High-Resolution Single-Cell DNA Methylation Measurements Reveal Epigenetically Distinct Hematopoietic Stem Cell Subpopulations

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SUMMARY

Increasing evidence of functional and transcriptional heterogeneity in phenotypically similar cells examined individually has prompted interest in obtaining parallel methylome data. We describe the development and application of such a protocol to index-sorted murine and human hematopoietic cells that are highly enriched in their content of functionally defined stem cells. Utilizing an optimized single-cell bisulfite sequencing protocol, we obtained quantitative DNA methylation measurements of up to 5.7 million CpGs in single hematopoietic cells. In parallel, we developed an analytical strategy (PDclust) to define single-cell DNA methylation states through pairwise comparisons of single-CpG methylation measurements. PDclust revealed that a single-cell epigenetic state can be described by a small (<1%) stochastically sampled fraction of CpGs and that these states are reflective of cell identity and state. Using relationships revealed by PDclust, we derive near complete methylomes for epigenetically distinct subpopulations of hematopoietic cells enriched for functional stem cell content.

INTRODUCTION

Hematopoietic stem cells (HSCs) are functionally defined cells that display evidence of extensive self-renewal of their ability to generate mature blood cells for the lifetime of the organism and following transplantation into myelosuppressed permissive hosts (Doulatov et al., 2012; Eaves, 2015). Clonal analyses of serially transplantable mouse HSCs have revealed that cells thus defined are composed of multiple distinct subpopulations that stably propagate specifically restricted abilities to produce different types of mature blood cell types (Benz et al., 2012; Dykstra et al., 2007; Kent et al., 2009; Sanjuan-Pla et al., 2013; Yamamoto et al., 2013).

Epigenetic modifications have been shown to be critical for the control of normal hematopoiesis as exemplified by the consequences of alterations incurred by disruption of de novo DNA methylation in primitive hematopoietic cells (Challen et al., 2012; Quivoron et al., 2011; Shlush et al., 2017). Moreover, in long-term HSC populations, lineage-specific enhancers appear to be epigenetically marked (Lara-Astiaso et al., 2014), and regulatory regions show gain or loss of DNA methylation during the differentiation of their progeny (Bock et al., 2012; Cabezas-Wallscheid et al., 2014). However, most of the epigenetic measurements underpinning these observations represent consensus values experimentally derived from thousands of cells partially enriched in HSCs or their progeny, thus failing to discern distinct epigenetic states within HSCs. Indeed, heterogeneity in methylation states of single CpGs is a common feature of cells assessed as bulk populations (Angermueller et al., 2016; Farlik et al., 2016; Hou et al., 2016; Hu et al., 2016; Qu et al., 2016). In addition, epigenetic heterogeneity has been observed across individual HSCs and clonally amplified HSC populations with preserved lineage potentialities (Farlik et al., 2016; Yu et al., 2016). Nevertheless, the degree to which heterogeneity in the methylome of HSCs is related to their defining properties remains poorly understood.

Assessment of the methylome of single cells is limited by measurement insensitivity and stochastic missing data. Current analytical strategies for single-cell DNA methylation measurements average DNA methylation in fixed genomic bins (Angermueller et al., 2016; Hou et al., 2016; Luo et al., 2017; Smallwood et al., 2014), or over defined genomic regions (Farlik et al., 2015, 2016; Hu et al., 2016). However, in many instances multiple regulatory regions are present within these genomic intervals and the relationship of their activity to average DNA methylation within an interval unknown. This is further complicated by the observations that the methylation state of a single CpG can affect transcription (Banet et al., 2000; Fürst...
et al., 2012; Hashimoto et al., 2013; Jinno et al., 1995; Mamrut et al., 2013; Nile et al., 2008; Tsuboi et al., 2017; Zhou et al., 2017) by altering transcription factor binding affinity (Rishi et al., 2010; Yin et al., 2017). Imputation strategies leverage sequence context along with CpG methylation states across single cells to increase the resolution of genomic intervals (Angermueller et al., 2017). However, inference across cells (as well as sequence context) assumes homogeneity across cells, which is at cross-purposes with the generation of single-cell molecular measurements through the potential to mask rare subpopulations.

