The Induction of Differentiation and Recruitment of PcG Proteins by RNAi

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April 23, 2010
1 Introduction

Chromatin structure is important in specifying a cell’s function. While it is unclear if epigenetic changes cause a cell’s expression profile to change, there is a strong correlation between the two. Moreover, a cell’s chromatin structure reflects its differentiated state by placing necessary and unnecessary genes in euchromatin and heterochromatin respectively\textsuperscript{1–3}. It can also allow a differentiated cell to “tweak” its function. For example, the nucleosome positioning profile of naive T-cells changes in response to the presentation of an antigen\textsuperscript{4,5}. Between their naive and learned state, the nucleosome profile shifts to turn on or off genes needed to express the appropriate proteins for the T-cell to be active. Hence,

The development of an organism and differentiation of tissues and cells relies, in part, on changes to the chromatin structure of dividing cells. Embryonic stem (ES) cells begin in a highly euchromatic state\textsuperscript{6,7}. As the cells differentiate, the former ES cells lose euchromatic markers and have their chromatin condense in specific areas. Hence, there is some process(es) by which a cell targets specific areas of the chromatin to fold into heterochromatin (or, conversely, to remain open). One interesting discovery, that occurs in several different species, has shown several, non-metabolic genes maintain a “bivalent” state (i.e., containing both heterochromatic and euchromatic markers) while in pluripotency\textsuperscript{8}. These bivalent domains tend to be genes that are either involved in development and/or specialized cell function. As a cell differentiates, each bivalent locus turns on or off by enriching itself with either the heterochromatic or euchromatic marker while losing the other marker. These sites allow for specific patterning of the gene expression profile of differentiated cells.

Part of this chromatin remodeling phenomenon during development relates to the Polycomb group (PcG) proteins. This protein family acts as a repressor by mediated binding to a Polycomb Response Element (PRE) and tri-methylating H3K27 of the downstream gene (a repressive chromatin mark). This group of portreins was first identified in \textit{Drosophila} in their role of silencing HOX genes\textsuperscript{9}. Since then, the PcG has been demonstrated to have an expanded role throughout the genome and to exist in many other species, including mice\textsuperscript{9} and humans\textsuperscript{10}. They are key in development of embryonic stem cells by being one the main chromatin remodeling signals in the cell.

Concurrently, small RNAs play a major role in the cell by regulating gene expression\textsuperscript{11}, although they are not typically associated with chromatin remodeling in accomplishing this task. The typical mechanism and proteins involved in creating siRNAs, targeting mRNAs, and degrading mRNAs is well-understood across many different eukaryotic species. Yet, it appears this siRNA pathway does more. For example, it has been shown in \textit{S. pombe} that siRNAs can direct chromatin modifying proteins to specific loci to create heterochromatin (generally by enriching an locus with H3K9me3)\textsuperscript{12,13}. Plants have a similar chromatin remodeling mechanism aided by siRNA as well\textsuperscript{14}. While regulating existing transcripts, siRNAs (and correspondingly miRNAs) can downregulate a gene by directly inhibiting its transcription by putting it in heterochromatin\textsuperscript{13,15}.

Given these two parts that are involved in gene regulation and chromatin remodeling, it is not hard to imagine that they may aid each other’s role. To utilize its repressive behavior, the PcG proteins must be directed to a PRE; however, this mechanism is not
fully understood in mouse or other mammals. An small RNA approach, especially one that already activates the chromatin modifying proteins, may just be the answer. It is known that ncRNA directs PcG in many different instances. For example, ncRNA drives X-inactivation and gene imprinting by recruiting PcG in mammals. An added approach for targeting these PcG proteins may be to use small RNAs. Currently, there is not many examples for this relationship, other than a set of specific cases in *Drosophila*. But the two mechanisms share common goals, and RNA already influences PcG proteins. Therefore, further investigation using a mouse model in examining of how to direct chromatin remodeling in embryonic stem cells and PcG recruitment would provide evidence for or against the correlation between these two pathways.

