Gene 635 (2017) 48–60

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

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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ARTICLE INFO

Keywords:
Umbilical cord blood derived mononuclear cell
Endothelial cells
Differentiation
DNA methylation
Promoter CpG islands
RNA seq
MeDIP seq
Gene discovery

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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http://dx.doi.org/10.1016/j.gene.2017.09.006
Received 7 February 2017; Received in revised form 29 August 2017; Accepted 4 September 2017
Available online 06 September 2017

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,

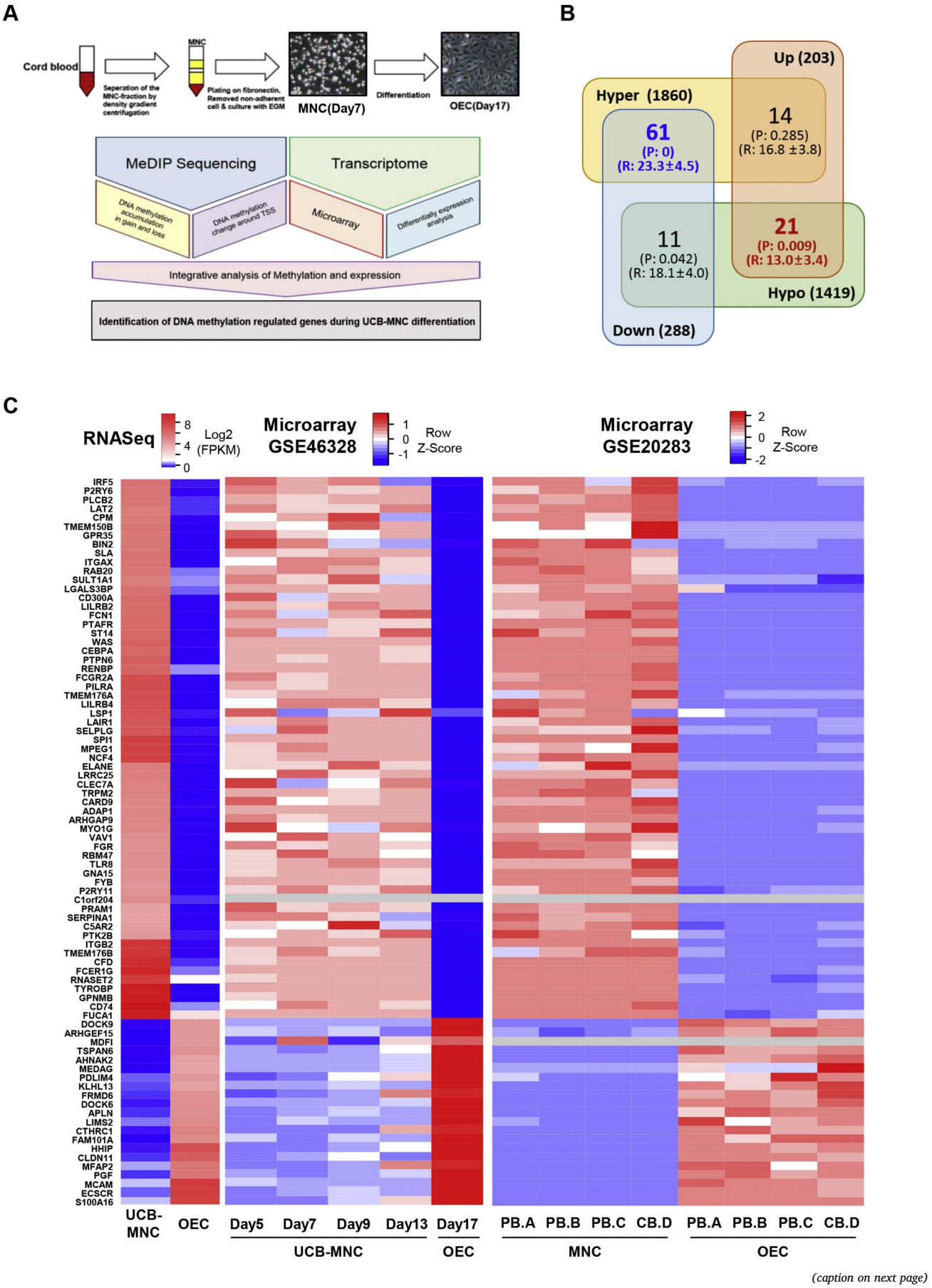

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Fig. 1. Integrative analysis of DNA methylation and gene expression during UCB-MNC to OEC differentiation. (A) Diagrams describing the profiling process for identifying differentially methylated and expressed genes in UCB-MNCs and OECs. (B) The overlap of differentially methylated and expressed genes is shown in the Venn diagram format. Numbers in the intersection regions are the number of genes satisfying both conditions, *p*-value of enrichment or depletion (marked as P), and average with standard deviation (marked as R) obtained from random simulation of 100,000 times. (C) RNA-seq analysis of gene expression profiles of UCB-MNC and OEC of our samples (left) and two publicly available microarrays (middle and right). The expression values of our focus genes are shown by the heat map. Genes are ordered by average linkage hierarchical clustering of the RNA-Seq dataset. The colour gradient of RNA-Seq and microarrays indicates Fragments Per Kilobase Million (log2 FPKM + 1) and z-score transformed across samples, respectively. In the heat map, red represents high expression, blue represents low expression, and gray represents undetectable expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.2 mM deoxynucleotide mix, 0.5 units Taq polymerase, and 0.2 μM each forward and reverse primers. The amplification conditions were as follows: 5 min at 95 °C, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were analyzed using 1.5% agarose electrophoresis.

2.8. Quantitative real-time PCR analysis (qRT-PCR)

The qRT-PCR reactions were performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) with a PikoReal™ Real-Time PCR System (Thermo Scientific) according to the manufacturer's recommended protocol. The PCR conditions for genes were as follows: 5 min at 95 °C, 50 cycles at 94 °C for 10 s, 58 °C for 20 s, 72 °C for 30 s, and final extension at 72 °C for 10 min. All results were normalized to the levels of human *GAPDH* mRNA.

2.9. Bisulfite sequencing

Genomic DNA (1 μg) was treated with bisulfite using the EZ DNA Methylation-Direction Kit (Zymo Research, Irvine, CA) and then subjected to PCR amplification. PCR conditions were as follows: 5 min at 95 °C, 50 cycles at 94 °C for 10 s, 58 °C for 20 s, 72 °C for 30 s, and final extension at 72 °C for 10 min.

OECs were treated at passages 3–5 with 5′-aza-2′-deoxycytidine (5′-aza-dc) (10 μmol/l) (Sigma-Aldrich) every 24 h for 7 days. Six days after isolation, UCB-MNCs were treated with 5′-aza-dc (Sigma-Aldrich) using these same conditions.

2.10. Lentivirus production and infection

TET1-depleted and control cells were generated by transducing the cells with TET1-shRNA or a scrambled shRNA incorporated into a pLKO.1 lentiviral particle (Santa Cruz Biotechnology, Inc.). Transduced OECs were selected and maintained in media containing 1 μg/ml puromycin. Four days later, the TET2-shRNA or control shRNA was used to transduce TET1-depleted cells. TET1- and TET2-depleted cells were selected from cultures containing 1 μg/ml puromycin in the medium.

2.11. Statistical analysis

All statistical analyses were performed using Graphpad Prism (version 5.0; Graphpad Software, La Jolla, CA). Tests for statistical significance were two-sided, and probability values < 0.05 were reflected significant. The Student's *t*-test or ANOVA was used to compare mean values, and results are presented as mean ± SEM or SD.

3. Results

3.1. Integrative profiling analysis identifies genes that are differentially methylated and expressed in UCB-MNCs and OECs

We previously reported that UCB-MNCs could be differentiated to EC lineage. Global gene expression change has been occurred during differentiation. Among them, we confirmed mononuclear cell markers, *CD133*, *CXCR4* and *CD45* were decreased as well as endothelial cell markers, *CD31*, kinase insert domain receptor (*KDR*), von-Willebrand factor (*vWF*) and *VE-cadherin* were increased (Kim et al., 2015; Jung et al., 2010; Maeng et al., 2015). To understand the relationship between DNA methylation change and gene expression regulation during UCB-MNC to EC differentiation, we performed MeDIP sequencing and RNA sequencing (Fig. 1A). Differentially methylated regions (DMRs) were defined as follows: i) > 2-fold change in mean methylation value (rms ratio > 2 or < 0.5), ii) rms methylation score within the highest 1% of either sample (quant = 0.99) (strong signals only), and iii) Wilcoxon test, p value $< 10^{-4}$. To identify DMRs with regulatory roles, we further filtered DMRs within the promoter regions (− 2 kb to + 0.5 kb) of protein coding genes available from UCSC RefSeq genes. Comparing OECs to UCB-MNCs, we identified 2180 hypomethylated regions in 1419 genes and 2694 hypermethylated regions in 1860 genes. DEGs were identified by considering the intersections of (i) edgeR (version 3.0.8), $p < 0.05$; (ii) DESeq (version 1.10.1), $p < 0.05$; (iii) DEGSeq (version 1.12.0), absolute log2 fold-change > 2 and $q < 10^{-5}$, and (iv) NOISeq (version 1.1.5), $q > 0.99$. This analysis identified 203 upregulated and 288 downregulated genes in OECs compared with UCB-MNCs (Supplementary Fig. 1).

