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Redefinition of the human mast cell transcriptome by deep-CAGE sequencing

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Key Points

- Generated a reference transcriptome for ex vivo, cultured, and stimulated mast cells, contrasted against a broad collection of primary cells.
- Identified BMPs as function-modulating factors for mast cells.

Mast cells (MCs) mature exclusively in peripheral tissues, hampering research into their developmental and functional programs. Here, we employed deep cap analysis of gene expression on skin-derived MCs to generate the most comprehensive view of the human MC transcriptome ever reported. An advantage is that MCs were embedded in the FANTOM5 project, giving the opportunity to contrast their molecular signature against a multitude of human samples. We demonstrate that MCs possess a unique and surprising transcriptional landscape, combining hematopoietic genes with those exclusively active in MCs and genes not previously reported as expressed by MCs (several of them markers of unrelated tissues). We also found functional bone morphogenetic protein receptors transducing activatory signals in MCs. Conversely, several immune-related genes frequently studied in MCs were not expressed or were weakly expressed. Comparing MCs ex vivo with cultured counterparts revealed profound changes in the MC transcriptome in in vitro surroundings. We also determined the promoter usage of MC-expressed genes and identified associated motifs active in the lineage. Befitting their uniqueness, MCs had no close relative in the hematopoietic network (also only distantly related with basophils). This rich data set reveals that our knowledge of human MCs is still limited, but with this resource, novel functional programs of MCs may soon be discovered. (*Blood*. 2014;123(17):e58-e67)

Introduction

Although mast cells (MCs) are commonly associated with the elicitation of immunoglobulin E–mediated allergic inflammation,[1,2] the spectrum of possible MC functions has greatly expanded over the last few years to now cover processes in which MCs assume potentially detrimental (autoimmune and inflammatory diseases, obesity, or tumors) or potentially beneficial roles (anti-inflammatory/tolerogenic effects, other types/stages of cancer, or antimicrobial defense).[3-10] With the exception of type I allergy (for which a large body of evidence also exists for humans), the roles that MCs play in human health and disease are undefined, because MC research to date strongly relies on studies in the mouse, even though MCs differ considerably between species.[11]

Research into the full range of functional programs of a cell requires information on its specific gene-activity profile. Previous transcriptome profiling efforts concentrated either on MCs alone (eg, stimulated vs baseline[12-14] and MCs cultured from different sources[15,16] or at different times of development[17]) or on comparisons between MCs and other leukocytes (either experimentally[18] or in silico[19]). Although these studies provided valuable insights into the transcriptional landscape of MCs, they did not allow direct comparisons with nonimmune cells and were limited by the MC model(s) employed.

In a natural setting, MCs develop exclusively in peripheral tissues like skin, gut, and lung. For research purposes, however, MCs are typically first expanded in culture, though it is currently unknown how well cultured MCs are representative of their in vivo counterparts.

Here, we used cap analysis of gene expression (CAGE) technology,[20] mapping transcription start sites (TSSs) and their relative expression levels, to obtain a comprehensive view of the human MC transcriptome. The major improvements are as follows: (1) we use skin MCs immediately ex vivo (and compare them directly with cultured skin-derived MCs); (2) transcriptome profiling is based on a quantitative technique combining cap trapping with high-throughput next-generation sequencing; (3) MCs are embedded in the Functional Annotation of Mammalian Genome 5 (FANTOM5) project, which provides transcriptome data on a large collection of 975 samples,[21] so that gene activity in MCs can be put immediately

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This article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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Table 1. Expression pattern of genes encoding well-defined MC markers

	MC ex vivo*			MC cultured†			Cultured + stimulated‡				
Gene	D2	D3	D4	D1	D8	D5	D1	D8	D5	(Next) Primary sample§	Mean all‖
KIT	3 272	3 633	3268	4 426	2906	3 125	2094	1 261	2195	548 (melanocyte)	16
IL1RL1	656	1 156	418	487	340	423	1631	488	1073	423 (lung)	8
FCER1A	811	657	1087	2 699	1774	2 405	1211	1 670	2983	3272 (immature LC)	19
MS4A2	1 163	1 717	1284	2 173	1109	2 827	1580	533	1759	60 (basophil)	1
ENPP3	312	454	273	890	700	920	3209	1 116	1084	157 (basophil)	3
HDC	994	1 091	864	1 516	772	925	496	400	707	255 (basophil)	5
TPSAB1-TPSB2	2 536	1 332	2650	671	250	991	0	57	492	76 (stimulated MΦ)	0.1
TPSD1	552	783	558	317	972	1 585	40	518	925	5 (gallbladder)	0.2
CMA1	1 841	1 919	1240	631	119	1 210	502	203	624	8 (left atrium)	0.2
CPA3	5 057	6 001	2824	7 089	7295	7 883	7305	5 884	4386	239 ($CD34^+$ diff. ery.)	4
CTSG	10 520	12 133	6902	14 981	7274	16 961	7000	10 752	9271	1278 ($CD34^+$ BM)	37
HPGDS	1 245	1 220	1220	4 097	3088	3 545	3489	2 587	3182	333 (immature LC)	5
LTC4S	199	270	226	1 830	3261	1 684	81	1 478	329	194 (immature LC)	7

BM, bone marrow; $CD34^+$ diff. ery. = $CD34^+$ cells differentiated along the erythroid pathway; D, donor; LC, Langerhans cell; MΦ, macrophage.
*Donor 2/donor 3/donor 4 (directly upon isolation from skin).
†Donor 1/donor 8/donor 5 (MCs expanded in culture for 4-5 weeks).
‡Donor 1 stimulated for 2.5 hours/donor 8 stimulated for 5 hours/donor 5 stimulated for 16 hours.
§Nontransformed cell/tissue sample with the (second) highest expression of the gene.
‖Mean expression of all FANTOM5 non-MC samples. The values are given in (rounded) tags per million tags.

into perspective; and (4) we provide additional information on preferential promoter usage and associated motif activity. Our study reveals that a multitude of nonannotated genes are particularly active in the lineage, several of which are only detectable in MCs ex vivo. Collectively, these findings underline that our current knowledge of human MCs is fairly limited. This paper can serve as a catalyst to spur further research into the functional programs of MCs and the specific roles these cells assume in health and disease in humans. This work is part of the FANTOM5 project. Data downloads, genomic tools, and copublished manuscripts are summarized online at http://fantom.gsc.riken.jp/5/.

Methods

Skin MC purification and culture

MC purification was performed as described previously.[22,23] The skin was obtained from cosmetic breast-reduction surgeries with informed consent of the patients. All experiments were conducted according to the Declaration of Helsinki Principles and approved by the ethics committee of the Charité Universitätsmedizin Berlin.

