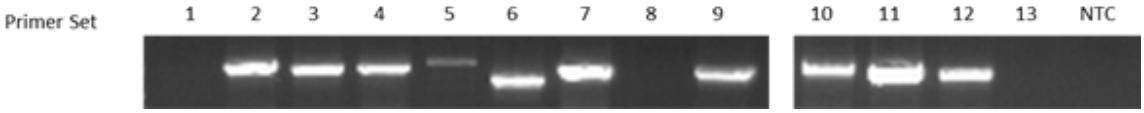


Figure 5: PCR amplification of Genomic DNA with primers described in Figure 4. NTC = no template control

In Figure 5a lack of bands can be seen for primers 1, 8 and 13, with a very weak band in lane 5, and the No Template Control (NTC). The lack of strong bands for these four primers indicates they do not amplify the expected product and therefore were not analyzed for subsequent analysis. The lack of bands in the NTC lane indicates that the primers were not contaminated with outside DNA.

PCR was then performed on the samples collected from ChIP while using the active histone mark H4ac antibody. Results can be seen in Figure 6**.**

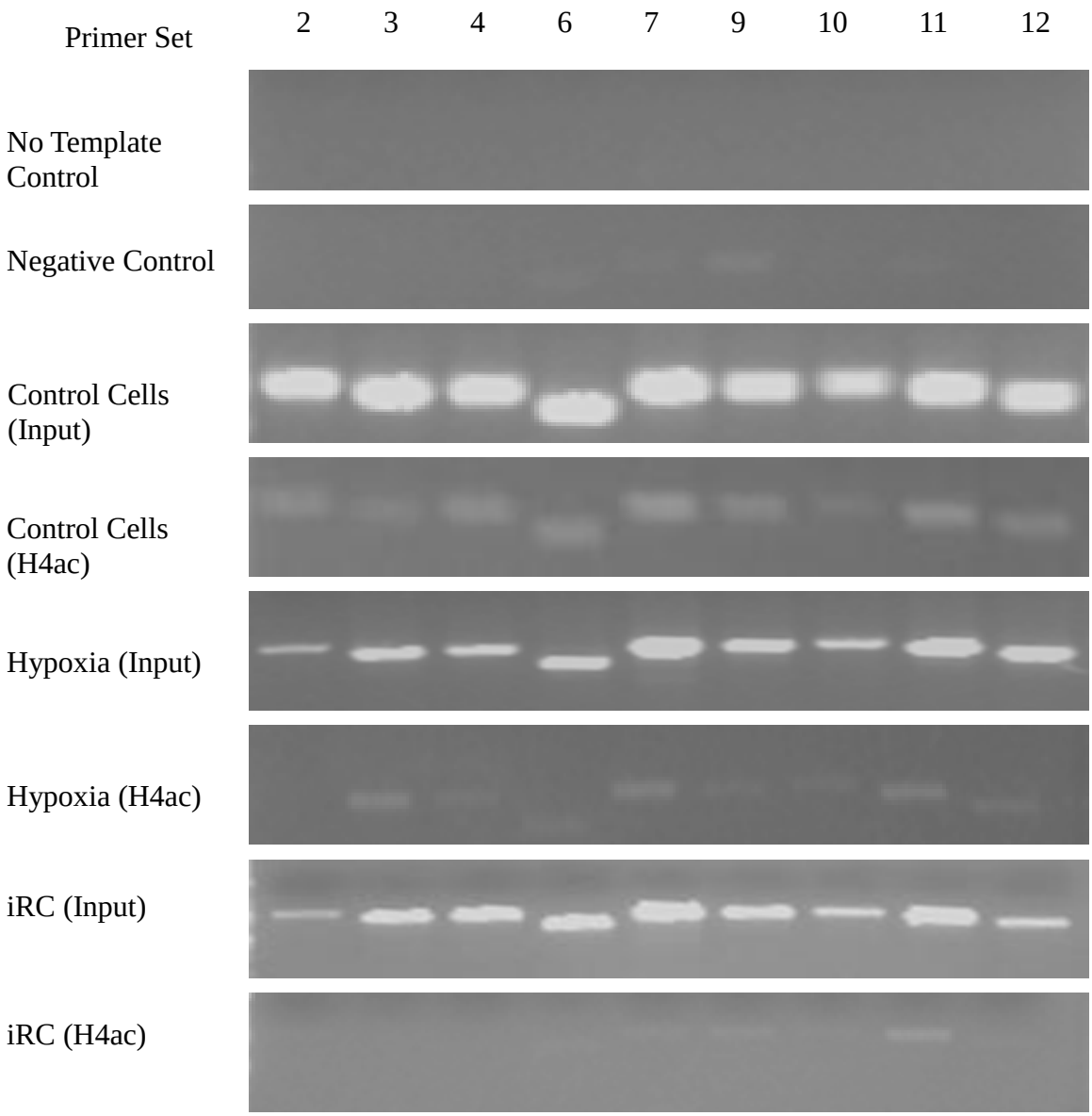


Figure 6: PCR amplification with primers described in Figure 4 after immunoprecipitation with antibody against acetylated histone H4ac.