To address these limitations, we developed an automated plate-based high-resolution single-cell methylation protocol that we call Post-Bisulfite Adapter Ligation (PBAL), and analyzed the resulting sequence reads with an analytical pipeline (Pairwise Dissimilarity Clustering: PDclust) that leverages the methylation state of individual CpGs. We applied this single-cell methylation framework to profile primitive hematopoietic cells of mouse and human origin to identify epigenetically distinct subpopulations. Deep sampling of the CpG content of individual HSCs allowed for the near complete reconstitution of regulatory states from epigenetically defined subpopulations of HSCs and revealed a high level of redundancy of CpG methylation states within these phenotypically defined hematopoietic cell types.

RESULTS

Post-Bisulfite Adapter Ligation

PBAL is an adaption of the post-bisulfite adapter tagging (PBAT) strategy (Miura et al., 2012) optimized for library diversity. Previous single-cell PBAT-like strategies have used random primers extended with Illumina sequences to enable direct amplification (Angermueller et al., 2016; Smallwood et al., 2014). When comparing this approach with untagged random priming, we observed that extended randomers generated shorter double-stranded DNA fragments compared with randomers alone, suggesting inefficient priming (Figure S1). To circumvent this we used untagged random primers and ligated Illumina sequencing adapters to the resulting double-stranded DNA fragments. Pooling of single-cell PBAL libraries allowed the number of PCR cycles to be reduced and hence increased library diversity without compromising the minimum yield requirements for Illumina sequencing (see Experimental Procedures).

We applied this protocol to EPCR⁺CD45⁻CD48⁻CD150⁻ (ESLAM) cells isolated by fluorescence-activated cell sorting from adult mouse bone marrow (see Experimental Procedures). This extremely rare, phenotypically defined population is of interest because it is the most highly purified (~40% pure) source of functionally defined mouse hematopoietic cells with durable (>4–6 months) repopulating activity in transplanted hosts (Benz et al., 2012; Kent et al., 2009). As a comparator population, we sorted a related but less HSC-enriched lineage-negative Lin⁻SCA¹⁺c-KIT⁺ (LSK) phenotype (~3% HSCs) (Osawa et al., 1996) (Figure 1A). We also applied our PBAL protocol to the analogously human HSC-enriched Lin⁻CD34⁻CD38⁻CD90⁻CD45RA⁻CD49f⁺ population (hereafter referred to as “CD49f cells”) isolated from two different cord blood donors and reported elsewhere (Knapp et al., 2017, 2018). Approximately 10% of these CD49f cells have long-term repopulating activity in transplanted immunodeficient mice, and contain all of those able to generate progeny with similar activity assessed in secondary hosts (Knapp et al., 2017, 2018; Notta et al., 2011).

Upon isolation, these mouse and human HSC-enriched cells were immediately lysed and fully unmethylated lambda and fully methylated T7 phage controls added, followed by bisulfite conversion and on-bead desulfonation (Domanico et al., 2013). The resulting single-stranded DNA was used as a template for random priming to generate double-stranded DNA for multiplexed library construction on an Agilent Bravo liquid handling platform (Figure 1B). Following library construction and low-cycle PCR amplification, failed wells were identified by qPCR targeted to repetitive sequences in the mouse or human sequences and removed (see Experimental Procedures) (Figures S2D and S2E). Libraries that passed this quality control (QC) threshold were pooled and sequenced together on an Illumina HiSeq platform.

An average of 38% of the resulting reads from all single cells sequenced aligned to the reference genome, compared with an average of 0.32% for no template controls (Figure S2A). Conversion of unmethylated cytosines was >99% efficient, while overconversion of methylated cytosines was <2% (Table S1). An average of 4 million sequence reads were generated per cell, enabling the measurement of the methylation states of 1.2 million CpGs per cell (Figure S2B). In silico merging of the resulting sequence reads confirmed that CpG recovery per cell is stochastic (Figure S2C). As an additional QC step, copy-number variation (CNV) in 5-Mb windows was assessed with Control-FREEC (Boeva et al., 2012) to identify and remove cells with uneven coverage that may represent biological or technical artifacts (Figures S2F and S2G). After removal of cells that failed any QC step (Supplemental Experimental Procedures), data for 64 LSK cells, 84 ESLAM cells, and 121 CD49f cells were available for analysis.