2 Aim 1: Profile the endogenous miRNAs within the cell and within the genome.

2.1 Rationale

The initial goal is to identify RNA seeds that would target genes in the genome. Since it is unclear exactly where these small RNAs could come from, a broad screen of the cell is necessary. The primary location for this type of information would be the mouse genome. Using annotation databases of miRNA genes, it would be useful to find existing miRNAs that would potentially fulfill this role. Since it is possible, though unlikely, this could produce a dearth of potential seeds, further genomic analysis of promoter regions and pre-mRNA transcripts would be needed to create more potential candidates. This is because since it is not fully clear if these small RNAs target genomic DNA or nascent transcripts, both should be gathered to cover both scenarios.

A more specific analysis that would reduce the above list would be to assay the actual presence of small RNAs within the differentiated cell type. Since the target of the project is to push ES cells towards skin stem cells, then it is necessary to pull down all small RNAs within both types to compare enrichment. Since the RNAi pathways are likely in a positive feedback loop, any small RNAs that targeted specific loci that were turned off could potentially be still floating within the nucleus, remaining around to continue to maintain the chromatic structure.

2.2 Plan

The first step would be to look through existing miRNA gene annotations within the mouse genome, such as those that could be found at the UCSC Genome Browser. These existing annotations would have what anticipated RNA seed the transcript would produce. Comparing these seeds against annotated promoter sequences and pre-mRNA transcripts, matching seeds would create an initial list of candidates. It is possible, although unlikely, that there would be no candidates derived from this step. A further step is to look at the annotated promoter and pre-mRNA transcripts and add sequences that would be good candidates for
To reduce the list of candidates, it would be helpful to look at the enrichment of miRNAs within differentiated cells relative to ES cells. For extracted skin stem cells and ES cells (as the baseline), I would extract the miRNAs using the mirVarna kit (Ambion) and sequence them. After obtaining the sequences from both extracts, I would look at the enrichment of the RNA seed in the skin stem cells relative to the ES cells. Enrichment of specific sequences would be prime candidates for further analysis in later aims. Since it is not completely possible to determine which seeds would be more likely to be involved in chromatin remodeling, all detected seeds would be taken and ordered by enrichment.

There is a possibility that there would be no enrichment of various small RNA seeds within the cell.

3 Aim 2: Assay miRNA-injected ES cells with ChIP-Seq for changes in chromatin state.

3.1 Rationale

After identifying small RNA sequences that would target the geneic regions in the genome, it follows to directly test their effects by injecting the sequences into ES cells. Injecting the cells with a set of siRNAs that target genes should cause the enrichment of H3K9me3 and H3K27me3 to increase around the targeted genes. These changes can be detected by running a CHiP-Seq assay against H3K9me3 and H3K27me3 of the induced cells. An increase in enrichment of either, ideally both, would point to the miRNAs inducing heterochromatic remodeling around specific regions. Additionally, any depletions at other non-targeted loci would suggest that there is some negative feedback mechanism: when one locus turns off, it induces another.

To test the involvement in development, an assay against mouse’s PcG proteins can be done to test if these siRNAs can recruit PcGs to PREs. Although the PRE sequence in mouse is not fully clear, genes near CpG rich regions or known developmental genes (e.g., HOX genes) can be tested directly, since PcG proteins are known to bind in these types of regions. Moreover, these target loci should also be bivalent domains. Any correlation between recruitment of PcG and RNAi injection would suggest some overlap in their mechanism.

3.2 Plan

To assess changes in H3K9me3 and H3K27me3, the profile of ES cells would first need to be assayed via ChIP-Seq for both markers, acting as a control/input for enrichment analysis. Concurrently, combinations of five (or more) small RNAs, gathered from the previous aim, would be injected into ES cells and allowed for the remodeling to take process. For each of these sets of injected ES cells, another ChIP-Seq assay targeting H3K9me3 and H3K27me3 would be run to gather the profile for each. Using spp, the enrichment for each set of injected cells for the markers would be calculated. If the remodeling worked, then there should
be an enrichment of H3K27me3 at the targeted loci. Presumably, all loci targeted would show increased enrichment, and each loci could be individually confirmed using qPCR. More interestingly, there could possibly be significant enrichment or depletion at non-targeted loci, suggesting some feedback relationship between the remodeling of different loci.