Briefly, skin was cut into strips and treated with dispase (Becton Dickinson, Heidelberg, Germany) at 4°C overnight. After removal of the epidermis, the dermis was chopped into small pieces and digested with collagenase (Worthington, Lakewood, NJ), hyaluronidase (Sigma, Deisenhofen, Germany), and DNase I (Roche, Basel, Switzerland) for 1 hour at 37°C. After 3 steps of filtration, the remaining tissue was subjected to a second digestion step. MC purification from the dispersates was achieved by selection with anti–human c-Kit microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an automated magnetic-activated cell sorting separation device. Viability (trypan-blue exclusion) and purity (acidic toluidine-blue staining) exceeded 99%. A total of 0.8 to 1.1×10^7 cells (from 1 donor) were immediately lysed in TRIzol and further processed for HeliScopeCAGE. MCs from 3 donors were used for the ex vivo analysis (donors 2, 3, and 4). To obtain the expanded samples, MCs were cultured for 4 to 5 weeks (donors 1, 5, and 8), as described previously.[24] Stimulation was achieved by FcεRI crosslinking with AER-37 at 2 μg/mL (eBioscience, San Diego, CA). Only samples showing >60% of activation (degranulation; *CD107a* upregulation) were included as stimulated samples in the deepCAGE analysis.

CAGE data, clustering, and gene assignment

The MC CAGE data and the entire FANTOM5 data set have been submitted to the DNA Data Bank of Japan database under accession numbers DRA000991 (samples from donors 1-4) and DRA001026 (samples from donors 5 and 8). Additional supplementary processed data files are available online at http://fantom.gsc.riken.jp/5/suppl/Motakis_et_al_2013. The details of the CAGE library generation and clustering are explained in the FANTOM5 main manuscript.[21] Briefly, ~4 million CAGE tags for each library were aligned to the genome (Hg19), neighboring tags were grouped into clusters, and decomposition-based peak identification (DPI) was applied to decompose clusters into independently regulated subregions (DPI peaks). The DPI peaks were annotated based on known transcript 5′ ends within 500 bases and summarized into regions (supplemental Methods, available on the Blood Web site).

The methods for RNA extraction, bioinformatics analysis, MC treatments, flow cytometry, histamine measurement, reverse-transcription quantitative polymerase chain reaction, and enzyme-linked immunosorbent assay are described in supplemental Methods.

Results

CAGE expression profiles of known MC marker genes

From the initial 184 827 FANTOM5 regions,[21] 15 643 annotated genes and 17 872 unannotated transcribed regions (33 515 in total) were expressed by MCs (supplemental Methods). Using this data set, we first focused on well-defined signature genes of the MC lineage, which were highly expressed by MCs and otherwise detected in a limited spectrum of cell types (Table 1). The most specific lineage markers were the MC-specific proteases. The profile was largely consistent with the expectation, although it has never been demonstrated for so many samples in direct comparison. There was no expression of lineage markers of other skin cells in the MC samples (*THY1* for fibroblasts, *KDR*/*CDH5* for endothelial cells, *KRT10*/*IVL* for keratinocytes, *TYR* for melanocytes, and *CD1A*/*CD207* for Langerhans cells), demonstrating an exceptional degree of MC purity.

Reproducibility among samples was statistically assessed by principal component and correlation analysis, further highlighting

Table 2. Genes with an MC-restricted expression pattern newly identified by FANTOM5

	MC ex vivo*			MC cultured†			Cultured + stimulated‡				
Gene	D2	D3	D4	D1	D8	D5	D1	D8	D5	(Next) Primary sample§	Mean all‖
MRGPRX2	1059	938	949	4	32	220	2	21	146	4 (fetal skin)	0.1
RGS13	942	1090	902	385	747	604	620	472	1636	89 (appendix)	2
C1orf150	1231	822	1059	1680	482	1256	6440	1949	2779	313 (basophil)	5
SIGLEC6	673	518	1190	1887	1634	1614	360	1166	1422	265 (placenta)	4
ERVFRD-1	65	89	75	734	176	315	1745	463	916	355 (retinal pigment epithelial cell), 132 (placenta)	2
SVOPL	31	52	40	173	105	217	104	93	113	18 ($CD34^+$ HPC)	0.6
C20orf118	46	80	69	100	27	172	19	22	107	46 (small intestine)	0.4
VWA5A	1400	1180	1308	1646	1469	1908	453	749	2079	257 (amniotic membrane)	14

Gene ranking by fold change = mean (MCs)*,†,‡ / mean (all)‖.
D, donor; HPC, hematopoietic progenitor cell.
*Donor 2/donor 3/donor 4 (directly upon isolation from skin).
†Donor 1/donor 8/donor 5 (MCs expanded in culture for 4-5 weeks).
‡Donor 1 stimulated for 2.5 hours/donor 8 stimulated for 5 hours/donor 5 stimulated for 16 hours.
§Nontransformed cell/tissue sample with (second) highest expression of the gene.
‖Mean expression of all non-MC FANTOM5 samples. The values are given in (rounded) tags per million tags; if <1, 1 decimal place is given.

the excellent quality of the data set, supported by an estimated biological coefficient of variation of 0.3846 for the MC samples.[25]

Identification of novel genes exclusively or preferentially active in MCs

The simultaneous availability of genome-wide transcriptome data for a large collection of samples allowed the search for additional genes with MC-restricted activity. The genes depicted in Table 2 had fold change = mean (MCs) / mean (all non-MC FANTOM5 samples) > 100, representing novel bona fide MC markers. The (putative) functions of the genes were searched in public databases (http://www.proteinatlas.org; http://www.genecards.org) if no specific references are given. Many genes identified herein have no annotated function so far.

c1orf150 was exclusively active in MCs and basophils, whereas expression of *C20orf118* was confined to MCs alone. The function of both genes is unknown.

ERVFRD-1 (*HERV-FRD*) originates from an ancient infection by retroviruses and can induce cell-cell fusion, a phenomenon found in placenta.[26] Its preferential expression in MCs raises the possibility of fusogenic capacity, as described for selected cell types.[27]

MRGPRX2 was exclusively active in MCs ex vivo and exhibited the highest fold change among candidates. It mediates MC degranulation by nonimmunologic secretagogues[28] and binds cathelicidin.[29] Thus, *MRGPRX2* may play important roles in skin MC activation.

Though not anticipated to be MC specific, *RGS13* has well-understood functions in MCs, operating as a negative regulator of MC signaling.[30]

SIGLEC6 is generally viewed as placenta specific[31] but was found here to be substantially more active in MCs than in placenta.

SVOPL has no known function but is related to the synaptic vesicle protein *SVOP*.