Methylation Adjacency in Individually Analyzed Cells

CpG methylation states derived from bulk cells are characterized by spatial correlation (Eckhardt et al., 2006; Zhang...
et al., 2015) and improvements in differentially methylated region (DMR) detection have been achieved through the development of algorithms that leverage spatial relationships (Hansen et al., 2012). Spatial correlation of CpG methylation state derived from bulk measurements has also provided a rationale for assigning the methylation state of single-CpG measurements to all CpGs within a genomic interval in single-cell methylation analyses (Farlik et al., 2015, 2016; Hou et al., 2016; Hu et al., 2016; Luo et al., 2017; Smallwood et al., 2014). However, the degree to which CpG methylation state is spatially correlated in individual cells has not been studied. To address this question, we calculated the probability that a CpG in single LSK cells was in the same methylation state with neighboring CpGs as a function of their genomic distance (Figure 2A) and, as a comparator, we generated adjacency measurements from PBAL datasets derived from 10,000 LSK cells using the calculated mean methylation difference between nearby CpGs to account for the continuous nature of bulk data.

This strategy revealed genome-wide concordance between CpGs up to 1 kb in cis in both the single cells and the bulk population, which then decreased to random chance at 2 kb (Figure 2B). Genome-wide CpG cis concordance was consistently higher in bulk compared with single-cell measurements and did not approach random chance at 5 kb, whereas single-cell concordance reached background levels at 4 kb (Figure 2B). Concordance between cis CpGs for single-cell and bulk-cell measurements was largely equivalent within CpG islands and promoters but was higher in bulk compared with single cells in all other genomic contexts (Figure 2C). In addition, we found that concordance for both single-cell and bulk samples decayed more rapidly within enhancers (Lara-Astiaso et al., 2014) compared with other genomic regions (Figure 2C).
Taken together, these findings suggest that analytical strategies that infer cytosine methylation within large bins (up to 100 kb, Luo et al., 2017) across the whole genome of single cells may lead to oversmoothening of CpG methylation, supporting a need for additional methodologies.

Identification of Epigenetic Subsets within HSC Populations at Single-CpG Resolution

Having established that spatial correlation of CpG methylation states decay rapidly toward random chance within 2 kb and are context specific within single cells, we sought to ask whether we could leverage the information content of single CpGs in single-cell methylation datasets. We (Gascard et al., 2015) and others (Bock et al., 2012; Hansen et al., 2012; Kundaje et al., 2015) have developed single-CpG resolution approaches for DMR detection from bulk DNA methylation measurements; however, these fail to address the sparse and stochastic methylation measurements characteristic of single-cell methylomes. To address this, we developed a measure of CpG methylation pairwise dissimilarity (PD) defined as the average of the absolute difference in methylation values at CpGs covered in each pairwise comparison (Figure 3A). We then calculated Euclidean
distances between each pair of cells using their PD values as features and performed hierarchical clustering (PDclust; see Experimental Procedures).

Application of this algorithm to the ELSAM and LSK single-cell datasets showed that ELSAM cells are epigenetically more similar to each other compared with LSK cells (p < 0.01), consistent with the knowledge that they are functionally less heterogeneous (Figure 3B). Unsupervised clustering on PDs derived from CpG sites within genomic regions previously implicated in the HSC-to-multipotent progenitor (MPP) transition (Cabezas-Wallscheid et al., 2014) generated two distinct subsets that were differentially enriched in single ELSAM and LSK cells (Figure 3C). Projection of PDs onto two-dimensional space with multidimensional scaling supported the existence of two epigenetic states defined by distinct DNA methylation signatures (Figure 3E)—one that was most enriched in the ELSAM population and the other in the LSK cells—with a proportion (13/64) of LSK cells showing an ELSAM-like epigenetic state.

Next, we enumerated PD values from CpGs genome wide to detect epigenetic subsets within the ESLAM and LSK populations.
LSK populations. When all available CpG sites were used, PDclust revealed a subgroup of cells with the highest relative homogeneity (group 1) compared with all other cells (group 2) (Figure 3D). Multidimensional scaling (MDS) analysis confirmed these relationships by revealing a highly similar population (group 1) and a dispersed population (group 2) (Figure 3F). Group 1 included a higher proportion of ESLAM cells than LSK cells (26/84 versus 3/64 or 31% versus 5%). Interestingly, these proportions closely resemble the published biologically defined HSC content of both of these phenotypically defined populations. MDS plots further revealed a gradual and continuously increasing heterogeneity in the CpG profiles of LSK cells (Figure 3F). As a negative control, we considered only CpGs within a genomic region set that would not be expected to be relevant in HSCs (cortex enhancers; Hon et al., 2013) and showed no discernible relationships (Figure S3). As an additional validation, we applied PDclust to previously generated methylome data for single human leukemic cells (Farlik et al., 2015) and single murine embryonic stem cells (Angermueller et al., 2016; Smallwood et al., 2014) and separated cells accurately by type and treatment, with an exception for HL60 cells treated with vitamin D (Figure 4). Taken together, these results suggest