As seen in Figure 6, the NTC produces no bands and negative control produces a negligible signal, indicating that there was no outside DNA contamination and the beads alone did not non-specifically bind to DNA. The presence of bands in the input samples shows that specified regions of the PRMT8 promoter are present in all three treatment groups. In the control

cells bands are existent in all lanes indicating H4ac occupies the entire examined region of 5' UTR of PRMT8 in control cells. The cells grown in hypoxic condition show bands for primer sets 3, 4, 6, 7, 9, 10, 11 and 12, which represent the majority of the 5' UTR of PRMT8 under hypoxic conditions. Lastly, the iRC cells show a band for primer set 11.This means H4ac only occupies the region of the PRMT8 5' UTR from 14,938bp to 15,848bp downstream of the transcription start site in iRC cells.

PCR was then performed on samples collected from ChIP pulled down with the H3K9me2 antibody as a representative of transcriptional repression. Results can be seen in Figure 7.

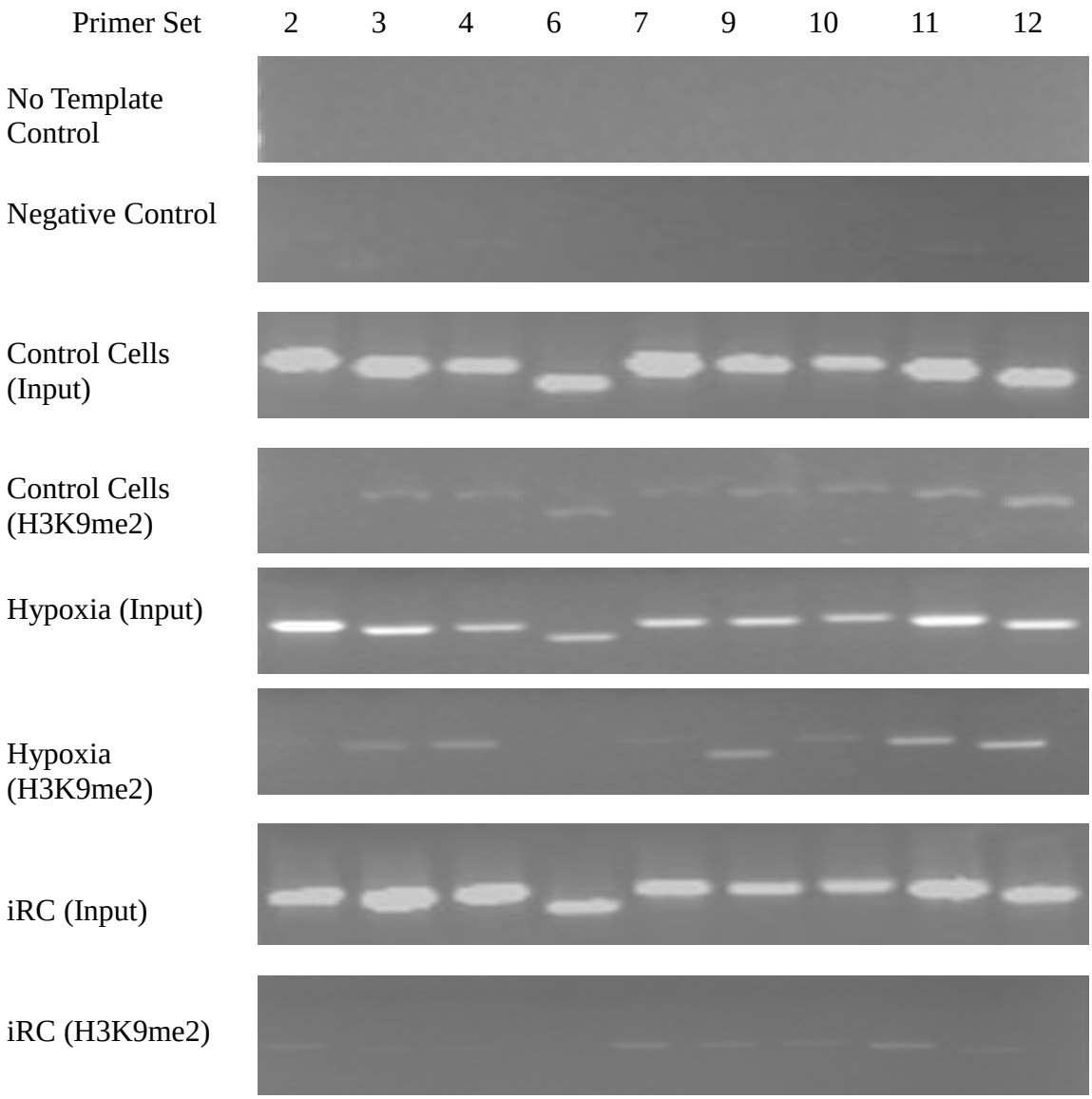


Figure 7: PCR amplification with primers described in Figure 4 after immunoprecipitation with antibody against dimethylated lysine 9 of histone H3K9me2

