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Research Article

The transcription factor profile of human mast cells in comparison with monocytes and granulocytes

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Abstract. Expression profiles of mRNAs and proteins for various transcription factors were determined for human skin mast cells (sMCs), leukemic HMC-1 MCs, monocytes and granulocytes. By quantitative RT-PCR, sMCs expressed lower levels of c-fos, PU.1, C/EBPα, and C/EBPε than monocytes and granulocytes, but higher levels of MITF, SCL, GATA-1 and GATA-2. At the protein level, MITF, SCL, GATA-2, Elf-1 and c-fos were clearly detectable in sMCs. With the exception of c-fos, these proteins were absent or expressed only slightly in monocytes and granulocytes. The expression of NF-E2p45, GATA-1, PU.1, Ets-1, C/EBPα and C/EBPε was below the detection limit in sMCs, but detectable in other myelocytes. The high expression of SCL and GATA-2 in sMCs is reminiscent of stem cells. The absence of C/EBPε in sMCs, but strong expression in HMC-1, suggests it may impair MC maturation. In summary, mature human MCs can be characterized as C/EBPα^{low}, C/EBPε^{-}, PU.1^{low}, GATA-1^{low}, GATA-2^{+}, SCL^{+}, MITF^{high}.

Key words. Transcription factor; mast cell; monocyte; granulocyte; myelopoiesis.

Cellular differentiation is a highly complex process defined by fundamental changes in actively transcribed genes. These shifts within the transcriptome of a cell are accomplished, in part, by altered expression of those genes that are placed upstream in the hierarchical network and code for regulatory proteins, in particular transcription factors (TFs). Their system and interplay will define the realization of cell-specific transcriptional programs. Rather than the mere presence (or absence) of one single factor, the communication of different TFs likely specifies lineage commitment and the fate of a cell at a given time. Although ectopic overexpression of single factors may be sufficient to induce lineage switching, determination of a particular cell type under physiological conditions likely results from the cross-talk of limited amounts of lineage-restricted and ubiquitous factors which positively regulate genes specifically expressed by the cell, while concomitantly suppressing those of other lineages.

Mast cells (MCs) are specialized hematopoietic cells relevant not only to allergic reactions but also to innate immunity [1–4]. Unlike other blood cells, MCs complete their maturation exclusively within tissues. While the molecular events driving transcriptional programs in human MCs are unresolved, a series of experiments with rodent MCs provided an invaluable insight into the role of specific factors during mastopoiesis, with a dominant role played by the mi transcription factor (MITF) [5], important contributions from GATA-1 and -2 [6–9] and

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some additional involvement of SCL/tal-1 [10] and NF-E2 [11, 12]. Since rodent MCs differ in many regards from their human counterparts [1], we need to clarify whether or not these factors are relevant to human MCs and determine other factors that may participate as positive or negative regulators directing formation and maintenance of the lineage.

In comparison to MCs, more data are available on other hematopoietic cells with which MCs share a common origin, including monocytes, neutrophils, eosinophils, erythrocytes and megakaryocytes [reviewed in refs. 13–16]. For example, C/EBPα and C/EBPε have both been assigned to distinct stages of granulocytic differentiation [17, 18], while monocyte development seems to depend particulary on PU.1 expression [19–21], and eosinophils can be characterized by high C/EBPα, moderate GATA-1 levels and the absence of FOG-1 [14]. In contrast, megakaryocytic and erythroid developments require, among others, the timely coordinated function of GATA-1, FOG-1 and NF-E2 [15, 16].

Human MCs are derived from $CD34^+$, c-kit^+, $CD13^+$ progenitors in the peripheral blood [22]. The molecular mechanisms that regulate lineage restriction and terminal differentiation of these cells as they mature within tissues remain unknown. The recent development of techniques to purify MCs directly from human tissue has led to a better characterization of cell surface markers and cytokines in the lineage [23, 24]. Although the cellular material is highly limited, this approach has allowed us to address the as yet unexplored question of TF expression in primary human MCs in comparison to an immature MC line. Twenty candidate TFs with known functions in murine mastopoiesis, and/or in the development of other hematopoietic lineages, factors involved in MC activation and factors coordinating transcription of hematopoietically restricted genes were selected. To put these data into a broader context and establish a relation between MCs and other myelocytes by TF pattern, monocytes and granulocytes were also included in the analysis.

Materials and methods

Cell lines

The immature human MC line HMC-1 was kindly provided by Dr. Butterfield [25]; its subclone 5C6 used for the experiments was generated in our laboratory [26]. Cells were routinely cultured in basal Iscove's medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, streptomycin, penicillin (all from Seromed, Berlin, Germany) and 10 µM monothioglycerol (Sigma, Taufkirchen, Germany). Other cell lines were as follows: HL-60, U-937, THP-1, K562, KU812 and Jurkat (all from American Type Culture Collection, Rockville, Md.) They were maintained in RPMI 1640 medium/10% FCS, 4 mM L-glutamine, streptomycin and penicillin.

