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Chromatin dynamics regulate mesenchymal stem cell lineage specification and differentiation to osteogenesis

Hai Wu [a,1], Jonathan A.R. Gordon [a,*,1], Troy W. Whitfield [b], Phillip W.L. Tai [a], Andre J. van Wijnen [c], Janet L. Stein [a], Gary S. Stein [a], Jane B. Lian [a]

[a] Department of Biochemistry and Vermont Cancer Center, University of Vermont, Burlington, VT, United States
[b] Department of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA, United States
[c] Department of Orthopedic Surgery, Mayo Clinic, Rochester, MN, United States

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ABSTRACT

Multipotent mesenchymal stromal cells (MSCs) are critical for regeneration of multiple tissues. Epigenetic mechanisms are fundamental regulators of lineage specification and cell fate, and as such, we addressed the question of which epigenetic modifications characterize the transition of nascent MSCs to a tissue specific MSC-derived phenotype. By profiling the temporal changes of seven histone marks correlated to gene expression during proliferation, early commitment, matrix deposition, and mineralization stages, we identified distinct epigenetic mechanisms that regulate transcriptional programs necessary for tissue-specific phenotype development. Patterns of stage-specific enrichment of histone modifications revealed distinct modes of repression and activation of gene expression that would not be detected using single endpoint analysis. We discovered that at commitment, H3K27me3 is removed from genes that are upregulated and is not acquired on downregulated genes. Additionally, we found that the absence of H3K4me3 modification at promoters defined a subset of osteoblast-specific upregulated genes, indicating that acquisition of acetyl modifications drive activation of these genes. Significantly, loss or gain of H3K36me3 was the primary predictor of dynamic changes in temporal gene expression. Using unsupervised pattern discovery analysis the signature of osteogenic-related histone modifications identified novel functional *cis* regulatory modules associated with enhancer regions that control tissue-specific genes. Our work provides a cornerstone to understand the epigenetic regulation of transcriptional programs that are important for MSC lineage commitment and lineage, as well as insights to facilitate MSC-based therapeutic interventions.

1. Introduction

Accumulating evidence from genome-wide studies have indicated that histone modifications, chromatin states and tissue-specific transcriptional regulators are inherited from progenitor cells and thus greatly influence phenotypic commitment and cell-specific gene regulation in progeny [1]. Post-translational modifications of histone proteins at lysine, arginine and serine residues dictate the activation or repressive state of a particular gene or genomic region, depending on the presence or absence of acetyl or methyl groups [2]. Several profiling studies examining constitutive histone modifications have demonstrated that chromatin states define genomic context for specific cell lineages [3]. In addition, dynamic changes in histone modifications can influence basal levels of gene expression and regulate expression of specific genes in tissue specification and differentiation programs [4,5]. However, for many tissues, the exact contribution of specific histone modifications controlling phenotypic gene expression during the stochastic progression of lineage commitment is not currently well understood.

Mesenchymal stromal cells (MSCs) derived from bone marrow are pluripotent progenitor cells that have the capacity to form and regenerate mature connective tissue, however the molecular heterogeneity of these cells is problematic for their wide-spread clinical use [6]. Under normal physiological conditions, growth factor signaling pathways (e.g. BMP/TGF FGF, Wnt) and downstream transcription factors influence decisions of these cells to commit to the formation of bone, cartilage, muscle or fat tissue [7]. During aging, these cell fate decisions are compromised resulting in the production of one tissue at the expense of another (e.g. increased adipose with decreased bone). In culture, MSCs can be readily differentiated into adipocytes, myoblasts, chondrocytes and osteoblasts and have also been shown to have a capacity to regenerate cardiac muscle and neurons under induced

* Corresponding author.
E-mail addresses: Hai.wu@uvm.edu (H. Wu), Jonathan.A.Gordon@uvm.edu (J.A.R. Gordon), Troy.Whitfield@umassmed.edu (T.W. Whitfield), Phillip.Tai@umassmed.edu (P.W.L. Tai), vanwijnen.andre@mayo.edu (A.J. van Wijnen), Janet.Stein@uvm.edu (J.L. Stein), Gary.Stein@uvm.edu (G.S. Stein), Jane.Lian@uvm.edu (J.B. Lian).
[1] Authors contribute equally to this work.

