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Transcriptomic profiling identifies a PU.1 regulatory network in macrophages

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ABSTRACT

PU.1 is a key transcription factor for hematopoiesis and macrophage differentiation. Using chromatin immunoprecipitation we have previously identified several PU.1 target genes in macrophages and microglia. With the aim to complement these studies, we performed a transcriptomic analysis of PU.1$^{-/-}$ progenitors after restoration of PU.1 activity. PUER cells committed to macrophage differentiation were analyzed with novel Affymetrix exon 1.0 ST arrays and Affymetrix 430 2.0 genome arrays for crosswise validation. We combined these genome-wide expression data with a publicly-available microarray dataset of PU.1-knockdown hematopoietic stem cells for an integrated analysis. Bibliographic gene connections, binding site prediction and ChIP-Chip data were used to define a multi-level PU.1 regulatory network in macrophages. Moreover, an alternative transcript of the novel PU.1 target gene Ptpro was identified by exon arrays and PU.1 binding to an intronic promoter was demonstrated. In conclusion, we present a PU.1 transcriptional network with novel validated PU.1 target genes.

Hematopoietic lineage development is regulated by a sophisticated interplay of a limited set of specific transcription factors [1]. PU.1, a member of the ETS family of transcription factors encoded by the gene *Sfpi1*, is required for the generation of early myeloid and lymphoid progenitors [2,3]. PU.1 also directs the differentiation of committed myeloid progenitor cells into macrophages and neutrophils [4,5]. Reduced PU.1 expression and lack of function are associated with a block in hematopoietic differentiation and increased proliferation leading to leukemia in humans [6] and mouse models [7].

PU.1 is also critically involved in the regulation of macrophage-specific basal transcription [8], including expression of the macrophage colony-stimulating factor (M-CSF) receptor encoded by the c-fms protooncogene [9]. Within the macrophage lineage, distinct cellular subsets can be defined based on the localization in specific tissues, activation status, effector functions, or marker proteins [10]. According to a new concept, macrophage cells have an extremely large plasticity, which is controlled by a regulatory network of transcription factors including PU.1 and various other nuclear proteins [11].

Microglia form an important population of macrophages in the CNS. However, little is known about the role of PU.1 in transcriptional programming of microglia activation. A previous study from our laboratory identified high PU.1 levels and several bioinformatically predicted PU.1 target genes in activated retinal microglia from retinoschisin-deficient (Rs1h$^{-/Y}$) mice [12]. Moreover, Walton et al. detected strong microglial PU.1 expression after hypoxic-ischemia brain injury [13]. The analysis of retinal or brain microglia from PU.1-deficient animals is hampered by the fact that null mutation of the *Sfpi1* gene in mice results in perinatal lethality [2,14].

Walsh et al. recently developed a tunable system in which transcriptional regulation by PU.1 can be investigated using PU.1-deficient myeloid progenitors and a conditionally activatable PU.1-estrogen receptor binding domain fusion protein PUER [15]. These myeloid progenitors undergo rapid cell cycle arrest and differentiate into macrophages upon addition of 4-hydroxy-tamoxifen (OHT) [16].

Using chromatin immunoprecipitation coupled to microarrays (ChIP-Chip) we have previously identified 1202 putative PU.1 target genes in RAW264.7 mouse macrophages [17], which partially overlap with differentially expressed genes of activated microglia [12]. To complement these previous studies we performed an integrated genome-wide analysis of the PU.1-dependent transcriptome in PUER cells committed to macrophage differentiation using Affymetrix mouse exon 1.0 ST arrays and Affymetrix 430 2.0 mouse genome arrays. We combined our data with a publicly-available microarray dataset of PU.1-knockdown hematopoietic stem cells and defined a novel PU.1 regulatory network in macrophages. Moreover, the in vivo binding of PU.1 to an intronic *protein tyrosine phosphatase receptor type O* (Ptpro) promoter was validated by ChIP and the corresponding alternative transcript was verified by quantitative real-time RT-PCR.

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Materials and methods

Cell culture. PUER cells were cultured in IMDM (Invitrogen, Carlsbad, CA, USA), supplemented with penicillin/streptomycin (10,000 U/ml), glutamine (200 mM), β-ME (50 μM), mouse IL-3 (5 ng/ml, Biosource, Camarillo, CA, USA), puromycin (1 μg/ml), and 10% FCS as described earlier [15]. To activate the PU.1-ER fusion protein, PUER cells were cultured in medium containing 0.1 μM 4-hydroxytamoxifen (OHT) for the indicated time points. RAW264.7 cells were cultured in DMEM, supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in 10% CO_2 in air at 37 °C.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed as described previously [12,17] using 2.5 μg polyclonal PU.1 antibody or IgG rabbit isotype control (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enrichment was analyzed by PCR with primers amplifying the alternative Ptpro promoter region (forward, 5′-CCC AAA TGT ATG TGG TGC AA-3′; reverse, 5′-ATG TGG GCT GGG GAG AAT A-3′).

RNA isolation and reverse transcription. Total RNA was extracted according to the manufacturer's instructions using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA). The RNA was quantified spectrophotometrically and then stored at −80 °C. First-strand cDNA synthesis was performed with the Reverse Transcription System from Stratagene (La Jolla, CA, USA).

Quantitative real-time RT-PCR. Amplifications of 50 ng cDNA were performed with the Taqman 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) in 20 μl reaction mixtures containing 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 200 nM primers, and 0.25 μl dual-labeled probe (Roche Universal Probe Library). Measurements were performed in triplicates and the results were analyzed with the $\Delta\Delta C_t$ method for relative quantitation. Normalization to three stable reference genes was performed as described earlier [18]. The primers for amplification of full-length Ptpro were F, 5′-GCC AGA AAC AGA AGG AGA GC-3′, and R, 5′-ACA GGT TTG CTT GAG TTC ACC-3′. The Ptpro short transcript form was quantified with primers F, 5′-TCC CAG TGT CCC TAC ATT CAT-3′, and R, 5′-CAC CAC GTT AGG GTT CAC CT-3′.

