A Protein Enrichment Assay to Study Temporal Post-Replication Binding of Transcription Factors

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Early in his freshman year, Abhinand Sudarshana heard Professor Downing give a presentation on epigenetics and the stem cell reprogramming work his lab was doing. Abhinand was fascinated by the topic and contacted Professor Downing about joining his lab. Throughout his research, he particularly enjoyed discussions with his mentors about how to design experiments and interpret the results. Abhinand found that he understood his project better when he was able to discuss it, and appreciated the opportunities to find creative ways to test his hypothesis. After graduation, Abhinand hopes to work in the research field for biotechnology companies to understand how the research industry operates.

Key Terms

- Amplification
- ChIP
- DNA Replication
- Human Embryonic Stem Cells (hESCs)
- Immunoprecipitation (IP)
- PCR/qPCR
- Transcription Factor (TF)

Abstract

DNA-protein interactions are crucial in directing cellular activities. Transcription Factors (TFs) are proteins that regulate gene expression and play a major role in maintaining a pluripotent, or undifferentiated, cell state in Human Embryonic Stem Cells (hESCs). The NANOG TF is known to coordinate gene regulation critical for pluripotency and its binding sites have been well-studied. However, its temporal activity has yet to be thoroughly explored. A new assay termed Repli-ChIP was developed to quantify TF-bound DNA during DNA replication. Two hESC cultures were treated with a synthetic nucleoside called BrdU, which labels nascent DNA. In one culture, immunoprecipitation (IP) using anti-NANOG antibodies was used to extract NANOG TFs at 1-hr post-BrdU treatment, termed the "early" or "0-hr" time point. In the second culture, BrdU treatment was stopped at 1 hour and NANOG was immunoprecipitated after an additional 16 hours of incubation in new media, termed the "late" or "16-hr" time point. Next, IP with anti-BrdU antibodies was used to isolate BrdU-labeled DNA. Quantitative PCR measured the amount of nascent DNA at the LHFP gene, which was used to infer the level of NANOG binding. Results imply decreased NANOG binding and activity late in replication. Findings for NANOG support the validity of Repli-ChIP in determining temporal TF binding changes.

Faculty Mentor

Statement to come.

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Introduction

Protein-protein and protein-DNA interactions are involved in several key cellular processes. Transcription factors are proteins that regulate DNA transcription by cooperating with other proteins and DNA regions, typically promoter sites (Spitz and Furlong, 2012). TFs commonly have motifs, or short nucleotide sequences, that they bind with strong affinity.

Binding motifs have been noted for various TFs in human cell lines (Lambert, 2018), but the temporal nature of TF binding has not been well studied. Therefore, we focused on investigating the temporal aspects of DNA-protein interactions during DNA replication.

Protein binding is largely dependent on the epigenetic nature of the chromatin landscape (Bach and Hegde, 2016). Chromatin Immunoprecipitation (ChIP) is currently the standard method of mapping DNA-protein interactions within the genome (Furey, 2012). Immunoprecipitation uses antibodies that bind to specific proteins to separate protein-DNA complexes from the rest of the chromatin in the cell nucleus. After DNA is separated from proteins, DNA sequencing provides high read counts, or strings of nucleotides, for DNA loci corresponding to protein binding. In addition, the time at which proteins bind post-DNA replication can also be deduced from analyzing sequenced DNA. Protein binding affinity can be measured by the number of ChIP-seq reads, or depth, for particular DNA regions.

We sought to develop a workflow, Repli-ChIP, that integrates ChIP and BrdU labeling to facilitate the placement of DNA-protein interactions in replication time. BrdU

(5-bromo-2'-deoxyuridine) is an analog of Thymidine that incorporates into newly synthesized DNA. Repli-ChIP aims to extract BrdU-labeled DNA bound to transcription factors through a series of experimental steps. The first assay, BrdU pulse-chase, was performed to specifically label nascent DNA with BrdU. BrdU treatment was performed to observe the loci of TF binding at different replication time points in order to infer binding duration. Transcription Factor-ChIP (TF-ChIP) was subsequently used to isolate TF-DNA complexes at the beginning (0-hour) and end (16-hour) of replication. BrdU-IP was used to recover nascent DNA from TFs using

anti-BrdU antibody. Quantitative methods such as Qubit and qPCR were used to determine and compare the amount of DNA bound to TFs. BrdU and TF-ChIP DNA may be sequenced in order to validate TF binding across all motifs in the genome.

