HHS Public Access

Author manuscript

Bone. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Bone. 2015 December ; 81: 733–738. doi:10.1016/j.bone.2015.03.013.

Multiple Levels of Epigenetic Control for Bone Biology and Pathology

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Abstract

Multiple dimensions of epigenetic control contribute to regulation of gene expression that governs bone biology and pathology. Once confined to DNA methylation and a limited number of post-translational modifications of histone proteins, the definition of epigenetic mechanisms is expanding to include contributions of non-coding RNAs and mitotic bookmarking, a mechanism for retaining phenotype identity during cell proliferation. Together these different levels of epigenetic control of physiological processes and their perturbations that are associated with compromised gene expression during the onset and progression of disease, have contributed to an unprecedented understanding of the activities (operation) of the genomic landscape. Here, we address general concepts that explain the contribution of epigenetic control to the dynamic regulation of gene expression during eukaryotic transcription.

Introduction

There is increasing appreciation for the contributions of genetic and epigenetic regulation to skeletal biology and evidence is accruing for perturbed epigenetic mechanisms in skeletal disease. Once principally restricted to DNA methylation and a limited series of post-translational histone modifications, the repertoire of epigenetic mechanisms is rapidly expanding with growing insight into both molecular and biochemical parameters of biological processes that are epigenetically mediated. With comprehensive understanding for the scope of epigenetic impact on skeletal gene expression and compromised epigenetic mechanisms in congenital and acquired skeletal disorders, the potential for epigenetic-based therapeutic targets is precipitously emerging.

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There are a number of authoritative reviews on specific dimensions of epigenetic control that collectively provide a comprehensive treatment of epigenetic biochemistry and regulatory biology [1–7]. These reviews effectively consider the challenges and opportunities encountered when interrogating epigenetic mechanisms within the context of normal cells, skeletal genesis, bone remodeling and bone metabolic disorders that are directly linked to genetic or acquired perturbations or are consequential to a spectrum of diseases and/or treatments that are standards of care. Here, we will illustrate options for the power of epigenetic mechanisms to support transformative insight in to skeletal biology and pathology. We will emphasize the convergence of skeletal epigenetic mechanisms that can provide insight in to regulatory networks that are pivotal for regulation of gene expression. Epigenetic control will also be explained in relation to the dynamic architectural organization of regulatory machinery from the perspective of nuclear structure-gene expression relationships. Similarly, we will consider extrapolations from the biology of skeletal epigenetic control to paradigm shifting options with the diagnosis and treatment of bone disease.

I. Multiple Dimensions to Epigenetic Control

A. Histone Modifications—From a historical perspective, epigenetic control was initially confined to DNA methylation [Reviewed in 8, 9] and three post translational histone modification designated acetylation [Reviewed in 10, 11], methylation [Reviewed in 12, 13] and phosphorylation [Reviewed in 14] with the assumptions that acetylation and phosphorylation are reversible while methylation is not under biological conditions. In the past several years, there has been a significant expansion in understanding the scope of complexity to histone acetylation, methylation and phosphorylation, with compelling evidence for the reversibility of these three classes of histone modifications. Table 1 summarizes the various enzymatic modifications that occur on specific histone protein residues and their functional implications in regulating transcription of a gene. Beyond expanded insight into the histone subtypes that are post-translationally modified and specific amino acid residues that undergo post-translational modifications, there has been significant progress in identification and characterization of the enzymology for histone modifications, both the addition and removal of acetate, methyl and phosphate groups [15, 16].

In addition to expanding understanding of the enzymology of the histone modifications, compelling evidence is emerging for association of particular histone modifications and specific histone modifying enzymes with specific biological circumstances, including developmental stages, cell cycle progression and disease-related changes [17–24]. The sequence of recruitment and engagement of the regulatory components remains to be determined and identification of the rate limiting factors for fidelity of histone modification is open ended. Stochastic relationships between components of the histone modification machinery can be mechanistically informative. Addressing these parameters of control will provide insight into the metabolism of histone modifications.