Figure 4. Pairwise Dissimilarity Applied to Existing Datasets Is Able to Distinguish Cells
Based on Cell Type and Treatment. (A and B) Clustering (A) and MDS scaling of PD values (B) calculated from HL60 and K562 cells (Farlik et al., 2015) separates cells by cell type, and reveals patterns of treatment-induced differentiation. Cells are shaded to represent the pairwise dissimilarity between each pair of cells, with red representing highly dissimilar and yellow representing highly similar. Meth, average genome-wide CpG methylation; cpg_count, number of distinct CpG sites recovered.

(C and D) Clustering (C) and MDS scaling (D) of embryonic stem cells (Smallwood et al., 2014; Angermueller et al., 2016) separates cells by the culture medium in which they were grown, and shows some cells in a transition state.
that the epigenetic state of a single cell can be accurately described by a small (<1%) stochastically sampled fraction of CpGs and that these states are reflective of cell identity and state.

Function of DMRs in Epigenetically Defined Subgroups of Mouse HSCs
To examine the functional significance of the epigenetically defined subsets of LSKs and ESLAM cells, we merged CpG calls in silico across all cells belonging to group 1 and separately to group 2 described above. We then inferred methylation values of all CpGs across the genome for these two groups using Bsmooth (Hansen et al., 2012) and identified DMRs between the two groups (Figure S4A and Experimental Procedures). This resulted in the identification of 9,922 DMRs that were hypomethylated in group 1 compared with group 2, and 10,047 DMRs that were the opposite state. For each DMR, we then used HOMER (Heinz et al., 2010) to annotate its overlapping genomic feature(s) as well as the nearest gene.

In general, CpG methylation is anti-correlated with expression when found in the promoter region or the first exon of a gene (Brenet et al., 2011), and active distal regulatory regions are typically hypomethylated (Stadler et al., 2011). We found that DMRs between groups 1 and 2 were enriched in promoters (±2 kb of transcription start site [TSS]), depleted in distal intergenic regions, and enriched in evolutionarily conserved genomic elements (Phastcon [TSS]), depleted in distal intergenic regions, and enriched within 20 kb from an annotated TSS (Kundaje et al., 2015). We assigned each DMR of this class to the subgroup that reported the lower methylation value and associated each DMR group with the nearest genes using HOMER (Heinz et al., 2010). As a control, we performed an in silico defined subsets of LSKs and ESLAM single cells. To identify genes uniquely associated with only group 1 or group 2, we removed those associated with DMRs from both populations and subjected the remaining group 1- or group 2-specific genes to gene set enrichment analysis (GSEA). Genes associated with hypomethylated DMRs in group 1 were significantly enriched (false discovery rate [FDR] adjusted q value < 0.1) in HSC proliferation terms as well as genes preferentially expressed in long-term HSCs and erythrocytes (Figure S4C). In contrast, genes associated with hypomethylated group 2 DMRs were enriched in genes specifically expressed in differentiated hematopoietic cells and genes that, when knocked out, lead to increased HSC numbers. Together these results suggest that DMRs specifically hypomethylated in group 1 compared with group 2 are associated with genes implicated in HSC function.

After identifying genes associated with hypomethylated DMRs, we sought to examine their expression in single ESLAM cells. To do this, we used previously generated single-cell RNA-sequencing data from Lin c-KIT”SCA1” CD34 FLT3” CD48 CD150” HSCs (Wilson et al., 2015) and compared the expression of the DMR-associated genes thus identified with all genes. This showed that the DMR-associated genes were expressed at higher levels in these cells as compared with all other genes (p < 0.01, Mann-Whitney test) (Figure S5A). Further investigation of the levels of expression of these DMR-associated genes within the ESLAM population (Wilson et al., 2015) showed that a significant number showed heterogeneous expression profiles as might be expected if they were composed of one or more subpopulations (hypergeometric p value < 0.01) (Figure S5B). To identify potential surface markers that might allow their physical separation, we identified genes encoding plasma membrane proteins (as defined by gene ontology) and associated these with DMRs that were hypomethylated in group 1. The resulting list included Cad2 (Figure S4A), a gene encoding a surface protein previously implicated in the maintenance of long-term HSCs in vivo (Hur et al., 2016).