Then the analysis can be more focused towards bivalent domains with a PcG binding site, primarily the HOX genes. As a baseline, a ChIP-Seq assay targeting PcG proteins at these loci in ES cells establishes where PcG proteins are. After the injection of siRNAs, as above, each group of cells can be assayed using ChIP-Seq for these PcG proteins. Since there is a circular correlation between the enrichment of H3K27me3 and the binding of PcG proteins, this injection should directly or indirectly induce PcG proteins to bind at the targeted loci. However, it is possible that this recruitment works for none or only some of the targeted loci (which has been the case in Drosophila). Yet, the widespread demonstration to control the heterochromatic remodeling of multiple loci concurrently would still be a useful tool, and showing which loci have a relationship between PcG proteins and RNAi may allow identification of specific proteins or DNA elements that allow this combination.

4 Aim 3: Drive ES cell to a differentiated state.

4.1 Rationale

After showing the ability to induce heterochromatic remodeling in ES cells, it becomes meaningful to show how this tool could be applied in development and in the lab setting to promote differentiation in a controlled way. By using skin stem cells' H3K9me3 and H3K27me3 profiles as a goal of chromatin structure, specific loci of ES cells can be targeted with these RNAi seeds to induced remodeling of these loci. By comparing the profile of these induced cells and its surface proteins, a rough picture of how well RNAi alone can induce this type of differentiation.

4.2 Plan

In previous steps, the control of ES cell H3K9me3 and H3K27me3 can be reused. Additionally, the profile of skin stem cells, the differentiation goal in this aim, for H3K9me3 and H3K27me3 will also be obtained by using ChIP-Seq. This profile, relative to the ES cells, would provide what loci would need to be turned off to push an ES cell towards this skin stem cell state. Using this list of loci, siRNAs would be designed to target these loci, ideally from those recovered from the first aim. These siRNAs would be injected into ES cells. Then these induced cells would be assayed for H3K27me3 and H3K9me3, and the profiles of the induced cells and the skin stem cells would be compared, likely using ChIPDiff. Ideally, the chromatin profiles of the injected cells and the actual skin stem cells would be very similar in enrichment. Ot is likely that, however, while closer than ES cells, the injected ES cells would not be completely like the skin stem cells in terms of their H3K9me3 and H3K27me3 profiles, suggesting there are other, non-RNAi steps necessary to get to that stage. Additionally, as a secondary measure of how well the induction worked, the injected cells can be
immunostained for epithelial surface proteins. Positive immunostaining of the injected cells would be another indicator that the remodeling of the chromatin induced internal changes, making it act more like a differentiated cell.

5 Summary and Significance

The basic biological importance of this work is the better understand the cellular mechanisms of driving a cell towards a particular type. There are two known mechanisms, the Polycomb complex and RNAi, that have worked, not always in relation with chromatin structure, parallel but have not generally been associated with each other. Yet, since both can modify the chromatin structure and PcG proteins need a targeting mechanism to reach PRE, it is not hard to imagine that these two components could be, if only sometimes, linked. By looking at specific small RNAs that target bivalent domains and looking at the enrichment of heterochromatin and PcG proteins nearby, a picture of how these two are linked can be made.

The expected result would be that small RNAs can target PcG proteins in mice to a wide array of targets. These results would be reflected in the increased enrichment of H3K9me3 and H3K27me3 with PcG proteins present near the loci. However, since PcG proteins tend to relate with enrichment of H3K27me3 and RNAi-induced chromatin remodeling relates with H3K9me3, it is possible that they overlap in only a limited set of cases. Furthermore, the limitation of this study is that it does not show the two mechanisms are directly linked, but that there tends to be a correlation between the two pathways. Nevertheless, the “tool” factor of the project (inducing chromatin remodeling) is useful, and it would show how closely the Polycomb pathway remains the same evolutionarily with *Drosophila* (in that there is some overlap between these two pathways).

Practically, this work is useful in synthetic biology and similar areas. This experiment provides a proof-of-concept tool in attempting to “program” a cell to become a certain type by altering the chromatin structure. Being able to use small RNAs to target specific loci to be put in heterochromatin could be useful in designing circuits that program cells to differentiate in a specific time-frame. Moreover, the widespread use of RNAi technology would make the deployment of this type of experiment relatively easy.
References