Next, we searched for negative correlations between the gene expression and DNA methylation data. Taking the intersections of 288 downregulated DEGs and the 1860 hyper-DMRs (Fig. 1B), we identified 61 downregulated genes with promoter hypermethylation. Similarly, we obtained 21 upregulated genes with promoter hypomethylation. Statistical analysis using random simulation showed that these negative correlations between the gene expression and DNA methylation were significantly enriched with the *p*-value of 0 and 0.009 for hyper-methylated and hypo-methylated cases, respectively. Depletions in the positive correlated bins were rather weak. The degree of enrichment/depletion was higher for downregulated genes, which is likely due to the fact that promoter DNA methylation typically acts to repress gene transcription.

Nevertheless, we focused on 82 DEGs with negative correlations with DMRs (Supplementary Tables 1 and 2), which should represent candidate genes that may play important roles in the differentiation of the endothelial lineage via promoter methylation. We further compared the expression of these focus genes using independent data sets. Our RNA-Seq data were highly consistent with the independent microarray datasets GSE46328 (Kim et al., 2015) and GSE20283 (Medina et al., 2010b) (Fig. 1C). These temporal data reveal that the expression levels of the focus genes did not vary significantly during the UCB-MNC state for 13 days and that the changes in expression occurred during terminal differentiation, likely through promoter methylation.

3.2. Gene ontology analysis indicates the biological functions of methylation-dependent genes that are differentially expressed during differentiation into the endothelial lineage

To determine the biological relevance of DEGs through a comparison of DNA methylation between UCB-MNCs and OECs, we subjected the focus genes to GO using the bioinformatics tool WebGestalt (WEB-based GEne SeT AnaLysis Toolkit). We analyzed lists of hypermethylated and hypomethylated genes separately. Biological processes enriched for 61 downregulated genes with hypermethylation (61 genes) were mainly associated with immune-related functions, including GO categories such as “defense response” (GO:0006952) and “immune response” (GO:0006952) (Fig. 2A and Supplementary Table 3A). In contrast, 21 upregulated genes with hypomethylation were enriched in

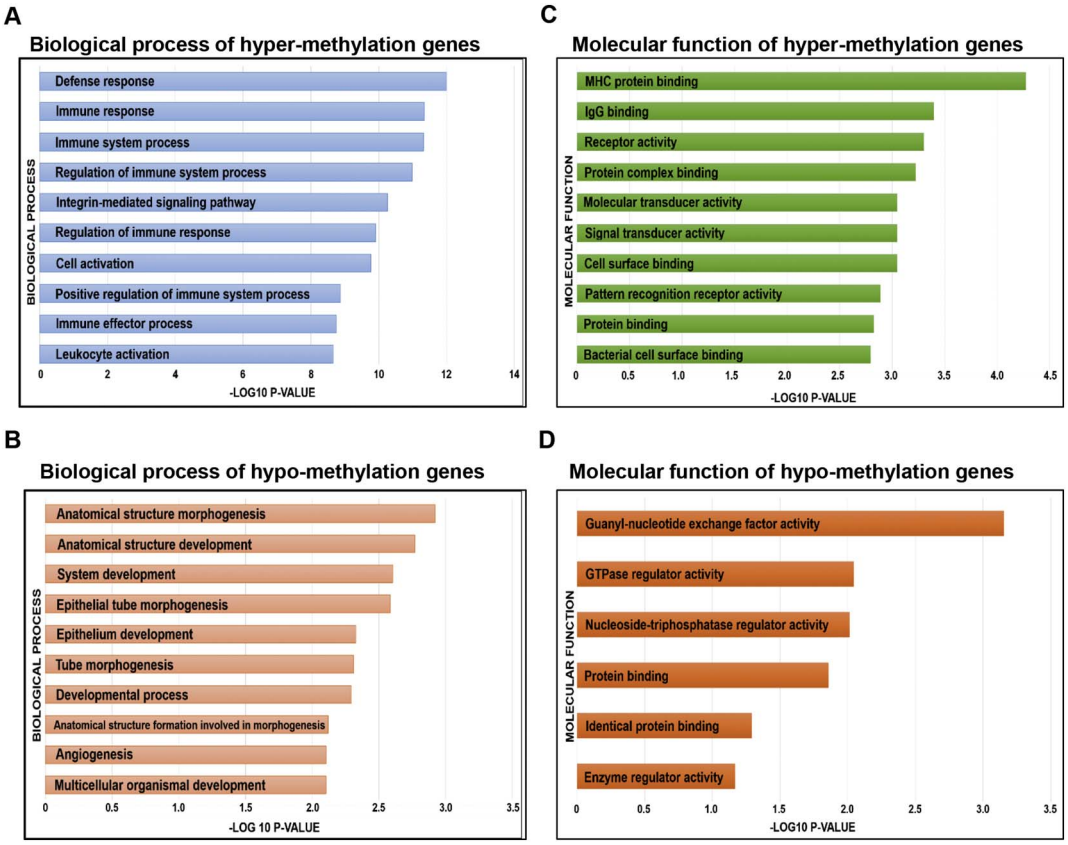

Fig. 2. Gene ontology (GO) analysis of significant differentially methylated and expressed genes. Gene ontology analysis using the WebGestalt Bioinformatics Resources was performed to identify biological processes and molecular functions associated with differentially expressed genes according to DNA methylation during UCB-MNC to OEC differentiation ($p < 0.05$). (A, B) Highest 10 biological process for hyper- and hypomethylated genes. The most significant ontology associated with hypermethylated and downregulated genes is immune system processes, and the most highly enriched process associated with hypomethylated and upregulated genes is related to development and angiogenesis. (C) The highest 10 molecular functions of hypermethylated and genes expressed differentially at lower levels. The most significant function is MHC protein binding essential for the immune system. (D) The highest six molecular functions for hypomethylated and upregulated genes. The most highly enriched molecular function is guanine nucleotide exchange factor activity (GEF) associated with morphogenesis.

GO functional terms associated with developmental process (GO:0032502) and angiogenesis (GO:0001525), which represent the major roles of ECs (Fig. 2B and Supplementary Table 3B).

GO analysis of molecular functions revealed that hypermethylated genes were most enriched in MHC and IgG binding associated with the immune response (Fig. 2C and Supplementary Table 3C). Moreover, hypomethylated genes were enriched in those encoding guanine nucleotide exchange factors that may play important roles in the morphological changes that occur during differentiation (Fig. 2D and Supplementary Table 3D). Thus, highly consistent results were acquired from analyses of molecular functions and biological processes. Moreover, the GO analysis results are consistent for UCB-MNCs and OECs derived from cord blood or peripheral blood (Medina et al., 2010b), indicating that differentiation is independent of the donor cell type of origin. Together, the differentiation from UCB-MNCs to OECs involves several important changes in biological processes such as the immune response and inflammation (characteristics of UCB-MNCs) and developmental process and angiogenesis (characteristics of OECs), which are likely regulated by DNA methylation.

3.3. RT-PCR validation of differentially expressed genes

We next determined the transcriptional states of focus genes associated with changes in promoter methylation. For this purpose, we conducted RT-PCR analysis to determine whether the RNA-Seq data reflected differences in expression. The expression of the 61 hypermethylated genes was downregulated and the expression of the 21 hypomethylated genes was significantly increased in OECs compared with UCB-MNCs (Supplementary Fig. 2A and B). We used qRT-PCR to quantitate the changes in expression of randomly selected genes representing the most enriched GO terms. The expression of genes related to defense and immune response-related genes *CD74*, *VAV1*, *TLR8*, *LILRB2*, *ITGAX*, *NCF4*, *LGALS3BP*, *LSP1*, *TRPM2*, and *ADAP1* was significantly decreased in OECs (Fig. 3A). In contrast, the expression of developmental process and angiogenesis-associated genes *ECSCR*, *MCAM*, *PGF*, *ARHGEF15*, *CLDN11*, *APLN*, *MDFI*, *CTHRC1*, *DOCK6*, and *DOCK9* was significantly increased in OECs (Fig. 3B). Therefore, we confirmed that differentially expressed genes identified using RNA-Seq analysis were differentially regulated in UCB-MNCs and OECs.

