Systematic Identification of Putative New Target Genes and Promoter Co-Occupancy Using Improved ChIP and Novel Sequential-ChIP Protocols

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ABSTRACT

Elucidation of molecular mechanisms underlying transcriptional regulatory networks, transcription factor (TF) interactions, and identification of their target genes has become imperative.

We have applied an improved, rapid, high sensitivity chromatin immunoprecipitation (ChIP) protocol that allows the systematic screening of 25 to 30 pre-selected target genes in a 96-well plate format and have identified putative new target genes for SOX2, NANOG, OCT-3/4, SOX17, BRACHYURY, RUNX2, OLIG2, SMAD2/3, BMI-1, and c-MYC in the human embryonic stem (hES) cell line BGO1V.

In addition, we have developed a novel Sequential ChIP-based assay to investigate *in vivo* promoter co-occupancy ("co-occupancy-ChIP"), which permits fast, highly sensitive analysis of promoter co-occupancy without antibody-antigen disruption.

ChIP/co-occupancy-ChIP analysis led to the identification of two TF complexes in hES cells: SOX2/NANOG/OCT-3/4/c-MYC and RUNX2/BMI-1/SMAD2/3 complexes. These two TF complexes control the expression of two different sets of target genes.

INTRODUCTION

Transcription factors (TFs) are encoded by approximately 10% of the human genome. The accurate identification of their target genes and the understanding of their complex interactions has become crucially important; however, only a small fraction of the *in vivo* target genes and TF-TF interactions have been elucidated.

The most used methods for TF-DNA and TF-TF interactions at DNA sites have been chromatin immunoprecipitation (ChIP) and its derivatives. Although high-throughput approaches, such as ChIP-on-chip and ChIP-SAGE are necessary for genome-wide analysis, traditional single-gene PCR remains the most reliable ChIP readout and continues to be used for the validation of genome-wide data.

In addition, high-throughput approaches are comparatively time-consuming, labor-intensive, and involve multiple steps that facilitate error introduction and elevate background levels that normally require complex statistical analysis.

Therefore, the development of ChIP and Sequential (co-occupancy) ChIP assays that combine PCR readout, speed, high sensitivity, and 96-well plate format adaptability, would provide a tool of great potential for small scale studies and for validation of high-throughput approaches.

MATERIALS AND METHODS

Cells and cell culture

Mouse ES cell line D3 (ATCC) and human ES cell line BGO1V (Novocell) were cultured according to the manufacturer's instructions. Mesendoderm and definitive endoderm differentiated BGO1V cells were obtained as described previously (D'Amour *et al*., Nat. Biotechnol. **24**:1392, 2006).

Immunocytochemistry

As described previously (Cai *et al*., BMC Dev. Biol. **5**:26, 2005).

Standard ChIP

The standard ChIP protocol is a combination of sensitive and fast ChIP protocols described previously (Nelson *et al*., Nat. Protocols **1**:179, 2006; Dahl & Collas, Nat. Protocols **3**:1032, 2008) with some modifications. Experiments were performed 3X, with independent samples. Representative results are shown.

Co-occupancy-ChIP

The 1st antibody added is a non-biotinylated antibody (overnight, 4 °C incubation), followed by protein A/G-agarose beads (2 hrs, 4 °C). Samples were washed, the 2nd antibody (biotinylated) was then added (overnight, 4 °C), followed by the addition of magnetic streptavidin beads. Beads were washed and the final DNA samples were obtained as in a standard ChIP assay. Experiments were performed 3X with independent samples. Representative results are shown. Diagram shown in Figure 3A.

PCR

DNA samples (containing about 20,000 cell equivalents), primers (1µM, IDT), and DMSO (5%) were added to the complementary PCR reagents (AmpliTaq® Gold Polymerase PCR kit and dNTP mix, Applied Biosystems) and subjected to the following cycle: 95 °C/9 min, 43X (95 °C/1 min, 60 °C/1 min), and 60 °C/10 min, using 96-well plates and Eppendorf Mastercycler®. PCR products were subjected to a 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and photographed. Images were saved as TIFF files for densitometric analysis with ImageJ. The primers used here have been validated previously.

AmpliTaq is a trademark of AppliedBiosystems. For research use only. Not for use in diagnostic procedures.

Figure 1.

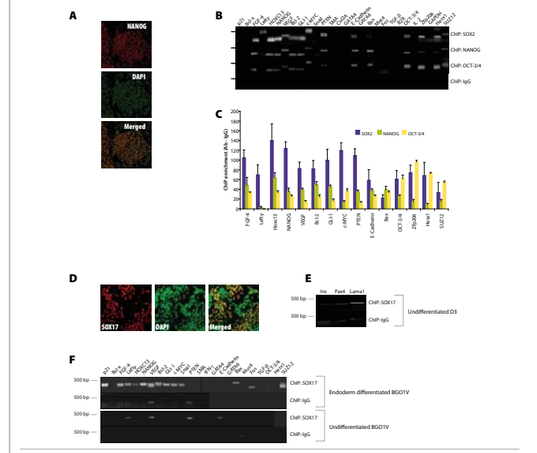

FIGURE 1: Identification of SOX2, NANOG, OCT-3/4 and SOX17 putative gene targets in BGO1V human ES cells. (**A** and **D**) Indicated antibody staining (red), DAPI (green), and merged images of undifferentiated (**A**) or endoderm-differentiated BGO1V cells (**D**) (20X). (**B**, **E** and **F**) PCR results from ChIP assays performed with the indicated antibodies, using previously validated primers for the indicated gene promoters and undifferentiated (**B**, **E**) or endoderm-differentiated BGO1V cells (**F**). (**C**) ImageJ densitometric analysis (ChIP enrichment: average antibody values minus average IgG values). Three independent experiments were performed for each ChIP assay. Representative results are shown.

