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Chromatin Preparation from Murine Eosinophils for Genome-Wide Analyses.

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Abstract

Dynamic gene expression is a major mechanism that directs hematopoietic lineage commitment and differentiation. Recent advances have revealed an association between chromatin signatures and functional genetic sequences such that these chromatin signatures can be used to predict regulatory elements such as enhancers that may direct lineage differentiation. Our understanding of the genetic elements that regulate eosinophil development is very limited, likely due to the technical challenges in working with a rare complex cell. Herein, we describe protocols to sort mature eosinophils from the bone marrow of mice and to prepare chromatin that can be used for ChIP studies for genome-wide mapping of histone marks and transcription factors in mature eosinophils. Comprehensive epigenomic profiling during critical stages in eosinophil development will ultimately aid in defining the gene regulatory networks necessary to regulate eosinophil production.

Keywords

Chromatin immunoprecipitation (ChIP); Gene regulation; Epigenetics

1 Introduction

Eosinophils participate in homeostatic and pathologic processes primarily through their ability to store and rapidly release an abundance of mediators, including cytokines, growth factors, and cytotoxic proteins [1, 2]. In the peripheral blood, eosinophils normally represent less than 5% of white cells, and eosinophilia is generally characterized as mild (450–1500 eosinophils per microliter), moderate (1500–5000 eosinophils per microliter), or severe (greater than 5000 eosinophils per microliter). In general, blood eosinophilia results from enhanced eosinophilopoiesis (i.e., increased production of mature cells from eosinophil

lineage-committed progenitors [EoPs] in the bone marrow). Dynamic gene expression is a major regulatory mechanism that directs hematopoietic lineage commitment and differentiation [3, 4]. Gene expression control is achieved, in part, via access of the transcriptional machinery to gene regulatory elements, such as promoters and enhancers.

Modifications to chromatin, a complex formed between genomic DNA and histone proteins, can profoundly influence gene expression via alterations in DNA accessibility [5]. Technological advances in recent years have revealed an association of specific patterns of histone modifications (i.e., chromatin signature or fingerprint) with functional genetic sequences such that these chromatin signatures can be used to predict regulatory elements such as enhancers that may direct lineage differentiation [6–9]. Our understanding of the genetic elements that regulate eosinophil development is very limited.

We previously identified genome-wide epigenome changes that occur at critical stages in eosinophil development—eosinophil-lineage commitment and eosinophil maturation. We performed chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) to identify genetic regulatory elements that are active during homeostatic eosinophil production in the bone marrow [10]. Our studies provided the first demonstration of stage-specific epigenomic signatures in the eosinophil lineage [10]. Previously, molecular assays involving chromatin required large numbers of purified cells (often as many as $10\text{--}50 \times 10^6$), which made studies focused on transcriptional regulation in rare cell populations, like eosinophils, very challenging and expensive to perform. In addition, eosinophils contain many highly charged granule proteins and enzymes that cleave DNA and RNA [11]; thus, isolation of quality chromatin from these complicated cells can be challenging. With our collaborators, we have developed a methodology to perform ChIP-seq reproducibly with $\sim 0.5 \times 10^6$ eosinophils. Herein, we describe protocols to sort mature eosinophils from the bone marrow of mice and to prepare chromatin that can be used for ChIP studies for genome-wide mapping of histone marks and transcription factors in mature eosinophils.

2 Materials

2.1 Equipment

1. Sterile mortar and pestle.
2. 70 μm cell strainer.
3. 50 mL conical tubes.
4. 15 mL conical tubes.
5. 1.5 mL microcentrifuge tubes.
6. FACS tubes: 5 mL polystyrene round bottom tubes.
7. Table top centrifuge.
8. Rocking platform.
9. Rotating platform.
10. Sonicator.

