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A Scalable System for Identifying Changing Patterns of DNA-Binding Proteins During Stem Cell Differentiation

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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2013
The Thesis of Erica Hsiao is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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2013
# TABLE OF CONTENTS

Signature Page ........................................................................................................ iii

Table of Contents .................................................................................................. iv

List of Abbreviations ............................................................................................ v

List of Figures ........................................................................................................ vi

Abstract .................................................................................................................. vii

1. Introduction ......................................................................................................... 1

2. Materials and Methods ...................................................................................... 5
   2.1. Cell culture and differentiation ................................................................. 5
   2.2. Protein extraction ...................................................................................... 5
   2.3. RNA extraction ......................................................................................... 6
   2.4. Quantitative PCR ...................................................................................... 6
   2.5. DNA oligonucleotides construction .......................................................... 6
   2.7. Electrophoretic mobility shift assay (EMSA) ........................................... 7

3. Results ................................................................................................................ 8
   3.1. Retinoic acid can induce neuronal differentiation in mouse ES cells, increase Pax6 activities, and decrease Nanog and Oct4 expressions ................................................................. 8
   3.2. Construction of end-labeled DNA probes ............................................... 8
   3.3. EMSA displays different protein binding patterns between ES and differentiated nuclear lysate with various oligonucleotides ..... 9
   3.4. DNA-protein complexes formation is dependent on monovalent salt, divalent salt, and glycerol concentration ................................................................. 10
   3.5. Labeling with Click-iT® EdU or ethidium bromide ................................ 12
   3.6. Protein-oligonucleotide complex formation is sequence specific for Oct4 oligonucleotides ................................................................. 13
   3.7. Confirmation of the protein-DNA identity through EMSA super shift assay with OCT4, SOX2, NANOG, and KLF4 antibodies .... 13

4. Discussion ......................................................................................................... 15

5. References ....................................................................................................... 25
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
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<tr>
<td>EMSA</td>
<td>Electrophoresis mobility shift assay</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
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<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>Mut</td>
<td>Mutant</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
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<td>PBS</td>
<td>Phosphoate-buffered saline</td>
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<tr>
<td>TBE</td>
<td>Tris/borate/EDTA</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Retinoic acid cultured cells are relatively differentiated in comparison to ES cells................................. 18

Figure 2. Construction of end-labeled DNA EMSA probes................................. 19

Figure 3. Changes in protein binding landscape between ES and differentiated cells with OCT4, SOX2, NANOG, or KLF4 binding sequences..................................................... 20

Figure 4. The effect of monovalent salt, divalent salt, and glycerol concentration on protein-DNA complex formation................................. 21

Figure 5. The efficiency of Click-iT® labeled oligonucleotides................................. 22

Figure 6. Binding specificity of protein to Oct4 oligonucleotides................................. 23

Figure 7. Super shift assay with OCT4, SOX2, NANOG, and KLF4 antibodies................................. 24
ABSTRACT OF THE THESIS

A Scalable System for Identifying Changing Patterns of DNA-Binding Proteins During Stem Cell Differentiation

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Master of Science in Biology

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Professor Benjamin D. Yu, Chair

Professor James T. Kadonaga, Co-Chair

Transcription factors determine cell fate during differentiation and are capable of reprogramming distinct cell types. They are regulated by multiple mechanisms, many of which control their stability and their ability to bind to DNA. Although a variety of approaches allow for the detection of the changing landscape of transcription factors during differentiation, the majority of these screening approaches, eg. microarray, RNA sequencing, quantitative PCR, western blot, do not address whether the detected transcription factors are stable or even capable of binding to DNA. Moreover, antibodies are not available or are insufficient at
characterizing thousands of different transcription factors. New advances have been made in nucleotide detection and these methods include end-labeled fluorescent dyes. Using this technology, we proposed an alternative electrophoretic mobility shift assay (EMSA) protocol using the Odessey® imaging system to supplement the shortcomings of the previously mentioned techniques. By using EMSA oligonucleotides with end-labeled fluorescent tags that can be visualized at a wavelength of 700nm with the Odessey® imaging system, results can be observed within three hours. This protocol provides a potentially scalable platform to screen for novel transcription factor binding profiles including those that function in embryonic stem cells or other cell lineages.
1. Introduction