Purification of primary cells

Skin MCs were isolated from the dermis of human foreskin, exactly as described recently [27]. MC purity in these preparations typically exceeded 95% by acidic toluidine blue staining and by flow-cytometric evaluation of c-kit and FcεRIα expression. Dermal fibroblasts (used as control) were recovered from the negative fraction. Monocytes and granulocytes were isolated from heparinized whole blood drawn from healthy volunteers. Following dextran sedimentation and Histopaque (Sigma) density gradient centrifugation, granulocytes were recovered from the pellet and contaminating erythrocytes removed by hypotonic lysis (around 99% positive for CD11b). Monocytes were purified from the PBMC fraction by negative selection with magnetic beads (Dynal, Hamburg, Germany), according to the supplier's protocol. Monocytes obtained by this method were very uniform, showing a distinct peak on cell counting (Casy 1, cell counter and analyzer; Schärfe System, Reutlingen, Germany) and were over 95% positive for CD14 and CD11c. Peripheral blood lymphocytes (used as positive control) were gained from PBMCs as the non-adherent fraction after three rounds of adhesion to plastic culture dishes. All primary cells were lysed immediately after isolation for downstream applications.

Semiquantitative and quantitative RT-PCR assay

Total cellular RNA was isolated from the different cells using the RNeasy Total RNA Kit, digested with RNAse-free DNAse (Qiagen, Hilden, Germany), and quantitated using the RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). Total RNA (0.5 µg per 20-µl reaction volume) was reverse transcribed with a first-strand synthesis kit (Roche Applied Science, Mannheim, Germany), using random priming, as detailed by the manufacturer. Aliquots of the reaction product were mixed with specific primers and a PCR Master mix (Roche Applied Science) containing all components required for PCR amplifications. For semiquantitative PCR, cDNA was typically diluted around 1:300 (the precise dilution depended on signal intesities obtained for β-actin), but in the case of negative results, much higher cDNA concentrations (up to a 1:10 dilution) were used. Optimum conditions (annealing temperatures and cycle numbers) were determined in preliminary tests. In general, two cycle numbers were run in parallel in each experiment. PCR was performed under the following conditions: 94 °C/5 min (1 cycle); 94 °C/45 s, variable annealing temperatures (50–70 °C)/45 s, 72 °C/1 to 2 min (19–40 cycles); 72 °C/10 min (1 cycle).

Quantitative PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science)

using cDNA from the different cells (with a minimum of eight cell preparations per cell type), that corresponded to 40 ng total RNA/assay. Reactions were run in a 20-µL final volume, at 3–4 mM $MgCl_2$ and 0.4 µM of each primer (final concentration) with a precycling hold at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 20 s, specific annealing temperatures (56° to 65 °C) for 4–5 s and extension at 72 °C for 8 s. Amplification specificity was checked using melting curve and gel analysis. Results were analyzed with the LightCycler Software (Roche Applied Science) using the second derivative maximum method to set C_T. Quantification of cDNA samples was performed automatically with reference to the standard curve.

For standard curve preparation, specific fragments were produced from cDNA by PCR, electrophoresed, the corresponding bands excised from the gels, and purified by the High Pure PCR Product Purification kit (Roche Applied Science). DNA was quantitated with the PicoGreen dsDNA Quantitation kit (Molecular Probes). To obtain a more complex DNA system (corresponding to the complex nature of cDNA), fragments were mixed with 100 µg/ml sheared herring sperm DNA (Gibco BRL Life Technologies, Eggenstein, Germany). No specific products were amplified when herring sperm DNA was used alone. Standard curves were freshly prepared from the stock solution each time, starting from 2.5×10^6 copies per assay and tenfold dilutions thereof down to 2.5×10^1 copies. The following primer pairs were used (LC in parentheses indicates when different primers were used for light-cycler PCR).