11. Gel electrophoresis equipment.

2.2 Eosinophil Purification

1. Red blood cell lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer).
2. PBS.
3. Fetal bovine serum (FBS).
4. Collection buffer (2% FBS in PBS).
5. FACS buffer (PBS with 0.5% BSA and 2 mM EDTA).
6. Sorting media (Iscove's Modified Dulbecco's Media with 20% FBS).
7. Fluorescent antibodies in FACS buffer (*see* Table 1).
8. Cell viability stain (diluted in PBS per manufacturer's direction).

2.3 Chromatin Immunoprecipitation Immunoprecipitation

1. 5 M sodium chloride (NaCl).
2. 10% sodium dodecyl sulfate (SDS) solution: 10% (w/v) SDS in ddH₂O.
3. 10% sodium deoxycholate (NaDOC) solution: 10% (w/v) NaDOC in ddH₂O.
4. 10% Triton-X solution: 10% (w/v) Triton-X in PBS.
5. Proteinase K.
6. 2.5 M glycine solution: 2.5 M glycine in PBS.
7. Glycerol.
8. TE buffer with 0.1% SDS.
9. 10× cross-linking solution: 8.8% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 0.5 M EGTA, 50 mM HEPES buffer in ddH₂O.
10. L1 buffer: 50 mM HEPES buffer, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X in ddH₂O.
11. L2 buffer: 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris, pH 8.0 in ddH₂O.
12. Protease inhibitor cocktail (10×): Mixture of AEBSF (104 mM), aprotinin (80 μM), bestatin (4 mM), E-64 (1.4 mM), leupeptin (2 mM), and pepstatin A (1.5 mM) in DMSO. Diluted 1:10 in L1, L2, and sonication buffers for use per manufacturer's instructions.
13. Sonication buffer: TE + 0.1% SDS in ddH₂O.
14. Chromatin QC solution: 0.3 M NaCl, 2 μL of 10% SDS, 0.5 μL of RNase A, and 2.5 μL of proteinase K to sterile ddH₂O (final volume 40 μL).
15. Agarose.

16. Chromatin immunoprecipitation (ChIP) buffer: 150 mM NaCl, 0.1% NaDOC, 1% Triton-X, 10% protease inhibitor cocktail in TE buffer with 0.1% SDS.
17. Magnetic protein A beads: magnetic beads with recombinant protein A covalently coupled to the surface for immunoprecipitation.

3 Methods

3.1 Eosinophil Purification by FACS

Murine eosinophils can be purified from whole bone marrow cells via cell sorting. Whole bone marrow cells ($\sim 10\text{--}15 \times 10^6$ per mouse) are collected from crushed femurs and tibiae (*see Note 1*). Mature eosinophils comprise 1–3% of whole bone marrow cells at homeostasis (*see Note 2*) and are identified by surface expression of Siglec-F and CCR3 (*see Fig. 1*).

1. Collect whole bone marrow cells from dissected and cleaned murine femurs and tibiae that were crushed in a sterile mortar and pestle containing collection buffer (*see Note 3*).
2. Push the collected cells through a 70- μm strainer into a 50 mL conical tube to remove debris and create a single-cell suspension.
3. Lyse red blood cells using lysis buffer per manufacturer's instructions. Centrifuge cells at $500 \times g$ -force at 4°C , discard supernatant, and resuspend pellet in 2 mL collection buffer. Count cells in the suspension.
4. Centrifuge cells at $500 \times g$ -force at 4°C , discard supernatant, and then resuspend cell pellet in 500 μL FACS buffer for every 100×10^6 live cells and transfer cell suspension to a 5 mL polystyrene round bottom tube (FACS tube).
5. Transfer 50 μL of the total cell suspension to another FACS tube for staining controls. Add 150 μL of FACS buffer to this cell suspension, mix gently, and transfer 50 μL of this diluted cell suspension to three FACS tubes for a total of four tubes for staining controls (*see Table 1*). Save the remaining 450 μL of cell suspension for sorting.
6. Add fluorescent antibodies to the cell suspensions in FACS tubes for staining controls and sample cells to be sorted (*see Note 4*).
7. Incubate cells at room temperature for 15–20 min in the dark (to protect antibodies conjugated with fluorochromes from the light), and then add cell viability stain for an additional 5 min.