3.4. DNA methylation status reveals a negative correlation of gene expression with differentiation

To determine if there was a negative correlation between validated gene expression and DNA methylation, we used the MeDIP-seq data to compare the methylation patterns of genes differentially expressed in UCB-MNCs compared with OECs (Fig. 4). The genes selected were those that exhibited the largest changes in DNA methylation between UCB-MNCs and OECs as well as to reveal their biological functions. *CD74*, *VAV1*, *TLR8*, *NCF4*, *ITGAX*, and *LILRB2* were not methylated in OECs at each CpG site of the proximal promoter region, whereas high levels of DNA methylation were detected in UCB-MNCs (Fig. 4A). Conversely, angiogenesis-related upregulated genes expressed by OECs (*ECSCR*, *MCAM*, *PGF*, *ARHGEF15*, *APLN*, and *CLDN11*) represented a low

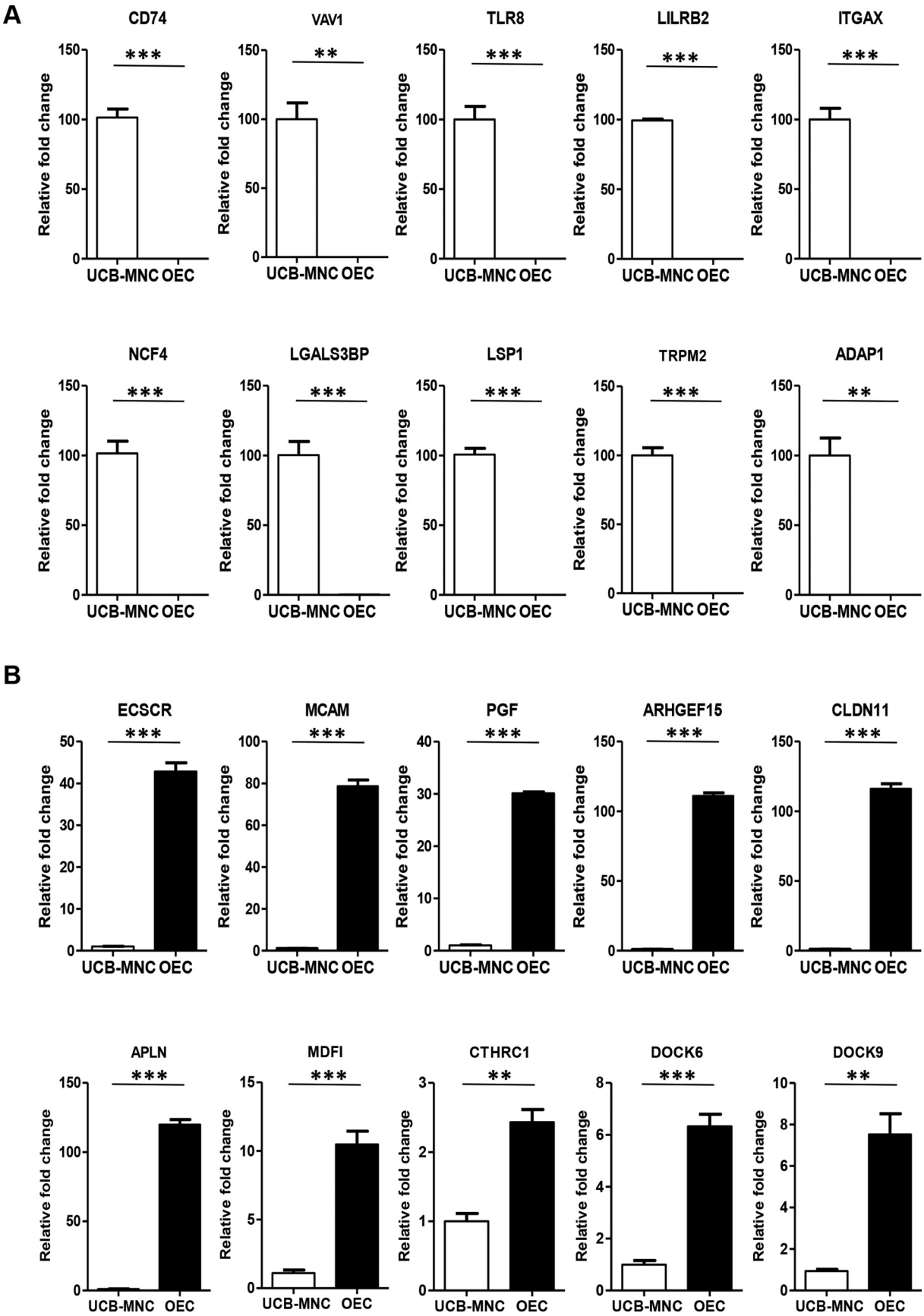

Fig. 3. Validation of mRNA levels of representative genes enriched GO terms. (A) qRT-PCR analysis of mRNA expression levels of representative hypermethylated genes. (B) Transcript levels of angiogenesis and developmental process-related hypomethylated genes. The transcript levels were normalized to that of GAPDH mRNA. $^{**}p < 0.01$, $^{***}p < 0.001$ versus UCB-MNC.

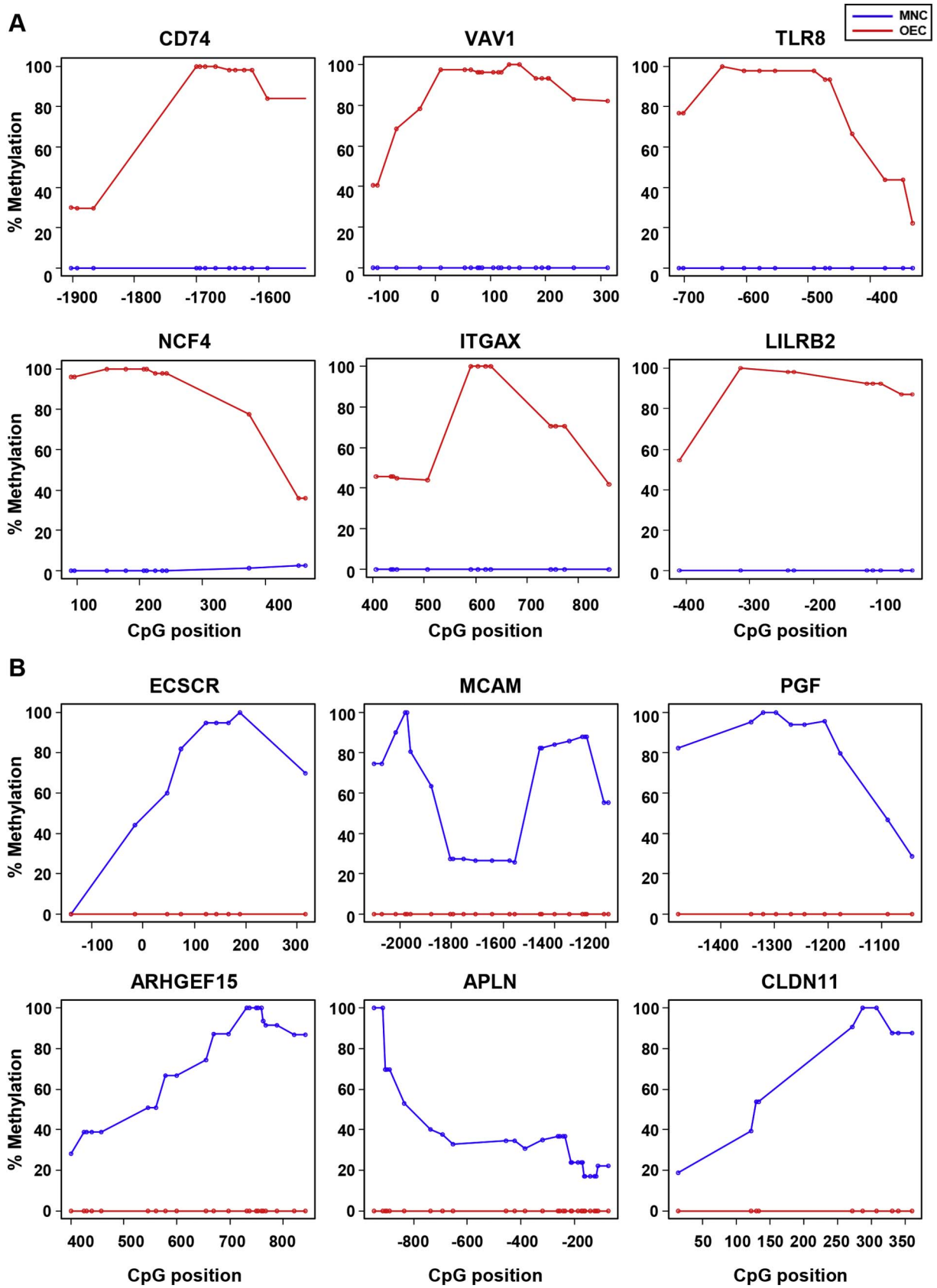

Fig. 4. Differential methylation levels of promoters of representative genes at each CpG site. Promoter methylation was determined using MeDIP-Seq results. (A) The promoters of *CD74*, *VAV1*, *TLR8*, *NCF4*, *ITGAX*, and *LILRB2* are unmethylated at each CpG site in UCB-MNCs (blue) and densely methylated in OECs (red). However, (B) the promoters of *ECSCR*, *MCAM*, *PGF*, *ARHGEF15*, *APLN*, and *CLDN11* are highly methylated in UCB-MNCs and methylated at low levels in OECs. The percentage methylation represents a relative score based on the highest methylation value. Open circles represent each CpG site. Blue line, UCB-MNC; red line, OEC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