Embryonic stem cells (ESCs), derived from the inner cell mass (ICM) of mammalian blastocysts, have the ability to differentiate into various cell lineages (Loebel et al., 2003). This pluripotency of ESCs is maintained and even regained from differentiated cells by a network of transcription factors such as OCT4, SOX2, KLF4, and NANOG (Kim et al., 2008; Takahashi and Yamanaka, 2006; Rodolfa et al., 2006; Loh et al., 2006). By binding to specific DNA consensus sequences, these transcription factors repress and activate transcription and alter gene expression profiles that result in phenotypic changes (Niwa et al., 2000). By understanding the binding pattern of these transcription factors, we can use them as biological markers to distinguish ESCs from differentiated cells and to categorize various cell types.

Transcription factors are regulated by several transcriptional, translational, and post-translational mechanisms that affect their stability and ability to bind to DNA (Whitmarsh and Davis., 2000; Lackner and Bahler., 2008). A majority of the approaches used to detect the changing landscape of transcription factors during differentiation including quantitative PCR, microarray, western blot, and RNA sequencing, do not indicate whether the transcription factors are capable of binding to DNA which is the primary function of transcription factors that leads to phenotypic changes. These approaches suffer from inadequacies that include 1) a lack of high throughput ability which limits the sample size 2) the use of radioactive reagents that also prevent high throughput applications, 3) a lack of sufficient antibodies against the vast number of uncharacterized transcription factors, and 4)
expensive fluorescent probes which often times can limit the scope of an experiment (Kerr., 1995).

Electrophoresis mobility shift assays (EMSA) are an easy and sensitive method for detecting the formation of protein-DNA complexes (Carey et al., 2009). The principle of this method is based on the altering of the mobility of a protein-DNA complexes when compared to the un-complexed molecules often times resulting in a slower migration pattern through a non-denaturing gel matrix (Kerr., 1995). EMSA is a useful method that accommodates a wide range of binding conditions for each protein and nucleic acid sizes, produces rapid results, and avoids the limitations resulting from the use of antibodies (Hellman et al., 2007; Fried et al., 1998).

Several factors in protein binding condition influence the formation of protein-DNA complexes and a successful EMSA result. The formation of protein-DNA complexes is salt dependent (Robidoux et al., 1992). Monovalent and divalent salts can neutralize the charges on protein molecules to induce proper folding and complex interactions (Alves and Cunha., 2012; Laniel et al., 2001). However, overabundance in cations can greatly reduce the negative charges on the DNA phosphate backbone. This decreases the electrostatic attractions between protein and DNA and alters the stability of protein-DNA complexes (Record et al., 1981). Glycerol is a neutral stabilizing osmolyte that can reduce the dissociation rate of protein-DNA complexes (Sidorova et al., 2010). Thus, in this paper, we experimented with various KCl (25mM and 50mM), MgCl₂ (4mM), and glycerol (1%) concentrations to find the most suitable binding condition for OCT4, SOX2,
KLF4, and NANOG (Sidorova et al., 2010; Lane et al., 1992). Furthermore, to avoid the use of radioactive materials, we proposed an alternative EMSA protocol using fluorophore end-labeled oligonucleotides that can be visualized at a wavelength of 700nm with Odessey imaging® system (LiCOR).

These oligonucleotides contain DNA consensus binding sequences for OCT4, SOX2, KLF4, and NANOG flanked by T7 and SP6 promoter sequences. These transcription factors are highly expressed in ESCs but are down regulated in differentiated cells (Pan et al., 2002; Loh et al., 2006; Chew et al., 2005; Zhang et al., 2010). These transcription factors also have known sequences of DNA that they preferentially bind to. OCT4 is known to bind to an octamer sequence ATTTGCAT (Scholer et al., 1989). NANOG optimally binds to 5’-TAAT[GT][GT]-3’ or 5’-[CG][GA][CG]ATTAN[GC]-3’ (GeneCards®) Lastly, the binding sequences for SOX2 and KLF4 are CCTTGTATGCAAA and GGGTGTGGC (JASPAR database)