Human HSCs and Their Derivatives Can Also Be Defined at Single-CpG Resolution
To determine whether single human hematopoietic cells could also be described using PDclust, we generated PBAL datasets for series of single human CD49f cells and obtained previously published datasets for all the major CD34+ phenotypes in human cord blood (Farlik et al., 2016). Application of PDclust to the latter datasets showed that a majority of megakaryocytes and selected CD49f cells and other CD34+ phenotypes had higher PD values and appeared to be outliers from the remaining cells (groups 2 and 3) (Figures S6A–S6C). These outlier cells also demonstrated significantly lower genome-wide average CpG methylation in comparison with all other cells, suggesting that they were either technical or biological outliers (Figure S6D). When they were removed, the single-cell methylome data clustered according to cell phenotype, with some overlap of the CD49f cells and MPPs (Figure S5A). Multidimensional analysis confirmed separation of CD49f cells and MPPs from other phenotypes and revealed a clear separation of progenitors of granulocytes and macrophages (GMPs) from all other phenotypes (Figure S5B). This analysis also showed that cells within the same phenotype had a lower dissimilarity as compared with other phenotypes (p < 0.01 for all comparisons) (Figure S5C). These results confirm that stochastic measurements of single-CpG states in single cells can accurately segregate multiple primitive phenotypically distinct human hematopoietic cell populations.
Sources of Epigenetic Heterogeneity within the Human CD49f Compartment

The CD49f cells were sorted from cord blood derived from two individual donors, which enabled an examination of donor-specific contributions to methylation states at the single-cell level. Interestingly, this analysis revealed that the data for single CD49f cells clustered separately by donor (Figure 5 A). To investigate whether these donor-specific epigenetic differences were a result of genetic differences between the two donors, we identified genetic variants by applying MethylExtract (Barturen et al., 2014) to the donor in silico merged datasets. After combining non-identical homozygous variants for both donors (excluding homozygous variants involving a C or G), we masked CpGs within 200 bp of the donor-specific variants and recalculated the PD values. This decreased both inter- and intradonor PD values, but donor-specific methylation states remained the major driver of variation within the CD49f population (Figures 6A and 6B). We also noted that the PD value increased when only CpGs within 200 bp of single-nucleotide variants (SNVs) were considered (Figure 6C). Taken together, this result suggests that genetic variation accounts only partly for the observed donor-associated epigenetic variation and that donor-specific epigenetic variation is a dominant feature across single and highly purified human CD49f cells.

Application of PDclust separately to the methylome data for the single CD49f cells from donors 1 and 2 then enabled the identification in each of a consistent subpopulation (group 1, comprising 9% and 11%, respectively, Figure 7A) that did not correlate with expression levels of the surface markers used to isolate them (CD3, 11b, 19, 34, 38, 90, 45RA, 49f) (Figure 7B). To test the sensitivity to the depth of sequence data obtained, we then sequenced the library from donor 1 to a 4-fold greater depth (an average of 7.8 million mapped reads per cell) (Figures S7 A and S7B). Interestingly, this did not significantly alter the results (Figure S7C), suggesting that the cell-to-cell variation was already accurately captured by the original dataset.

Figure 5. Pairwise Dissimilarities of Human Hematopoietic Cells with Different Phenotypes

(A) PD of CD49f and other CD34+ subsets separate cells by phenotype with some overlaps. The rows and columns are symmetrical and represent single cells. Cells are shaded to represent the PD between each pair of cells, with red representing highly dissimilar and yellow representing highly similar.

(B) MDS of PD values shows that CD49f cells cluster in the middle, with GMPs, CLPs, and MLPs branching out in separate directions.

(c) Single cells have lower PD values compared with cells of the same type versus cells of a different phenotype. The distribution of PD values when a cell is compared with either a cell of the same or different phenotype is plotted as a box plot.

