Journal of
Carcinogenesis & Mutagenesis

Syeed et al., J Carcinog & Mutagen 2014, 5:3
DOI: 10.4172/2157-2518.1000168

Review Article Open Access

Molecular Enhancers; Remote Controls the Differential Gene Expression during Development

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Received date: Jan 20, 2014; **Accepted date:** March 24, 2014; **Published date:** March 27, 2014

Abstract

Enhancers are the DNA elements, which belong to class of regulatory sequences that can influence the transcriptional output independently of their location, distance or orientation with respect to the promoter of the genes they control. These regulatory DNA elements throughout the genome monitor the spatial and temporal expression patterns of specific sets of genes during the course of development. In the recent past, various studies suggest that the discrete chromatin characteristics of enhancer sequences are involved in directing the varied signalling molecules to distinct DNA regions that drive the differential gene expression program during the development. These diverse chromatin features contribute to the differential epigenetic patterning of enhancers which is regulated by the complex interaction between the DNA methylation status, the binding of specific transcription factor to enhancers and existing histone modifications. Herein, we present insights into the epigenetic mechanisms of enhancer functions, which eventually contribute to the repertoire of cellular mechanisms to facilitate the altered patterns of gene expression and cell differentiation choices during developmental processes.

Keywords: Enhancer; Chromatin; Epigenetics; Differentiation; Development

Introduction

The establishment of spatial and temporal patterns of gene expression has a pivotal role in the development of multicellular organisms. During the development, stem cells must differentiate into a broad choice of specialized cells though containing the same DNA. This significant achievement is accomplished by the presence of different regulatory DNA elements in the genome called enhancers, which contain the DNA sequences with various transcription factor binding sites [1]. The transcription of eukaryotic genes is extremely complex process that needs various protein complexes to interact with specific DNA sequences [2]. Enhancers are the regulatory DNA elements involved in activating the transcription irrespective of their location, distance or orientation with respect to promoters of genes they regulate [3]. Sometimes they can influence the expression of genes present in a different chromosome [4]. Conventionally, enhancers are the clusters of DNA sequences able to engage combinations of transcription factors (TFs) that later interact with components of Mediator complex or TFIID. The recruitment of RNA polymerase II (RNAP II) to the respective promoter is driven by the complexes assembled at the enhancers by looping out the intervening sequences, elucidating the ability of the enhancers to act in distance independent manner [5,6]. In addition, as the activation of eukaryotic genes needs the de-compaction of the chromatin fibre, enhancer bound transcription factors have been shown to recruit histone-modifying enzymes, or ATP-dependent chromatin remodelling complexes to alter chromatin structure and increase the accessibility of the DNA to other interacting proteins at the promoter in order to facilitate the transcription initiation or elongation [7-9]. The activity of the enhancers in monitoring the expression of specific sets of gene is highly regulated by an another class of DNA sequence elements called as *"Insulator"* which possess a common ability to protect genes from inappropriate signals emanating from their surrounding environment. The insulator blocks the enhancer functioning when it is situated between the enhancer and the promoter, not if it is placed elsewhere.

Recent advances in molecular and computational biology techniques like genome-wide location analysis (GWLA), FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements), DNAseI hypersensitivity mapping, chromatin immunoprecipitation, array technologies and more recently high-throughput sequencing have allowed the application of genome-wide mapping of different histone modifications, transcription factors and other chromatin signatures that has made a significant contribution in understanding enhancer structure and function [10]. These studies have shown that the epigenetic information is embedded within the enhancer sequences in the form of specific histone modifications. The unique chromatin signatures at enhancers offer the distinct epigenetic patterning required for regulating the cell differentiation programme during development. In addition, it appears that the enhancer sequences not only provides the landscape for the binding of transcription factors but they are also transcribed into non-coding RNAs, which together with the cohesion, may play an important role in regulating the transcription by stabilizing the long range enhancer-promoter interactions [11-16]. These findings have eventually begun to explain the possible mechanisms through which the enhancers might precisely regulate the transcription irrespective of distance and orientation with respect to promoters. Studies on varied developmental model systems have suggested that the combination of distinct patterns of histone modifications within these sequences makes the enhancers functionally different. These distinct unique patterns results from a complex interaction specific DNA binding factors, unique DNA sequences and the methylation status of DNA. The histone modification pattern in turn provides the binding site for the

transcription factors in response to the differentiation signals during development.