VWA5A is functionally undefined but ranks in the top position among genes influencing longevity.[32]

MCs are excellently suited to delineate the biological significance of these poorly defined genes in future studies.

In addition, we identified a substantial host of genes with highest activity in MCs and enrichment by at least 10-fold compared with the mean of all 893 FANTOM5 samples (supplemental Table 2). Although several genes are associated with MC biology (such as granule biosynthesis/function[33,34]), the function of others is currently ignored. This indicates that our view of MCs is largely incomplete.

FANTOM5 profiling data are excellently suited to stimulate research into new functional programs of MCs: BMPR1A as a modulator of MC function

Of the genes preferentially expressed by MCs, *BMPR1* appeared attractive for further analysis because of its well-documented role in hematopoietic stem cells (HSCs),[35] with which MCs share common features (see below).

We initially verified that skin cells produced *BMPR* ligands (*BMP2/4*), so that MCs were exposed to these mediators in their natural surroundings. Because *BMPR* activation stimulates lineage-specific transcription factors in HSCs,[36] we tested the impact of *BMPR* stimulation on the expression of markers and transcription factors (TFs) of the lineage.[37] *FcεRIα* and *GATA-1* transcripts were selectively increased by *BMP* signaling (Figure 1A), probably through direct activation of lineage-specific TFs by Smads.[37]

In addition, *BMP4* signaling increased the sensitivity to FcεRI aggregation (Figure 1B). After stimulation, MCs become refractory to a second stimulus, but *BMP4* assisted in MC recovery from refractoriness, increasing responsiveness to subsequent FcεRI crosslinking after the first stimulus (Figure 1C).

Ligation of *BMPR* also prolonged MC survival, and this effect required low concentrations of *BMP2* (Figure 1D; *SCF* is given as positive control). In summary, rational use of the FANTOM5 data sets allowed us to uncover *BMPR1A* as a new functional receptor of human MCs that crosstalks with the FcεRI pathway.

Genes with unexpectedly weak activity in MCs

Next, we focused on genes commonly investigated in MCs. There is controversy as to whether MCs serve as antigen-presenting cells.[38] Here, MCs were found to express negligible levels of the different MHCII members (Table 3). Additionally, ex vivo MCs expressed virtually no chemokine receptors. The inconsistency with previous reports[39] may be a result of the MC subset employed, because in vitro–expanded MCs showed increased expression of several receptors (Table 3).

Finally, the expression pattern of *TLR*s, by which MCs supposedly contribute to immune surveillance, was re-examined.[8,9] Surprisingly, skin MCs expressed minute amounts of *TLR* genes if compared directly with other immune cells (Table 3). Although *TLR*s may activate MCs in selected microenvironments, the data conclusively explain our previous inability to detect *TLR2* and *TLR4* in MCs ex vivo