With this alternative EMSA protocol, the synthesis of EMSA probes is simplified and made economical. Since the binding sequences are flanked by T7 and SP6 promoter sequences, EMSA probes can be made through polymerase chain reaction (PCR) amplification with fluorophore end-labeled T7 and SP6 primers. Overall, this method provides an increasingly efficient technique for synthesizing oligonucleotides for use in EMSA to detect the direct protein-DNA interaction. Additionally, this EMSA protocol eliminates radioactive reagents, and avoids the limitations of using antibodies against the protein of interest and allows for the visualization of results in 3 hours. These advantages make this modified EMSA
protocol faster, safer, and more economical than the creation of signature transcription factor binding profile.
2. Materials and Methods

2.1. Cell culture and differentiation

E14 mouse ES cells were cultured in 0.1% gelatin-coated petri dish with KnockOut DMEM (GIBCO) supplemented with 15% FBS (HyClone Tested Lot), 100x Pen-Strep, 100x Na Glutamine, 100x Na Pyruvate (0.11mg/ml), 100x Non-essential Amino Acids, 50x LIF, and 1000x B-mercaptoethanol (5.5uM). Differentiated cells were cultured in the same media as mES cells with the absence of LIF and the addition of 10uM of retinoic acid. Cells were maintained in respective media at 37°C with 5% CO₂ for 6 days before harvesting.

2.2. Protein extraction

Cells from mES and differentiated culture were treated with trypsin for 5 min and harvested in phosphate-buffered saline (PBS). After centrifugation, cells were vortexed in cytoplasmic lysis buffer (10mM Hepes, pH8, 10mM KCl, 0.1mM EDTA, pH8, 0.1mM EGTA, pH7, 0.5% NP40, 1mM dithiothreitol [DTT], and Complete EDTA-free protease inhibitor cocktail tablet [Roche]), incubated on ice for 15 min, and spun at 8000 rpm for 5 min at 4°C. Nuclear lysates were obtained by re-suspending pelleted nuclei in nuclear lysis buffer (20mM Hepes, pH8, 400mM NaCl, 1mM EDTA, pH8, 1mM EGTA, pH7, 1% NP40, 1mM DTT, and Complete EDTA-free protease inhibitor cocktail tablet [Roche]), incubated on ice for 30 min, and centrifuged at 14000rpm for 10 min.
2.3. RNA extraction

Total RNA was extracted from mES and differentiated cultures by using TRIzol® Reagent RNA isolation protocol (Invitrogen).

2.4. Quantitative PCR

50uL of total RNA extracted from mES and differentiated cultures were treated with 10x DNase I buffer and 0.5uL DNase I (New England BioLabs) for 30 min at 37°C. DNase I was inactivated with 1uL 250mM EDTA and was incubated at 65°C for 10 min. cDNA was made from 1ug of DNase I treated RNA with qScript™ cDNA synthesis kit from Quanta Biosciences™. 2.5uL cDNA (1:10 dilution) was analyzed by LightCycler® 480 II (Roche) with 2x SYBR Green (Fermantas) and 2uL of 1pmoles primer mix for specific marker genes in 10uL of reaction. Each marker gene was run in triplicates. PCR was performed in a cycle of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec.

2.5. DNA oligonucleotides construction

DNA oligonucleotides were amplified through PCR with Accupower® HotStart PCR premix (Bioneer), 10pmol of template oligonucleotides, 10pmol of T7 and SP6 fluorophore end-labeled primers, and 17uL of DNase/RNase free water in 20uL of reaction. Amplified oligonucleotides were purified with QIAEX II Gel Extraction Kit (Qiagen).

Click-iT® oligonucleotides were synthesized through PCR with Taq DNA polymerase (NEB), 10pmol of template oligonucleotides, 10pmol of unlabeled T7
and Sp6 primers, 2.5mM nucleosides, and 2.5mM 5-ethynl-2’-deoxyuridine (EdU). Synthesized oligonucleotides were purified with QIAEX II Gel Extraction Kit (Qiagen), activated with Alexa Fluor®647 azide according to Click-iT® EdU HCS Assays, and purified again before loading on to agarose gel or used in EMSA binding reactions.