Studying temporal TF-DNA interactions is important in identifying the relationship between TF activity and gene expression over the span of replication. Regulation of TF activity is critical in the transition from the pluripotent to differentiated cell state (Tsankov et al., 2015). Understanding the temporal changes in TF activity that occur in the conversion of normal to cancerous cells may reveal useful information about stages of cancer development. Due to the possibility of several such applications, Repli-ChIP has the potential to facilitate important discoveries in various biological contexts.

In this study, we used the NANOG transcription factor to demonstrate the feasibility and validity of Repli-ChIP and lay the experimental foundation for determining temporal TF activity in hESCs. NANOG has been shown to play an important role in maintaining pluripotency in hESCs (Pan and Thomson, 2007), making it a suitable candidate for Repli-ChIP. We hypothesized that Repli-ChIP could be a useful method to reveal differential NANOG binding at 0-hour and 16-hour replication time points.

Materials and Methods

Cell Culture

Human Embryonic Stem Cells (HUES64) were used in BrdU treatment and ChIP experiments. The hESCs were grown in Geltrex (Thermo Fisher Scientific). Cells were fixed in 4% PFA and stored in -80ºC prior to preparation for ChIP and BrdU treatment.

BrdU Pulse-Chase

BrdU (BD Biosciences) was used to label nascent DNA strands in pulse-chase fashion in proliferating hESCs. After a 1-hour pulse period, the media was changed to remove the presence of BrdU and the cells were washed and collected at 0-hr and 16-hr time points. Cells were resuspended in PBS and fixed in 4% PFA and stored at -80ºC prior to ChIP. Cultured cells were aliquoted into tubes of 10 million cells each.

Chromatin Immunoprecipitation

A stock of hESCs (15 million cells) was aliquoted into five samples containing three million cells per immunoprecipitation (IP) sample. Chromatin fragmentation was carried out by high frequency ultrasound sonication. For standard ChIP (short-read), each sample underwent sonication with the settings at 40% amplitude and a total sonication time

of 8 minutes divided into two rounds of 4-minute sonication (0.7s on, 1.3s off). Each IP sample received 1.5mg of the target protein antibody or rabbit anti-IgG antibody (ThermoFisher). Human anti-NANOG antibody was purchased from R&D systems. Whole cell extract (WCE) samples did not receive antibody treatment. Immunoprecipitated samples were purified and eluted by AMPure XP beads after overnight incubation. DNA extraction was performed using acid-base extraction with chloroform. Equal volume phenol chloroform was added to each sample and the top layer was removed. Further extraction was performed using chloroform. Samples were washed with 70% ethanol and 1X TE buffer was used to elute DNA.

BrdU-IP

Three samples each of 0-hr and 16-hr-treated BrdU cells were thawed on ice in preparation for ChIP. Sonication was carried out with 40% amplitude and a total time of 4 minutes (0.7s on, 1.3s off). Both 0-hr and 16-hr samples were treated with Human anti-NANOG antibody (R&D Systems), and the 0-hr and 16-hr control samples were treated with IgG.

Double-antibody immunoprecipitation protocol as in Charlton et al. (2018) was used to retrieve nascent ssDNA from each IP sample using mouse anti-BrdU (BD Biosciences pharmingen). Nascent DNA was incubated with anti-BrdU antibody overnight at 37ºC and purified using phenol chloroform extraction.

PCR amplification

PCR amplification of the BrdU-IP DNA was conducted to verify the presence of the NANOG binding site on the gene LHFP. Phusion High-Fidelity polymerase mix (New England Biolabs) was used as part of a total reaction volume of 25uL. Each PCR reaction contained an equal input volume of 1uL DNA. 2%-Agarose gel was used in gel electrophoresis. Quantitative analysis of PCR gels was conducted using ImageJ.