Dynamic Chromatin Signatures at Enhancers Depict Specificity

The advances in the chromatin biology have led to the concept that the gene expression is significantly affected by the posttranslational modifications of histones and the presence of specific histone variants. Hence, the eukaryotic transcription is immensely influenced by the nucleosome dynamics [17]. Recent studies on genome-wide mapping of epigenetically marked nucleosomes and transcriptional regulators, together with functional assays of cells and transgenic animals, have provided the insights into chromatin landscape of enhancers [10]. The in vivo mapping of several thousand p300 (histone acetyltransferase) binding sites in various mouse tissues has accurately identified novel enhancers that are involved in tissue-specific gene expression patterns in transgenic mouse assays [18,19]. For instance, predicted putative enhancers for p300 is highly enriched for histone H3 mono and dimethylated at Lys4 (H3K4me1, H3K4me2) and acetylated at Lys27 (H3K27ac) but depleted for histone H3 trimethylated at Lys4 (H3K4me3) unlike in active promoters where trimethylation is predominant at lysine 4 [20]. These results depict that enhancers are frequently associated with regions of low nucleosome occupancy termed as 'nucleosome free or depleted regions'. In addition, various well characterized enhancer regions are associated with highly unstable nucleosomes containing histone variants H3.3 and H2A.Z unlike in promoter nucleosome which is enriched in histone H3, suggesting the possibility of variant H3.3 as substrate for methylation [21-25]. The nucleosome containing enhancer regions has been found to be highly dynamic and varied histone modifications has been witnessed at putative enhancers in different cell types augmenting the functional activity of enhancers in cell type specificity [23,26]. For example, in human CD4T cells 20% of putative enhancers are associated with at least six types of histone modifications including H2A.Z, H3K4me1/2/3, H3K9me1 and H3K18ac [21], while as enhancers like CD28 responsive element (CD28RE) and the CNS22 are associated with more than 10 different histone modifications [21].

Since the enhancers are characterized by varied patterns of histone modifications so this observation has raised the question of what consequences of these histone modifications have on the regulatory activity of enhancers. In human and mouse embryonic stem cells (ESCs), it appears that enhancers of actively transcribed genes are marked by the presence of H3K4me1 and H3K27ac (Figure 1A; [26–28]). However, the enhancers of many developmental poised differentiation genes for future activation contain repressive histone H3 trimethylated at Lys 27 (H3K27me3) instead of the H3K27ac mark (Figure 1B; [26]). Likewise, in human primary haematopoietic stem cells or progenitor cells (HSCs/HPCs), enhancers of several genes involved in subsequent differentiation are enriched for H3K9me1 and H3K27me1 modifications, in addition to the H3K4me1 mark (Figure 1C; [29]). Furthermore, recent findings in *Drosophila* have shown that the H3K27ac and H3K79me3 modifications are predominant in active enhancers of mesoderm tissues (Figure 1D; [30]). These findings suggest that the pre-patterning of enhancers by H3K9me1 and H3K27me1marks might be involved in turning their target genes active, and changes in histone modification patterns of enhancers correlate with their regulatory activity. In addition, enhancers with distinct epigenetic signatures strongly correlate with particular cell type gene expression unlike in promoter regions where the characteristics of chromatin largely remains invariant across various cell types [31].

Together, these data suggest that the presence of histone variants is an essential feature of enhancers that contribute to specific cell type gene expression. Furthermore, from a variety of experimental studies it is possible to propose a model in which at least some enhancer sequences carry epigenetic information that regulates the programming of cell differentiation during development. The fundamental features of enhancers are possibly defined by the presence of H3K4me1/2 and H3.3/H2A.Z. Additional modifications might also come into play to hamper the enhancer activity and restrict their potential to activate genes.