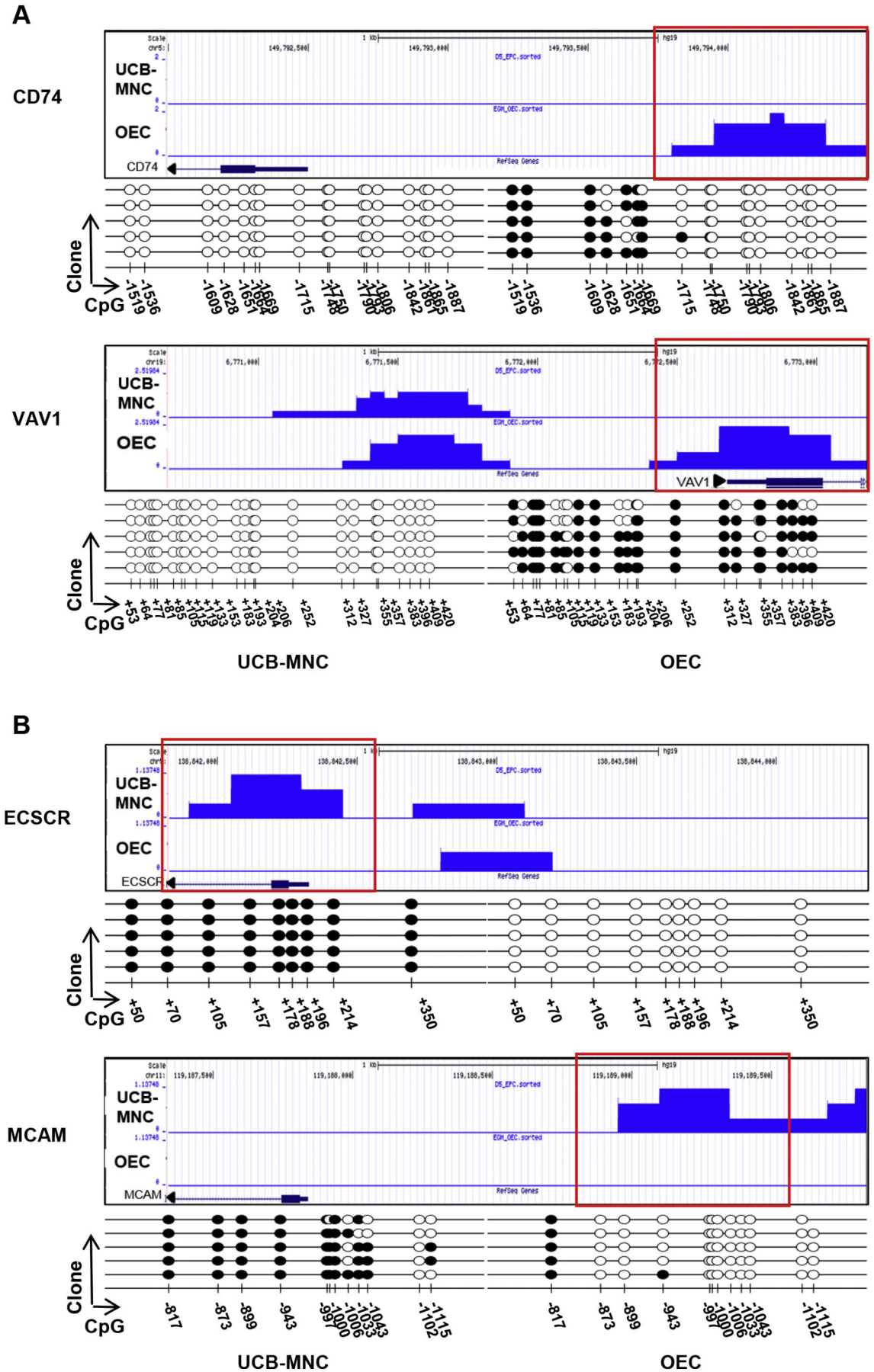

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Fig. 5. Promoter methylation of immune response- and angiogenesis-related genes. (A–B) Bisulfite sequence analysis of specific CpG promoter sites (red box) proximal to the transcription start sites of representative genes (*CD74*, *VAV1*, *ECSCR*, and *MCAM*). Unmethylated CpGs (Open circles), methylated CpGs (filled circles) of 5 independent CpG clones encompassing the region of interest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percentage of DNA methylation in OECs at each CpG site compared with UCB-MNCs (Fig. 4B). These results indicate that hyper- or hypomethylation negatively regulates gene expression.

3.5. Bisulfite sequencing reveals DNA methylation patterns of representative genes

To confirm these results of statistical analysis acquired from DNA methylation sequencing, we performed bisulfite sequencing of *CD74*, *VAV1*, *ECSCR*, and *MCAM*. CD74 is highly expressed by UCB-MNCs. Previous expression analysis of peripheral blood-derived eEPCs and OECs shows that CD74 mediates the immune response (Medina et al., 2010b) and that VAV1 mediates hematopoiesis (Katzav, 2009). We selected regions with the largest changes in promoter methylation (Fig. 5A and B red box). The promoters of *CD74* and VAV1 were not methylated in UCB-MNCs but were densely methylated in OECs (Fig. 5A). In the hypomethylated dataset, the angiogenesis-related genes *ECSCR* and *MCAM* are closely associated with VEGF signaling, which mediates EC function (Kilari et al., 2013; Jiang et al., 2012). Moreover, ECSCR enhances VEGFR2 activation and promotes EC migration (Verma et al., 2010), and MCAM mediates EC proliferation, adhesion, and migration (Jiang et al., 2012; Bardin et al., 2001). Comparison of bisulfite sequencing data for UCB-MNCs to OECs reveals that the *ECSCR* and *MCAM* promoters were not methylated in OECs but were highly methylation in UCB-MNCs (Fig. 5B). Thus, these data confirm the accuracy of the profiling data and indicate that DNA methylation contributes to the alteration of gene expression.

3.6. DNA methyltransferase directly regulates gene expression and methylation patterns during the differentiation of UCB-MNCs to OECs

To understand the regulatory mechanism of transcriptional inactivation mediated by increased promoter methylation, we investigated the effects of DNA methyltransferases (DNMTases) on epigenetically silenced genes in OECs. We first determined the expression levels of *DNMT3A*, *DNMT3B*, and *DNMT1* in UCB-MNCs and OECs. Expression of each gene increased in OECs compared with UCB-MNCs (Fig. 6A), indicating that gain of DNA methylation is more common than its loss during the differentiation of mononuclear cells into terminally differentiated cells.

We treated OECs with the DMMT inhibitor 5′-aza-dc and found that among 61 hypermethylated downregulated genes, the transcription of genes related to the immune system was reactivated (Fig. 6B). To determine whether reactivation was caused by DNA demethylation, we performed bisulfite sequencing. The methylation of *VAV1* was reduced in 5′-aza-dc-treated OECs (Fig. 6C). The promoter region of the representative UCB-MNC enriched gene CXCR4 was not methylated in UCB-MNCs or OECs. Further, *CXCR4* expression was unchanged following treated with 5′-aza-dc (Fig. 6B). These findings suggest that DNA methylation is not involved in the transcriptional regulation of CXCR4. Taken together, we conclude that DNMTases regulates DNA methylation during differentiation and silence the expression of immune-related UCB-MNC enriched genes.