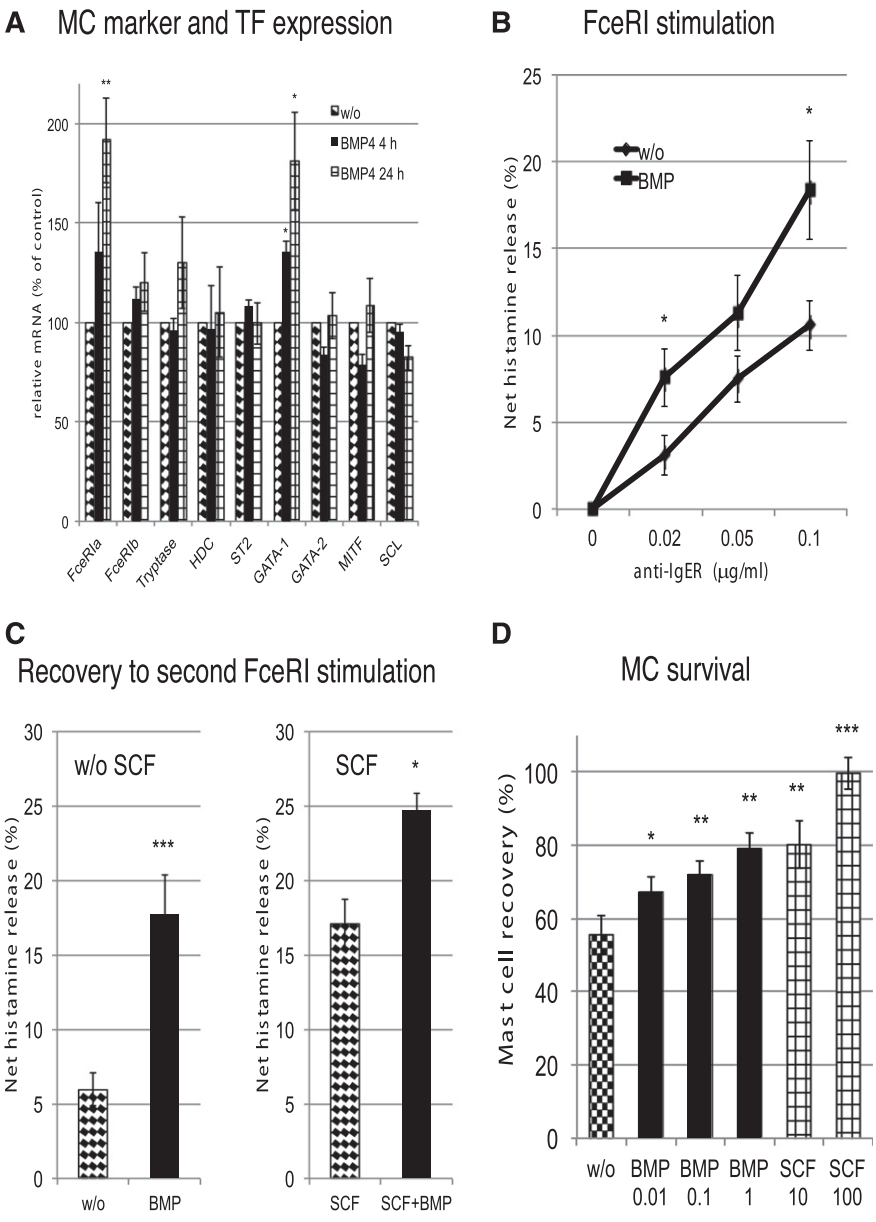


Figure 1. *BMPR1* regulates MC effector functions and increases MC survival. (A) MCs were stimulated with *BMP4* at 20 ng/mL for the times indicated and the expression of selected MC marker genes quantified by reverse-transcription quantitative polymerase chain reaction and normalized to β-actin. Data are shown in relation to cells kept in the absence of bone morphogenetic protein (BMP) for the same times. (B) Cultured MCs (last addition of *SCF* 5-7 days earlier) were washed and replated in fresh media with or without *BMP4* (20 ng/mL) for 24 hours, after which time histamine release was elicited by FcεRI crosslinking with different concentrations of AER-37. IgER, immunoglobulin E receptor. (C) Cultured MCs were stimulated by AER-37 (0.1 μg/mL) for 2 hours, washed, and replated in fresh media with or without *BMP4* (20 ng/mL) ± stem cell factor (SCF) (100 ng/mL) for 48 hours. After this time, cells were subjected to a second round of stimulation by AER-37. MCs are largely refractory to a second stimulation for prolonged times, but BMP accelerates this recovery. Although SCF alone also accelerates recovery, the effects from *BMP* and *SCF* are additive. (D) MCs after isolation were cultured in the presence or absence of the indicated factors for 10 days, and recovery of viable cells was assessed. The numbers indicate growth factor concentrations in ng/mL. All data in this figure are the mean ± standard error of the mean from 5-7 independent assays. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

or to activate them by specific ligands (M.B. and S.G., unpublished data).

MCs change their transcriptome in in vitro surroundings

We found significant changes in the transcriptome of cultured vis-à-vis ex vivo MCs. Out of 33 515 expressed regions, 5826 differentially expressed targets were identified (1802 RefSeq annotated; Figure 2A). A total of 85.5% of all differentially expressed (and 70.1% of the annotated) targets were downregulated, implying that various transcripts, which accumulate in MCs in vivo, are not maintained outside of the tissue. Functional classification by Gene Ontology revealed that cultured MCs were enriched in selective enzymes (oxidoreductases and transferases), indicating metabolic shifts occurring simultaneously with cell-cycle progression[24] (Figure 2B). Accordingly, genes involved in energy metabolism and biosynthetic pathways (respiratory chain, cholesterol/nucleotide biosynthesis, pentose phosphate shunt; supplemental Table 3) were overrepresented, and so were cell-cycle regulators. In contrast, TF genes strongly dominated in the ex vivo samples (Figure 2A-B). Because TFs determine the expression of gene networks, it is reasonable that their expression coordinates the activity of multiple downstream genes, explaining the greater diversity in the gene repertoire active in MCs in the tissue.

Strikingly, several genes of other blood lineages were induced in cultured MCs (multilineage markers along with the platelet gene *ITGA2B* and the erythroid regulator *GFI1B*[40]), suggesting that MCs may undergo partial de- or transdifferentiation in vitro. This was further supported by the de novo appearance of genes from other tissues, in particular *UTS2* (expressed by cultured MCs and parts of the nervous system) and *MAOA* (highest in cultured MCs and adipocytes).

Changes in the MC transcriptome following activation

The most commonly studied (and clinically relevant) activation pathway for MCs is FcεRI aggregation. Here, we found 260 upregulated and 84 downregulated targets (supplemental Table 1; Figure 2C). Signaling components and carrier proteins specifically

Table 3. Transcripts of important immune receptors underrepresented in MCs

Gene	MC ex vivo*			MC cultured†			Cultured + stimulated‡			First primary sample§	Mean all‖
	D2	D3	D4	D1	D8	D5	D1	D8	D5		
Receptors involved in antigen presentation											
HLA-DRA	45	30	25	0.1	0	0	0	0	0.1	26 120 (migratory LC)	798
HLA-DPA1	28	20	16	8.8	22	18	10	27	16	13 278 (migratory LC)	408
HLA-DPB1	16	13	17	2	4.6	7.5	0.9	13	4.2	13 319 (migratory LC)	239
HLA-DQA1	0.5	1	6.2	0	0.4	0	0	0	0	17 246 (migratory LC)	143
HLA-DQA2	26	1.4	4	0	0	0	0	0	0.1	3 963 (migratory LC)	38
Chemokine receptors											
CCR1	0.2	0	0	0	0	0	0	0.7	0	705 (stim. monocyte)	18
CCR2	0	0.4	0.2	0	1.2	0.1	0.3	0.7	0	178 (monocyte)	5.3
CCR3	0	0	0	0	0	0	0	0	0	190 (whole blood)	2.3
CCR4	2.6	0.5	1.4	21	6.6	80	1.3	3.5	27	1 384 (memory Treg)	19
CCR5	0	0	0.2	0.4	2.5	0.7	0	0.7	0.8	199 (stim. monocyte)	7.8
CCRL2	0	0	0.2	0.4	0.2	1.2	3.4	0.7	1	470 (stim. monocyte)	8
CXCR3	0.7	0.4	0.5	4.2	0.2	5.6	4.1	7.7	40	359 (plasmacytoid DC)	5.9
IL8RA/CXCR1	0	0	0	0	0	0	0	0	0	1 216 (whole blood)	13
Pattern recognition receptors											
TLR1	0.9	1.2	1.2	0.3	1.9	0.4	0	0.7	2.4	434 (neutrophil)	12
TLR2	4.7	3.9	2.3	0	1	0	0	0	0.4	896 (eosinophil)	29
TLR3	1.4	0.7	0.5	3.9	2.5	3.4	0.3	0.7	3.6	114 (placenta)	6.5
TLR4	3.5	11	5.4	75	22	44	28	16	28	1 085 (stim. monocyte)	55
TLR7	0.9	0.4	0.8	0.1	0.2	0	0	0	1.5	343 (plasmacytoid DC)	7.7
TLR9	0	0	0	0	0	0	0	0	0	162 (plasmacytoid DC)	1.9

DC, dendritic cell; LC, Langerhans cell; stim., stimulated; Treg, regulatory T cell.
*Donor 2/donor 3/donor 4 (directly upon isolation from skin).
†Donor 1/donor 8/donor 5 (MCs expanded in culture for 4-5 weeks).
‡Donor 1 stimulated for 2.5 hours/donor 8 stimulated for 5 hours/donor 5 stimulated for 16 hours.
§Nontransformed cell/tissue sample with (second) highest expression of the gene.
‖Mean expression of all non-MC FANTOM5 samples. The values are given in (rounded) tags per million tags; if <10, 1 decimal place is given.

decreased, whereas oxidoreductases increased upon stimulation (Figure 2D). These changes likely contribute to desensitization (ie, unresponsiveness to a second stimulus) and survival, because a striking number of genes were associated with apoptosis and cell-cycle progression (with proapoptotic genes being coordinately downregulated and antiapoptotic genes upregulated). Therefore, stimulated cells seem to protect themselves from apoptosis but also halt the cell cycle to fully recover from the incisive event of stimulation (supplemental Table 4).