The recent observation of epigenetically bivalent genes in stem cells, that is, genes including both activation and suppression histone “marks” (H3K4me3 and H3K27me3, respectively), provide an additional dimension to histone-mediated epigenetic regulation [for a recent

review, see 25]. The restriction of bivalency to pluripotency is rapidly evolving to recognition of a broader presence. While the functional significance for bivalency needs to be further established, a viable possibility is that the simultaneous representation of activating and suppressing histone modifications at a particular promoter poises the downstream gene to subsequently acquire an epigenetic landscape. In response to developmental, phenotypic or physiological regulatory queues, conditions are established leading to either gene transcription or silencing and resulting in a committed cell phenotype. Recent evidence supports cell cycle-stage specific bivalency that can reinforce regulatory competency for responsiveness to regulatory signals required for establishing, sustaining and/or modifying phenotype [26, 27, and Grandy R et al., submitted manuscript].

Mechanistic contributions of histone modifications to biological control are emerging from developments in technologies able to analyze whole genomes. These approaches include chromatin immunoprecipitation using well characterized antibodies to most histone modification, followed by massive parallel sequencing (ChIP-Seq) or a ChIP-on-chip procedure to identify interacting proteins with the enzyme that mediates the histone modification. The RNA-seq identifying the cell's gene expression profile allows determining histone modifications profiles on genes or for genome-wide screens to comprehensively establish histone modifications associated with individual genes or cohorts of genes [28, 29]. The availability of web-based programs to align GWAS data and SNP sequences with epigenetic profiles are leading to possible mechanisms for their deregulated activity of the linked gene with the epigenetic modification as a potential therapeutic target [30].

From another perspective, histone modifications combined with strategic time course approaches both *in vitro* and *in vivo*, are increasing capabilities to dissect temporal sequences of epigenetic and histone modifications. It is feasible to visualize association of modified histones with specific genes or particular sets of genes in response to multiple physiological stimuli. Combining the capabilities of these approaches with genomic and proteomic analyses provides the basis for defining epigenetic control within a three dimensional context of the cell nucleus where this regulatory machinery is architecturally organized. The composite perspective that results from cellular, biochemical, molecular and genomic analysis is a platform for understanding and experimentally addressing epigenetic engagement in regulatory networks, the dynamics of assembly and activity of epigenetic and regulatory machinery and the integration of specific epigenetic pathways with the components of nuclear structure and function.

B. Epigenetic Control beyond Histone Modifications—The pivotal contributions of epigenetic regulation to control of gene expression is illustrated by expansion of understanding for epigenetic control that is provided by DNA methylation, non-coding RNAs and mitotic bookmarking.

1. DNA Methylation: There is appreciation for the importance to functionally define mechanisms that support cross talk between DNA and histone modifications. In part, DNA methylation establishes competency for protein-DNA interactions that are obligatory for selectively modifying histones associated with target genes in response to endogenous and

exogenous regulatory signals. DNA methylation provides long-term “regulatory directions” for transcriptional control and generally “instructions for responsiveness”. In contrast, histone acetylation generally governs gene activation while histone methylation can activate or suppress transcription in a context-depended manner. DNA methylation contributes to the context in which histones are modified by establishing conditions where genes and associated histones, become effective substrates for the addition or removal of acetate, methyl and phosphate groups [31, 32].

DNA methylation has been studied in skeletal disorders, largely for osteoporosis and osteoarthritis. In general many genes that are silenced by methylation in CpG islands that are needed for homeostasis of bone and cartilage tissues. As a consequence, the tissues undergo degeneration [33–35]. The ageing process itself appears to involve an “epigenetic drift ” where gradual demethylation occurs across the genomic, while hypermethylation is observed in CpG islands in promoter regions [36].

The DNA methylation/demethylation associated with diseased states is further contributing to changes in the histone modifications that regulate gene expression, but a better understanding of these mechanisms are needed.

2. Non-coding RNAs: There is extensive and far reaching knowledge of contributions by non-coding RNAs (ncRNAs) to biological control as well as to compromised gene expression that is associated with numerous diseases [37–39]. A paradigm for understanding ncRNA-mediated control is provided by XIST that is responsible for X-chromosome inactivation [40]. During the past several years there has been extensive investigation of micro-RNAs as post-transcriptional inhibitors and more recently, long non-coding RNAs (lncRNAs) that exhibit both positive and negative control are being increasingly studied [41–44]. The regulatory involvement of small and long non-coding RNAs in skeletal biology, is reviewed in the article by Hassan and colleagues in this series.

3. Mitotic Bookmarking: Retention of transcription factors at target gene loci of mitotic chromosomes accompanies transcriptional regulatory machinery from parental to progeny cell during cell division [45]. This process designated mitotic bookmarking, epigenetically supports gene expression by two mechanisms. While genes are transcriptionally inactive during mitosis, retention of transcription factors at target gene loci supports competency for resumption of cell type specific transcription in G1. Also, the chromatin organization of mitotic chromosomes epigenetically poises bookmarked genes for post-mitotic transcriptional reactivation [46, 47].