¹For the highest quality of chromatin, the eosinophils need to be sorted from fresh whole bone marrow preparations that have not undergone any bead-mediated depletion or enrichment. It has been our experience that cells that have been incubated with beads yield poor quality and amount of chromatin.

²We typically sort eosinophils from the pooled bone marrow of ten mice ($\sim 100 \times 10^6$ total cells) to typically yield around 1×10^6 mature eosinophils.

³Alternatively, the femurs can be flushed with collection buffer and pooled prior to pushing thru a cell strainer. Total cell yield is smaller but collection is faster.

⁴Each antibody lot and viability dye should be titrated for optimal staining. We typically use the antibodies directed against Siglec-F (PE-conjugated) and CCR3 (FITC-conjugated) at 1:200 dilutions. We have found that the viability dyes are very potent and use at a dilution of 1:3000. We make a 1:1000 dilution in PBS of the original stock and add one-third of the final volume to the cell suspension in FACS buffer.

8. Centrifuge at $500 \times g$ -force for 5 min. Discard the supernatant.
9. Wash cell pellets by resuspending cells with 500 μ L FACS buffer and centrifuge at $500 \times g$ -force for 5 min.
10. Resuspend pelleted bone marrow cells to be sorted at a density of 20×10^6 cells per mL of FACS buffer. Cell pellets for staining controls can be resuspended in a final volume of 100–200 μ L FACS buffer.
11. Live mature murine eosinophils are identified from whole bone marrow suspensions via surface expression of Siglec-F and CCR3 (*see* Fig. 1). Sort cells directly into 5 mL sorting media in a 15 mL conical tube that has been coated with the sorting media by gently inverting the tube several times (*see* Note 5).

3.2 Chromatin Fixation

1. Make note of the final volume of sorted eosinophils in the collection tube (*see* Note 6).
2. Add freshly made 10 $\times$ cross-linking solution directly to the collection tube containing sorted murine eosinophils, and mix gently by inversion (*see* Note 7).
3. Incubate the cells on ice for 8 min.
4. To stop the cross-linking reaction, add one-twentieth of the final volume of a 2.5 M glycine solution to each tube. Place the tube on a rocking platform at a slow/medium speed at room temperature for 5 min.
5. Centrifuge at $2000 \times g$ -force for 10 min at 4°C. Discard the supernatant.
6. Gently resuspend the cell pellet in 1 mL ice-cold PBS, and transfer the suspension into a fresh 1.5 mL microcentrifuge tube.
7. Centrifuge cells at $2000 \times g$ -force for 5 min at 4°C, and carefully decant and discard the supernatant.
8. Resuspend the cell pellet in 1 mL ice-cold PBS.
9. Repeat step 7.
10. Centrifuge the cell pellet again at $3000 \times g$ -force for 5 min to dry the pellet. Remove and discard any residual liquid in the tube.
11. Freeze and store at -80°C until ready for use (*see* Note 8).

⁵We use fluorescence minus one (FMO) controls to identify and gate cells when multiple fluorochromes are used in a given panel. To set the gates for sorting, we use the unstained initially, then FMO3 to set the gate for live cells, and then FMO1 and FMO2 to set gates for Siglec-F and CCR3-positive cells, respectively. We sort cells using a 70 μ m nozzle on the FACSARIA. Coat the 15 mL conical tube with the sorting media by rotating and inverting the tube after adding 5 mL sorting media. This prevents the sorted cells from sticking to the side of the tubes.

⁶Since the cross-linking buffer is added at 10 $\times$, the total volume that the sorted cells are suspended in needs to be recorded.

⁷One-tenth of the final volume of the cross-linking buffer is added to the cells suspended in the sorting media. For example, if the cells are sorted in 1 mL, then 0.11 mL of the 10 $\times$ cross-linking buffer would be added to cells directly. The final concentration of formaldehyde is 0.8% and should not exceed 1%.

