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Research paper

# Integrative analysis of DNA methylation and mRNA expression during differentiation of umbilical cord blood derived mononuclear cells to endothelial cells

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ABSTRACT

Differentiation of umbilical cord blood derived mononuclear cells to endothelial cells is accompanied by massive changes in gene expression. Although methylation and demethylation of DNA likely play crucial roles in regulating gene expression, their interplay during differentiation remains elusive. To address this question, we performed deep sequencing of DNA methylation and mRNA expression to profile global changes in promoter methylation and gene expression during differentiation from mononuclear cells to outgrowing cells. We identified 61 downregulated genes with hypermethylation, including *CD74*, *VAV1*, *TLR8*, *and NCF4*, as well as 21 upregulated genes with hypomethylation, including *ECSCR*, *MCAM*, *PGF*, *and ARHGEF15*. Interestingly, gene ontology analysis showed that downregulated genes with hypermethylation were enriched in immune-related functions, and upregulated genes with hypomethylation were enriched in the developmental process and angiogenesis, indicating the important roles of DNA methylation in regulating differentiation. We performed polymerase chain reaction analyses and bisulfite sequencing of representative genes (*CD74*, *VAV1*, *ECSCR*, and *MCAM*) to verify the negative correlation between DNA methylation and gene expression. Further, inhibition of DNA methyltransferase and demethylase activities using 5′-aza-dc and shRNAs, specific for TET1 and TET2 mRNAs, respectively, revealed that DNA methylation was the main regulator of the reversible expression of functionally important genes. Collectively, our findings implicate DNA methylation as a critical regulator of gene expression during umbilical cord blood derived mononuclear cells to endothelial cell differentiation.

## 1. Introduction

Umbilical cord blood derived mononuclear cells (UCB-MNCs) could be differentiated to the endothelial lineage (Asahara et al., 1997; Eggermann et al., 2003). The phenotypes of UCB-MNC derived outgrowth endothelial cells (OECs) such as proliferative potential, migration, and tube formation, are similar to those of other endothelial cell (EC) types such as LECs, HUVECs, and HUAECs (Medina et al., 2010a; Patan, 2004). Differentiation of circulating UCB-MNCs is associated with altered global gene expression. In particular, fully differentiated UCB-MNCs downregulate the expression of stem cell antigens such as CD34 and CD133 and increase the expression markers of mature ECs such as CD31, KDR, vWF, and e-NOS (Hristov et al., 2003). Recent studies indicate that altered chromatin organization can affect cell type-specific gene expression by changing the accessibility of genes to transcription factors in either a positive or a negative manner (Voss and Hager, 2014). Two major classes of such modifications include DNA methylation and histone methylation, acetylation, or both. DNA methylation is associated with gene silencing (Schubeler, 2015) and regulates tissue-specific gene expression (Jones and Takai, 2001; Jaenisch et al., 1994). In mammalian cells, CpG methylation is catalyzed by a family of DNA methyltransferase enzymes (DNMTases) comprising

*Abbreviations:* UCB-MNC, umbilical cord blood derived mononuclear cell; OEC, outgrowth endothelial cell; HSC, hematopoietic stem cell; ESC, embryonic stem cell; MeDIP, methylated DNA immunoprecipitation; DNMTases, DNA methyltransferases; TETs, ten-eleven translocation; DMR, differentially methylated region; DEG, differentially expressed gene; 5′-aza-dc, 5′-aza-2′-deoxycytidine

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Dnmt1, Dnmt3a, and Dnmt3b (Hermann et al., 2004; Okano et al., 1999). In hematopoietic stem cells (HSCs), *Dnmt3a*-null mice exhibit the upregulation of HSC multipotency genes and downregulation of differentiation genes; further, their progeny exhibit global hypomethylation and insufficient repression of HSC-specific genes (Challen et al., 2012). Loss of Dnmt3a and Dnmt3b from embryonic stem cells (ESCs) leads to the loss of differentiation potential with cell passage, although the potential for self-renewal is maintained (Chen et al., 2003). Conversely, DNA demethylation is catalyzed by Ten-eleven translocation (TET) proteins TET1, TET2, and TET3. These processes comprise two ways that activate and passive DNA demethylation. TETs promote DNA demethylation by catalyzing the conversion of methylcytosine (5mC) to 5-hydroxymethylcytosine (5-hmC) or 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Bhutani et al., 2011). In mouse ESCs, TET1 regulates transcription by promoting demethylation at CpG sites in promoters and by repressing transcription through binding to the Polycomb group of target genes (Williams et al., 2011). Moreover, TET2 plays a role in hematopoiesis by limiting the self-renewal capacities of HSCs and by regulating differentiation. Here, we aimed to determine how promoter DNA methylation regulates gene expression during UCB-MNC to EC differentiation (Ko et al., 2010; Pronier et al., 2011).

By integrating methylated DNA immunoprecipitation-sequencing (MeDIP-Seq) with RNA-Seq analyses of UCB-MNCs and OECs, we identified a significant set of genes regulated by DNA methylation during the differentiation of UCB-MNCs to OECs. We identified the biological functions of these genes and verified their expression and methylation patterns. Further, we demonstrated direct regulation of gene expression through DNA methyltransferase and demethylase. Our results show that DNA methylation is involved in endothelial lineage-specific gene expression, indicating a role for epigenetic mechanisms in the regulation of the differentiation of UCB-MNCs to OECs.