5′-AATCTCATCTTGTTTTCTGCG and 5′-CCTTCCTGGGCATGGAGTCCT for β-actin [28], 5′-ATCGACCACTACCTGGGCAA and 5′-TTCTGCATCACGTCCCGGA for glucose-6-phospate dehydrogenase (G6PDH), 5′-CCCTTATTCCATCCACGGGTCTC and 5′-ATACTGCTCCTCCGGCTGCTTGT for MITF, 5′-GAGGCAGGGAGTCGGGATGA and 5′-CGGGGCACAAGGAAGATGGTC for NF-E2 p45 [29], 5′-GAAGTGCTCCCCTCTGAAAGTT and 5′-GGCTATCTCTCCTCTGACCTCG for SCL, 5′-ACAGGAGCAGCCGAAGGACC and 5′-GAGGAGAAGGACACCACCCC for GATA-1 [29], 5′-CAGGAGCAGCCGAAGGACCA and 5′- ATGGGGCCTGTGGGGAAGGA for GATA-1 (LC), 5′-TTCACCCCTAAGCAGCGCAG and 5′-TAGGTGCCATGTGTCCAGCC for GATA-2 [29], 5′-ACCCCACTGTGGCGGCGAGA and 5′-GACACCACAGTGAGCTCCTT for GATA-3, 5′-GCCGACTACCACGAGTGCA and 5′-TTGTGGGCGATGAAGGTGGA for FOG-1, 5′-CTGGTTGCTCACCCACC and 5′-GCCATTCTTAGGCTGATGC for Elf-1, 5′-GCATACATTGACCCCGGTGC and 5′-CTTCTACTCACATCCAAAAACGC for Elk-1, 5′-TACCCCTCGGTCATTCTCCG and 5′-ATCACTCGTCGGCATCTGGC for Ets-1, 5′-TGGAAGGGTTTCCCCTCGTC and 5′-TGCTGTCCTTCATGTCGCCG for PU.1 [29], 5′-GAATCTCCTAGTCCTGGCTC and 5′-GATGAGAACAGCAACGAGTAC for C/EBPα [30], 5′- ATGGGCAACTGCGCGTGA and 5′- AAGTCCGGCGCAGAGGAA for C/EBPα (LC), 5′-CAGACAGGAAGGCGCTGGG and 5′-CGGCAGTGGCCAAAGGGGCCTT for C/EBPε [30], 5′-AGCCCGCTCCGTGCCAGACA and 5′-GTGGGCAGCTGCACAGCTGG for c-fos [31], 5′-AGATGCCCGGCGAGACACCG and 5′-AGCCCCCGACGGTCTCTCTT for c-jun [31], 5′-AGAAACTCGGCTCCAGAATCC and 5′-TGGTTGCCCTCATGTTGTTTTT for NFATp [32], 5′-GCCGCAGCACCCCTACCAGT and 5′-TTCTTCCTCCCCGATGTCCGTCTCT for NFATc [32], 5′-CCAAGGATGAGGCTTTCCGG and 5′-ACCCAGGAGTAGGTGGGGATAGG for STAT6, 5′-ACAGCAGGTGGAGAGGA and 5′-GATGCCACTGTTGGCAA for Sp1, 5′-GAGTGGCTCACAGCCTGTCA and 5′-TGGGGTGTCTGGCCTTGAGA for Sp1 (LC), 5′-AACCTGATCCTGAAGAGTGGC and 5′-TGGCGGAAGTATTAACAGTTCC for Sp3.

Preparation of nuclear extracts

Typically, 1×10^7 cells were used per preparation with 150–300 µl of lysis buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.6% NP-40 (added 10 s prior to centrifugation)] and 75–100 µl of nuclear extraction buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 25% glycerol). Both buffers contained 0.5 mM PMSF, and complete protease inhibitor cocktail (Roche, Basel, Switzerland). Incubation with each buffer was 15 min on ice, in the case of buffer B, under continuous agitation. Monocytes and granulocytes were occassionally lysed at up to five times higher cellular concentrations to obtain more concentrated extracts. Extracted proteins were stored in aliquots at –80 °C until use. The following rough amounts of nuclear protein were recovered from 1×10^7 cells: 180 µg (skin MCs), 190 µg (monocytes) and 140 µg (PMNs). Protein concentrations in all preparations were determined with the Coomassie plus protein determination kit (Pierce).

Western blot analysis

In preliminary tests, total cell extracts, cytoplasmic lysates and nuclear extracts were run in parallel. The TFs under study were either exclusively or predominantly located in the nucleus. Since the aim was to detect cell-to-cell differences as close as possible to the biological function, we decided to use nuclear proteins for comparison. Protein loadings allowing for semiquantitative analysis, antibody concentrations and film exposure times were optimized first. Striking differences were found between proliferating (leukemic) and non-proliferating cells, with the same amounts of protein from the former yielding substantially higher signals in a manner largely indepen-

dent of the precise cell line and TF (provided the factor was expressed at all). This may be due to a higher ratio of functional to structural proteins in actively proliferating cells and/or a less compact chromatin structure, allowing for preferential TF extraction. As a result, only systems equivalent by these criteria were used for comparative analysis. For SDS-PAGE, 7.5 or 10% gels were used. After electrophoresis, proteins were transferred to a PVDF membrane for 30–50 min, using a semidry transfer cell. Membranes were blocked with 5% non-fat dried milk in PBS for 1 h, then probed with different primary antibodies (rabbit or goat, dilutions ranging from 1:500 to 1:3000 in PBS/5% non-fat dried milk) at 4 °C overnight. After extensive washing, blots were incubated with goat anti-rabbit or rabbit anti-goat secondary peroxidase conjugates (dilution 1:2000; both from Dako, Glostrup, Denmark) for 1 h at room temperature, washed and signals then detected with the ECL enhanced chemiluminescence system and Hyperfilm ECL Plus (Amersham Pharmacia, Flensburg, Germany). The following primary antibodies directed against TFs were used: MITF (sc-11002), SCL (sc-12982), NF-E2 p45 (sc-291), GATA-1 (sc-1233), GATA-2 (sc-9008), Elf-1 (sc-631), Elk-1 (sc-355), Ets-1 (sc-111), PU.1 (sc-352), c-fos (sc-52), c-jun (sc-1694), C/EBPα (sc-61) (all from Santa Cruz, Heidelberg, Germany) and C/EBPε [33].

Results

Comparative analysis of TF expression in mature skin MCs, HMC-1 5C6 cells, monocytes and granulocytes

Figure 1 gives a broad overview of the TF-specific mRNA pattern of skin MCs in comparison with HMC-1 5C6 cells, monocytes and granulocytes. Two out of five quadruplets (corresponding to the four cell types) are shown. The precise quantities of TF-specific mRNAs in the different cell types are given in table 1, while immunoblots showing nuclear protein expression are presented in figure 2.