3.7. DNA demethylation activates gene expression during the differentiation of UCB-MNCs to OECs

We next treated UCB-MNCs with 5′-aza-dc to determine whether silenced expression of OEC-enriched genes was induced by DNA methylation. The qRT-PCR results show that 5′-aza-dc activated the transcription of the OEC-enriched genes *ECSCR*, *PGF*, *MCAM*, and *ARHGEF15* in UCB-MNCs (Fig. 7A). Thus, increased DNA methylation induced by DNMTases maintained the silenced expression pattern of angiogenesis-related genes in UCB-MNCs. Therefore, we sought to explore the role of DNA demethylase in the activation of silenced genes.

We first determined the expression of DNA demethylase family genes *TET1*, *TET2*, and *TET3* in UCB-MNCs and OECs. If all *TET* family genes were expressed in OECs, *TET2* and *TET3* expression levels decreased. In particular, *TET3* was significantly reduced compared with *TET2*. However, *TET1* expression was increased in OECs compared with UCB-MNCs (Fig. 7B). Therefore, we cotransduced OECs with a lentiviruses expressing shRNA targeting *TET1* or *TET2*. Knockdown of *TET1* and *TET2* reduced the levels of *TET1* and *TET2* transcripts by 60% (Supplementary Fig. 3). After knockdown of *TET1* and *TET2* expression, qRT-PCR analysis detected decreased expression of the angiogenesis-related genes *ECSCR* and *PGF* and the developmental process-associated genes *MDFI and CTHRC1* and *DOCK9* (Fig. 7C). Although *VEGFR2* is highly expressed in OECs and associated with angiogenesis, its methylation did not change during differentiation. Accordingly, *VEGFR2* expression was unchanged in UCB-MNCs treated with 5′-aza-dc or in OECs cotransduced with *TET1* and *TET2* shRNAs (Fig. 7C). These results suggest that DNA methyltransferase and demethylase directly regulate certain cell type-specific genes by changing DNA methylation patterns during UCB-MNC to OEC differentiation and that they play a role in maintaining the expression patterns of terminally differentiated cells.

4. Discussion

In this study, we determined that DNA methylation was significantly involved in regulating UCB-MNC to OEC differentiation. We used MeDIP-Seq and RNA-Seq to profile the changes in promoter methylation and gene expression during the differentiation of UCB-MNCs to OECs. We identified 82 genes that showed negative correlation between methylation and gene expression.

Functional analysis revealed that the characteristics of UCB-MNCs, i.e., immune-associated functions, were silenced by hypermethylation and the characteristics of OECs, such as developmental process and angiogenesis, were activated with hypomethylation during differentiation. Furthermore, we identified that DNA methylation regulators (DNMTases and TETs) directly regulated gene expression through the reactivation of silenced genes. Our findings suggest that DNA methylation and demethylation are specific regulators for determining the gain and loss of biological function during UCB-MNC to OEC differentiation.

Thus, we provide a new insight that epigenetic mechanisms are involved in UCB-MNC to OEC differentiation. Accumulating evidence shows that DNA methylation plays important roles in regulating gene expression and cell differentiation. For example, DNMT3a-deficient HSCs increase self-renewal by upregulating multipotency genes that are crucial for HSC functions (*Gata3*, *Runx1*, *Pbx1*, and *Cdkn1a*) and by downregulating differentiation-related genes (*Flk2*, *Sfpi1*, and *Mef2c*) (Challen et al., 2012). Loss of Dnmt3a and Dnmt3b from HSCs inhibits proliferative potential, although these cells retain differentiation potential (Challen et al., 2014). Moreover, the loss of *TET2* results in impaired hematopoietic differentiation and preferential myeloid commitment (Ko et al., 2010; Figueroa et al., 2010), suggesting that de novo methylation is important for self-renewal and differentiation of HSCs. HSCs are multipotent cells that give rise to erythrocytes, lymphocytes, monocytes, and granulocytes (Seita and Weissman, 2010). HSCs and UCB-MNCs are derived from a common precursor (hemangioblast) (Pardanaud et al., 1989; Flamme and Risau, 1992). In particular, CD31- and CD34-positive HSCs have similar capacities of differentiation to EC

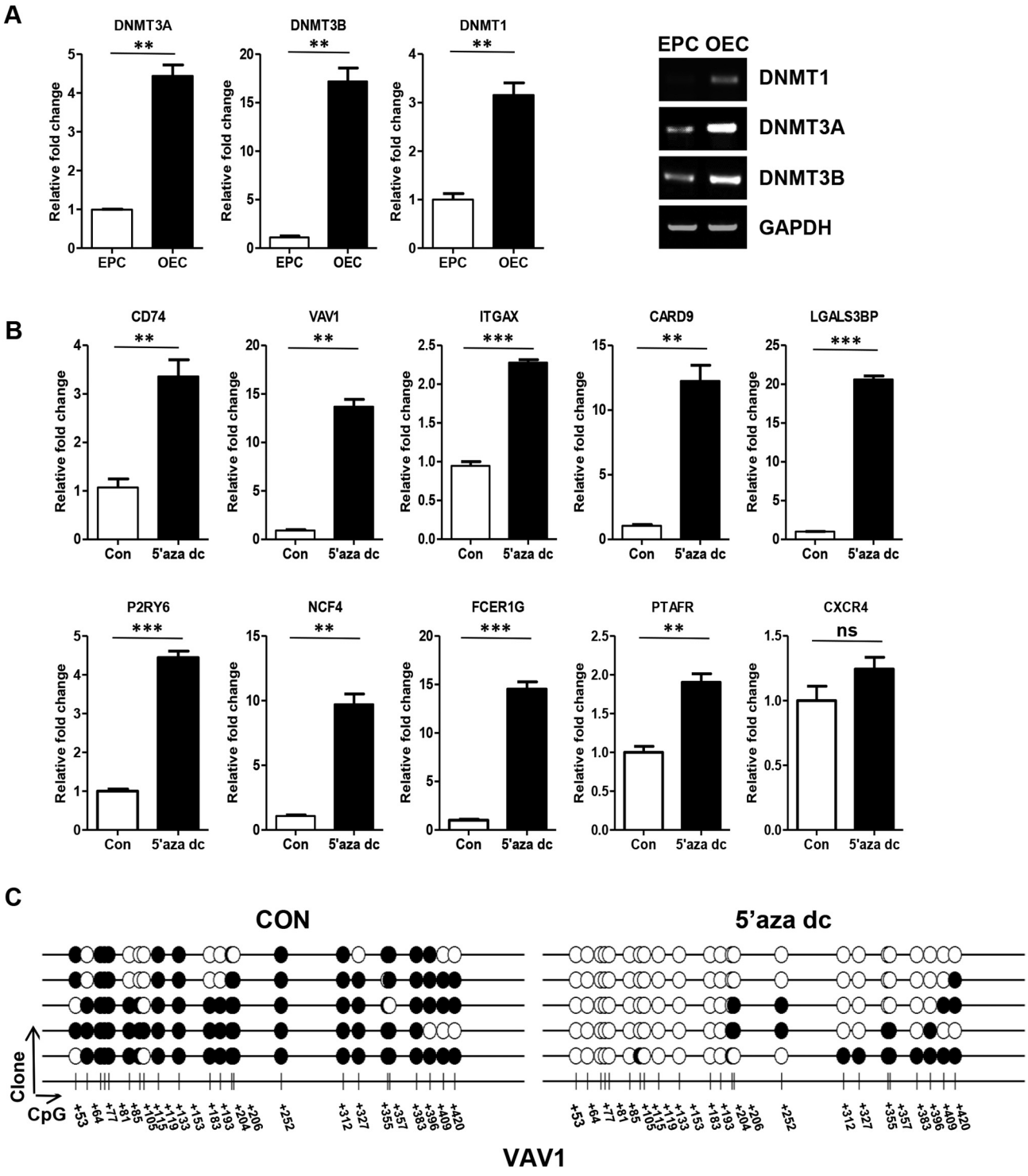

Fig. 6. Inhibiting DNMT activity affects the transcriptional levels of UCB-MNC enriched genes through decreasing DNA methylation. (A) qRT-PCR (left) and RT-PCR (right) analyses of mRNA levels of *DNMT3A*, *DNMT3B*, and DNMT1 in UCB-MNCs and OECs. (B) We treated OECs with 50% acetic acid (Con), 5′-aza-2′-cytidine dissolved in 50% acetic acid (5′-aza-dc; 10 μm, 7 days, 24 h) followed by qRT-PCR analysis of MNC-enriched genes (*CD74*, *VAV1*, *ITGAX*, *CARD9*, *LGALS3BP*, *P2RY6*, *NCF4*, *FCER1G*, and *PTAFR*). *CXCR4* served as the negative control. (A–B) qPCR data were normalized to the levels of *GAPDH* mRNA. $*p < 0.1$, $**p < 0.01$, $***p < 0.001$. ns, not significant. (C) Bisulfite sequencing of specific CpGs within the *VAV1* promoter of OECs treated with the control and 5′-aza-dc. Open and filled circles indicate unmethylated and methylated CpGs, respectively.

