pluripotent cells, while Tet3 (presumably in combination with Tet1 or Tet2) may function to control hydroxylation in differentiated cells. Nonetheless, Koh et al. leave us with several lingering issues that need to be addressed before we will understand how DNA methylation and DNA hydroxylation are functionally embedded in the epigenetic and genetic network control of pluripotency. First of all, it will be important to precisely determine the location of 5hmC modifications in ESCs, as well as the gene specificity of individual Tet enzymes. Furthermore, the targeting mechanisms that direct individual Tets to specific regions/genes need to be investigated. Finally, it remains to be seen how hydroxylation itself modulates gene expression.

Toward these goals, Song et al. (2011) have recently presented one approach designed to locate 5hmC modifications in the genome. Their study suggests that in adult brain cells, which express Tet3, 5hmC is enriched, relative to 5mC, in intergenic regions and both upstream and downstream of the transcription start site (TSS). However, the distribution of this mark is likely to be distinct in ESCs and in actively differentiating cells, given that Tet1 and Tet2 are the predominant enzymes expressed in these populations and, relatively speaking, Tet3 is absent.

Technologies that achieve simultaneous detection and location of both 5mC and 5hmC are only beginning to be developed, but with these new tools the field may soon see ESC profiles that will hopefully point to answers for these questions. ChIP and mass spectrometry experiments with individual Tet enzymes in ESCs will provide insight into interacting partners and enzyme binding locations. RNA-Seq in combination with direct 5hmC mapping techniques (e.g., through hMeDIP) in ESCs depleted for either of the Tet enzymes will provide further answers as to possible mechanistic links. But as stated by Koh et al. (2011), the relation between Tet function and DNA methylation is (apparently more) complex and not necessarily always promoter directed. It remains an open question as to which direct or indirect mechanisms link the presence and absence of 5hmC to the control of gene expression. Despite the lengthy list of unresolved questions, the (re)discovery of the sixth base and of the regulation of Tet enzymes will greatly influence our understanding of epigenetic control in stem cells. The time for new epigenetic concepts in stem cell research is on the horizon.

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A Comprehensive Transcriptional Landscape of Human Hematopoiesis

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Recently in *Cell*, Novershtern et al. (2011) reported a comprehensive transcriptome analysis of human hematopoiesis, combined with sophisticated bioinformatics analysis and high-throughput DNA binding data for multiple transcription factors. The resulting map of regulatory interactions controlling stem cell differentiation provides a valuable resource for identification of novel hematopoietic regulators.

All stem cells share the properties of self-renewal and multilineage differentiation, and understanding how these processes are controlled is a central question of developmental biology. The hematopoietic system serves as an excellent model for delineating the regulatory networks that control self-renewal and differentiation of stem cells owing to its easy isolation and manipulation and because dysregulation of this system results in severe diseases including immune deficiency and leukemia. Hematopoietic stem cells (HSCs) give rise to at least eight distinct cell lineages through a process in which self-renewal potential is lost as HSCs differentiate into progressively restricted multipotent progenitors that ultimately become restricted to a specific cell fate (Adolfsson et al., 2005). Multiple transcription factors are known to be required for development of specific hematopoietic lineages or for self-renewal of HSCs, and their perturbation through gene

deletion or ectopic expression has allowed their placement into regulatory networks that control essential decision points (Orkin and Zon, 2008). While the analysis of single transcription factors has been highly informative, such studies allow us to build relatively small networks with a limited number of transcription factor interactions and a few defined targets. The development of genome wide approaches, including gene expression profiling and chromatin immunoprecipitation followed by deep-sequencing (ChiP-seq), has revolutionized the scale on which regulatory networks and transcription factor targets can be identified. A new study reported in *Cell* (Novershtern et al., 2011) takes advantage of these approaches, combined with sophisticated bioinformatics analysis, to provide an unprecedented view into the depth and breadth of the regulatory networks controlling human hematopoiesis.