Results for the distinct groups or families of TFs are given below.

TFs associated with rodent MCs

MITF has been considered to play a dominant, yet complex role for MC maturation and maintenance in the mouse [5]. Binding motifs for this factor are present in the promoters of several murine MC proteases (with known homologies to human tryptase and chymase) and the SCF receptor c-kit [34, 35].

Highest levels of the MITF transcript were detected in skin MCs and HMC-1 5C6 cells (fig. 1, table 1). Low levels were found in monocytes, while granulocytes were negative. MITF protein (30 kDa) expression was also detected in skin MC nuclear extracts using very low protein amounts (fig. 2). MITF protein was not detected in other primary cells. Low levels were found in HMC-1 5C6 (three forms of around 52, 60 and 72 kDa), when high protein amounts were loaded (fig. 2). Numerous isoforms of MITF are known to exist in different tissues, and a novel isoform has been recently described in mouse MCs [36]. The sizes found in HMC-1 5C6 are compatible with

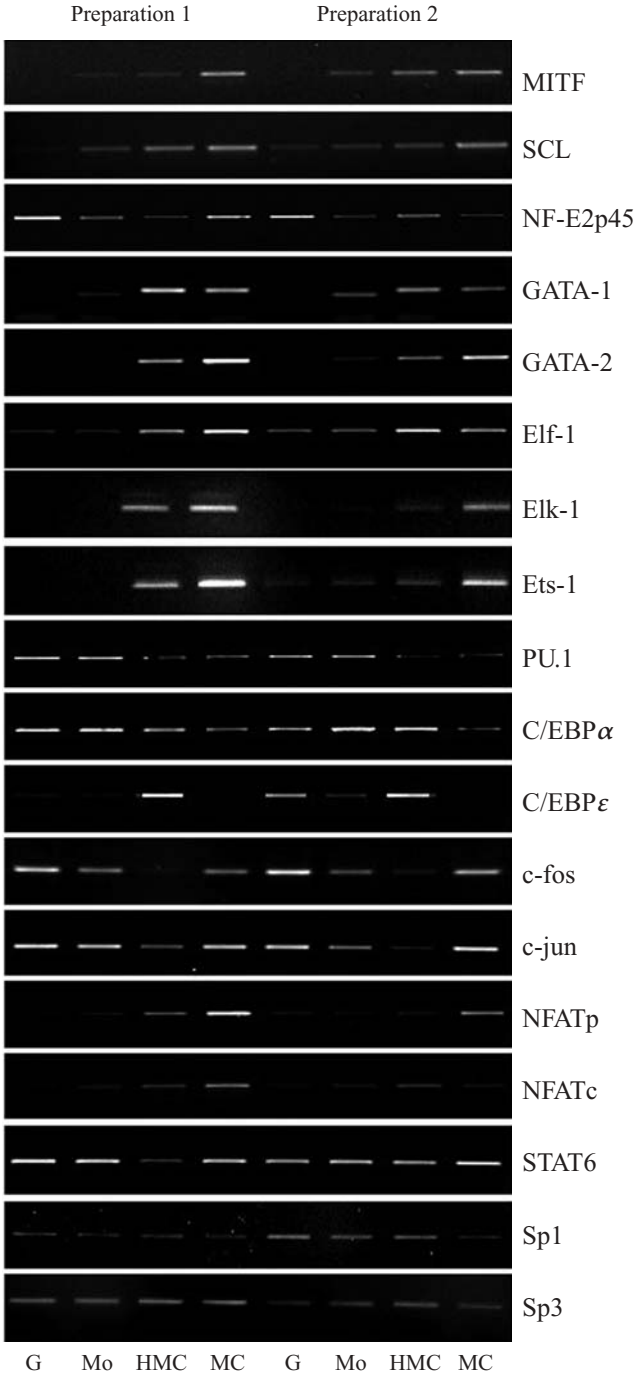

Figure 1. Comparative expression of TFs by granulocytes (G), monocytes (Mo), HMC-1 5C6 cells (HMC) and skin MCs (MC). Two representative out of five preparations are shown.

Table 1. Quantitation of transcription factors at the mRNA level by real-time RT-PCR. RT-PCR was run on the Light-Cycler using the SybrGreen system, as described in Materials and methods. A minimum of eight individual cell preparations was used for each cell type. The numbers of transcripts are expressed as thousands and are the mean ± SE of 8–45 experiments, each corresponding to 40 ng of total RNA (after normalization to the housekeeping gene G6PDH). This amount of cDNA gave an average signal of 22,700 copies of G6PDH (independently of the cell type); the value of 22,700 was set at 1 and the transcripts obtained for the different factors normalized by the following equation: TF transcripts in sample/(G6PDH transcripts in sample/22,700). Where the value given is 0, the number of copies was below 50, no specific product was detectable on gel and/or in melting peak analysis.