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conditions [8]. It is likely that there is a change in the normal underlying epigenetic landscape of the progenitor population and committed phenotypes that are associated with changes in histone modifications. Although MSCs are a widely used model for lineage commitment, unique changes in histone modifications that establish progression from a progenitor to a defined cell lineage has not been extensively defined.

Osteogenesis is a highly regulated program of commitment and differentiation progressing through well-defined phenotypically distinct cell populations from MSCs to the committed osteoprogenitor, mature osteoblasts and the terminally differentiated osteocyte embedded in mineral [9]. This developmental program is recapitulated in vitro as four distinct stages that include the proliferation, commitment, matrix formation and mineralization which are based on gene expression profiles of each subpopulation of osteoblast lineage cells. Previously, epigenetic regulation of bone formation by histone modifications have been identified from studies reporting the actions of HDACs on chromatin during skeletal development, as well as other histone and chromatin modifying enzymes [10,11].

In this study, using osteogenesis as a model, we are testing the hypothesis that distinct constitutive and dynamic changes in histone modifications are associated with MSC commitment. We further postulate that the pattern of histone modifications will define distinct mechanisms contributing to repression and activation of genes required for temporal stages of osteogenesis. Our study utilizes a homogenous cell population of bone marrow-derived MSCs induced into committed osteoprogenitors, sequentially differentiated to osteoblasts (OB) and ultimately into osteocytes that are embedded in a developing mineralized matrix during culture. Each of these stages represent a molecular and functionally distinct population of the osteogenic lineage in vivo that has defined roles in bone formation [12].

The genome-wide patterns of 7 different histone marks, H3K4me1, H3K4me3, H3K9me3, H3K9acetyl, H3K27me3, H3K27acetyl and H3K36me3 modifications were profiled and combined with profiles of gene expression (RNA-Seq) during MSC differentiation, in order to define patterns of histone modifications that are indicative of gene expression during MSC differentiation. In general, during osteogenic commitment H3K27me3 is removed from genes that are upregulated and are not acquired on genes that are downregulated, suggesting that repression by this mark is not a major contributor to gene regulation during differentiation. Loss or gain of the transcriptional elongation marker, H3K36me3 was associated with dynamic changes in temporal gene expression. In addition, in a subset of osteoblast-related genes we have discovered that the absence of the H3K4me3 signal is associated with proximal promoters of upregulated genes, indicating that acquisition of acetyl modifications drives activation of these genes. Using segmentation analysis based on a combination of histone modifications and transcription factor binding, we have defined novel genomic regions that have osteoblast-related regulatory potential throughout the MSC genome. Many of these identified enhancer regions are likely bone-specific as they overlap with previously identified enhancers regulating craniofacial development [13] as well as their proximity to osteoblast-related genes, including the SIBLING gene cluster.

These discoveries and characterization of epigenetic signatures provide novel insight into gene regulatory mechanisms governing MSC commitment and osteogenic differentiation. These findings are relevant to other mesenchymal lineages and developmental processes and have promise of identifying fresh approaches for tissue regeneration and disease intervention.