II. The Dynamic Architectural Organization of Skeletal Epigenetic Regulatory Machinery

A. Strategic Localization of Epigenetic Domains

1. Epigenetically Responsive Promoter Sites: A wealth of studies have shown that epigenetic regulation of gene expression plays a key role in lineage commitment and maintenance. For example, epigenetic control of bone tissue-specific gene expression is principally mediated by the RUNX2 transcription factor at multiple sites on target gene promoters and enhancers (or cis-regulatory modules) where this regulatory protein provides

a scaffold for the strategic localization of regulatory machinery for histone modifications and chromatin remodeling [48–51]. Developmental and Vitamin D responsive regulation of the chromatin organization for the bone-specific osteocalcin gene promoter directly illustrates functional linkage of chromatin organization with requirements for skeletal gene expression. The necessity for epigenetic integrity of the osteocalcin gene promoter elements that support basal tissue-specific transcription and vitamin D-responsive enhancement directly establish the requirement of epigenetic mechanisms for biological control in bone [52–54].

The complexity of organization and activity for regulatory complexities that epigenetically control skeletal gene expression is biologically and mechanistically relevant. The RUNX transcription factors that occupy the proximal and upstream promoter sites, as well as intragenic regions of bone-specific genes (e.g., osteocalcin), interact with cohorts of co-regulatory proteins that are both similar and different, supporting physiologically responsive requirements for epigenetic control of transcription [48–51, 55]. Obligatory relationships between epigenetically mediated chromatin remodeling of bone-specific promoters and transcriptional responsiveness to developmental and homeostatic cues have been demonstrated experimentally *in vitro* and *in vivo* with systematically constructed mutations to directly establish functionality.

The strategic placement of epigenetic regulatory complexes at multiple sites of bone target gene promoters is not confined to the osteocalcin gene. A similar regulatory landscape for physiologically responsive epigenetic control has been established for several skeletal genes and for tissue-specific gene expression in general [see 56].

2. Intranuclear Domains: The mammalian nucleus is a highly organized cellular compartment where genetic and epigenetic regulatory machineries interact with each other in a precise and timely fashion for physiologically relevant outcome. Several subnuclear compartments have been described and extensively studies. For example, RNA Polymerase II is organized in punctate nuclear foci that interact with actively transcribing genes, thus resulting in the formation of transcription factories. Similarly, DNA replication takes place at defined sites within the nucleus, where the DNA replication machinery resides. Splicing of nascent messenger RNA is often confined to Splicing Speckles as characterized by localization of SC-35 splicing factors to specific nuclear domains. Nucleolus, the site of ribosomal RNA synthesis, is yet another nuclear entity that offers a paradigm for understanding the nuclear structure-function relationship. We and others have shown that several lineage restricted transcription factors are also confined to punctate nuclear microenvironments where they interact with and regulate target genes.

Fluorescence analysis of bone cells with antibodies for the RUNX2 bone tissue-specific transcription factor strikingly illustrates a punctate organization [57, 58]. The 150–300 foci exhibit resistance to high salt and detergent extraction indicating that the regulatory machinery for bone tissue-specific transcription is architecturally associated. This is not a unique observation. Rather, the punctate intranuclear organization of RUNX2 transcription factors is consistent with growing evidence for compartmentalization of regulatory machinery for transcription, replication and repair within the nucleus [59–61]. Confirmation

of the punctate intranuclear localization of the RUNX transcription factor by time-lapse fluorescence microscopy of cells with a fluorescent-tagged RUNX protein provides *in situ* validation of the punctate intranuclear organization [62]. Similar punctate intranuclear distribution has been confirmed for the vitamin D receptor (VDR) and co-regulatory molecules in bone-related cells [63, 64], indicating that transcriptional control of osteoblastic genes in response to vitamin D is also architecturally organized.