On the other hand, soluble mediators were the most potently induced genes following FcεRI aggregation (eg, *CCL1* and IL-5; supplemental Table 4). Receptors responsible for the communication with T cells were likewise upregulated (supplemental Figure 2A shows *OX40L* and *TNFRSF9/ILA* at the protein level). Apart from several exceptions, there was good overall agreement with previous profiling efforts, implying that this pathway is fairly consistent among MC subsets.[12-16]

IL-31 was appreciably expressed in only one of the stimulated MC samples, suggesting particularly tight control. Testing MCs from 10 individuals, we found highly variable *IL-31* protein levels among donors (supplemental Figure 2B). This result adds to the importance of MCs in the pruritus network,[41] whereas inter-individual variability may contribute to diseases characterized by chronic itch.

In addition to the essentially expected pattern of soluble mediators and coreceptors, unexpected genes were likewise induced by stimulation. The most striking examples were organ-specific genes, such as *XIRP1* (heart[42]) and *AQP2* (kidney[43]).

In summary, activation of MCs induces not only expected transcripts but also selected markers of unrelated tissues.

Hierarchical clustering and heatmap analysis

We employed hierarchical clustering to identify patterns of genes coexpressed with significant TFs (see Babina et al[36] and references therein) and with the newly discovered marker *MRGPRX2*.

Gata1/Gata2 cluster. *GATA1*/*GATA2* belong to the large cluster shown in Figure 3A. A variety of genes with MC-selective expression that tended to be upregulated in culture were found in this group (color distribution in Figure 3A; red predominates in the MC samples on the left). In addition to *GATA1*/*GATA2*, this cluster contains additional TF genes (eg, *HOXB2*, *LYL1*, and *SOX13*), and numerous genes of this cluster are validated targets of these TFs (eg, *GATA2*/*KIT*[44] and *GATA1*/*HOXB2*[45]). Further TF-gene pairings may be uncovered based on this analysis.

MITF cluster. In contrast to *GATA1*/*GATA2*, the MITF cluster contained virtually no genes with MC-restricted expression (Figure 3B). Genes found here were active not only in monocytes/MCs but also in melanocytes (*IDH1*, *NQO1*, and *TSPAN4*), although melanocytes were not included in the cluster analysis, providing independent validation of the clustering procedure. The osteoclast/melanoblast marker *GPNMB*, a well-defined *MITF*-target,[46] was captured by our clustering analysis.

MRGPRX2 cluster. The *MRGPRX2* cluster turned out to be particularly informative, because it encompassed newly identified genes exclusively or particularly active in MCs as well as several TFs (*TAL1*, *PBX1*, *MEIS2*, and *ERG*; Figure 3C).

Indeed, *PBX1* and *MEIS2* are known to cooperate, regulating a variety of genes, and they can also form complexes with *KLF4*[47] (a TF with particularly high activity in MCs, as discovered here). *PBX1* is involved in development and Mullerian-duct formation,[48] and