⁸The fixed cell pellets can be stored indefinitely at -80°C . We have successfully performed ChIP on pellets stored for up to 6 months.

3.3 Nuclei Preparation

1. Prepare L1 and L2 buffers. Add protease inhibitor cocktail to each buffer. Keep L1 buffer on ice and L2 buffer at room temperature until ready to use.
2. Thaw the frozen cell pellet by incubation on ice.
3. Resuspend the cell pellet in 1 mL of L1 buffer with added pro-tease inhibitor cocktail. Incubate on a rotating platform at 4 °C for 10 min.
4. Centrifuge at $5000 \times g$ -force for 5 min at 4 °C.
5. Carefully remove and discard the supernatant using a micropipette or vacuum.
6. Resuspend the cell pellet in 1 mL of L2 buffer with added pro-tease inhibitor cocktail.
7. Incubate on a rotating platform at room temperature for 10 min.
8. Centrifuge at $5000 \times g$ -force for 5 min at 4 °C. Carefully remove and discard the supernatant using a micropipette or vacuum.
9. Wash the pelleted nuclei by adding 0.5 mL of sonication buffer to lift the pellet up from the bottom of the tube without resus-pending into a suspension (*see* Note 9). Do not disrupt the pellet.

3.4 Chromatin Shearing

The sonication intensity, length, and number of cycles needed for optimal chromatin shearing should be determined experimentally for each cell type and cell density and will vary between machines.

1. Resuspend the pelleted nuclei in 130 μ L of sonication buffer with added protease inhibitor cocktail.
2. Transfer the nuclei suspension into a small vial or microcentrifuge tube for sonication. The suspension should be loaded into vials without bubbles for proper and reproducible sonication.
3. Sonicate the nuclei to generate sheared chromatin that is between 200 and 500 base pairs in size. The length, intensity, and number of cycles will need to be determined experimentally as it will vary by machine (*see* Note 10).
4. Collect the chromatin suspension into a 1.5 mL microcentrifuge tube and transfer 10 μ L for quality control testing.
5. Add 5 μ L of 100% glycerol (5% glycerol final concentration) to the rest of the suspension (~100 μ L) and store at -80 °C until ready for use.

⁹.If the pellet dissolves in the buffer, centrifuge the suspension at $5000 \times g$ for 10 min to repellet the nuclei and move on to shearing.

¹⁰.In our laboratory, we use a Covaris sonicator to shear the chromatin with the nuclei suspended in small vials. The settings that we have determined to be optimal when starting with $\sim 1 \times 10^6$ mature eosinophils are 10% duty cycle, 5 intensity (equals 105 pulse intensity), and 200 burst for 45 s.

3.5 Chromatin Quality Control Testing

1. To the microcentrifuge tube with the chromatin for quality control, add 40 μ L of chromatin QC solution to the 10 μ L of sheared chromatin.
2. Incubate the quality control chromatin at 37 °C for 60 min. Incubate at 65 °C for an additional 4–5 h up to overnight (*see* Note 11).
3. Run the chromatin sample on an agarose gel (1%) to determine the size of sheared chromatin (*see* Fig. 2). Sheared chromatin should be between 200 and 500 base pairs.

3.6 Chromatin Preparation for Immunoprecipitation

1. If sonication looks appropriate, thaw the chromatin sample on ice.
2. Add 100 μ L of ChIP buffer to the sample.
3. Centrifuge at 20,000 $\times$ *g*-force for 10 min at 4°C.
4. To preclear the chromatin solution, add 5 μ L protein A-coated magnetic beads (same beads will be used for the immunoprecipitation) to the sample and incubate between 30 and 60 min at 4°C on a rotating rack.
5. Put tubes on magnet for 1 or 2 min, and collect the negative fraction that contains the pre-cleared chromatin. Adjust total volume up to 200 μ L with ChIP Buffer.
6. Keep 2 μ L of the negative fraction (or 1% of total) for input control.
7. Transfer 198 μ L of chromatin suspension into tube. Chromatin suspension can be stored with added glycerol (final 5% glycerol content) at –80°C until needed for future ChIP studies (*see* Note 12).