2. Materials and methods

2.1. Cell culture

Primary MSCs were isolated from femurs and tibias of 6–8 week old SMAA-mCherry mice [14], which were kindly provided by Drs. I. Kalajzic and D. Rowe. The bone marrow was flushed into ascorbic acid-free MEM-alpha medium (Hyclone, Novato, CA, USA) supplemented with 1× Pen/Strep antibiotics (Invitrogen, Carlsbad, CA, USA). The crude suspension was twice passed through 20 gauge syringes to dissociate cell clumps, followed by hypotonic lysis of erythrocytes. Cells were gently pelleted and resuspended in MSC growth media (ascorbic acid-free α-MEM medium supplemented with 1× Pen/Strep antibiotics and 18% FBS (Hyclone)). Single cell suspension was obtained by filtering resuspended bone marrow cells through 70 μm metal mesh, and then plated at 10 million cells per 10 cm^2 tissue culture plate (Corning Inc., Corning, NY, USA). Bone marrow stromal cells attached to the plate surface after 72 h were kept and cultured for an additional 4 days before enriching for SMAA-mCherry positive cells. SMAA-positive MSCs were plated at 1.5×10^5 cells per 10 cm culture plate for the expansion in the MSC growth media. BMSCs of passage 6 to 10 were used for all the experiments in this study. All animal work was reviewed and approved by UMASS and/or UVM IACUC (protocol number JL-512).

2.2. Flow cytometry

Cell surface markers on isolated MSCs at passage 8 to 10 were determined by flow cytometry on BD FACSCalibur (BD Biosciences). Briefly, cells were lifted by 0.05% Trypsin-EDTA solution (Invitrogen) and pelleted at 1000*g* for 3 min, washed thrice with 1× PBS supplemented with 2% FBS (Hyclone) and 10 mM sodium azide (Sigma-Aldrich, St. Louis, MO, USA), incubated with antibody conjugated with FITC for 20 min on ice. After antibody incubation, cells were pelleted at 1000*g* for 3 min and washed thrice with 1× PBS supplemented with 2% FBS (Hyclone) and 10 mM sodium azide (Sigma-Aldrich). Cells were kept in 1× PBS supplemented with 2% FBS (Hyclone) and 5 mM sodium azide (Sigma-Aldrich) before analysis at 4 °C. Antibodies against cell surface markers were: anti-CD11b-FITC, anti-CD45-FITC, anti-Sca-1-FITC, and anti-CD29-FITC; proper IgG isotype controls were used to assess background signal. All antibodies were purchased from BioLegend (San Diego, CA, USA) and used at the dilution ratios recommended by manufacturer.

2.3. RNA isolation, quantitative PCR, and RNA-Seq libraries

RNA isolation and quantitative PCR were performed as previously described [15]. RNA-Seq libraries were built with TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) following the manufacturer's instruction. Four biologically independent RNA-Seq libraries were prepared for each time point during MSC differentiation. All RNA-Seq libraries were pair-end sequenced (PE100) on a HiSeq-1500. Base calls and sequence reads were generated by bcl2fastq software (version 1.8.4, Illumina).

2.4. Chromatin immunoprecipitation sequencing (ChIP-Seq)

At day 0, 7, 14, and 21 of differentiation, approximately 5×10^7 MSCs were washed with PBS and then fixed on plate with 1% formaldehyde for 10 min at room temperature to crosslink DNA-protein complexes. Chromatin were prepared and sheared as previously described [15]. Sheared chromatin was used for immunoprecipitation with Runx2 antibody (M-70, Santa Cruz), Ctcf (07-729, Millipore, Billerica, MA, USA), Smc1a (A300-055A, Bethyl Laboratories, Montgomery, TX, USA), H3K4me (ab8895, Abcam, Cambridge, MA, USA), H3K4me3 (ab1012, Abcam), H3K9me3 (Ab8898, Abcam), H3K9ac (39137, Active Motif), H3K27me3 (07-449, Millipore), H3K27ac (07-360, Millipore), H3K36me3 (ab9050, Abcam), ap300 (sc-585 X, Santa Cruz) or Ezh2 (A304-196A, Bethyl Laboratories) or immunoglobulin G (IgG) (12-370, Millipore) followed by purification using Protein-G Dynabeads (Invitrogen). ChIP-Seq libraries from immunoprecipitated DNA were constructed as previously described [15]. DNA libraries were sequenced on an Illumina Genome Analyzer II or Illumina HiSeq-1500. Base calls