TF	Transcript copies (in 1000)			
	Granulocytes	Monocytes	HMC-1 5C6 cells	Skin MC
MITF	0***	0.6 ± 0.1***	3.7 ± 0.6	3.9 ± 0.4
SCL/tal-1	0.1 ± 0.01***	1.4 ± 0.2***	11.9 ± 0.9	13.8 ± 1.7
NF-E2	30.1 ± 4.9*	8.8 ± 2.5*	13.1 ± 4.0	19.5 ± 3.8
GATA-1	0.7 ± 0.1***	0***	15.3 ± 1.2***	6.3 ± 0.8
GATA-2	1.8 ± 0.3***	8.1 ± 0.3***	28.6 ± 3.5***	117.7 ± 16.9
GATA-3	0	0	0	0
FOG-1	0	0	0	0
Elf–1	2.8 ± 0.4***	8.1 ± 1.2*	10.9 ± 0.9*	17.5 ± 3.1
Ets-1	1.6 ± 0.2**	6.1 ± 0.3*	7.0 ± 1.3	14.4 ± 3.7
PU.1	12.5 ± 2.3**	9.7 ± 1.3***	2.5 ± 0.2***	0.9 ± 0.1
C/EBPα	1.2 ± 0.2***	7.0 ± 1.2***	0.6 ± 0.1	0.3 ± 0.1
C/EBPε	1.7 ± 0.2***	1.6 ± 0.1***	4.0 ± 0.8***	0
c-fos	247 ± 63**	355 ± 67***	4 ± 1***	30 ± 7
c-jun	186 ± 14	379 ± 39***	85 ± 9**	160 ± 24
Sp1	16.8 ± 1.3	14.9 ± 0.7	17.0 ± 1.1	15.1 ± 1.0
Sp3	4.6 ± 0.3	8.7 ± 0.8*	7.3 ± 0.5	5.8 ± 0.6

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significantly different (lower or higher) from skin MCs.

those in melanoma cells [37], while the protein of skin MCs seems fairly small. Since high expression of this protein was detected in seven of seven MC preparations examined, it is clearly specific for skin MCs. One cannot exclude that a fragment produced posttranslationally is functionally active in these cells. Together, the data indicate that MITF is preferentially expressed by MCs compared with other myelocytes. While high protein levels are found in primary human MCs, transcript levels in skin MCs and HMC-1 5C6 are virtually identical, indicating that posttranscriptional events may play a role in dictating the isoform(s) and/or quantity of the nuclear protein.

SCL/tal-1 is another factor linked to the MC lineage in rodent systems [10]. Levels of SCL transcript were highest in skin MCs and HMC-1 5C6, but lower amounts were also detected in monocytes (fig. 1, table 1). Similar results were obtained on immunoblotting, where high amounts of SCL were extracted from skin MCs, while monocytes displayed around one-fifth of MC specific reactivity. Granulocytes were negative for SCL protein. In contrast, SCL expression could be detected in most myeloid cell lines, in accordance with the preferential expression of the factor in less mature hematopoietic cells [10, 38], with the exception of MCs which appear to maintain high-level expression during terminal differentiation.

The factor NF-E2 (nuclear factor-erythroid 2) is composed of two subunits, MafK (or MafG) and p45. While MafK is ubiquitously expressed, p45 is restricted to some hematopoietic lineages, inluding rodent MCs, with particularly clear functional implications in megakaryocytes [12, 16]. In this study, the p45 transcript was detected in all myeloid cell types tested with highest amounts unexpectedly found in granulocytes (fig. 1, table 1). Additionally, its broad distribution in myeloid cells was confirmed at the protein level, since it was detected in all the cell lines used (fig. 2), while no signal was detected in skin fibroblast nuclear extracts (which served as negative control, not shown). Among primary cells, the protein was found in PMNs and monocytes, but not in skin MCs under the same conditions (fig. 2). Taken together, NF-E2 p45 seems broadly expressed in myeloid cells and is obviously not specific for human MCs.

GATA TFs

This family appears particularly important for MC development and function. In the mouse, GATA-2 was found indispensable for early stages of development, but disappeared at later stages [6, 7, 39], while expression of GATA-1 seemed to increase with differentiation [40]. Recently, GATA-1 underexpression was associated with high numbers of morphologically abnormal immature