2.6. Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed with 10mM Tris, pH 7.5, 1mM DTT, 1ug polydI/dC (Thermo Scientific), 80nM DNA oligonucleotides, 10ug-20ug nuclear lysate in 20uL of reaction. Extra 2.5mM DTT and 0.25% Tween20 were added to each reaction to stabilize the fluorescent signals on the oligonucleotides as indicated in LiCOR EMSA protocol. KCl and MgCl₂ concentrations vary within each experiment. Electrophoresis was run in a 4% polyacrylamide gel (5mL 40% polyacrylamide stock, Polyacrylamide-BIS ratio = 29:1, 2mL 1M Tris, pH 7.5, 7.6mL 1M Glycine, 160uL 0.5M EDTA, 26mL H₂O, 200uL 10% APS, and 30uL TEMED in 40mL mix). The gel was first pre-run in 1xTBE at 100V for 20min. After loading the samples, the gel was run at 100V for 20min then 120V for another 20min or until the running dye reach the bottom ¼ of the gel cassette. The gel was removed from the cassette and was visualized with Odyssey® imaging system 700nm.

Super shift assay was performed with 1ug of OCT4 antibody (SC-9801), SOX2 antibody (SC-20088), NANOG Alexa Fluor® 488 antibody (eBioscience), and KLF4 antibody (SC-20691).
3. Results

3.1. Retinoic acid can induce neuronal differentiation in mouse ES cells, increase Pax6 activities, and decrease Nanog and Oct4 expressions

Previous studies showed that ES cells, when cultured with retinoic acid (RA) and without LIF, would predominantly differentiate into neuronal lineages with increasing activity from a neuroectoderm marker, Pax6. (Gajovic et al., 1997). Thus, to verify that the RA cultured cells were relatively differentiated in comparison to ES cells, we examined RNA expressions of Pax6, Nanog, and Oct4 in both ES and differentiated cultures by qPCR. Upon differentiation, expression of Pax6 was up-regulated approximately 9-fold whereas Nanog and Oct4, markers for ES cells, were down-regulated by around 25 folds (Fig. 1).

3.2. Construction of end-labeled DNA probes

Template oligonucleotides with consensus binding sequences for targeted protein flanked by T7 and SP6 promoter sequences were synthesized. Following the PCR cycling protocol starting with a denaturing step at 95°C for 1 min, followed by 25 cycles of 95°C for 10 sec, 40°C for 20 sec, and 72°C for 20 sec, and a final extension time of 72°C for 3 min with fluorophore end-labeled T7 and SP6 primers, double stranded oligonucleotides were synthesized with fluorophore tagged at the ends of T7 and SP6 promoter sequences (Fig. 2).
3.3. EMSA displays different protein binding patterns between ES and differentiated nuclear lysate with various oligonucleotides

To examine whether EMSA is capable of detecting the changing landscape of protein binding patterns between ES and differentiated (RA) nuclear lysate, experiments were performed with 10x binding buffer (50mM KCl), 10ug and 20ug nuclear lysate, and 80mM end-labeled oligonucleotides with OCT4, SOX2, NANOG, and KLF4 consensus binding sequences. Lanes 1, 4, 7, 10, 13, 16, 19, and 22 with 0ug of nuclear lysate were used as negative controls to eliminate complexes formed by molecules in binding reagents or by oligonucleotides aggregation (Fig. 3).

In reactions with ES nuclear lysate Oct4, Sox2, and Nanog oligonucleotides, one major complex and a minor complex around the same size were formed creating a doublet (Fig. 3, lane 3, 9, and 15). The smaller complex in the doublet was determined to be the result of protein degradation since its intensity increased with increasing number of lysate freeze and thaw cycles. With Klf4 oligonucleotides, only a singlet was observed (Fig. 3, lane 20 and 21). When comparing the band intensities with oligonucleotides in ES nuclear lysate, the complexes with Oct4 oligonucleotides were the most abundant, followed by Sox2, Nanog, and the weakest in Klf4 (Fig. 3, lane 3, 9, 15, and 21). The band intensities can be used as a measure of the relative protein expression levels in ES lysate.

As cells differentiate (RA), a two to three folds decrease in doublets and singlet band intensity can be observed (Fig. 3, lane 6, 12, 18, and 24). Moreover, with Oct4 oligonucleotides, six more complexes that migrated faster than the doublet were observed with differentiated nuclear lysate. All of these complexes were visualized
with stronger intensities in comparison to ES lysate (Fig. 3, lane 2, 3, 5, and 6). In the case of Sox2, a very intense signal from a smaller complex was visualized only in differentiated lysate (Fig. 3, lane 11 and 12).