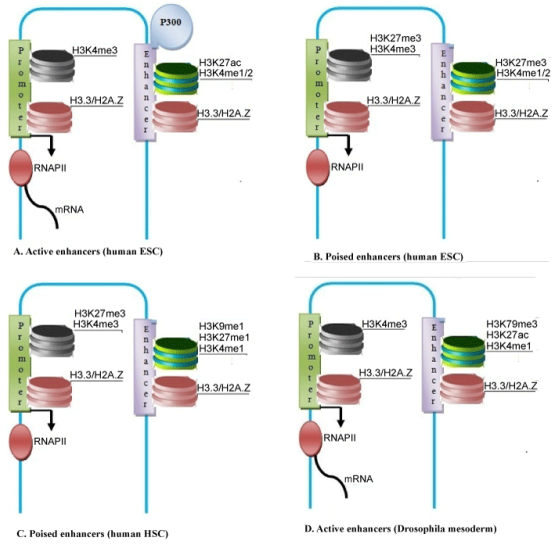

Figure 1: Distinct epigenetic signatures are related to enhancer functions in different cell types. Many enhancers are characterized by the presence of H3K4me1/2, H3.3/H2A.Z and the absence of an H3K4me3 mark. (A) In human ESCs, enhancers of actively transcribed genes are marked with H3K27ac. However, enhancers associated with poised genes contain (B) H3K27me3 in human ESCs or (C) H3K9me1 and H3K27me1 in human haematopoietic stem cells. (D) In Drosophila mesoderm cells, enhancers of actively transcribed genes are marked with H3K27ac and H3K79me3. Dark grey nucleosomes contain canonical histones. Pink nucleosomes are highly dynamic and contain histone variants. Green nucleosomes contain modified histones that are associated with many enhancers. ESC, embryonic stem cell; SC, stem cell.

Chromatin Features at Enhancers Act as Epigenetic Signals for Gene Activation

Studies on differential gene expression patterns during development of multicellular organisms have led to the question of what regulates the binding of particular transcription factors in different cell types in response to varied differentiation signals. On the basis of strong correlation between DNase I hypersensitive sites (DHS) and gene regulatory sequences, the transcriptional factors preferentially bind to histone depleted chromatin regions [32,33].

Recent genome wide findings have also supported the view that transcription factors were found to bind preferentially to their target DNA sites located within nucleosome depleted regions. On the contrary, the recognition sites occupied by the nucleosomes are often inaccessible for the binding of putative transcription factors [34-36].

The presence of unstable H3.3 and H2A.Z histone variants containing H3Kme1/2 modifications as well as other specific histone variants and modifications at enhancers might provide the specific binding sites for the transcription factors. Indeed, the transcriptional efficiency of *MYOD1* gene was shown to be dependent on the H3K4me1 modification at its enhancer within the nucleosome depleted regions [25]. The nucleosome depleted regions at enhancers are associated with predetermined chromatin signatures involved in determining the cell type specificity. Consistent with this view, cell-selective glucocorticoid receptor occupancy patterns appear to be comprehensively predetermined with chromatin accessibility patterns. Different set of glucocorticoid receptor (GR) recognition sites are hypersensitive to DNase I in different cell types. Upon hormone induction 95% of genomic binding of the GR is targeted to pre-existing region of accessible chromatin marked by DNase I hypersensitivity [37]. However, the pioneer transcription factor foxhead box proteinA1 (FoxA1) might have evolved a slightly different tactics to execute cell specific gene expression, as it has been shown to bind to nucleosomal DNA [38]. In MCF7 cells, enhancers are marked with H3K4me1/2 have estrogen receptor binding sites whereas in LNCaP cells, H3K4me1/2-marked enhancers have androgen receptor binding sites. Therefore, the differential binding of FoxA1 to selected H3K4me1/2-marked enhancers is followed by recruitment of either oestrogen or androgen receptor to activate the oestrogen and androgen-responsive programmes respectively [39].

Together, the present data suggest that enhancers are epigenetically modified before gene activation, and the recruitment of different transcription apparatus as well as chromatin modifying enzymes is driven by H3K4me1/2 or H3.3/ H2A.Z marks present at enhancers. In addition, the enhancer sequences contain epigenetic information that changes in complexity and records the differentiation history of cells during development. At each juncture, the epigenetic information can be interpreted by cell-type specific transcription factors. Therefore, the idea that the cell-specific distribution of epigenetic marks at enhancers might be responsible for particular signalling output during differentiation is sustained by the presence of H3k4me2 marks at enhancers of developmentally poised haematopoietic genes in multipotent haematopoietic cell line [40].