lineage (Wijelath et al., 2004; Lian et al., 2014). However, DNA methylation has not been studied in the differentiation of UCB-MNCs and HSCs to EC lineage. Our integrative analysis data indicate that epigenetic mechanisms are involved in UCB-MNC to EC differentiation and newly found differentially methylated and expressed genes in UCB-MNCs and OECs. Furthermore, this is the first study to demonstrate that representative biological functions of UCB-MNCs or OECs were regulated by DNA methylation and demethylation. Previous transcriptome profiling studies of UCB-MNCs and ECs showed that PB-derived early EPCs are hematopoietic cells with a typical monocyte phenotype, are enriched in transcripts involved in inflammation and immune responses, and PB EPC-derived OECs possess angiogenic properties (Medina et al., 2010b). Our GO data for methylation-regulated differentially expressed genes reveals that an UCB-MNC specific

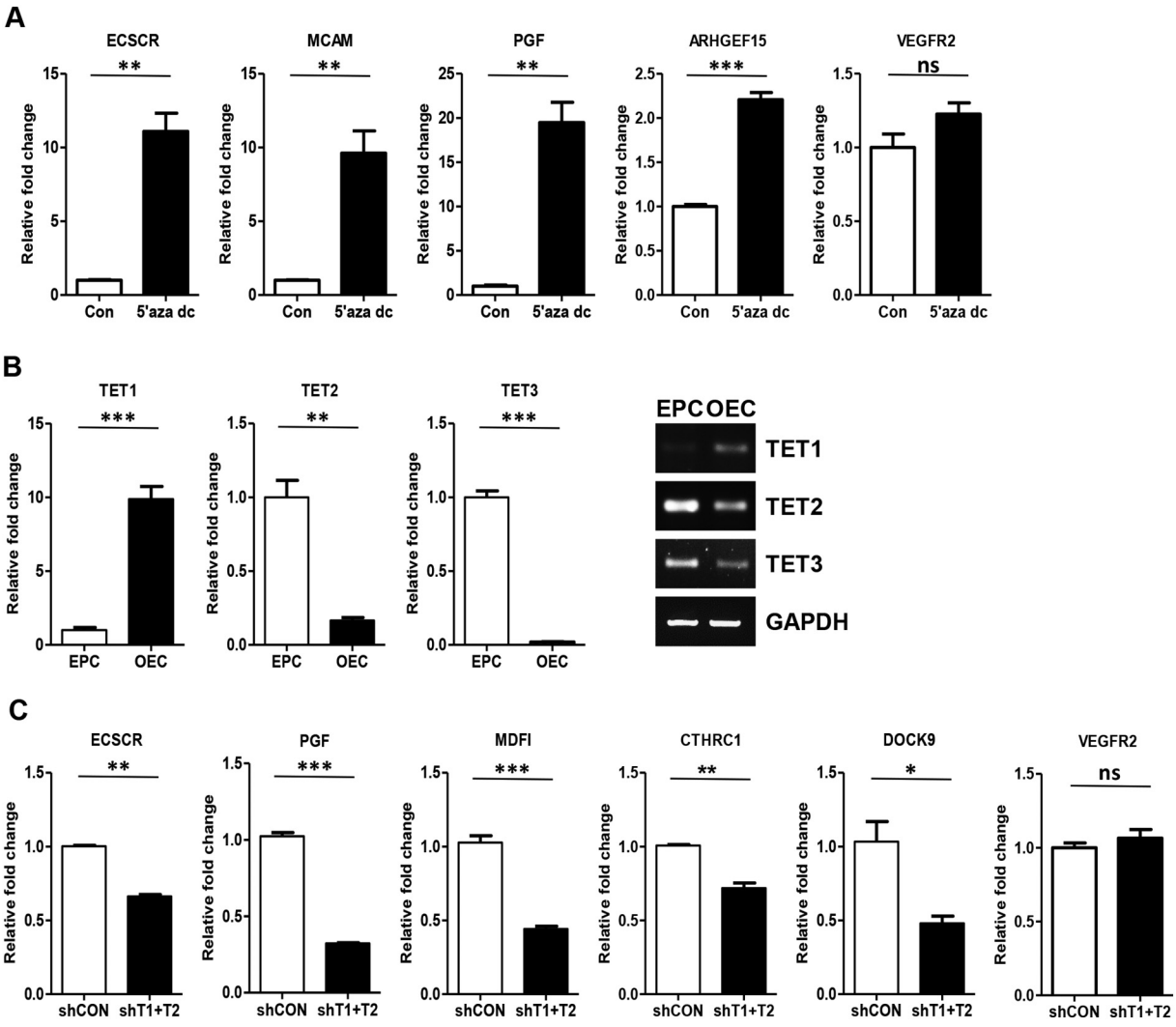

Fig. 7. DNA demethylase directly affects the transcriptional levels of OEC-enriched genes. (A) After exposure of UCB-MNCs to 5′-aza-dc (10 μmol/l) every 2 days for 14 days, relative expression levels of OEC-enriched genes (*ECSCR*, *MCAM*, *PGF*, and *ARHEGF15*) were assessed by qRT-PCR. (B) The mRNA levels of DNA demethylase family genes *TET1*, *TET2*, and *TET3* in UCB-MNCs and OECs were analyzed using qRT-PCR (left) and RT-PCR (right). (C) OECs were transfected with a control shRNA or shRNA-TET1 and shRNA-TET2 lentivirus. After culture for 14 days, relative mRNA levels of OEC-enriched genes (*ECSCR*, *PGF*, *MDFI*, *CTHRC1*, and *DOCK9*) were analyzed using qRT-PCR. All results were normalized to *GAPDH* mRNA levels. $^{**}p < 0.01$, $^{***}p < 0.001$ (*t*-test). ns, not significant. *VEGFR2* mRNA served as the negative control.

gene cluster is hyper-methylated in OECs and was highly enriched immune response-associated functions, reflecting the hematopoietic character of mononuclear cells compared with terminally differentiated ECs. In contrast, OEC-enriched hypomethylated genes were associated with developmental process and angiogenesis, indicating that specific UCB-MNC and OEC phenotypes are regulated by DNA methylation during differentiation.

We confirmed the negative correlation of promoter methylation and gene expression. Our work highlights representative genes included in GO biological process categories. CD74 is a receptor for the proinflammatory cytokine macrophage migration inhibitory factor (Schwartz et al., 2009). This ligand/receptor complex activates CXCR4 signaling pathways, which is a marker of UCB-MNCs, which trigger the synthesis and secretion of proinflammatory factors and cell adhesion molecules (Doring et al., 2014). VAV1 is a guanine nucleotide exchange factor for Rho/Rac GTPase and is specifically expressed by cells of the hematopoietic system. The *VAV1* promoter is fully demethylated in lymphocytes where VAV1 is normally expressed, and VAV1 controls hematopoiesis through binding to the hematopoietic transcription factor MYB (Ilan and Katzav, 2012). We determined that these UCB-MNC specific enriched genes were silenced by hypermethylation during differentiation.

Conversely, ECSCR and MCAM play crucial roles in ECs. ECSCR and MCAM are cell-surface proteins expressed by ECs, which play roles in EC migration, apoptosis, and proliferation (Verma et al., 2010; Jouve et al., 2015). Their function is associated with VEGF-triggered signaling. Loss of ECSCR from primary ECs reduces tyrosine phosphorylation of the VEGF receptor 2/kinase insert domain receptor (KDR) (Verma et al., 2010). Further, MCAM deficiency decreases the expression of VEGFR-2/Ve-cadherin and alters focal adhesion kinase (FAK) activation in response to VEGF (Jouve et al., 2015). Our RT-PCR and bisulfite sequencing results show that OEC-enriched genes, particularly those that are angiogenesis-related, are hypomethylated state with increased expression levels during differentiation.

Analyzing microarrays to compare the expression of our focus genes in other EC lineages such as HUVEC, HUAEC, and LEC, these data are consistent with our previous finding on mRNA sequencing profiling (Supplementary Fig. 4). Methylation of the promoters of EC marker genes encoding eNOS, vWF, CD31, and VE-cadherin is reduced in HUVECs compared with the non-EC cell type AoVSMC (Shirodkar et al., 2013; Ohtani et al., 2011). Similarly, we detected hypomethylation of these genes in OECs. However, the genes encoding vWF, CD31, and VE-cadherin were also hypomethylated in UCB-MNCs. Moreover, when we compared DNA methylation of UCB-MNC derived mesenchymal stem

cells (MSCs) and OECs, the genes encoding eNOS, CD31, and VE-cadherin were hypermethylated in MSCs, indicating that the methylation of these EC-enriched genes may differ between ECs and non-ECs, but these patterns do not always occur during UCB-MNC to OEC differentiation.