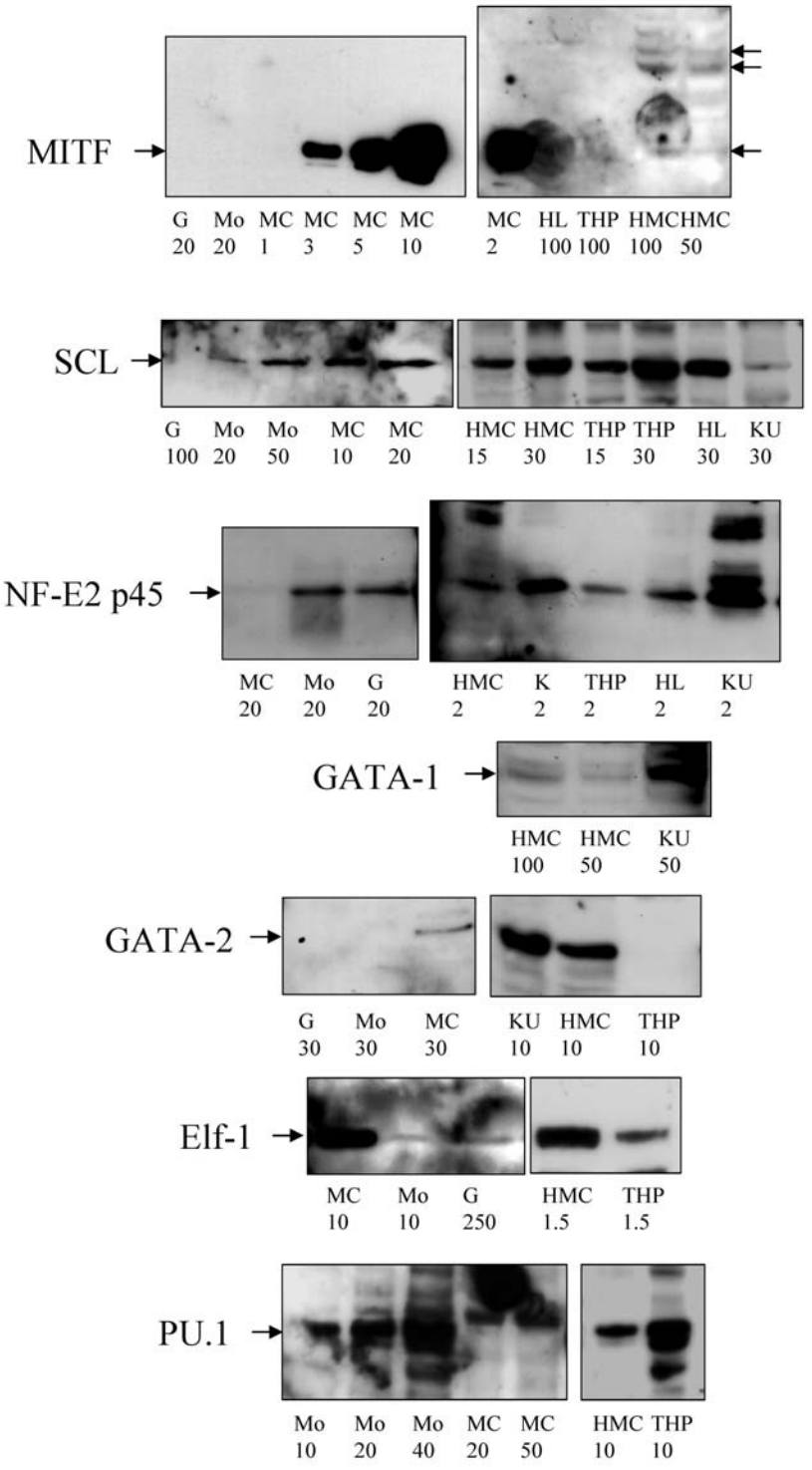

Figure 2. Expression of TFs in nuclear extracts of different myeloid cells. Representative blots of three to ten individual cell preparations are shown. Cell purification and preparation of nuclear extracts were performed as described in Materials and methods. HMC, HMC-1 5C6 cells; K, K562 cells; KU, KU812; HL, HL-60 cells; THP, THP-1 cells; Ju, Jurkat cells; G, granulocytes; Mo, monocytes; MC, skin MCs; PBL, peripheral blood lymphocytes. The values indicate the amounts of protein loaded (in μg).