Cross-Talk between Pioneer Transcription Factors and DNA Methylation Status Determine Enhancer Patterning

During cell differentiation the pre-existing histone modifications at various enhancer sequences regulate the gene expression in response to various signalling inputs. However, the enigma of how enhancer sequences attain the specific patterns before cell fate decision is poorly understood. Although the enzymes involved in various histone modifications and H3.3/H2A.Z deposition are well characterized [41-43], their spatial and temporal interaction with DNA regulatory elements remains mostly unclear. Genome-wide mapping of H3K4me3 and H3K27me3 marks in zebra fish embryo has shown that these marks are deposited at promoters of both active and inactive genes in the absence of sequence-specific transcriptional activators or stable association of RNAPII, suggesting that enhancers acquire chromatin modifications before diverse transcriptional programmes are turned on in embryonic stem cells (ESCs). Recent analysis of chromatin modifications, transcription factor occupancy and DNA methylation status in various developmental model systems have provided the insights into how the recruitment of chromatin modulators and DNA methylation status are involved in determining the enhancer patterning.

Methylation state and histone modifications

One of the key mechanisms of epigenetic regulation in multi cellular organisms is DNA methylation [44]. In mammals, DNA methyltransferases establish and maintain methylation of cytosine residues in DNA within CpG dinucleotides. CpG islands (CGIs) are short genomic regions highly enriched in CpG dinucleotides. Interestingly, CpGs located within CGIs tend to be unmethylated compared with other sites across the genome. The mapping of epigenetic changes that occur during haematopoietic development has revealed a complex interdependence between DNA sequence, histone modifications and developmental gene function [45]. For instance, enhancers of haematopoietic lineage-specific genes are unmodified (H3K4me2-/me3-) in ESCs. These enhancers acquire H3K4me2 marks only upon their commitment to multi-potent haematopoietic stem cells, poising their genes for future expression during terminal differentiation. The association of known haematopoietic transcription factors—for example, PU.1—with these poised enhancers suggests that PU.1 might be the key player involved in establishing the H3K4me2 modification upon ESCs differentiation. However, CGI-containing genes are largely composed of either poised developmental regulators (H3K4me2+/me3–) or constitutively active housekeeping genes (H3K4me2+/me3+) in ESCs [41]. The correlation between histone modification and CGI status suggests that DNA methylation might influence the H3K4me state of enhancers. Consistent with this assumption, recent comparative studies on CD4+ conventional T cells and regulatory T cells have shown that more than 100 differentially methylated regions (DMR) are present in cell type-specific genes with differential patterns of histone H3 lysine 4 methylation. Interestingly, the majority of DMRs were located at promoter-distal sites, and many of these areas harbour DNA methylation-dependent enhancer activity in reporter gene assays [45]. Furthermore, an inverse relationship between cell-specific modifications and DNA methylation has been observed at enhancers of ESCs and differentiated IMR90 lung fibroblasts [46]. Although these results does not provide a significant insight into the functional relationship between histone modifications and DNA methylation at the enhancers but suggest that lineage-specific enrichment of HK4me1 at enhancers might be in part regulated by a low level of DNA methylation.

ESC factors and epigenetic marks

Recent studies have shown that the epigenetic state of enhancers is intricately orchestrated in a stepwise fashion by multiple transcription factors during development. In ESCs, the pluripotent state is maintained by the transcription factors Oct4, Sox2 and Nanog [47]. In addition, Oct4 and Sox2 also have a role in germ-layer fate selection apart from maintaining ESC identity. Oct4 suppresses neural ectodermal differentiation and promotes mesendodermal differentiation, whereas Sox2 acts in an opposite fashion. Therefore, the differentiation signalling inputs continuously and asymmetrically

modulate Oct4 and Sox2 protein levels, altering their interaction with the DNA, and leading to cell fate decisions [48].