Several other smaller complexes were also formed with Nanog ES and differentiated nuclear lysate. But the locations of these complexes were too low to be the protein of interest (Fig. 3, lane 14, 15, 17, and 18).

Although the identities of these complexes are not yet determined, there are clear differences in protein binding patterns between ES and differentiated cells.

3.4. DNA-protein complexes formation is dependent on monovalent salt, divalent salt, and glycerol concentration

Concentrations of salt and other components used in DNA-protein binding buffers are critical for proper complex formation. Additionally, these conditions are specific for the proper folding of each protein. EMSA was performed with five different binding buffers (buffer A-E) to determine the most favorable binding condition for each oligonucleotide. In 20uL reaction, these buffers contain 10mM Tris, pH 7.5, with various concentrations of monovalent salt (25mM or 50mM of KCl), divalent salt (4mM of MgCl₂), and glycerol (1%). 20uL of indicated nuclear lysate and 80mM of oligonucleotides were used in each reaction.

The protein-Oct4 oligonucleotides complex (doublet) is most stable in ES nuclear lysate with 50mM of KCl (Fig. 4A, lane 3). As the KCl concentration decreased to 25mM, the doublet intensity also decreases (Fig. 4A, lane 4). After the addition of 4mM of MgCl₂, only the lower band in the doublet can be seen (Fig. 4A,
lane 4). When these results are compared with ES nuclear lysate, there is approximately a 2 to 5 folds decrease in the band intensities in RA induced differentiated nuclear lysate (Fig. 4A, lane 2-12).

For Sox2 oligonucleotides, the result is similar. The most apparent band is observed in ES nuclear lysate with 50mM of KCl. The band intensity decreases with the addition of 4mM of MgCl₂ and further decreases with 1% glycerol (Fig. 4B, lane 1-6). With differentiated nuclear lysate, the upper band intensity was decreased by approximately 2-fold (Fig. 4B, lane 6-12).

In terms of Nanog, a doublet is only observed with 50mM of KCl (Fig. 4C, lane 3). With 25mM KCl only the upper band is visible while the addition of 4mM of MgCl₂ only allowed the lower band to be seen. The bands disappear with differentiated nuclear lysate (Fig. 4C, lane 6-12).

Lastly, with Klf4, only a very faint band was observed in ES nuclear lysate with 50mM KCl (Fig. 4D, lane 3). No band at the same position was observed with differentiated nuclear lysate (Fig. 4D, lane 6-12).

These results suggest that the addition of 4mM of MgCl₂ in the binding reaction increases the accumulation of smaller protein-oligonucleotide complexes in the gel (Fig. 4A-D, lane 11-12) while glycerol decreases total band intensity (Fig. 4A-D, lane 6 and lane 12).

Overall, 50mM of KCl in the binding buffer produced the strongest signal in all oligonucleotides with ES nuclear lysate and appears to be the ideal salt concentration to perform further EMSA experiments.
3.5. Labeling with Click-iT® EdU or ethidium bromide

When comparing to radioactive probes, fluorophore end-labeled oligonucleotides have weaker signals. It is possible that proteins of interest that were bound to fluorophore end-labeled oligonucleotides did not produce a sufficient signal to be detected by Odyssey® Imaging System (Li-COR Biosciences). Thus, several different labeling techniques such as Click-iT® Edu Alexa Fluor® HCS Assay and ethidium bromide were utilized to increase the number of labeled nucleotides in the probes to intensify the resulting signals.

Click-iT® EdU Alexa Fluor® HCS Assay labels newly synthesized DNA with 5-ethynyl-2’deoxyuridine (EdU), a thymidine nucleoside analog. EdU contains an alkyne that can be activated by azide from the Alexa Fluor® 647 dye though a copper catalyzed covalent reaction. After PCR amplification with unlabeled T7 and Sp6 primers, EdU was successfully incorporated into EMSA oligonucleotides and activated for visualization with Alexa Fluor® 647 prior to the purification process. After purification, 50ng Click-iT® labeled Oct4 oligonucleotides were used in EMSA with a 50mM KCl binding buffer and 10 or 20ug of ES nuclear lysate. No protein-DNA complex was observed (Fig. 5A). Experiments with 100ng of end-labeled purified probe, Click-iT® unpurified probe, and Click-iT® purified probe on a 2% agarose gel detected by Odyssey® Imaging System showed that oligonucleotides were lost after purification with QIAEX II gel extraction kit (Qiagen) (Fig. 5B, lane 3).
Ethidium bromide staining was done after EMSA. 4% polyacrylamide gel was separated from the cassette, incubated with 5uL of ethidium bromide at room temperature for 30min, and visualized with UV light. The whole gel was stained.