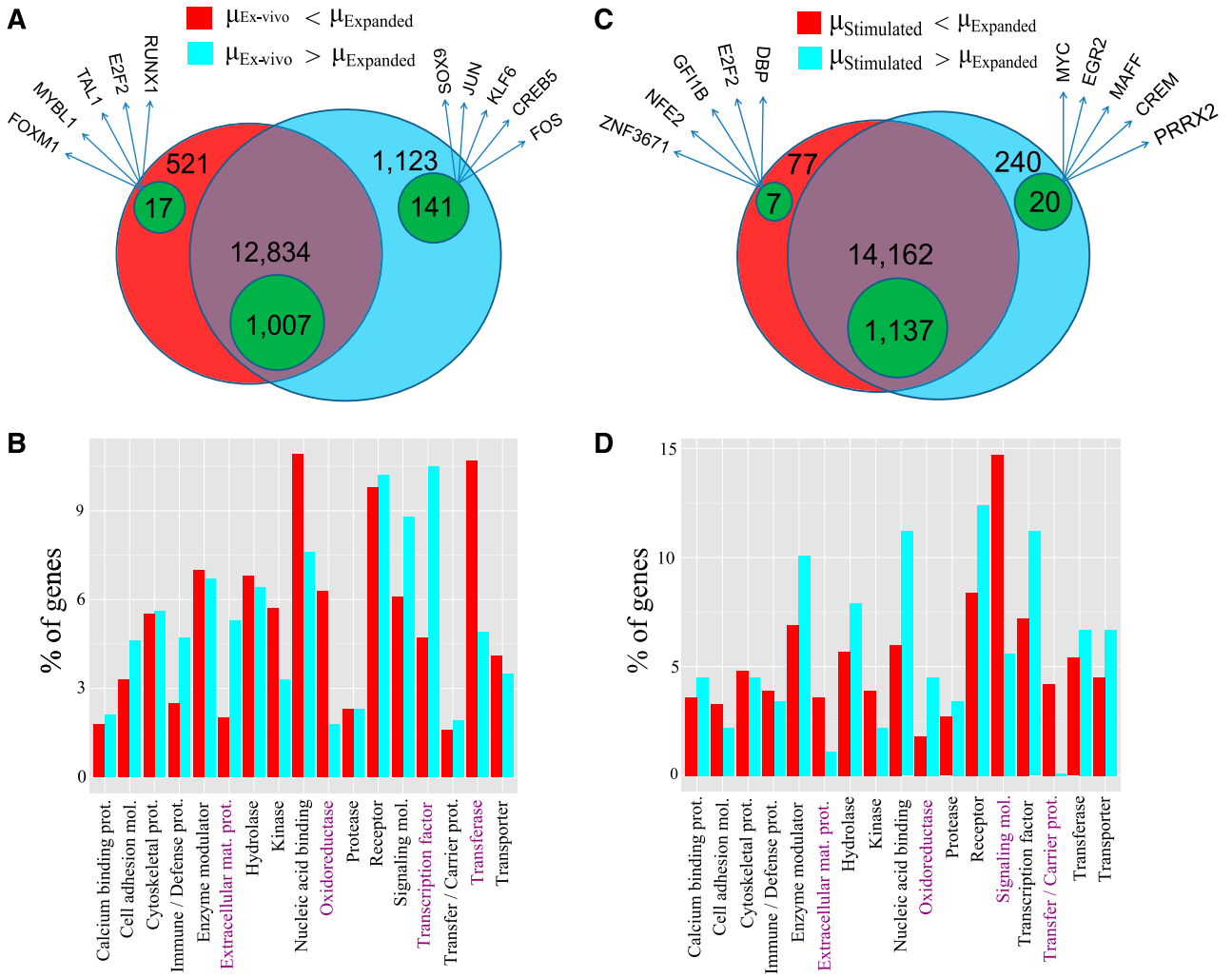


Figure 2. MCs change their transcriptome in in vitro surroundings and upon FcεRI crosslinking. Comparison of the different MC samples by Venn diagrams (A,C) and protein class Gene Ontology analysis (B,D). (A) Venn diagram with the number of RefSeq genes expressed at lower level in ex vivo MCs (red/green-within-red areas) and at higher level in ex vivo MCs (cyan/green-within-cyan areas) as well as those nondifferentially regulated (purple/green-within-purple areas). The genes are specified in supplemental Table 3. The green areas denote the number of TF genes in each group. Five indicative TFs from the differentially regulated lists are also given. (B) Significant protein class terms ($\alpha = 5\%$) of the differentially regulated groups. Pronounced differences are highlighted in purple. (C) Venn diagram with the number of genes expressed at lower/higher level in stimulated as opposed to expanded MCs in analogy to panel A. The genes are specified in supplemental Table 4. (D) Significant protein class terms in stimulated vs expanded MCs in analogy with panel B.

AMHR2 expression (receptor for anti–Mullerian hormone and part of the cluster) was unexpectedly highest in MCs. These TFs may therefore well contribute to the MC-selective gene signature and warrant further exploration. In keeping with the much higher expression of *MRGPRX2* in ex vivo MCs, the majority of the genes in this cluster were preferentially active in ex vivo MCs (Figure 3C; red predominates among the MC samples on the right).

More detailed, higher-resolution versions of these 3 clusters are provided in supplemental Figures 3-5.

MC-specific promoters and motif activity. The above analyses concentrated on gene-expression patterns in MCs vs non-MCs to identify gene-activity programs that distinguish MCs from other cells. Contrary to microarrays, however, CAGE not only quantifies gene expression but also precisely maps TSSs, thereby identifying the specific promoter(s) used (included in supplemental Tables 3 and 4). In addition to genes overexpressed by MCs, we found selective promoters particularly active in MCs also in more broadly expressed genes. Supplemental Table 5 gives promoters hyperactive in the lineage (expression 50-fold higher in MCs vs mean of non-MCs) and contrasts them against the overall expression of the respective genes; at the top of these lists are examples (*LHX3*, *STX3*, and *C11orf49* for ex vivo MCs) for which MC selectivity of a particular promoter surpassed MC selectivity at the gene level by more than 1000-fold.

We also found that promoter preference can change in dependence of the microenvironment. *EXOC6B* is an interesting example, because it forms part of the exocytosis machinery (substantially more active in cultured vis-à-vis ex vivo MCs[24]). *EXOC6B* was highly expressed by MCs, with no change between subsets at the gene level but striking difference at the promoter level (supplemental Figure 6), perhaps giving rise to transcripts with altered translational activity or stability. Similar promoter swaps were detected in several other genes upon culture (supplemental Table 6), whereas no such genes could be identified upon stimulation.

The mapping of TSSs in MC transcripts allowed identification of motifs preferentially enriched in active MC promoters. By using the previously published motif activity response analysis approach,[49] which fits the CAGE expression profiles to computationally predicted regulatory sites for TFs, we found important regulators that explain the CAGE data variation and predict the regulatory role and