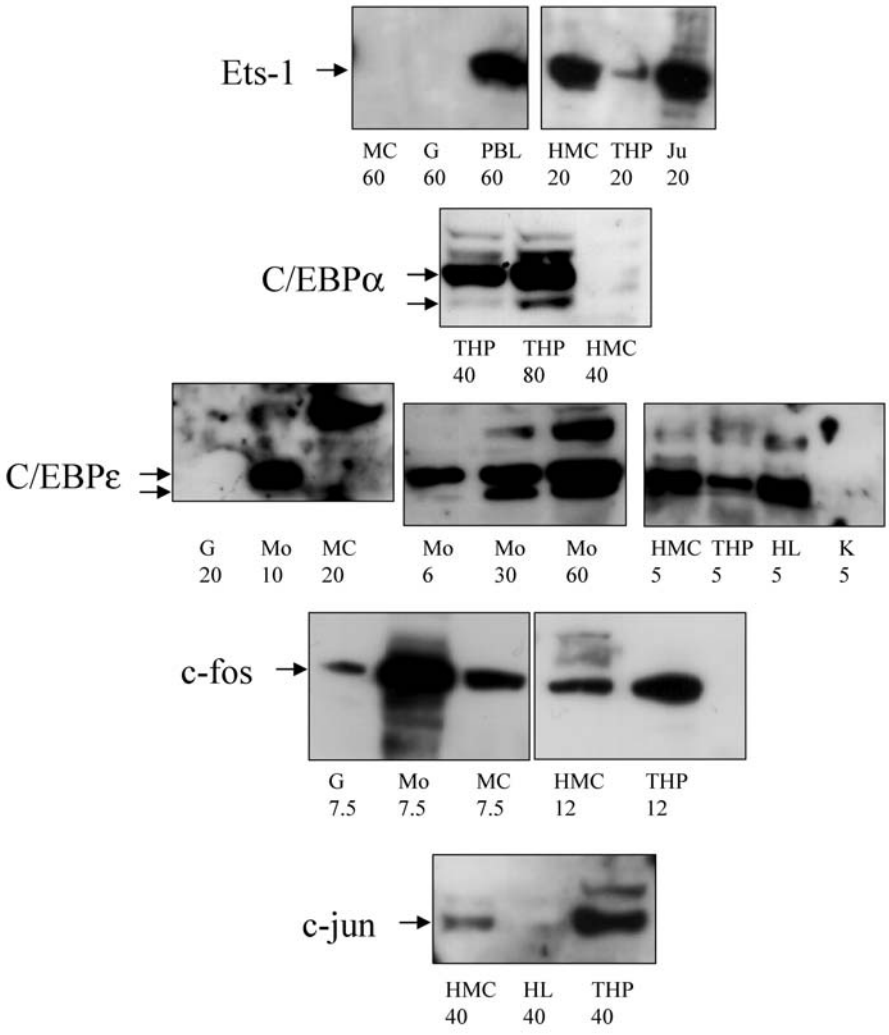

Figure 2 (continued)

MCs [9], and the factor has been described to participate in the transcription of the FcεRIα and β genes [41, 42].

Both skin MCs and HMC-1 5C6 cells were found to express the GATA-1 transcript, but higher levels were associated with a lower degree of maturation (fig. 1). This was particularly striking on quantitative evaluation where the difference between HMC-1 and skin MCs was highly significant (table 1). Low amounts were found in PMNs likely reflecting eosinophilic expression. With the exception of immature cells like KU812 (that exert characteristics of multiple lineages, including erythroblastic and basophilic ones [43]), all other cell lines tested (THP-1, HL-60, U937) were negative or expressed negligible amounts of GATA-1 mRNA (not shown). KU812 cells represented the only system in which GATA-1 protein could be unambigously detected (fig. 2), while in primary cells, GATA-1 expression remained undetectable at protein loadings which were achievable with the limited cellular material (up to 100 μg of skin MCs, not shown).

GATA-2 was one of the most abundant TF transcripts with over 100,000 copies per 40 ng RNA in skin MCs, and thus substantially enhanced versus HMC-1 5C6 cells (table 1). PMNs and monocytes expressed low levels only. Likewise, among primary cells, only skin MCs expressed GATA-2 protein, while in cell lines, GATA-2 was found in HMC-1 5C6 and KU812 cells, but not in THP-1 cells. These data support critical involvement of this TF family in human MC biology as well as a highly restricted expression of GATA factors among myelocytes. However, GATA-2 (rather than GATA-1) seems to be associated with a higher differentiation stage of human MCs, opposite to the situation in the mouse.

GATA-3 is mainly involved in transcription of typical Th2-type cytokine genes [44]. While GATA-3 was undetectable in the four myeloid systems, a transcript was found in PBLs used as a positive control (data not shown). Likewise, FOG-1 (involved in megakaryocytic and erythrocytic differentiation [16]) mRNA remained

undetectable in all primary cells and cell lines used in the study (table 1 and data not shown).

Ets TFs

The family comprises numerous members implicated in developmental processes [45], four of which were included in the study. Elf-1 was originally proposed to participate in a positive way in regulation of the FcεRIα gene promoter [41]. Although this was later corrected [46], Elf-1 mRNA was preferentially associated with skin MCs, followed by HMC-1 5C6 and monocytes, whereas negligible levels were found in granulocytes (table 1). By immunoblotting, Elf-1 was clearly strongest in MCs both among primary cells and cell lines (fig. 2). Thus, high-level expression of Elf-1 protein seems characteristic of the MC lineage.

The expression of PU.1 which is indispensable for monocytic, granulocytic and dendritic/Langerhans cell maturation [13, 19–21, 47–49], was as expected highest in monocytes and granulocytes at the mRNA level in the present study (table 1). Lower, yet detectable, expression was found in HMC-1 5C6, followed by skin MCs, in accordance with its importance in MC progenitor formation in the mouse [8], and the potentially positive role at the FcεRIα promoter [46]. At the protein level, high amounts were detected in monocyte nuclear extracts, but not in skin MCs with up to 50 μg of nuclear protein (fig. 2). However, the factor was detectable in HMC-1 5C6 cells in addition to monocytic THP-1 cells (fig. 2). This latter finding together with the clearly detectable mRNA expression in all of the skin MC preparations used (even if at around 10% of the amount found in monocytes), suggests that PU.1 protein may be present in mature MCs, albeit at much lower levels than in monocytes.

The transcript for Elk-1, implicated as a downstream target in the signaling cascade via FcεRI [50], was quite specifically expressed by MCs (fig. 1). With the antibody used, Elk-1 protein, however, remained undetectable in all primary cells (up to 50 μg of nuclear protein) and cell lines (up to 100 μg of nuclear protein).