DNA-microarray analysis. Generation of probes, hybridization to Affymetrix GeneChip Mouse Genome 430 2.0 Arrays and Affymetrix GeneChip Mouse Exon ST 1.0 Arrays, washing, and scanning were performed according to the Affymetrix standard protocol. Triplicate microarrays of both types were carried out with RNA from PUER cells isolated before (0 h) and after (24 h) induction of the PU.1-ER fusion protein with 100 nM OHT. Minimum information about a microarray experiment (MIAME) criteria were met [19]. The microarray datasets of this study are publicly-available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as series records GSE13125 and GSE9011.

Bioinformatic data analysis. The Affymetrix Expression Console Software Version 1.0 was used to create summarized expression values (CHP-files) from 3′ expression array and exon array feature intensities (CEL-files). The Robust Multichip Analysis (RMA) algorithm was applied to both microarray types. Exon arrays were analyzed at the extended gene level limiting the analysis to transcripts with exon-level probe sets that map to cDNA alignments. Integrative analysis of genome-wide expression activities from PUER cells

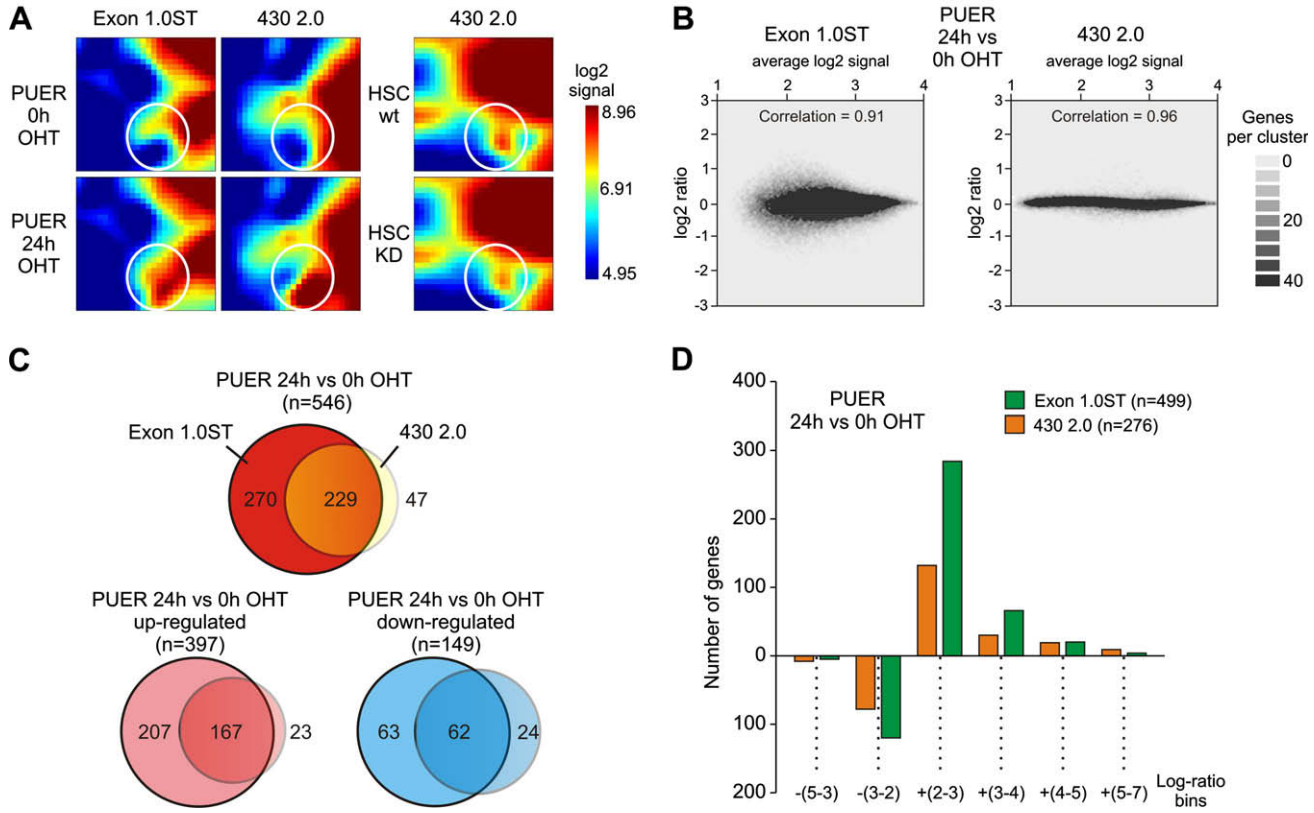

Fig. 1. Microarray expression analysis after modulation of PU.1 activity. (A) Comparative GEDI analysis of Affymetrix microarray expression signals from PUER cells before and after 24 h OHT induction (left panel) and PU.1 knockdown (KD) in hematopoietic stem cells (right panel). The HSC dataset was retrieved from GEO accession number GSE5654. (B) MvA plot of mean expression data from triplicate Exon 1.0 ST or 430 2.0 arrays from PUER cells before and after OHT treatment. (C) Genomatix ChipInspector single probe analysis of differentially expressed genes in PUER cells using data from Exon 1.0 ST or 430 2.0 arrays with a FDR <0.1%, 3 significant probes and log2 ratios >2. (D) Log2 ratio distribution of differentially expressed genes of Exon 1.0 ST versus 430 2.0 arrays.