See also Figure S6.
We then took advantage of the more deeply sequenced dataset to perform an in silico merging of the cells previously identified as group 1 (n = 6) and group 2 (n = 63) cells. In silico merging of the data from the first group 1 resulted in a nearly complete recapitulation of the human methylome (17.1 M CpGs in group 1 and 27.1 M CpGs in group 2) allowing for comprehensive epigenetic annotation of the two groups. As a comparator, we performed in silico merging of all available CD49f cell data as well as the published hematopoietic progenitor cell (HPC) data (common lymphoid progenitors [CLPs], common myeloid progenitors, GMPs, multilymphoid progenitors [MLPs], and MPPs) (Farlik et al., 2016). As before, we estimated the smoothed methylation values of all CpG sites in the genome for each group and called DMRs. This resulted in the identification of 18,035 DMRs that were hypomethylated in group 1 compared with group 2, and 15,283 DMRs that were in the opposite state. DMRs were enriched in promoters (i.e., sequences between 2 kb upstream and 500 bp downstream of coding gene TSSs), depleted in intergenic regions, and highly enriched in evolutionarily conserved elements (Phastcon score [Siepel et al., 2005]; two-sided t test, p ~ 0), suggesting that these DMRs have functional relevance (Figure 7C). GSEA of genes associated with DMRs that were hypomethylated in CD49f cells compared with HPCs showed these were enriched in pathways implicated in HSC differentiation (Figure 7D). Interestingly, DMRs hypomethylated in group 1 compared with group 2 were enriched in genes that are upregulated in leukemia (Casorelli et al., 2006) and genes whose expression is upregulated in hematopoietic progenitors (Ivanova et al., 2002) (Figure 7D). For example, SERPING1, a gene that is upregulated in acute promyeolocytic leukemia (Casorelli et al., 2006) and a prognostic marker for acute myeloid leukemia (Laverdière et al., 2016), was found to contain a DMR that was hypomethylated in group 1 cells (Figure 7E).

**DISCUSSION**

We describe an automated methodology for single-cell DNA methylation profiling of single cells optimized for genomic coverage. The increased coverage afforded by our methodology allowed epigenetically distinct subsets within highly purified phenotypes to be identified and distinguished from developmentally both closely and
more distally related cell types. We therefore anticipate that it will have utility in dissecting biologically important, but potentially subtle, epigenetic changes in cells that drive as yet poorly understood developmental and disease processes.

Existing single-cell DNA methylation experimental protocols can be broadly categorized into those that include random priming-based genomic preamplification and those that do not. In general, single-cell experimental protocols that involve rounds of random genomic priming (e.g., five in single-cell bisulfite sequencing [scBS-seq], two in PBAL, one in single-nucleus methylcytosine sequencing [snmC-seq], versus none in single-cell whole-genome bisulfite sequencing [scWGBS]) result in libraries...
with increased fragment diversity independent of the number of subsequent PCR cycles (Luo et al., 2017). However, challenges associated with single-cell random priming include a consistent decrease in aligned sequence fraction and increased per-cell cost. Rounds of random priming are inversely correlated with lower mapping efficiency (average 25% in scBS-seq, 38% in PBAL, 53% in snmC-seq, versus 55% in scWGBS), suggesting that external nucleic acid sources are amplified in addition to the target genome. Additional sequencing of the resulting library pools can circumvent this problem as in the case of PBAL. We have designed the PBAL approach with these limitations in mind to optimize fragment diversity through a combination of two rounds of random priming and low-cycle PCR. We have developed a qPCR-based assay that identifies failed wells from all causes prior to pooling and sequencing. Taken together, these optimizations allow for the generation of single-cell methylomes at lower cost with diversities meeting or exceeding existing published strategies.

To identify epigenetic states from stochastic and sparse measurements characteristic of single-cell methylation datasets, we have developed an analysis package (PDclust; https://github.com/hui-tony-zk/PDclust) that leverages the information content of single CpGs without the need to infer the methylated states of missing data. We demonstrated its application to CpGs within regulatory regions (Cabezas-Wallscheid et al., 2014) through the identification of an epigenetically distinct subpopulation of cells in two different compartments of adult mouse bone marrow (LSK and ESLAM cells) that match their known content of HSCs defined by long-term repopulating assays: ~3% in LSKs (Osawa et al., 1996) and 40% in ESLAM cells (Benz et al., 2012; Kent et al., 2009). Analysis of the resulting DMRs further revealed a statistically significant number of hypomethylated regions in this subgroup that were associated with genes implicated in mouse HSC function, a majority of which have also been found to be transcriptionally active in mouse HSCs (Wilson et al., 2015). This supports previous evidence of a connection between CpG methylation status and expression examined at a single-cell level (Angermueller et al., 2016; Hu et al., 2016). A significant proportion of DMR-associated plasma membrane genes were also heterogeneously expressed among individual ESLAM cells including Cd82, a previously annotated marker of HSCs (Hur et al., 2016), suggesting that this might be useful in conjunction with the ESLAM protocol to further enrich for HSCs from adult mouse bone marrow.