The study by Novershtern et al. (2011) is centered on the analysis of the gene expression profiles of 211 human samples representing 38 distinct hematopoietic populations. These populations included mature lymphocytes, monocytes, granulocytes, erythroid cells, and megakaryocytes and their lineage restricted and multipotent but non-self-renewing progenitors as well as HSCs. The resulting gene-expression profiles were compared by multiple clustering methods and revealed a set of 80 distinct profiles, or modules, some of which were cell-type specific while others were observed in multiple populations. Ten modules showed lineage-specific expression and contained transcription factors shown previously to regulate development of the associated lineage. The remaining modules were divided between those with HSC and multipotent progenitor expression and those that come on later in development and are reused in multiple lineages. These expression modules have the potential to reveal important regulators of human hematopoietic differentiation. For example, there were eight transcription factors expressed in the granulocyte plus macrophage module, but only 2 of these, CEBPA and PU.1, have been described as regulators in these cells (Laslo et al., 2008). By association, the remaining six transcription factors may play a role in granulocyte or macrophage differentiation, although this remains to be tested directly. Gene-expression profiling has been done previously to compare mouse hematopoietic populations, and the conclusions from these studies are highly congruent (Chambers et al., 2007). For example, lineage priming, the low-level activation of lineage-associated genes in multipotent progenitors, is a common feature of both mouse and human hematopoietic progenitors (Mansson et al., 2007; Yoshida et al., 2010). In addition, in both mice and humans, the gene expression profiles of HSCs and multipotent progenitors are highly distinct from mature cells, as different as the expression profiles of different tissues (Chambers et al., 2007).

While gene expression profiles are informative and frequently reveal processes vital to the function of the cell, an important question is how these gene expression modules are established and maintained. To investigate the regulatory mechanisms controlling gene expression modules, Novershtern et al. (2011) enlisted both an ''expression-based'' and a ''sequence-based'' analysis. In the expression-based analysis, transcription factors that were expressed in the modules were analyzed using the ''modules network algorithm'' to identify factors that ''explain'' the expression profile of the module. They identified 220 transcription factors that were associated with at least one module, but remarkably, only 15 of these factors were previously known to function in hematopoiesis. The model predicts that when a chosen transcription factor, or set of transcription factors, is present, the module will be expressed. When these key regulators are placed within the hematopoietic tree, clear points of regulation emerge.

In the sequence-based analysis, promoters for the genes in a module were examined for common motifs using six motif-finding algorithms and a motif-clustering pipeline. These motifs were then compared to multiple databases of known transcription factor binding sites. Both techniques have limitations. For example, the expression-based analysis will fail to identify factors that are not differentially expressed, whereas the sequence-based analysis may fail to identify important motifs if they are rare. Notably, the concordance between these approaches was not high, potentially due to these limitations. The authors proceeded to combine the expression-based and sequence-based analysis results with known transcription factor interactions to connect the factors expressed in the modules to their potential transcription factor targets. The resulting networks reveal dense transcriptional circuits in HSCs, which decline during differentiation, while new but less intricate circuits emerge. While the networks were quite crowded, the number of factors involved in establishing the modules is likely an underestimate because 66 of the motifs identified in the sequenced-based analysis were for unknown binding factors. The abundance of networks in HSCs is noteworthy, particularly in light of the observation that mouse HSCs have a larger transcriptome than their differentiated progeny (Chambers et al., 2007).

Importantly, Novershtern et al. (2011) tested their model using multiple high-throughput approaches. They identified targets of four transcription factors, MEIS1, TAL1, PU.1, and IKAROS, in HSCs using ChiP-seq and found a good correlation with the targets predicted in their model, demonstrating self-regulation, feed-forward interactions, and coregulation of target genes. Intriguingly, they also found ''anticipatory'' regulation, that is, interactions with target genes that are not expressed in HSCs but, rather, at later stage of development. This anticipatory binding may underlie lineage priming, in which lineage-associated genes are transcribed a low abundance in progenitors prior to lineage specification (Akashi, 2005).