Quantitative PCR (qPCR)

Quantitative PCR was used to determine the enrichment of NANOG binding. Known binding sites were amplified in immunoprecipitated DNA along with non-binding sites. Relative amplification, or enrichment, of binding site compared to non-binding site was calculated by using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001). These quantified fold enrichment levels reflect the degree of protein binding at specific loci. The SsoAdvanced SYBR Green Supermix (Bio-Rad) was used in each qPCR reaction. Each reaction mix consisted of 20uL in total with the same amount of template DNA (ug) used in each reaction. Primers were designed using the Primer-BLAST (NCBI) tool. Transcription factor binding sites were located using previously published ChIP data visualized in IGV (Integrative Genomics Viewer).

ChIP data accession number: NANOG: GSM1124070

Table 1:

qPCR primer regions

Positive region: previously established binding site
Negative region: non-binding site

	Positive (+) region	Negative (-) region
NANOG	*LHFP*	*ZWINT*

Table 2:

qPCR primers: Positive and negative primer sets used in qPCR

	Forward (+)	Reverse (+)	Forward (-)	Reverse (-)
NANOG	ATAAGAACCT GGG GCTGG GAA	CTTGGCAGG AGCTGAATT GT	GACAGGGA ACATCCTTA CCTCC	GATGAGAG CGAGCCTT TCAGA

Table 3:

Antibodies used in ChIP experiments

Antibodies	**Source**	**Catalog Number**
NANOG	R&D Systems	AF1997
Anti-rabbit IgG	Thermo Fisher Scientific	A11035

Results

A modified ChIP workflow to isolate nascent DNA following BrdU pulse-chase

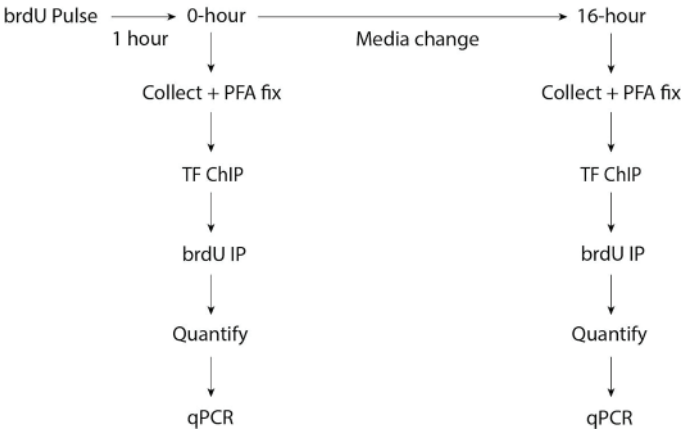

Figure 1:

The sequence of experimental steps performed to isolate nascent DNA, termed Repli-ChIP.

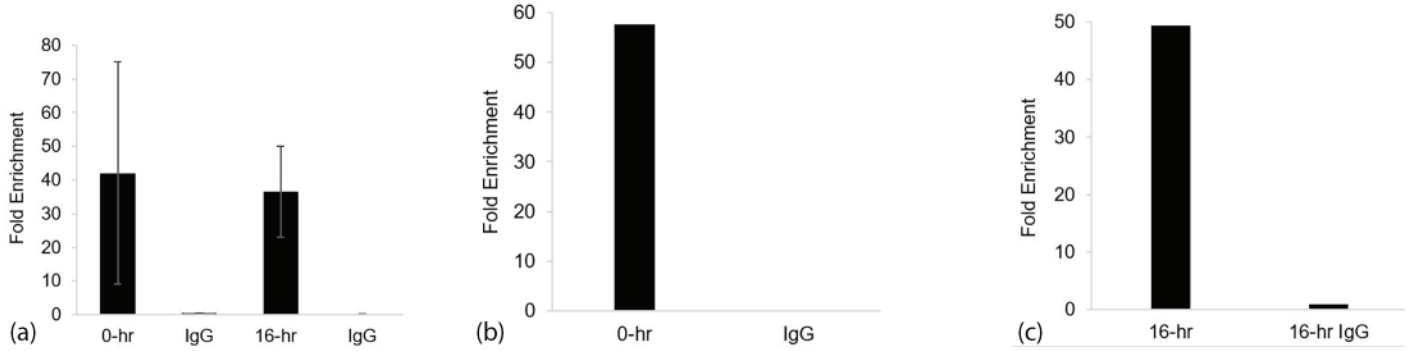

Figure 2:

(a) Quantitative PCR shows a high enrichment of the NANOG binding site in the LHFP gene across samples of the 0-hr (n=3) and 16-hr (n=3) time point conditions in hESCs. The 0-hr condition (42.0+33.0) had a 14.8% greater enrichment than the 16-hr condition (36.6+13.4) but not significantly (p=0.742) (unpaired t-test). Average enrichment in the 0-hr condition (42.0+33.0) was greater than the 0-hr IgG (0.4+0.0). Average enrichment in the 16-hr condition (36.6+13.4) was greater than the 16-hr IgG (0.3+0.0). (b) and (c) Quantitative PCR shows an overall high enrichment of LHFP for NANOG in aggregate 0-hr and 16-hr samples. The 0-hr and 16-hr conditions both showed enrichment greater than their respective IgG samples in hESCs.

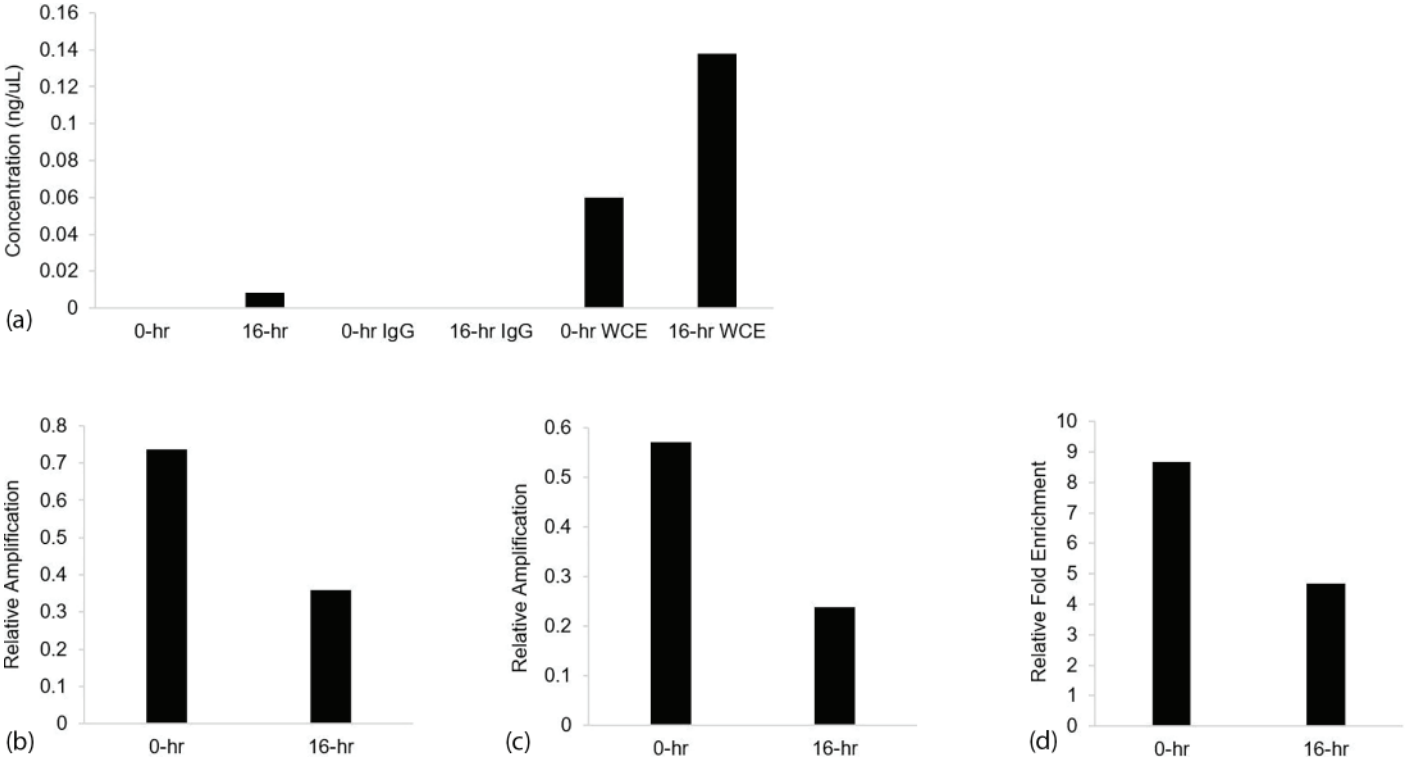

Figure 3:

(a) The detectable Qubit DNA concentrations (ng/uL) of samples recovered from BrdU immunoprecipitation. The 0-hr, 0-hr IgG, and 16-hr IgG samples showed no detectable concentration of immunoprecipitated DNA. The 0-hr (0.060) and 16-hr (0.138) whole cell extract (WCE) samples showed greater DNA concentrations than the 16-hr condition (0.008). (b) The relative PCR amplification of LHFP in 0-hr and 16-hr BrdU IP conditions over respective (0-hr and 16-hr) WCE conditions. The 0-hr sample (0.736) (n=1) showed a 105.6% greater relative amplification than the 16-hr sample (0.358) (n=1). (c) The relative amplification of the NANOG negative region (ZWINT) in 0-hr and 16-hr BrdU IP samples over respective (0-hr and 16-hr) WCE conditions. The 0-hr sample (0.570) (n=1) showed a 139.5% greater relative amplification than the 16-hr sample (0.238) (n=1). (d) Quantitative PCR shows the relative fold enrichment of LHFP in the 0-hr and 16-hr BrdU IP samples over the fold enrichment of their respective (0-hr and 16-hr) IgG conditions. There was a 46% decrease in the enrichment of nascent LHFP in the 16-hr sample (4.7) (n=1) compared to the 0-hr sample (8.7) (n=1).

Short-read NANOG ChIP indicates significant NANOG binding at early and late replication stages

BrdU pulse-chase was used to locate and isolate nascent DNA in proliferating hESCs at specific time points. TF-ChIP at 0-hr and 16-hr time points quantified NANOG binding. TF-ChIP qPCR (Figure 2a) showed that NANOG binding at LHFP at the 0-hr condition (42.0+33.0) was similar to the 16-hr condition (36.6+13.4) ($p=0.742$) (unpaired t-test). The 0-hr and 16-hr conditions had greater enrichment values than the 0-hr and 16-hr IgG conditions, respectively. Concentrations of individual 0-hr and 16-hr samples were too low for future sequencing preparation, so these samples were combined to create one 0-hr and one 16-hr sample.

Quantitative PCR was performed on these aggregate samples to verify proper enrichment (Figures 2b and 2c) similar to the previous step (Figure 2a). The qPCR results showed that the 0-hr and 16-hr conditions had high fold enrichment, both greater than their respective IgG conditions. The IgG condition exhibited very little enrichment in all BrdU experiments. From these results alone, it is not possible to determine whether NANOG has sustained or fluctuating binding characteristics over the 16-hr time period. Using more time points between the 0-hr and 16-hr time points may provide higher resolution in dynamic NANOG binding during replication.

BrdU immunoprecipitation indicates increased NANOG binding to nascent DNA early in replication

BrdU immunoprecipitation (BrdU IP) was carried out to selectively extract BrdU-labeled DNA from the 0-hr and 16-hr samples. Normally, TF-ChIP is unable to differentiate protein binding of nascent DNA from parent DNA, whereas BrdU IP provides information on nascent DNA specifically. The amount of DNA recovered from BrdU IP was believed to reflect the abundance of NANOG binding. Qubit was used to calculate the detectable DNA concentration (ng/uL) across all samples of equal volume. Qubit data (Figure 3a) showed detectable concentrations of 0-hr (0.060) WCE, 16-hr (0.008), and 16-hr WCE (0.138) conditions. The 0-hr, 0-hr IgG, and 16-hr IgG samples showed no detectable DNA concentration. DNA samples may contain NANOG binding sites other than LHFP, so the concentrations do not necessarily reflect relative binding to LHFP alone. Higher DNA concentration in the 16-hr WCE compared to the 0-hr WCE may be the result of more replicated DNA present at the time of collection. PCR amplification of LHFP was used to determine amplification relative to WCE conditions (Figure 3b). Amplification in the 0-hr and 16-hr conditions were normalized to their respective WCE conditions. The 0-hr condition (0.736) showed a greater relative amplification than the 16-hr sample (0.358). Since there was greater relative amplification in the 0-hr condition, this indicates a higher level of NANOG binding to LHFP at the 0-hr time point. PCR amplification of ZWINT (NANOG non-binding site) was conducted to determine amplification relative to WCE (Figure 3c). After normalization to WCE, the 0-hr sample (0.570) showed a greater relative amplification than the 16-hr sample (0.238). Quantitative PCR of the BrdU IP samples was conducted to obtain more accurate enrichment of LHFP. Fold enrichment of 0-hr and 16-hr conditions was normalized to their corresponding IgG conditions (Figure 3d). The 0-hr (8.7) showed greater relative fold enrichment than the 16-hr condition (4.7). The greater relative enrichment in the