Insights into the relation of chromatin signs and pluripotency have raised the question of how Oct4 or Sox2 affect the epigenetic pattern of enhancers. Studies in B-cell and neural lineage development support a 'factor relay model' in which ESC factors establish active epigenetic signatures at tissue- specific elements before being replaced by cell-type-specific factors as ells differentiate [49]. For instance, the deposition of H3K4me2 mark in the enhancers of various B-cell differentiation genes in ESCs is facilitated by Sox2 factor. The replacement of Sox2 by the lineage-specific transcription factor Sox4 at these enhancers leads to specific gene expression as ESCs differentiate into pro-B cells [49]. Likewise, Sox2 preselects for neural-lineage-specific genes in ESCs destined to be bound and activated by Sox3 in neural precursor cells [50]. In addition to histone modification, the DNA occupancy of varied signalling molecules at distinct enhancers to execute cell-type-specific responses might also be regulated by lineage/master transcription factors. [51,52]. For example, binding of the transcription factors Smad2/3 to their DNA recognition sequences is governed by Oct4 in ESCs, PU.1 in proB cells and Myod1 in myotubes.

The organization of epigenetic marks at diverse enhancers by other transcription factors has been shown in several other model systems. Geminin was initially characterized as a nuclear protein that could regulate the expansion of the neural plate in early *Xenopus* embryos and inhibit DNA replication origin licensing [53,54]. It was recently revealed that Gemini is involved in restraining the mesodermal and endodermal lineage commitment in the early *Xenopus* embryo by recruiting the Polycomb-group protein Ezh2 is necessary [55]. However, during development of the ectoderm, Geminin promotes the neural fate acquisition of mouse ESCs by maintaining the chromatin of lineage-specific genes in an accessible and hyper acetylated state [56]. These results suggest that the transcription factors might have distinct roles in regulating chromatin signatures at different enhancers.

Relationship between TF binding and DNA methylation

Studies on GR gene expressions have provided the insights into interesting interplay between the roles of DNA methylation and transcription factor binding in the establishment of epigenetic features at enhancers [57]. In GRresponsive cells, GR can bind to pre-programmed DHS (accessible) chromatin sites, or at non-DHS *de novo* binding sites. Pre-programmed DHS sites are enriched for CpG whereas *de novo* binding sites are located in regions with low CpG density. Interestingly, CpG demethylation at pre-programmed GRbinding sites correlates with cell-type-specific DHS sites. However, the mechanism underlying the functional association between DNA methylation and DHS sites at the pre-programmed GR sites remains unclear. It was recently revealed that DNA methylation patterns might be influenced by the nucleosome positioning and DNA methyltranferases prefer to target nucleosome-bound DNA [58]. This suggests that the presence of pre-existing DHS sites—that is, nucleosome-depleted regions—might precede the removal of DNA methylation. In addition, DNA methylation can be reduced by GR binding at the de novo binding sites [57], suggesting that pioneer transcription factors such as GR can create unmethylated DNA regions at enhancers [57]. Consistent with this idea, FoxA1 can bind to both highly methylated CpG sites as well as cell-specific hypomethylated enhancers during the neural differentiation of P19 cells. FoxA1 binding in turn leads to DNA demethylation and deposition of the H3K4me2 modification at enhancers [59]. Similarly, the fork-head family member FoxD3 is essential for maintaining the unmethylated CpG mark at the enhancer of the lineage-specific gene *Alb1*in ESC cells [60]. Together, these results suggest that the establishment of epigenetic signatures at enhancers might be regulated by the intricate interplay between the status of DNA methylation, histone modifications and other chromatin factors. The pattern at a subset of enhancers of a particular cell type might be a transient end-result of this interplay, where any of the three components is likely to be a catalyst of the patterning process.