References

1. Lee J, Rosenberg HF (2013) Eosinophils in health and disease, 1st edn. Elsevier/Academic Press, London; Waltham, MA
2. Rosenberg HF, Dyer KD, Foster PS (2013) Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* 13(1):9–22. 10.1038/nri3341 [PubMed: 23154224]
3. Kee BL (2011) A comprehensive transcriptional landscape of human hematopoiesis. *Cell Stem Cell* 8(2):122–124. 10.1016/j.stem.2011.01.006 [PubMed: 21295265]
4. May G, Soneji S, Tipping AJ, Teles J, McGowan SJ, Wu M, Guo Y, Fugazza C, Brown J, Karlsson G, Pina C, Olariu V, Taylor S, Tenen DG, Peterson C, Enver T (2013) Dynamic analysis of gene expression and genome-wide transcription factor binding during lineage specification of multipotent progenitors. *Cell Stem Cell* 13(6):754–768. 10.1016/j.stem.2013.09.003 [PubMed: 24120743]
5. Maston GA, Evans SK, Green MR (2006) Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet* 7:29–59. 10.1146/annurev.genom.7.080505.115623 [PubMed: 16719718]

¹¹. A PCR machine can be used for the heating cycles, but we recommend that the last cycle be set for 15 °C to avoid precipitating the SDS in the solution.

¹². We typically yield ~1.5 μ g chromatin from 1×10^6 sorted eosinophils. We use ~1–1.5 μ g of chromatin for every ChIP. The ratio of chromatin to antibody needs to be determined experimentally as the optimal ratio depends on the antibody used for the immunoprecipitation.

6. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ, Zhao K (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 40(7):897–903. 10.1038/ng.154 [PubMed: 18552846]
7. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* 39(3):311–318. 10.1038/ng1966 [PubMed: 17277777]
8. Hon GC, Hawkins RD, Ren B (2009) Predictive chromatin signatures in the mammalian genome. *Hum Mol Genet* 18(R2):R195–R201. 10.1093/hmg/ddp409 [PubMed: 19808796]
9. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. *Cell* 129(4):823–837. 10.1016/j.cell.2007.05.009 [PubMed: 17512414]
10. Bouffi C, Kartashov AV, Schollaert KL, Chen X, Bacon WC, Weirauch MT, Barski A, Fulkerson PC (2015) Transcription factor repertoire of homeostatic eosinophilopoiesis. *J Immunol* 195(6): 2683–2695. 10.4049/jimmunol.1500510 [PubMed: 26268651]
11. Marichal T, Mesnil C, Bureau F (2017) Homeostatic eosinophils: characteristics and functions. *Front Med (Lausanne)* 4:101 10.3389/fmed.2017.00101 [PubMed: 28744457]

Fig. 1.

Eosinophil gating strategy. A representative gating strategy to identify mature eosinophils from naive murine bone marrow is shown and starts with pregating on viable single cells and then further gating on cells that coexpress Siglec-F and CCR3. Percentage of parent gate is shown

Fig. 2.

Chromatin q uality control testing. The size of sheared chromatin from two independent experiments is shown on 1% agarose gel. The left panel shows a smear of DNA between 200 and 500 base pairs. This chromatin was used for chromatin immunoprecipitation. The right panel shows uneven shearing, and this chromatin was not used for further experiments

Eosinophil Chromatin Preparation

Table 1

Control and sample staining for mature murine eosinophils

Tube	Anti-CCR3	Anti-Siglec-F	Live/dead stain
<i>Unstained</i>			
FMO1—Siglec-F gating	X		X
FMO2—CCR3 gating		X	X
FMO3—Live/dead gating	X	X	
Sample	X	X	X