Application of PDclust to single human hematopoietic cell datasets separated them according to established phenotypes and identified rare epigenetically distinct subpopulations from which near complete reconstitution of the methylome was achieved. Interestingly, within the highly purified CD49f population we identified donor as a significant source of consistent epigenetic heterogeneity, which was reduced but not eliminated by correcting for personal genetic variants. This observation is consistent with previous reports that showed genetic diversity as related to but not accountable for all DNA methylation differences (Gertz et al., 2011; Xie et al., 2012) and suggests that in utero environmental differences may be encoded within the HSC compartment, as has been reported for bulk analysis (Bommarito et al., 2017; Provençal and Binder, 2015).

The ability to detect distinct epigenetic states that distinguish phenotypically defined hematopoietic cell types from a few thousand stochastically sampled CpGs in single cells is an intriguing observation with potentially broad implications. One possible explanation for this observation is that phenotype-specific methylation signatures are characterized by extensive redundancy such that distinct epigenetic states can be accurately described by only a small fraction of single-CpG methylation states. In support of such a notion, the unique components of a DNA methylation “age” signature are contained in ~353 CpGs sites, presumably representing a random sample of a total age signature that involves many more sites not detected using the reduced representation strategies from which these signatures have been derived (Horvath, 2013). Regardless of the mechanism, the ability to describe epigenetic states in single cells from single-CpG measurements without the need to infer adjacent methylation states has significant implications for the design and analysis of single-cell methylation studies.

**EXPERIMENTAL PROCEDURES**

This study involved animal research and is approved by the University of British Columbia under ACC certificate number A14-0091. Human cord blood cells were obtained with informed consent from mothers of normal babies according to UBC-approved protocols (UBC REB Certificate H07-01945 to C.J.E.).

**Sample Preparation**

Murine bone marrow of 8- to 10-week-old mice was stained using LSK or ESLAM staining cocktails (Table S1). HSCs were then sorted using a FACSAria Fusion (Becton Dickinson, Franklin Lakes), flash-frozen, and stored at −80°C until processing. Human cord blood was obtained from two normal full-term deliveries. Cells were stained and sorted with the CD49f cocktail as described elsewhere (Knapp et al., 2017, 2018). Full details can be found in Supplemental Experimental Procedures.

**PBAL Library Construction**

All reagents were DNaseI or UV treated to remove ambient contaminants, and all samples were processed with negative controls. All liquid handling steps were carried out on the Bravo Automated Liquid Handling Platform (Agilent Technologies, G5409A). Plates
containing single cells were lysed, spiked-in with bisulfite conversion controls, and subject to automated bead-based bisulfite conversion (Domanico et al., 2013). The resulting single-stranded DNA was subject to random priming with random hexamers (3’-phosphothioate, NNNNNN*N*N) and Klenow exo- (NEB, M0212M) twice. The double-stranded DNA was then ligated with forked Illumina adapters (Lorzadeh et al., 2016) and PCR amplified for eight cycles. Successful wells were identified by qPCR targeted to repetitive regions of the mouse or human genome, pooled, and sequenced on an Illumina HiSeq 2,500. Full details can be found in Supplemental Experimental Procedures.

Data Processing
The first six bases of read1 and read2 were trimmed using Trimgalore v0.4.0 and aligned using Novoalign V3.02.10 (www.novocraft.com) to the mouse assembly GRChm38 (mm10) or human assembly GRCh37 (hg19). Duplicate reads were discarded using Picard V1.31 (http://picard.sourceforge.net), and methylation states were called using Novomethyl V1.01 (www.novocraft.com). Fractional methylation was merged for each CpG dinucleotide by taking a weighted average of each cytosine using bedtools (Quinlan and Hall, 2010). In most cases, only one base within a CpG dinucleotide had coverage. In these cases, the methylation information of the covered base was extrapolated to the other base. Processed CpG calls were imported into R (V3.3.2) for downstream analysis. The first six bases of read1 and read2 were trimmed using Trimgalore v0.4.0 and aligned using Novoalign V3.02.10 (www.novocraft.com). The resulting single-stranded DNA was subject to random priming with random hexamers (3’-phosphothioate, NNNNNN*N*N) and Klenow exo- (NEB, M0212M) twice. The double-stranded DNA was then ligated with forked Illumina adapters (Lorzadeh et al., 2016) and PCR amplified for eight cycles. Successful wells were identified by qPCR targeted to repetitive regions of the mouse or human genome, pooled, and sequenced on an Illumina HiSeq 2,500. Full details can be found in Supplemental Experimental Procedures.