Figure 7 shows both NTC and Negative Control did not produce an amplicon, indicating a lack of DNA contamination, while the inputs produced bands for every primer, showing the specified region of the PRMT8 5' UTR is amplified in these treatments. In control cells H3K9me2 occupies the 5'UTR of PRMT8 from 5,191bp downstream of the transcription start site to 16,621bp downstream which is shown by the presence of bands for primers 3, 4, 6, 7, 9,

10, 11 and 12. Cells grown in hypoxic conditions show occupancy very similar to that of control cells, though they lack H3K9me2 occupancy in the region represented by primer sets 9 and 12. Occupancy of H3K9me2 in iRC cells is slightly less than the other two types of cells, lacking occupancy in the regions represented by primers 3, 4 and 6. However, unlike the other cells types, the iRC cells do show H3K9me2 occupancy in the region of 544bp to 1,543bp downstream of the transcription start site, with primer set 2.

Histone occupancy was established using Chromatin Immunoprecipitation to precipitate the portions of DNA bound to these specific residues and the results were analyzed using PCR. The results of the PCR were compiled and are pictorially represented in Figure 8**.**This figure shows the occupancy of both studied residues in all three cells types.

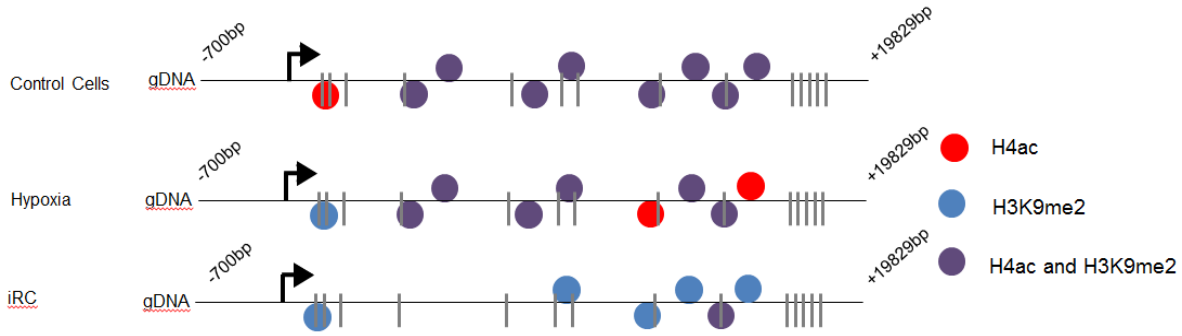


Figure 8: Histone Occupancy of 5' UTR of PRMT8 methyltransferase.

From this figure it can be seen that in control cells the promoter region and 5' UTR of PRMT8 is heavily occupied by H3K9me2, as expected. The occupancy of H3K9me2 is lessened in hypoxic cells and the occupancy is even lower in iRC cells. This progression of the loss of the repressive mark due to changes in culture conditions indicates that H3K9me2 is playing some role in the repression of PRMT8 in control cells.

Unexpectedly, this portion of the PRMT8 promoter and 5' UTR in control cells is heavily occupied by not only H3K9me2 but also by the active mark H4ac. This points to the conclusion that H4ac is not responsible for the activation of PRMT8 in iRC cells. Further supporting this conclusion is that iRC cells show almost no occupancy of H4ac.

Now that the histone occupancy of the PRMT8 5' UTR for these two residues has been determined it is important to look at other residues since there are other marks believed to confer activation and repression. Additional histone marks must be studied in order to determine which, if any, are causing the upregulation of PRMT8 in iRC cells. It should also be determined if other repressive marks add to the repression of PRMT8 in control cells.

Chromatin modification enzymes outside of PRMT8 should also be studied for histone occupancy in order to determine what else could be mediating the change in phenotype between control and iRC cells. Current research shows that other chromatin modification enzymes, such as PRMT6, are down regulated from control cells. Histone occupancy could be used in order to determine what is causing the down regulation of certain enzymes in iRC cells.

Conclusions

The goal of this project was to determine the histone occupancy of the 5' UTR region of PRMT8 for histone residues demonstrated to represent either transcriptional activation or repression. The two histone residues studied were H4ac, a mark of activation, and H3K9me2, a mark of repression. We have demonstrated that PRMT8 gene expression may be regulated by differential occupancy of PRMT8 5'UTR region by differently modified histones H3 and H4. Functional relevance of these changes for transcriptional activity, however, still needs to be determined.

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