There are important regulatory implications for the observed compartmentalization of RUNX and VDR transcription regulators that are applicable to *in situ* mechanistic understanding of regulatory mechanisms supporting transcription, replications and repair in nuclei of intact cells. Focal organization and endogenous levels of regulatory proteins that can be directly observed without amplification or overexpression suggests focal concentrations at a limited number of sites within the nucleus. Such focal concentrations of proteins is consistent with threshold levels to facilitate binding at promoter sites on target gene loci that mediate the organization and assembly of factors to support histone modifications and chromatin remodeling for epigenetic mechanisms that conformationally poise genes for transcription and support recruitment of factors and co-regulatory proteins that directly control transcription. Such reasoning is in agreement with co-localization of RUNX foci with antibodies to active (hyper phosphorylated) RNA polymerase II, BrUTP-labeled regions and co-regulatory proteins that support transcription [65, 66]. Important for epigenetic control, the factors that are responsible for histone modifications have been shown to be punctately organized, architecturally associated and co-localized with genes that are transcriptionally active [61]. The emerging perspective is a multidimensional “nucleome” with the components for genetic and epigenetic parameters of control architecturally configured.

More than observations that are documented by molecular, cellular and biochemical criteria, there is *in vitro* and *in vivo* genetic evidence for a RUNX intranuclear trafficking signal that is necessary and sufficient to support fidelity of RUNX regulatory protein localization at intranuclear sites that support transcription [62].

III. Epigenetic Crosstalk

A. Intrachromosomal—Components of epigenetic control are temporally, spatially and architecturally integrated to support physiologically responsive regulation of gene expression. The three dimensional organization of the bone-specific osteocalcin gene provides a paradigm for conceptually understanding and experimentally defining the integration of epigenetic components to control within the multiple levels of nuclear organization [53]. Conceptually direct, but difficult to mechanistically address, is the functional organization of the cohorts of co-regulatory proteins that associate with the active transcription factors at the proximal (basal, transcriptional control) and immediate upstream (enhancer) sites. There are requirements to support histone modifications within and between the basal transcriptional and enhancer promoter domains. While formidable challenges, recent advances and rapidly evolving strategies in genomic chromatin immunoprecipitation analysis are increasing our capabilities to functionally characterize epigenetic control that is confined to specific promoter domains and operative through

communication between genomic sites. Recent analysis of genome-wide binding profiles of the bone specific RUNX2 protein using ChIP-seq has identified numerous regulatory sites that may have implications for epigenetic regulation of commitment [51, 55]. In addition analysis of the Runx2 gene locus, by chromatin capture approaches [67], has provided insight into higher order architectural organization that contributes to control a gene expression and provides a roadmap for exploring long-range components of architectural genomic organization that can be informative. The power of chromatin capture approaches includes the options to employ candidate strategies that are designed for addressing specific interactions and to utilize unbiased screens for identifying interactions that can provide novel insight into regulatory crosstalk.

B. Interchromosomal Communication—There is emerging evidence from chromatin capture strategies for functional connectivity that can contribute to “collaborative control” of gene expression. Unbiased screens can identify interactions that are not intuitively evident. Candidate approaches may be particularly important to interrogate crosstalk within the repeated copies of the ribosomal genes that reside on five acrocentric chromosomes and address context-based communications between the ribosomal gene clusters residing on the five acrocentric chromosomes. Interchromosomal communication can enhance understanding of interphase chromosomal translocations and provide an unbiased approach to identify interphase translocations [68, 69]. Examining nuclear localization of chromosomes that frequently undergo reciprocal translocations can expand regulatory insight into the molecular etiology of leukemias.

IV. Sustaining the Epigenetic Landscape

Contributions of histone modifications and DNA methylation to epigenetic control of gene expression are well documented. A recently described epigenetic mechanism – mitotic bookmarking, which is retention of transcription factors at target gene loci on chromosomes during mitosis – provides long sought insight into mechanisms that sustain competency for tissue-specific gene expression during cell division [45]. The initial observation that provided a basis for establishing mitotic bookmarking as an epigenetic mechanism for retaining transcriptional regulatory complexes with target genes during mitosis was a doubling of RUNX protein content and the number of RUNX regulatory foci during the cell cycle [70]. Equivalent distribution of RUNX proteins and RUNX foci from parental to progeny cells was observed at the completion of mitosis. Visualization of RUNX foci on chromosomes provided a direct indication that phenotypic transcription factors are mitotically inherited. Expression profiling, together with chromatin immunoprecipitation analysis, established RUNX foci that are symmetrically associated with chromosomal foci on sister chromatids are complexed with target gene loci. These findings accommodate stringent criteria for epigenetic control, establishing mitotic bookmarking by the RUNX transcription factor as a novel dimension to epigenetic regulation.