Methylation of Adjacent CpGs in Single Cells
For single cells, we randomly sampled up to 100,000 CpG sites either genome wide or within relevant genomic regions. For each randomly sampled CpG site (CpG1), we analyzed 100 CpG sites with coverage >0 before and after CpG1 and calculated the genomic distance to CpG1 in base pairs. We also recorded whether or not each CpG1 had the same methylation status as CpG1, resulting in a 2-column table containing the distance and equality status for each CpG for near CpG1. After combining all of the data in these tables for every CpG1, we binned distances into 100-bp bins and calculated the mean concordance as the fraction of CpGs in each bin that were equal. For single LSK cells, we did this for the ten single cells with the most CpG coverage. For the bulk LSK cell data that were continuous, we instead calculated the absolute difference in methylation between CpG1 and all nearby CpGs, using only CpGs with coverage ≥5 to avoid low coverage biasing potential CpG methylation values.

Pairwise Dissimilarity Clustering Using PDclust
For each pair of single cells, we calculated the average difference in DNA methylation of all pairwise-common CpG sites to obtain PD values. For genome-wide analysis, we took into consideration all CpGs, while for each genomic region sets we only considered CpG sites that lie within those respective regions. To group cells together with similar PD values, we calculated Euclidean distances between each pair of cells using the their PD values as features and performed hierarchical clustering with Ward’s linkage (ward.D2 in R). We used PD directly as input to multidimensional scaling (cmdscale in R) for visualization of cells in 2D space. Cell groups were annotated manually based on their visual distinctiveness and their resulting hierachical clustering patterns. PDclust has been packaged into an R package, which can be downloaded from https://github.com/hui-tony-zk/PDclust.

Differentially Methylated Region Analysis
To group cells that belonged to the same cluster, we treated coverage of every CpG site as the number of cells with coverage at that site, and treated methylation fraction as the fraction of cells that had a methylated CpG at that site. We used Bsmooth (Hansen et al., 2012) to obtain estimated CpG methylation at all CpG sites in the genome. For each CpG comparison between the two groups, we calculated the Z score converted that into a two-tailed p value assuming a normal distribution (pnorm function in R) with the null hypothesis that the methylation is not different between the two CpGs. p values were multiple test-corrected using an FDR estimate, and CpGs with q value of <0.1 were called as dCpGs. We grouped dCpGs together into DMRs if they were within 500 bp of each other and only considered DMRs with ≥3 CpGs. We split these DMRs into two groups depending on which population had the lower methylation in each pair of comparisons, and associated DMRs to the nearest protein-coding transcript. After filtering for those DMRs in intergenic regions, exon1, or the promoter (+2 kb/–0.5 kb), we removed DMRs that associated with the same gene in both groups. We then performed GSEA for all DMR-associated genes for each group using a supervised set of ontologies. Full details can be found in Supplemental Experimental Procedures.

ACCESSION NUMBERS
Single-cell bisulfite sequencing (raw reads and CpG methylation calls) can be accessed from the Gene Expression Omnibus at GEO: GSE89545. Bulk LSK data can be accessed at GEO: GSE95697. Human CD49f CpG methylation data can be accessed at GEO: GSE106957. Raw reads for the human CD49f data can be conditionally accessed from the European Genome-Phenome Archive (EGAS00001002789).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.07.003.

AUTHOR CONTRIBUTIONS
T.H. and Q.C. developed the single-cell methylation protocol and generated the single-cell libraries. T.H. developed the analytical framework, analyzed and interpreted the data, and wrote the paper. K.O. helped with the CpG adjacency analysis. J.W.-W., C.A.H., and D.J.H.F.K. isolated the cells analyzed and assisted in...
data interpretation. E.L. sorted test single cells for technology development. M.M. assisted with technology development, interpreted the data, and wrote the paper. S.A. and A.K. interpreted data. M.H. designed the study, interpreted data, and, with C.J.E., wrote the final draft of the manuscript.

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