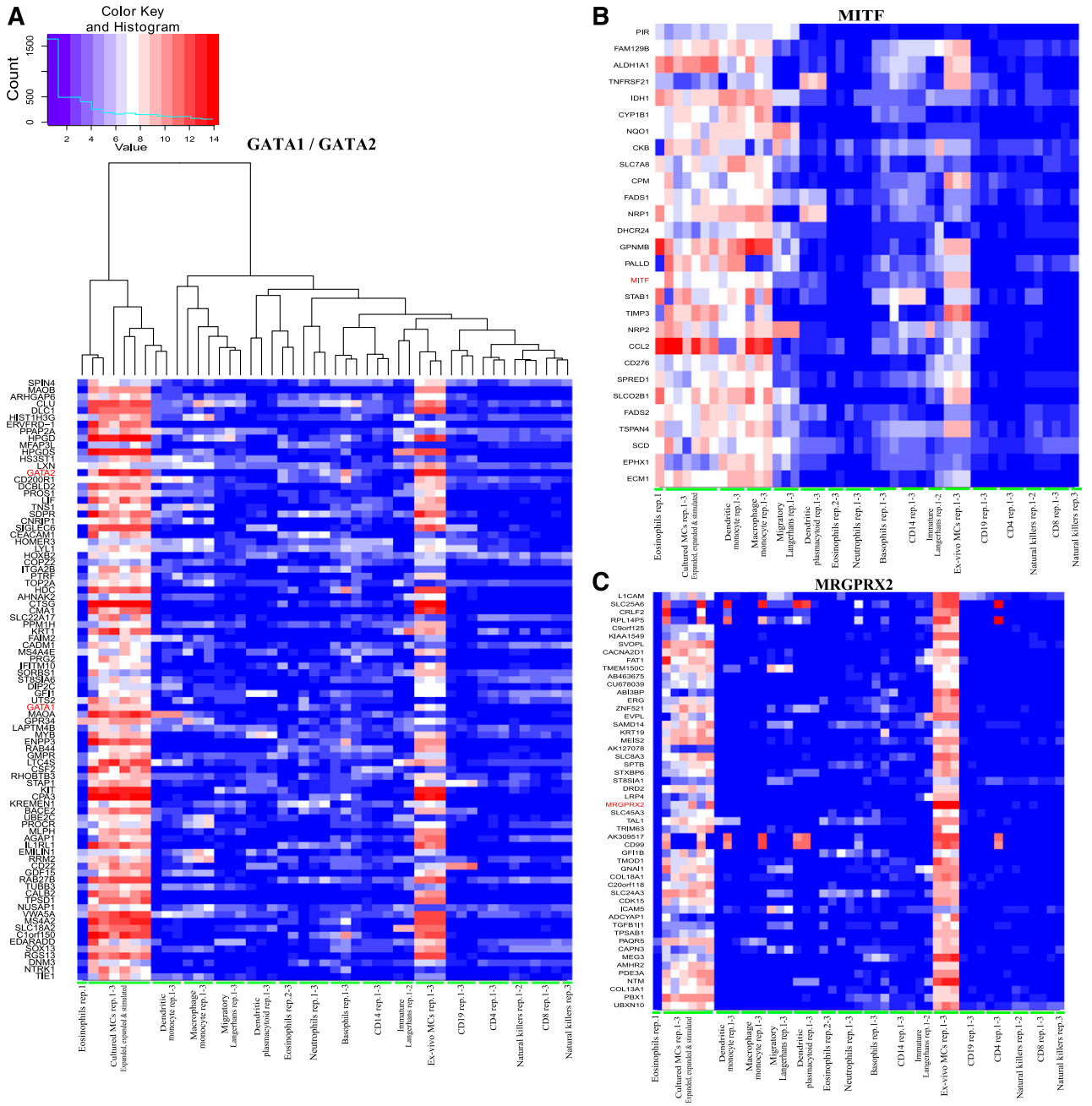


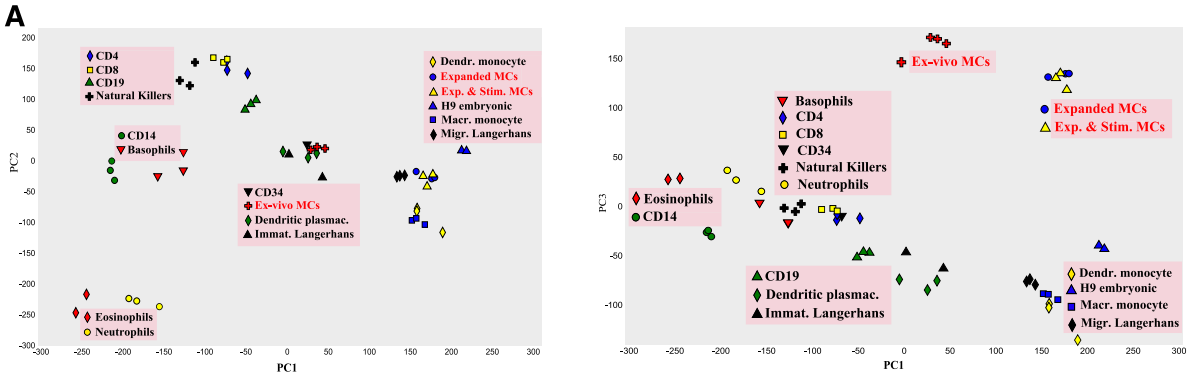
Figure 3. Clusters of MC-relevant genes. The hierarchical clustering of 49 FANTOM5 hematopoietic lineage samples using all data and following extraction of subclusters for (A) *GATA1*/*GATA2* (tree shown in panel A is that same as in panels B and C), (B) *MITF*, and (C) *MRGPRX2*. The algorithm is described in the supplemental Methods. More detailed, higher-resolution versions are provided as supplemental Figures 3-5.

importance of each regulator (supplemental Methods). Supplemental Table 7 ranks these motifs according to their activity.[49] Interestingly, the motif most active in ex vivo MCs turned out to be *PATZ1*, which so far has been associated with spermatogenesis and testis development.[50] In fact, *PATZ1* expression was higher in MCs than in most other blood cells, and MCs unexpectedly expressed several testicular genes. Another interesting motif was *FOXJ1*, with fundamental roles in developmental processes,[51,52] whereas nothing is known about its role in mastopoiesis. The *FOXJ1* target gene calpastatin[51] correlated with *FOXJ1* expression in MCs. The preferential expression and activity of several FOX family TFs (*FOXI2* and *FOXJ1*) suggests that they may have fundamental yet hitherto-overlooked roles in the MC lineage.

Although multiple regulatory levels exist between the transcription of a TF gene and activity of the respective protein in the nucleus, various TFs with particularly active motifs were also highly expressed by MCs (eg, *KLF4*, *CREB1*, and *ELF1*), indicating that at least one part of their activity stems from transcriptional regulation.

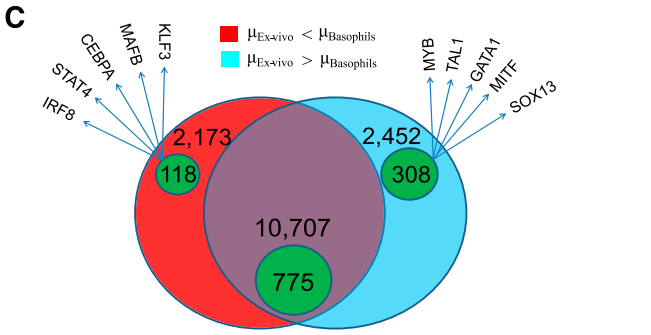
MCs in the hematopoietic network

There is consensus that human MCs are derived from HSCs, but their relationship with other bone-marrow derived cells is barely defined, and MCs were not included in efforts examining the global architecture of hematopoiesis.[53] Thus, the FANTOM5 data set provides the unique opportunity to position MCs relative to other lineages. To



B

	Ex-Vivo MCs	Cultured MCs	Basophils	Eosinophils	Neutrophils	Monocyte	MΦ	Dendr. Monocyte	Dendr. Plasmacytoid	Immat. Langerhans	Migrat. Langerhans	CD4+	CD8+	Natural Killers	CD19+	H9 embr.	CD34+ HSCs
Ex-vivo MCs	0.967	0.839	0.693	0.538	0.537	0.586	0.705	0.692	0.712	0.718	0.729	0.692	0.686	0.687	0.695	0.668	0.736
Cultured MCs	0.839	0.968	0.646	0.502	0.526	0.517	0.805	0.781	0.737	0.691	0.788	0.682	0.671	0.656	0.685	0.763	0.723



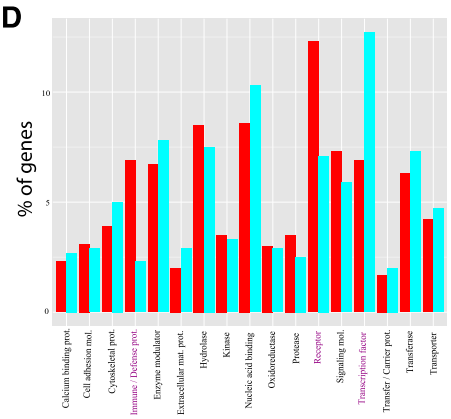


Figure 4. MCs in the hematopoietic network. (A) Principal component analysis of 49 blood samples of the FANTOM5 data set (H9 embryonic stem cells are included for further comparison) as described in supplemental Methods. The first 3 principal components (PCs) explain 82.3% of the total variance (see supplemental Methods). (B) Means of all pairwise Pearson correlation coefficients for each sample pair as described in supplemental Methods. The mean coefficients of MCs vs other blood samples that are >0.7 are depicted in red. (C) Venn diagram with the number of genes expressed at higher level (red/green-within-red areas), and at lower level in basophils vs ex vivo MCs (cyan/green-within-cyan areas) and nondifferentially expressed RefSeq genes (purple/green-within-purple areas). The genes are specified in supplemental Table 8. The green areas denote the number of TF genes in each group. Five indicative TFs are given for each cell. (D) Significant protein class terms (α = 5%) of the differentially expressed groups.