0-hr condition compared to the 16-hr condition indicates preferentially higher NANOG binding at the 0-hr time point. These results are consistent with the higher relative amplification of LHFP in the 0-hr condition (Figure 3b). Differing qPCR enrichment values of LHFP between the 0-hr and 16-hr conditions reflect time-dependent binding tendencies of NANOG.

Discussion

Short-read NANOG ChIP experiments validated the Repli-ChIP approach for studying TF-bound DNA (Figure 1). NANOG enrichment at early (0-hr) and late (16-hr) replication time points supported the hypothesis (Figure 2). Strong NANOG enrichment in hESCs is supported by previous studies describing its integral role in maintaining stem cell pluripotency (Boyer et al., 2005). NANOG enrichment should be analyzed at other binding sites to gain a more comprehensive view of overall NANOG activity early and late in replication. Additionally, the expression of NANOG target genes may be affected by NANOG binding changes throughout replication. BrdU DNA quantification demonstrated the ability to compare TF binding to nascent DNA at different replication time points (Figure 3). Although NANOG was shown to preferentially bind early in replication, its non-binding site ZWINT was unexpectedly also present post-BrdU IP (Figure 3c). This may indicate non-target binding by NANOG or ZWINT contamination in the BrdU immunoprecipitation step. Nonetheless, NANOG TF-ChIP and subsequent BrdU IP results (Figures 2 and 3) imply that NANOG may be consistently expressed and active throughout replication in hESCs. This may also suggest that TFs associated with NANOG (Boyer et al., 2005) are upregulated at corresponding replication phases. In this deductive manner, the binding duration of TFs in the same

regulatory networks can be studied. The implication that NANOG is active throughout replication can be validated by BrdU IP extraction at multiple time points throughout replication. This would also allow analysis of NANOG binding across motifs other than LHFP.

Furthermore, other information such as epigenetic modifications can be obtained from studying TF-ChIP DNA. For example, DNA methylation, the addition of a methyl (-CH3) group most commonly to adjacent Cytosine-Guanine (CpG) nucleotides, can be detected from DNA sequencing. DNA methylation plays a crucial role in modulating the expression of genes, a process known as epigenetic regulation. DNA methylation, in turn, impacts TF binding. CpG methylation data from TF-bound DNA may elucidate the relationship between TF binding affinity and motif DNA methylation. Although studies have explored TF motifs and their DNA methylation (Héberlé et al., 2019), a well-defined relationship has not been thoroughly characterized in hESCs so far.

Previous literature on the role of NANOG in pluripotency has not deeply explored its temporal activity states, which may provide further insight into how pluripotency is maintained across generations of hESCs. Repli-ChIP offers a way to study protein-DNA interactions in many potential contexts. Specific genes may be investigated in how temporal changes in TF binding correlate to gene expression. In the context of differentiation, TF binding changes that occur in order to achieve various cell fates can be monitored. The potential implications of Repli-ChIP highlight its importance in characterizing protein-DNA relationships.

Acknowledgements

This research was supported by funding from Dr. Timothy L. Downing and UROP (Undergraduate Research Opportunities Program). BrdU pulse-chase experiments were conducted with help from Ph.D. candidates Navied Akhtar and Julien Morival of the Downing Lab.

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