We show here the reversibility of DNA methylation, which emphasizes the importance of DNA methylation regulators in gene expression. Inhibition of promoter methylation induced the demethylation and reactivation of UCB-MNC specific immune-related genes in OECs, in which the gene was hypermethylated. However, certain genes were not reactivated. Our GO analysis reveals that *TLR8*, which encodes a member of the Toll-like receptor family, was enriched in immune-related processes (Takeda and Akira, 2005). *TLR8* was not reactivated in nonexpressing cells (OECs) after inhibiting DNMTase activity, although bisulfite sequencing showed reduced methylation of *TLR8*, suggesting the possibility that gene activation requires transcription factors, although DNA methylation is decreased. In the hypomethylated gene set, we detected reduced expression of certain genes related to angiogenesis, such as *PGF*, *ECSCR*, *CTHRC1*, and *DOCK9* in long-term cultures of OECs after inhibiting the DNA demethylases TET1 and TET2. These data are consistent with the possibility that DNA demethylase promotes the hydroxymethylation of the promotor of these genes, facilitating demethylation and promoting transcription. In contrast, we observed that TET1 and TET2 depletion did not affect the expression of other genes such as MCAM and ARHGEF15. Our data suggest that the MCAM and ARHGEF15 promoter is normally silenced by DNA methylation, and these demethylations using 5′-aza-dc reactivated the expressions in UCB-MNCs. However, it is clear that MCAM and ARHGEF15 expressions were affected by DNA methylation, although further studies are required to ascertain whether TET directly binds to *MCAM*, *ARHGEF15*, or other genes and affects the expression of these genes.

Altogether, the data suggest that DNA methylation is a specific factor that controls the cell-type expression of specific genes during UCB-MNC to OEC differentiation, and the changes induced by DNMTases and demethylase directly regulated gene expression and were maintained in terminally differentiated cells.

5. Conclusion

DNA methylation and demethylation are crucial regulators of gene expression during cell differentiation. Understanding of interrelation between DNA methylation and gene expression provide a new insight that epigenetic mechanisms are involved in UCB-MNC to OEC differentiation. Here, we profiled global changes of promoter methylation and gene expression during the differentiation of UCB-MNCs to OECs and identified a number of genes with negative correlation between methylation and gene expression. Functional analysis revealed that the characteristics of UCB-MNCs were silenced by hypermethylation and the characteristics of OECs were activated with hypomethylation during differentiation. Further, the reversibility of DNA methylation emphasizes the importance of DNA methylation regulator in gene expression. Our findings suggest that DNA methylation is a specific factor that controls the cell-type expression of specific genes during UCB-MNC to OEC differentiation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.09.006.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology [NRF-2015R1A2A1A05001859; NRF2013M3A9B6046563; NRF-2015K1A4A3047851; NRF-2015M3A9B6066967], by the Bio and Medical Technology Development Program of the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (MEST) [NRF-2015M3A9B6066967], by a grant from the Korea Health 21 R & D Project, Ministry of Health and Welfare of the Republic of Korea [H16C1501], and by the Technology Innovation Program of the Ministry of Trade, Industry and Energy, Republic of Korea [Grant 10050154].

Conflict of interest

None.

References

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11 (10) (doi: ARTN R10610.1186/gb-2010-11-10-r106. PubMed PMID: WOS:000287378900008).

Asahara, T., Murohara, T., Sullivan, A., Silver, M., vanderZee, R., Li, T., et al., 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275 (5302), 964–967. http://dx.doi.org/10.1126/science.275.5302.964. (WOS:A1997WH38800041).

Bardin, N., Anfosso, F., Masse, J.M., Cramer, E., Sabatier, F., Le Bivic, A., et al., 2001. Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. Blood 98 (13), 3677–3684. http://dx.doi.org/10.1182/blood.V98.13.3677. (PubMed PMID: WOS:000172604700023).

Bhutani, N., Burns, D.M., Blau, H.M., 2011. DNA Demethylation Dynamics. Cell 146 (6), 866–872. http://dx.doi.org/10.1016/j.cell.2011.08.042. (PubMed PMID: WOS:000295258100010).

Challen, G.A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J.S., et al., 2012. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat. Genet. 44 (1), 23–31. http://dx.doi.org/10.1038/ng.1009. (PubMed PMID: 22138693; PubMed Central PMCID: PMCPMC3637952).

Challen, G.A., Sun, D., Mayle, A., Jeong, M., Luo, M., Rodriguez, B., et al., 2014. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. Cell Stem Cell 15 (3), 350–364. http://dx.doi.org/10.1016/j.stem.2014.06.018. (PubMed PMID: 25130491; PubMed Central PMCID: PMCPMC4163922).

Chavez, L., Jozefczuk, J., Grimm, C., Dietrich, J., Timmermann, B., Lehrach, H., et al., 2010. Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage. Genome Res. 20 (10), 1441–1450. http://dx.doi.org/10.1101/gr.110114.110. (PubMed PMID: 19451168; PubMed Central PMCID: PMCPMC2705234).

Chen, T.P., Ueda, Y., Dodge, J.E., Wang, Z.J., Li, E., 2003. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol. Cell. Biol. 23 (16), 5594–5605. http://dx.doi.org/10.1128/Mcb.23.16.5594-5605.2003. (PubMed PMID: WOS:000184527100010).

Doring, Y., Pawig, L., Weber, C., Noels, H., 2014. The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease. Front. Physiol. 5 (doi: ARTN 21210.3389/fphys.2014.00212. PubMed PMID: WOS:000347060500001).

Eggermann, J., Kliche, S., Jarmy, G., Hoffmann, K., Mayr-Beyrle, U., Debatin, K.M., et al., 2003. Endothelial progenitor cell culture and differentiation in vitro: a methodological comparison using human umbilical cord blood. Cardiovasc. Res. 58 (2), 478–486. http://dx.doi.org/10.1016/S0008-6363(03)00252-9. (PubMed PMID: WOS:000183190300023).

Figueroa, M.E., Abdel-Wahab, O., Lu, C., Ward, P.S., Patel, J., Shih, A., et al., 2010. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18 (6), 553–567. http://dx.doi.org/10.1016/j.ccr.2010.11.015. (PubMed PMID: 21130701; PubMed Central PMCID: PMCPMC4105845).

Flamme, I., Risau, W., 1992. Induction of vasculogenesis and hematopoiesis invitro. Development 116 (2), 435–439 (PubMed PMID: WOS:A1992JX00200015).

Hermann, A., Goyal, R., Jeltsch, A., 2004. The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites. J. Biol. Chem. 279 (46), 48350–48359. http://dx.doi.org/10.1074/jbc.M403427200. (PubMed PMID: WOS:000224957000110).

Hristov, M., Erl, W., Weber, P.C., 2003. Endothelial progenitor cells - mobilization, differentiation, and homing. Arterioscler. Thromb. Vasc. Biol. 23 (7), 1185–1189. http://dx.doi.org/10.1161/01.Atv.0000073832.49290.B5. (PubMed PMID: WOS:000184100900009).

Ilan, L., Katzav, S., 2012. Human Vav1 expression in hematopoietic and cancer cell lines is regulated by c-Myb and by CpG methylation. PLoS One 7 (1) (doi: ARTN e29939 10.1371/journal.pone.0029939. PubMed PMID: WOS:000301355700067).

Jaenisch, R., Li, E., Laird, P., Jacksongrusby, L., Juttermann, R., Beard, C., 1994. The role of DNA methylation in mammalian development. J. Cell. Biochem. 186 (PubMed PMID: WOS:A1994MV41200629).

Jiang, T.X., Zhuang, J., Duan, H.X., Luo, Y.T., Zeng, Q.Q., Fan, K.L., et al., 2012. CD146 is a coreceptor for VEGFR-2 in tumor angiogenesis. Blood 120 (11), 2330–2339. http://dx.doi.org/10.1182/blood-2012-01-406108. (PubMed PMID: WOS:000309044200025).

Jones, P.A., Takai, D., 2001. The role of DNA methylation in mammalian epigenetics. Science 293 (5532), 1068–1070. http://dx.doi.org/10.1126/science.1063852. (PubMed PMID: 11498573).