this end, principal component analysis was applied to 49 samples for the purpose of deriving components representing large fractions of data variability. PC1-3 cumulatively explains >80% of the variance (supplemental Methods). PC1, the dominant component, segregated the samples mainly by source (blood, tissue [ie, bone marrow/skin], or culture), whereas PC2 segregated the samples by cell type (lymphoid, myeloid long-lived/plastic, or myeloid short-lived/end stage) (Figure 4A left). Accordingly, ex vivo MCs clustered with Langerhans cells and HSCs (cells from skin or immediately after exit from bone marrow), whereas cultured MCs clustered with other cultured cells in PC1. In PC2, MCs were found to belong to the myeloid/plastic subset. Of particular interest, however, was the separation of MCs from all other samples in PC3, potentially explaining the uniqueness of MCs (Figure 4A right). No other cell showed a separation of this kind. In keeping with principal component analysis, MCs had likewise no close neighbor by Pearson correlation (Figure 4B). The best concordance for ex vivo MCs was again found with HSCs (perhaps as a result of similar TF patterns[54]) and Langerhans cells. MCs showed a more-than-expected relation with all lymphocytes (Figure 4A-B), which fits previous observations.[55] Therefore, MCs may be tentatively positioned between myeloid and lymphoid cells (Figure 4A), which would favor an early separation of the MC lineage.

The similarity between MCs and basophils seemed fairly limited. Because developmental relationships between the cells in humans have remained obscure, we analyzed this relationship in greater detail. Of a total of 15 332 annotated (non-TF) genes, 4625 were differentially expressed, with slightly more genes being overrepresented in MCs (Figure 4C). The almost equal distribution at the level of non-TF genes was skewed in favor of MCs when TF genes were regarded (72.3% higher in MCs, 27.7% higher in basophils), further highlighting that greater TF diversity is a hallmark of MCs.

A closer inspection of the differential genes (supplemental Table 8) revealed that MCs overexpressed not only typical lineage markers but also genes shared between MCs and other cell/tissue subsets, including the brain (eg, *CALB2*, *DIP2C*, *EPB41L1*, *KIAA1549*, *L1CAM*, and *NTM*). Basophils, on the other hand, expressed a gene set typically associated with immune/inflammatory responses (eg, *S100* family, *TREM*, and *HLA-DRA*). The most differential genes on comparison with MCs were expressed by other myelocytes, not just by basophils, putting basophils closer to other

blood cells (Figure 4A). In fact, barely any truly basophil-specific genes could be identified, in stark contrast with MCs (also underscored by the divergence in the number of specific promoters: 39 in basophils vs 542 in ex vivo MCs; supplemental Table 5).

Taken together, basophils represent typical myeloid cells dedicated to host defense and immune modulation (Figure 4D). Conversely, MCs express more genes exclusively active in the lineage or shared by cells/tissues not primarily dedicated to immune function. As a result, various genes are specifically overexpressed by MCs, but only few are specific for basophils. Overall, this speaks against a common dual precursor of MCs/basophils in humans, in contrast to what was found in the mouse.[56] In fact, mouse MCs have many characteristics of (human) basophils, including dependency on *IL-3* for their development.[11]

Discussion

FANTOM5 has generated the most comprehensive transcriptome data collection using sophisticated technical and bioinformatics tools. The first-time-ever inclusion of human MCs in a global expression atlas has unequivocally demonstrated the individual character of MCs and suggested their participation in as-yet-undiscovered physiological processes. The unique character of MCs is owed to several "private genes" expressed strongly by MCs but only weakly (or not at all) by other constituents of the human body. Both well-characterized MC markers (Table 1) and genes newly discovered as MC specific in this study (Table 2) fall into this category. Moreover, MCs express a plethora of other genes at the highest levels (supplemental Table 2) and are enriched in entire networks (eg, G-protein–coupled receptors, associated signaling intermediates/negative regulators and G-protein–regulated TFs as well as genes related to the *TGF* network).

In addition, MCs not only express a multitude of genes not associated with the lineage before but also use MC-specific promoters to express non–MC-specific genes. This finer level of resolution is only achievable with deep sequencing.

Although MCs are derived from HSCs, their distinctiveness is underlined by the absence of several transcripts, which normally accumulate in (innate) immune cells, by the absence of a near neighbor in the hematopoietic network and by the profound difference vis-à-vis basophils, despite their overlap regarding few genes not shared by other cells. Although basophils seem typical myelocytes dedicated to host defense and immune regulation, several overexpressed transcripts of MCs are actually signature genes of (unrelated) organs, especially brain (which may be explained by the similarity in their exocytosis machineries and the presence of neurotransmitter/neurotransmitter receptors in MCs) and reproductive tissues (epididymis, testis, ovary, and placenta [eg, *ADAM12*, *HERV-FRD*, *SIGLEC6*, *SPAG8*, *SPATA16*, and *WNK3*]). Considering the extremely limited expression of some of these genes, their activity in MCs is remarkable, and research into their implication in the lineage may identify unanticipated functions of both the cells and the genes.

The similarity with reproductive tissues, together with the relationship of MCs with HSCs and even embryonic stem cells (Figure 4A-B), implies that MCs may be equipped with a certain degree of "stemness" and that their plasticity goes beyond what is currently assumed.[38]

In fact, we show directly that MCs experience profound changes when exposed to an altered microenvironment (Figure 2A-B). Because human MC research relies almost exclusively on cultured MCs, gene-expression patterns and functional properties of the lineage may have been missed so far.

Understanding the molecular pathways effective in MCs is a prerequisite to modulate deregulated MC activities. The unique gene signature of MCs offers the possibility to specifically target these cells in therapeutic settings without serious side effects. Therefore, our study also offers an excellent basis for the identification of MC-specific targets for pharmacologic intervention.

In summary, FANTOM5 has created an outstanding resource for the MC community. The presented data sets will undoubtedly spur further research to decipher the programs that orchestrate lineage commitment, differentiation, maintenance, and functional spectra of this unique cell subset.

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Authorship

Contribution: A.R.R.F. and M.B. designed the study; S.G. isolated the MCs and performed most experiments; M.B. performed several experiments; Y.I. isolated RNA; E.M. performed all bioinformatics analysis of MCs; M.I. was responsible for data production; T.L. was responsible for tag mapping; M.d.H. helped with the motif activity analysis; H.K. managed the data handling; P.C., Y.H., and A.R.R.F. were responsible for FANTOM5 management and concept; M.B. analyzed and interpreted the data and wrote the manuscript; and S.G., E.M., T.Z., and A.R.R.F. helped with interpretation and the writing of the manuscript.

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