Enhancers in Epigenomic Reprogramming

The epigenetic pre-patterning of enhancers via distinct histone modifications has a major role in cell specific gene activation during ESC differentiation. As the changes in epigenetic patterns at enhancers depict the cell-type specificity [29,40,61], reprogramming of somatic cells to Induced pluripotent stem cells (iPSCs) would impose the reversion of these changes. The pre-patterning of enhancers in ESCs, derived from the inner cell mass at the blastocyst stage, is likely to occur during the early stage of embryonic development. The information embedded in the enhancer patterns of ESCs might be inherited from epigenetic signatures carried in the mature egg and sperm. Therefore, on the other way it might be re- switched during spermatogenesis and oogenesis, and re-established after fertilization in the zygote. Genome wide analysis of chromatin characteristics in sperm suggests that both events might take place at different loci. Consistent with this view, the overlapping distributions of histone modifications and DNA methylation patterns has been observed across different genomic regions of sperm and ESCs [62-64]. For instance, similar epigenetic patterns (H3K4me3/H3K27me3 and hypomethylated DNA) are carried by the genes involved in developmental processes whereas genes involved in specific functions —for example, spermatogenesis and *HOX* clusters—exhibit significant variations. A similar observation was made in zebra fish sperm, in which genes activated after mid-blastula transition are pre-patterned by specific histone modifications [64]. However, the dynamic or unstable nature of epigenetic marks present in the sperm is indicated by the low levels of histone modifications in pre-mid-blastula transition after fertilization [65,66].The insights into the remodelling of epigenetic marks at enhancers during normal development might be applicable to the understanding of the mechanisms underlying the establishment of iPSCs. These cells can be obtained through the reprogramming of somatic cells by ectopic expression of defined transcription factors [67]. Although the reprogramming of various cells has been improved by using certain chemicals compounds that alter DNA methylation or chromatin modifications [68], recent comparison of iPSCs and ESCs revealed that the former retained significant somatic epigenetic patterns in the form of DNA methylation and histone modifications [69,70].Similar observations were made in the analysis of the H3K4me1 mark in iPSC enhancers [28]. It therefore remains a challenge to understand how different technical methodologies affect the quality of iPSCs in terms of epigenetic features, transcriptional marks, and genomic integrity. In addition, evidences accumulating from the studies of enhancer function indicate that epigenetic outcomes can be highly context-dependent and dynamic. For instance, the binding of Oct4 or Myod1 to the same permissive enhancers might result in entirely different epigenetic outcomes [25]. Myod1 activates its own transcription by binding first at the enhancer, which then leads to the formation of a transcription-permissive nucleosome-depleted region at its associated

promoter. However, the binding of Oct4 to the enhancer converts the monovalent H3K27me3 mark at its cognate promoter into a bivalent state characteristic of stem cells [25].This suggests that pluripotency transcription factors can coordinate the epigenetic states of enhancer–promoter pairs throughout the genome. Together, these studies suggest that enhancers might act as the signal-integrating sites for reprogramming the epigenome. Further insights are needed to understand how different reprogramming factors alter the epigenetic state of enhancers during developmental processes.

Conclusions and Perspectives

Advances in genomic technologies have provided unprecedented opportunities to advance the understanding of enhancer features and functions. Accumulated evidence indicates that enhancers play a pivotal role in regulating the developmental processes by contributing to the epigenetic mechanisms responsible to determine cell fate choices and competency during development. The emerging evidences suggest that enhancers impart epigenetic memory and dictate context-dependent signalling outcomes through their unique chromatin characteristics. Histone modification patterns at the enhancers may determine cell-fate choices by fine-tuning the transcriptional output via differential recruitment of transcriptional factors and other chromatin-modifying enzymes during the developmental processes. The future challenges in this field are clear. Many questions remain to be answered. How these varied histone modifications co-ordinate in determining the cell type specificity?. One of the major challenges will be to understand the regulatory mechanisms that maintain or edit chromatin modification patterns at enhancer sequences during cell differentiation. To better understand the mechanisms of enhancer function during development, it will also be valuable to identify the chromatin characteristics of enhancers associated with various signalling pathways like TGF-β Notch, Hedgehog, Hippo and Wnt. It is very apparent that the epigenetic states of enhancers are highly dynamic and regulated by varied mechanisms during development. Therefore, it will be interesting to find out whether the epigenetic marks of enhancers are also regulated by other strategies like RNAi machinery/ non coding RNA, Posttranslational modifications of particular transcription factors and nuclear organisation. Furthermore, future progress towards the goal of understanding how chromatin features at specific enhancers is regulated and coordinated during development will greatly benefit from ongoing improvements in genomics, as well as from the ability to combine studies of development with studies of somatic cell reprogramming.

Acknowledgment

We thank the Dr. Mohammad Saleem Bhat for critical reading of the manuscript. Due to space limitations, we apologize to those colleagues whose publications could not be cited.

Conflict of Interest

The contributing Authors have no financial or any non-financial competing interests.

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