Mitotic bookmarking is not confined to the bone specific RUNX transcription factor, but has been similarly observed for other transcriptional regulatory proteins including translocation-fusion proteins that control aberrant gene expression in leukemia cells [71]. Another dimension to mitotic bookmarking that extends beyond traditional thinking about

mechanisms that govern transcription is the observation that phenotypic transcription factors, including the RUNX regulatory proteins (RUNX 1, 2 and 3), MyoD (controls myogenesis), CEBP (controls adipogenesis) and AML/ETO (controls the leukemia phenotype) co-regulate RNA polymerase II target genes as well as ribosomal genes that are transcribed by RNA polymerase I. An epigenetic mechanism for coordinate control of cell growth and phenotype is indicated that includes regulation of normal biological processes as well perturbations in gene expression that are associated with acquisition of a transformed phenotype in cancer cells [45, 72, 73].

While mitotic bookmarking increases the cellular options to sustain epigenetic management of the transcriptional regulatory machinery, there are open-ended components of control that require resolution. The extent that the complete cohort of complete co-regulatory proteins is retained in mitotic bookmarking complexes remains to be comprehensively determined. And, from a biological perspective, the modifications in mitotic bookmarking to comply with requirements for asymmetric cell division is open-ended and relevant to mitotic division of pluripotent stem cells during development as well as to lineage committed stem cells during tissue remodeling, repair and replacement.

Concluding Remarks

Here, we have presented an overview of how bone-related gene expression is controlled at multiple levels of epigenetic regulation. We have discussed the contributions of histone modifications and DNA methylation – two well established mechanisms of epigenetic regulation – within the context of bone cell differentiation. Contributions of nuclear organization of gene regulatory machinery is a recently recognized key parameter of gene regulation that we have discussed. Finally, we have described an emerging epigenetic mechanism – mitotic bookmarking – that ensures sustained lineage commitment of mesenchymal stem cells to osteoblast lineage. It is necessary to establish a complete landscape of various epigenetic mechanisms in osteoblasts for a comprehensive understanding of bone-related gene expression. An integrated approach to establishing the osteoblast epigenome within the three-dimensional nuclear architecture will provide avenues for targeted therapies in osteopathologies where epigenetic mechanisms have been compromised.

Acknowledgments

This work has been supported by grants from FONDAP 15090007 and FONDECYT 1130706 (to MM), NIH/NCI PO1 CA082834 (to GSS), NIH R01 AR039588 (to GSS), NIH/NCI R03 CA167726 (to SKZ), and VCC/LCCRO Program Grants (to SKZ).

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Table 1

Transcripcion-associated covalent histone modifications

Residue	Modification	Relation with gene expression	Localization
Histone H3			
K4	me1	Inactive or active [74]	Widespread[75]; Enhancers[74, 76]
	me2	Active[77, 78]	TSS[75]
	me3	Active[75–80]; Inactive(Poised)[81, 82]	TSS[75, 76, 80]
R8	Me	Inactive [83, 84]	
K9	Ac	Active[75, 77, 79, 80, 85–87]	TSS[75, 80, 85]
	me3	Inactive[79, 82, 88, 89]	Heterochromatin [90]
S10	P	Active[91, 92]	TSS [92]
K14	Ac	Active[75, 77, 85–87]	TSS[75, 85]
K16	Ac	Active[79]	
R17	me1/me2-asym	Active [93]	
K18	Ac	Active[87, 93]	
K20	me1	Inactive [88]	
K23	Ac	Active[87, 93]	
K27	Ac	Active [74]	Enhancer [74]
	me3	Inactive[79, 81, 82, 88, 89]	
K36	me3	Active[80, 82, 85]; Hallmark of elongation[79, 85]	Coding[80]
K79	me1	Inactive[94]	TSS or Intergenic[94]
	me2	Active[94]	Coding[80]; TSS[94]
	me3	Active[94]	TSS[94]
Histone H4			
R3	me2 (sym)	Inactive[83, 95, 96]	TSS[95]
K5/K8/K12/K16	Ac	Active[78]	Widespread[75]
K20	me3	Inactive [82]	

Abbreviations: *ac*: acetylation; *K*: lysine residues; *me*: methylation, where me1, 2, 3 denotes mono-, di-, or trimethylation; *P*: phosphorylation; *R*: arginine residue; *(a) sym*: (a) symmetrical; *TSS*: Transcription Start Site