All of these approaches taken together reveal potential regulatory networks that control hematopoietic differentiation. However, the real test of the model was to show that the networks identified are essential in differentiating cells. To address this issue, Novershtern et al. (2011) developed a multiplex bead PCR assay to confirm the expression profiles of 33 transcription factors during HSC differentiation to the erythroid or myeloid-monocytic lineages, and they used this assay to determine the functionality of shRNAs against each factor. The selected set of factors could distinguish the two lineages both in vivo and in vitro using progenitors from the adult or cord blood. Moreover, the requirements for 17 factors were tested in an in vitro differentiation assay using shRNA-mediated

knockdown. Notably, 9 of these factors affected erythroid development whereas 8 factors affected myeloid development. More than half of these factors were predicted to regulate the specific lineage based on the sequence or expression-based model. Moreover, two of these factors, HIF3A and AFF1 (encoding the AF9 protein), were not previously implicated in erythropoiesis but emerged from this study based on their expression profile. The scope of the analysis performed in this study on human hematopoietic progenitors provides a rich resource for identifying regulatory networks controlling HSC self-renewal, differentiation, and lineage determination.

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What Your Heart Doth Know

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Combining embryological insight with careful analysis of early stage cardiomyocyte differentiation, Kattman et al. (2011) in this issue of *Cell Stem Cell* have defined minimal culture conditions to efficiently produce cardiomyocytes from hESCs and hiPSCs. The lessons learned are applicable to the derivation of other organotypic cell types.

The promise of stem cell biology as an inexhaustible source of differentiated cells for research applications and disease therapies is predicated on the ability to efficiently produce organotypic cells. A complementary body of knowledge from over a century of experimental embryology offers many insights into fundamental mechanisms of differentiation. The two fields have come together nicely in a paper by Kattman et al. in this issue, describing the efficient and controlled differentiation of pluripotent stem cells (PSCs) into cardiomyocytes (Kattman et al., 2011). The trick to working out the protocol was combining the use of embryological markers with careful titrations of signals that drive cells along discrete steps toward terminally differentiated, functional cardiomyocytes. The novelty resides not in the particular molecules that were used to drive the production of cardiomyocytes but, rather, in the approach they took to establish ideal concentrations and exposure windows. Although embryological development turned out to be a good guide, significant differences between PSC lines, even from the same species, necessitated devising a systematic approach that relied on parsing development into discrete steps and optimizing the use of a small number of embryological signals in each. These findings epitomize the extrapolation of experimental embryology to stem cell biology and reinforce the idea that embryonic development holds many clues for controlled differentiation of stem cells.

The strategy taken by Kattman et al. was enabled by their characterization of the VEGF receptor-2 (called Flk-1 or KDR) and PDGF receptor-α (PDGFRα) together as enriching cardiogenic mesoderm, which can form cardiomyocytes, endothelial cells, and smooth muscle, as distinct from hematopoietic progenitors in the KDR$^+$, PDGFRα^- population. Since the divergent TGFβ molecules Nodal and BMP, along with Wnt, induce cardiac mesoderm in embryos (Mercola et al., 2010), Kattman et al. optimized the concentration and duration of Activin (as a surrogate of Nodal) and BMP treatment (Figure 1). Strikingly, small differences in concentrations affect the yield of KDR$^+$, PDGFRα^+ cells dramatically and, consequently, reduce the yield of cardiomyocytes. Although anticipated by embryologists knowledgeable of threshold effects that subtle gradations in Activin concentration have on inducing different types of mesoderm, originally shown in *Xenopus* (Green et al., 1992) and later generalized to the mouse and zebrafish (Freeman and Gurdon, 2002), the finding is puzzling from a signal transduction perspective because it is not clear how a small concentration difference is reflected in qualitatively different genomic and developmental responses. Timing was as crucial as dose, because greatly reduced Activin and Wnt signaling were subsequently