3.6. Protein-oligonucleotide complex formation is sequence specific for Oct4 oligonucleotides

Binding specificity of protein to Oct4 oligonucleotides is determined by reacting nuclear lysate with probes that were mutated with two nucleotides in Oct4 consensus binding sequences (Mut). Lanes with no nuclear lysate added were used as negative controls (Fig. 6, lane 1, 4, 7, and 10). Once the consensus sequence was mutated, the upper band in the doublet disappeared and the lower band decreased by 10-fold (Fig. 6, lane 2, 3, 5, and 6). This mutation is critical to the protein-oct4 oligonucleotides complex formation.

3.7. Confirmation of the protein-DNA identity through EMSA super shift assay with OCT4, SOX2, NANOG, and KLF4 antibodies

Antibodies, when bound to the protein-DNA complex, will increase the size of the complex and slow its migration through a gel. This phenomenon called super shift is commonly used to identify proteins that bind to oligonucleotides. In this experiment, EMSA was performed in 50mM KCl binding buffer with Oct4, Sox2, Nanog, and Klf4 antibodies in ES and differentiated nuclear lysate. Lane 4 and lane 9 in figure 7 A-D served as negative controls to eliminate the bands formed by
antibodies and oligonucleotides. No super shift was observed with any of the oligonucleotides (Fig 7, A-D).
4. Discussion

An efficient assay that detects the changing landscape of transcription factor binding patterns in ESCs and different cell lineages is a powerful tool that has been lacking. Electrophoretic mobility shift assay (EMSA) detects the direct interaction between proteins and DNA and can potentially fill this void. Although EMSA is usually radioactive, non-radioactive oligonucleotides containing consensus binding sequences for proteins and fluorescent end-labeled dyes can be used with Odyssey® Imaging system to visualize the protein-DNA complexes using an excitation wavelength of 700nm. This alternative method eliminates the safety hazard of using radioactive reagents and allows oligonucleotide synthesis to be more economical.

The stability of protein-oligonucleotide complex formation is influenced by various monovalent salt, divalent salt, and glycerol concentrations in binding conditions. With Oct4, Sox2, Nanog, and Klf4 oligonucleotides, the most stable complexes were formed in the binding buffer with 50mM KCl where changes in protein binding patterns between ES and differentiated lysate were observed (Fig. 3 and 4). Using mutated Oct4 oligonucleotides, we showed that the complex formation was specific to Oct4 consensus binding sequences (Fig. 6).

However, all the complexes, regardless of the differences in protein size, shared the same mobility and were not super shifted by OCT4, SOX2, NANOG, and KLF4 antibodies (Fig. 7). This data suggest that proteins other than OCT4, SOX2, NANOG, and KLF4 formed the observed complexes. To identify these unknown proteins, super shift assay can be done with antibodies for proteins that have been shown to associate with the same
consensus binding sequences as OCT4, SOX2, NANOG, and KLF4 in previous studies. This experiment might give us insights into what other protein or proteins have the ability to bind to the same consensus sequences and influence the process of differentiation and cell lineage determination.

It is also plausible that the antibodies used were unable to recognize their respective proteins or were denatured by the high concentrations of dithiothreitol (DTT), a denaturant, in binding buffer (Okuno and Kondelis., 1978). Further experiments, eg. western blots, can be performed to investigate the ability of antibodies to recognize the proteins of interest followed by EMSA with a decreasing gradient of DTT to determine the threshold concentration of DTT for proper antibody activities.

Alternative oligonucleotides labeling methods with Click-iT® or ethidium bromine were not able to increase the signal intensities or provide a better visualization of DNA-protein complexes in polyacrylamide gels. The addition of azide into the oligonucleotides during “activation” can interfere with the binding of DNA to QIAEX II beads. As a result, Click-iT® probes were lost during oligonucleotides purification with QIAEX II gel extraction (Fig. 6). Alternative purification process must be used to avoid the lost of Click-iT® probes.