Jouve, N., Bachelier, R., Despoix, N., Blin, M.G., Matinzadeh, M.K., Poitevin, S., et al., 2015. CD146 mediates VEGF-induced melanoma cell extravasation through FAK

activation. Int. J. Cancer 137 (1), 50–60. http://dx.doi.org/10.1002/ijc.29370. (PubMed PMID: WOS:000353297600005).
Jung, J.H., Choi, H.J., Maeng, Y.S., Choi, J.Y., Kim, M., Kwon, J.Y., et al., 2010. Mel-18, a mammalian Polycomb gene, regulates angiogenic gene expression of endothelial cells. Biochem. Biophys. Res. Commun. 400 (4), 523–530. http://dx.doi.org/10.1016/j.bbrc.2010.08.086. (PubMed PMID: 20801102).
Katzav, S., 2009. Vav1: A hematopoietic signal transduction molecule involved in human malignancies. Int. J. Biochem. Cell Biol. 41 (6), 1245–1248. http://dx.doi.org/10.1016/j.biocel.2008.11.006. (PubMed PMID: WOS:000264669300005).
Kilari, S., Remadevi, I., Zhao, B.F., Pan, J., Miao, R., Ramchandran, R., et al., 2013. Endothelial cell-specific chemotaxis receptor (ECSCR) enhances vascular endothelial growth factor (VEGF) receptor-2/kinase insert domain receptor (KDR) activation and promotes proteolysis of internalized KDR. J. Biol. Chem. 288 (15), 10265–10274. http://dx.doi.org/10.1074/jbc.M112.413542. (PubMed PMID: WOS:000317565000008).
Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14 (4), R36. http://dx.doi.org/10.1186/gb-2013-14-4-r36. (PubMed PMID: 23618408; PubMed Central PMCID: PMCPMC4053844).
Kim, J., Kim, M., Jeong, Y., Lee, W.B., Park, H., Kwon, J.Y., et al., 2015. BMP9 induces cord blood-derived endothelial progenitor cell differentiation and ischemic neovascularization via ALK1. Arterioscler. Thromb. Vasc. Biol. 35 (9), 2020–2031. http://dx.doi.org/10.1161/ATVBAHA.115.306142. (PubMed PMID: 26229139).
Kirov, S., Ji, R., Wang, J., Zhang, B., 2014. Functional annotation of differentially regulated gene set using WebGestalt: a gene set predictive of response to ipilimumab in tumor biopsies. Methods Mol. Biol. 1101, 31–42. http://dx.doi.org/10.1007/978-1-62703-721-1_3. (PubMed PMID: 24233776).
Ko, M., Huang, Y., Jankowska, A.M., Pape, U.J., Tahiliani, M., Bandukwala, H.S., et al., 2010. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 468 (7325), 839–843. http://dx.doi.org/10.1038/nature09586. (PubMed PMID: 21057493; PubMed Central PMCID: PMCPMC3003755).
Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25 (14), 1754–1760. http://dx.doi.org/10.1093/bioinformatics/btp324. (PubMed PMID: 19451168; PubMed Central PMCID: PMCPMC2705234).
Lian, X.J., Bao, X.P., Al-Ahmad, A., Liu, J.L., Wu, Y., Dong, W.T., et al., 2014. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Rep. 3 (5), 804–816. http://dx.doi.org/10.1016/j.stemcr.2014.09.005. (PubMed PMID: WOS:000345118600011).
Maeng, Y.S., Kwon, J.Y., Kim, E.K., Kwon, Y.G., 2015. Heterochromatin protein 1 alpha (HP1alpha: CBX5) is a key regulator in differentiation of endothelial progenitor cells to endothelial cells. Stem Cells 33 (5), 1512–1522. http://dx.doi.org/10.1002/stem.1954. (PubMed PMID: 25588582).
Medina, R.J., O'Neill, C.L., Humphreys, M.W., Gardiner, T.A., Stitt, A.W., 2010a. Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy. Invest. Ophthalmol. Vis. Sci. 51 (11), 5906–5913. http://dx.doi.org/10.1167/iovs.09-4951. (PubMed PMID: WOS:000283558400065).
Medina, R.J., O'Neill, C.L., Sweeney, M., Guduric-Fuchs, J., Gardiner, T.A., Simpson, D.A., et al., 2010b. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. BMC Med. Genet. 3 (doi: Artn 1810.1186/1755-8794-3-18. PubMed PMID: WOS:000278383900001).
Ohtani, K., Vlachojannis, G.J., Koyanagi, M., Boeckel, J.N., Urbich, C., Farcas, R., et al., 2011. Epigenetic regulation of endothelial lineage committed genes in pro-angiogenic hematopoietic and endothelial progenitor cells. Circ. Res. 109 (11), 1219-U71. http://dx.doi.org/10.1161/Circresaha.111.247304. (PubMed PMID: WOS:000296872200009).
Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99 (3), 247–257 (PubMed PMID: 10555141).
Pardanaud, L., Yassine, F., Dieterlenlievre, F., 1989. Relationship between vasculogenesis, angiogenesis and hematopoiesis during avian ontogeny. Development 105 (3), 473–485 (PubMed PMID: WOS:A1989T746200004).
Patan, S., 2004. Vasculogenesis and angiogenesis. Cancer Treat. Res. 117, 3–32 (PubMed PMID: 15015550).
Pronier, E., Almire, C., Mokrani, H., Vasanthakumar, A., Simon, A., Mor, B.D.R.M., et al., 2011. Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. Blood 118 (9), 2551–2555. http://dx.doi.org/10.1182/blood-2010-12-324707. (PubMed PMID: WOS:000294476400027).
Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26 (1), 139–140. http://dx.doi.org/10.1093/bioinformatics/btp616. (PubMed PMID: WOS:000273116100025).
Schubeler, D., 2015. Function and information content of DNA methylation. Nature 517 (7534), 321–326. http://dx.doi.org/10.1038/nature14192. (PubMed PMID: 25592537).
Schwartz, V., Lue, H.Q., Kraemer, S., Korbiel, J., Krohn, R., Ohl, K., et al., 2009. A functional heteromeric MIF receptor formed by CD74 and CXCR4. FEBS Lett. 583 (17), 2749–2757. http://dx.doi.org/10.1016/j.febslet.2009.07.058. (PubMed PMID: WOS:000270149800009).
Seita, J., Weissman, I.L., 2010. Hematopoietic stem cell: self-renewal versus differentiation. Wires Syst. Biol. Med. 2 (6), 640–653. http://dx.doi.org/10.1002/wsbm.86. (PubMed PMID: WOS:000283713500002).
Shirodkar, A.V., St Bernard, R., Gavryushova, A., Kop, A., Knight, B.J., Yan, M.S.C., et al., 2013. A mechanistic role for DNA methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing. Blood 121 (17), 3531–3540. http://dx.doi.org/10.1182/blood-2013-01-479170. (PubMed PMID: WOS:000321831600030).
Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17 (1), 1–14. http://dx.doi.org/10.1093/intimm/dxh186. (PubMed PMID: WOS:000225675800001).
Tarazona, S., Furio-Tari, P., Turra, D., Di Pietro, A., Nueda, M.J., Ferrer, A., et al., 2015. Data quality aware analysis of differential expression in RNA-seq with NOISeq R/Bioc package. Nucleic Acids Res. 43 (21) (doi: ARTN e14010.1093/nar/gkv711. PubMed PMID: WOS:000366410900003).
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., et al., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7 (3), 562–578. http://dx.doi.org/10.1038/nprot.2012.016. (PubMed PMID: WOS:000300948500013).
Verma, A., Bhattacharya, R., Remadevi, I., Li, K.G., Pramanik, K., Samant, G.V., et al., 2010. Endothelial cell-specific chemotaxis receptor (ECSCR) promotes angioblast migration during vasculogenesis and enhances VEGF receptor sensitivity. Blood 115 (22), 4614–4622. http://dx.doi.org/10.1182/blood-2009-10-248856. (PubMed PMID: WOS:000278372600041).
Voss, T.C., Hager, G.L., 2014. Dynamic regulation of transcriptional states by chromatin and transcription factors. Nat. Rev. Genet. 15 (2), 69–81. http://dx.doi.org/10.1038/nrg3623. (PubMed PMID: WOS:000331894200007).
Wang, L.K., Feng, Z.X., Wang, X., Wang, X.W., Zhang, X.G., 2010. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26 (1), 136–138. http://dx.doi.org/10.1093/bioinformatics/btp612. (PubMed PMID: WOS:000273116100024).
Wijelath, E.S., Rahman, S., Murray, J., Patel, Y., Savidge, G., Sobel, M., 2004. Fibronectin promotes VEGF-induced CD34(+) cell differentiation into endothelial cells. J. Vasc. Surg. 39 (3), 655–660. http://dx.doi.org/10.1016/j.jvs.2003.10.042. (PubMed PMID: WOS:000227387100024).
Williams, K., Christensen, J., Pedersen, M.T., Johansen, J.V., Cloos, P.A.C., Rappsilber, J., et al., 2011. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature 473 (7347), 343–U472. http://dx.doi.org/10.1038/nature10066. (PubMed PMID: WOS:000290722400041).