Figure 2.

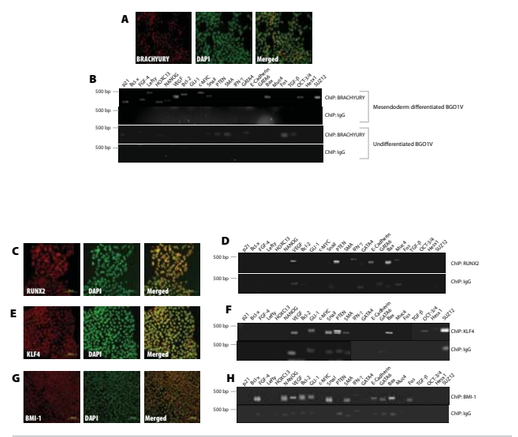

FIGURE 2: Identification of BRACHYURY, RUNX2, KLF4, and BMI-1 putative gene targets in BGO1V human ES cells. (**A**, **C**, **E** and **G**) Indicated antibody staining (red), DAPI (green), and merged images of mesendoderm-differentiated (**A**) or undifferentiated BGO1V cells (**C**, **E** and **G**) (20x). (**B**, **D**, **F** and **H**) PCR results from ChIP assays performed with the indicated antibodies, using previously validated primers for the indicated gene promoters in mesendoderm-differentiated (**B**) or undifferentiated BGO1V cells (**D**, **F** and **H**). Three independent experiments were performed for each ChIP assay. Representative results are shown.

Figure 3.

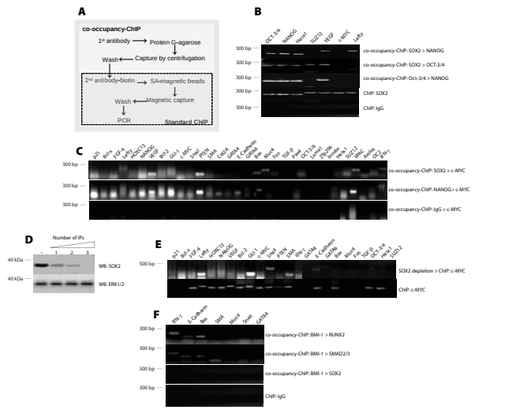

FIGURE 3: The SOX2/OCT-3/4/NANOG/c-MYC and RUNX2/BMI-1/SMAD2/3 complexes in BGO1V cells. (**A**) Schematic representation of the "co-occupancy-ChIP" assay. (**B**) Detection of promoter co-occupancy using SOX2, OCT-3/4 and NANOG antibodies (in the indicated combinations). SOX2 ChIP as positive control and IgG ChIP as negative control. (**C**) Detection of promoter co-occupancy using SOX2, NANOG and c-MYC antibodies (in the indicated combinations). IgG followed by c-MYC (bottom panel), as negative control. (**D**) SOX2 depletion by subsequent SOX2 IP in BGO1V lysates. (**E**) SOX2-depleted or normal BGO1V lysates were submitted to c-MYC ChIP. (**F**) Detection of promoter co-occupancy using BMI-1, RUNX2, and SMAD2/3 antibodies with the co-occupancy-ChIP assay. IgG as negative control. Representative results are shown.

Table 1.

	Transcription Factors										
	SOX2	NANOG	OCT-3/4	c-MYC	SOX17*	BRACHYURY**	RUNX2	KLF4	BMI-1	SMAD2/3	OLIG2
Target Genes											
p21					●	●					
Bcl-x											
FGF-4	●	●	●	●	●	●			●		
Lefty	●			●	●	●					
Hoxc13	●	●	●	●	●	●					
NANOG	●	●	●	●	●	●			●		
VEGF	●	●	●	●	●	●	●	●	●	●	
Bcl-2	●	●	●	●	●	●			●		
GLI-1	●	●	●	●	●			●	●		
c-MYC	●	●	●	●	●	●					●
Snail				●	●	●		●	●		●
PTEN	●	●	●	●			●	●		●	●
SMA				●				●	●	●	●
IFN-γ							●		●	●	●
GATA4											
E-Cadherin	●	●	●	●			●		●	●	
GATA6									●		●
Bax	●	●	●	●	●	●	●	●	●	●	●
Muc4							●			●	●
Fos					●				●	●	●
TGF-β											
OCT-3/4	●	●	●	●	●	●		●			
Hesx1	●	●	●	●							
SUZ12	●	●	●	●	●	●		●			●

* in endoderm differentiated BGO1V cells. **in mesendoderm differentiated BGO1V cells. All others in undifferentiated cells.

CONCLUSIONS

The approach used here, a combination of ChIP and co-occupancy-ChIP (Sequential ChIP) assays, is a powerful tool for small-scale studies of transcription regulation. Our specific proof-of-principle set of experiments indicated the existence of two TF complexes in human ES cells, a SOX2/OCT-3/4/NANOG/c-MYC complex and a RUNX2/BMI-1/SMAD2/3 complex.

These two complexes regulate two different groups of genes among those analyzed (Table 1). In addition, Table 1 shows that the group of TFs comprised of RUNX2, KLF4, BMI-1, SMAD2/3, and OLIG2 bind to a different set of promoters than others analyzed. BMI-1 seems to be the only TF involved with both groups of genes (Table 1), suggesting that BMI-1 might play a more significant role than previously thought in ES cells.

The major advantage of the ChIP/co-occupancy-ChIP approach is that it can be situated between genomic analysis studies, such as ChIP-on-ChIP (can be more expensive, include higher background levels, is more labor-intensive, and may require complex statistical analysis) and those approaches that rely on the analysis of a single or a few genes (lack broader applicability).