## 2. Materials and methods

### 2.1. UCB-MNC isolation and sample preparation

UCB-MNCs were isolated from human cord blood using Ficoll-Paque density gradient centrifugation. Cells were seeded onto six-well plates coated with fibronectin (Sigma-Aldrich, Inc., St Louis, MO) and cultured in EBM™-2 (Clonetics Cell Systems, St Katharinen, Germany). Media were supplemented with 20% heat-inactivated fetal bovine serum (FBS) and EGM™-2 SingleQuots® (Clonetics Cell Systems). After 3 days, nonadherent cells were removed, and fresh culture medium was added. Cultures were maintained for 30 days. Each sample was prepared for RNA and MeDIP sequencing from five cord blood samples. In addition, MNCs and OECs were used from another cord blood sample as experimental evidence to support our findings.

### 2.2. Genomic DNA isolation and methylation sequencing and profiling

Genomic DNA was isolated using a NucleoSpin-Tissue Kit (Macherey-Nagel) and fragmented to a median length of 150 bp using a Covaris-S2 (Covaris). Methylated DNA was isolated from fragmented, genomic DNA via binding to the methyl-CpG binding domain of human MBD2. Libraries were prepared according to the manufacturer's instructions (Illumina Chip DNA Prep Kit). Methylated DNA was amplified using PCR. The libraries were purified and size-selected using 2% agarose gel electrophoresis. The purified products were quantitated before seeding the clusters in a flow cell of a 2100 Bioanalyzer and QPCR. Clusters were generated in the flow cell of a cBot automated cluster generation system (Illumina), and the flow cell was loaded on a HISEQ 2000 sequencing system (Illumina) that generates 1 × 50-bp reads. Sequence reads were aligned to the human genome (hg19 from the UCSC genome database) using BWA (version 0.7.5a) (Li and Durbin, 2009) after a standard quality check and trimming using FastQC and Fastx-toolkit, respectively. The MEDIPS package (version 1.8.0) (Chavez et al., 2010) of the BioConductor software project was used to identify the methylated DNA segments. Specifically, the targeted data resolution and the extension range for smoothing were defined as 50 bp and 400 bp (i.e., bin_size = 50 and extend = 400), respectively. The mean methylation value is expressed as the relative methylation score (rms) was obtained for each nonoverlapping window of 500 bp.

### 2.3. RNA sequencing and analysis

Total RNA was isolated using an RNeasy Kit (Qiagen), and integrity was checked using an Agilent Technologies 2100 Bioanalyzer using an RNA Integrity Number (RIN) value > 8. Libraries of mRNA sequences were prepared using the Illumina Truseq RNA Prep Kit, and mRNA was purified (two rounds) and fragmented from total RNA (1 μg) using oligo-T bound to magnetic beads. RNA sequencing was performed using an Illumina HiSeq 2000 (101-bp paired-end runs). Sequence reads were aligned to the human genome reference sequence (hg19 from the UCSC genome database) using Tophat2 (version 2.0.9) (Kim et al., 2013) after a standard quality check and trimming using FastQC and Fastx-toolkit (version 0.0.13.2), respectively. Cufflinks (version 2.1.1) (Trapnell et al., 2012) was used to quantify mRNA abundance. Genes that were differentially expressed by UCB-MNCs and OECs were identified using edgeR (version 3.0.8) (Robinson et al., 2010), DESeq (version 1.10.1) (Anders and Huber, 2010), DEGSeq (version 1.12.0) (Wang et al., 2010), and NOISeq (version 1.1.5) (Tarazona et al., 2015).

### 2.4. Correlation between DNA methylation and gene expression

To explore the regulatory impact of DNA methylation on gene expression, statistical tests of enrichment and depletion were performed for negative and positive correlations between DNA methylation and gene expression, respectively. The matching numbers of differentially methylated and differentially expressed genes (i.e., 3,279 DMR genes and 491 DEGs) were randomly selected. Hyper/hypomethylated genes and up/downregulated genes were selected in a mutually exclusive manner. Then, the number of genes was counted with negative (hyper-down and hypo-up) and positive (hyper-up and hypo-down) correlations. This procedure was repeated 100,000 times to estimate the P-value of the enrichment and depletion of negative and positive correlations in the observed values, respectively.

### 2.5. Microarray data

UCB-MNC and OEC RNA-Seq data were compared using the publically available microarray databases Gene Expression Omnibus (GEO) database (GSE46328 (Kim et al., 2015) and GSE20283 (Medina et al., 2010b)). Quantile values of gene expression data were normalized and used to generate heat maps of differentially expressed genes (DEGs).

### 2.6. Gene-set analysis

Gene-set analysis of gene ontology (GO) terms was performed using the WebGestalt web server (Kirov et al., 2014). Two gene sets of up-regulated DEGs with hypo-methylated DMRs and downregulated DEGs with hyper-methylated DMRs were included in the analysis to deduce the functions of the DEGs.

### 2.7. RNA isolation and RT-PCR analysis

Total RNAs were isolated from UCB-MNCs and OECs using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) served as template for cDNA synthesis using M-MLV reverse transcriptase (Promega, Munich, Germany) according to the manufacturer's instructions. RT-PCR was performed using Taq polymerase (Intron Biotechnology, Seoul, Korea) in a reaction mix containing 1 × reaction buffer, 1.5 mM MgCl2,