One major challenge during the process of determining the most favorable binding condition for increased signal intensities, and protein identification was the inconsistency in EMSA results and the lack of a positive control. If EMSA was performed with recombinant or purified OCT4, SOX2, NANOG, and KLF4, the identity of the protein in protein-DNA complex could be established and the most
favorable binding condition for each protein identified. As for signal intensities, radioactive probes can be used as a supplement to fluorescent end-labeled oligonucleotides to eliminate the possibility of signals given by protein-DNA complexes that are too weak for visualization.

In conclusion, changes in protein binding patterns to Oct4, Sox2, Nanog, and Klf4 oligonucleotides can be observed between ES and differentiated cells. Moreover, the binding of protein to Oct4 oligonucleotides is specific to OCT4 consensus binding sequences. If further experiments can be done to confirm the identity of the complex or to increase non-radioactive signal intensities, this alternative EMSA protocol can provide an efficient and affordable method for gaining insight into cell lineage determinations.
Figure 1. Retinoic acid cultured cells are relatively differentiated in comparison to ES cells. qPCR: The addition of retinoic acid and the deletion of LIF in cell culturing media result in ~25 folds decrease in Oct4 and Nanog expressions and ~9 folds increase in Pax6 expression. RNA samples were collected from ES and differentiated (RA) cells after 6 days of culturing.
Figure 2. Construction of end-labeled EMSA probes. Template oligonucleotides were synthesized by flanking protein consensus binding sequences with T7 and Sp6 promoter sequences. Through polymerase chain reaction (PCR) with fluorophore labeled T7 and Sp6 primers, double stranded, end-labeled DNA EMSA probes were made.
Figure 3. Changes in protein binding landscape between ES and differentiated cells with OCT4, SOX2, NANOG, or KLF4 consensus binding sequences. 10ug and 20ug of ES or differentiated (RA) nuclear lysate was used with each oligonucleotide. With Oct4, Sox2, and Nanog oligonucleotides, a doublet was observed (arrow). However, with Klf4 oligonucleotides, there was only one complex (arrow). As cells differentiate, the intensities of these doublets and singlets were decreased by 2 to 3 folds. Other smaller additional complexes were also visualized in differentiated lysate. Free oligonucleotides that were not bound by proteins were seen at the bottom of the gel. 0ug of nuclear lysate was performed to eliminate complex formed by the aggregation of oligonucleotides or with other molecules in binding buffer.
**Figure 4.** The effect of monovalent salt, divalent salt, and glycerol concentration on protein-DNA complex formation. EMSA was performed with 20ug of ES and differentiated nuclear lysate and 5 various buffers in each oligonucleotide. Buffer A: LiCOR 10x buffer; Buffer B: 50mM KCl; Buffer C: 25mM KCl; Buffer D: 25mM KCl and 4mM MgCl$_2$; Buffer E: 25mM KCl, 4mM MgCl$_2$, and 1% Glycerol. 0ug of nuclear lysate was used to eliminate formation of complex due to oligonucleotide aggregation or other molecules in binding buffer.
Figure 5. The efficiency of Click-iT® labeled oligonucleotides. (A) 50ng of Click-iT® labeled Oct4 oligonucleotides was used in EMSA reaction with binding buffer containing 50mM KCl and 10ug and 20ug of ES nuclear lysate. No signal was observed except the free incomplete PCR product at the end of the gel. (B) 100ng of fluorophore end-labeled purified probe, Click-iT® unpurified probe, and Click-iT® purified probe was ran on a 2% agarose gel and was visualized by Odyssey® imaging system. After purification, Click-iT® EMSA oligonucleotides was lost.
Figure 6. Binding specificity of protein to Oct4 oligonucleotides. 10ug and 20ug of ES and differentiated (RA) nuclear lysate were used in binding reaction with 50mM KCl and 50ug of wild type (WT) or mutated (Mut) oligonucleotides. Mutated oligonucleotides sequences: ATTAGTTT vary from the WT by 2 nucleotides: ATTAGCAT. With mutated oligonucleotides, the upper band in the doublet disappeared and the lower band decreased by 10 folds.
Figure 7. Super shift assay with OCT4, SOX2, NANOG, and KLF4 antibodies. 1ug of antibody was added to respective oligonucleotide with 20ug of ES and differentiated (RA) lysate. No shift in the bands was observed.
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