In addition to its role in T cells, Ets-1 has been linked to MC function [51]. The levels of Ets-1 transcript among the four systems were on average highest in MCs, but significant amounts were also found in monocytes (fig. 1, table 1). In comparison with PBLs which expressed extremely high levels of Ets-1, the amounts detected in MCs were however substantially lower (data not shown). Similarly, Ets-1 remained below the detection limit in skin MCs up to 60 μg of nuclear protein, while the protein was easily extracted from PBLs which served as positive control (fig. 2). Ets-1 was however detectable in most cell lines, with high expression in HMC-1 5C6 (fig. 2 and data not shown). In conclusion, Ets-1 is obviously expressed by MCs, but amounts in mature MCs are much lower than in primary lymphocytes.

C/EBP TFs

C/EBP family members have important roles in the differentiation and function of hematopoietic cells [52]. C/EBPα is expressed by monocytic and granulocytic cells, and is indispensable both for early stages of granulopoeisis and eosinophilic development [14, 18, 53]. C/EBPε is essential for later stages of granulopoiesis and is also involved in eosinophilic and macrophage gene expression [54–57]. Conversely, C/EBP factors have a negative impact on dendritic/Langerhans cell maturation, and this is at least partially due to an inactivation of PU.1 [48, 49]. Of the cells tested here, monocytes expressed the highest level of C/EBPα mRNA, followed by granulocytes. Expression in MCs was low, with HMC-1 5C6 cells showing higher levels of C/EBPα transcript than skin MC (table 1). At the protein level, C/EBPα expression was below the detection limit in monocytes and granulocytes (skin MCs were not tested) using up to 100 μg of nuclear protein (not shown). The significance for monocytes rather than MCs was, however, confirmed by the exclusive detection of C/EBPα protein in THP-1 (but not in HMC-1 5C6) cells (fig. 2), where two bands were found at 40 and 30 kDa, which likely correspond to the two described isoforms [52]. The relatively high level of C/EBPα expression in monocytic cells is in line with its transactivating potential at monocyte-specific promoters [58, 59], although at earlier stages, C/EBPα blocks monopoiesis and favors granulopoiesis instead [60].

C/EBPε transcripts were detected in all cell types tested (fig. 1 and data not shown). The only exception was skin MCs which were consistently negative. Surprisingly, the levels found in HMC-1 5C6 cells were higher than in any other cell line (with the exception of HL-60 cells, used as positive control) both by RT-PCR (table 1 and data not shown) and by immunoblotting (fig. 2 and data not shown). Skin MCs were negative by immunoblotting, while monocytes were found to express fairly high levels of the factor (two bands of about 35 and 30 kDa, which likely represent the two largest of the four known isoforms [54]). Granulocytes were virtually negative at the protein level, where only at very high protein loadings was a faint band occasionally detected (not shown). The absence in end-stage neutrophils is in line with a recent study showing that C/EBPε is highest at the myelocyte stage, being downregulated there after [61].

In conclusion, members of the C/EBP family are expressed to only a minor degree (C/EBPα) or are entirely absent (C/EBPε) from primary human MCs.

AP-1 TF

AP-1 impacts cell cycle regulation [62], but depending on the cell type, can favor either differentiation or proliferation. Both c-fos and c-jun transcripts were among the most highly expressed TF transcripts with up to around 300,000 copies. While detectable in all the myelocytes

tested, untransformed cells expressed much higher levels of c-fos than transformed HMC-1 5C6 and THP-1 cells (table 1 and data not shown), such that a higher degree of differentiation rather than proliferation seems clearly associated with high AP-1 levels in the myeloid system.
By Western blot analysis, highest levels of c-fos expression were found in monocytes, but the factor was also easily detected in granulocytes and skin MCs at about equivalent levels (fig. 2 and data not shown). In cell lines, THP-1 cells expressed higher amounts of both fos and jun protein than HMC-1 5C6 cells (fig. 2). The c-jun protein, however, remained below detection levels in all primary cells with the antibody used (not shown).
In conclusion, AP-1 (in particular c-fos) seems to increase in the course of myeloid differentiation. As in monocytes [13], AP-1 may thus play some part in the maintenance of MC maturity and/or survival [63]. However, the substantially lower levels of c-fos in skin MCs clearly distinguish these cells from monocytes and granulocytes.

TFs associated with MC signaling

Nuclear factors of activated T cells (NFATs) regulate many immunologically relevant genes [summarized in ref. 64] and are involved in rodent MC signaling [65, 66].
In the present study, NFATp and NFATc transcripts were preferentially linked to the MCs with slightly higher amounts in skin MCs with the rank of expression being skin MCs > HMC-1 5C6 > monocytes > granulocytes for both factors, with NFATp being hardly detectable in the latter two cells (fig. 1). Levels of NFATp and NFATc transcripts were, however, higher in PBLs than in MCs (data not shown). STAT6 is known to transduce signals from the interleukin-4 receptor into the nucleus, IL-4 being a cytokine with profound effects on MCs [23, 27]. All cells investigated expressed STAT6 at the transcript level with no pronounced cell-to-cell variation, although slightly lower levels were detected in HMC-1 5C6 (fig. 1).

Sp1 and Sp3

Sp1 and Sp3 have a ubiquitous distribution and are viewed as housekeeping-like TFs [67]. While many other housekeeping genes are regulated by Sp1/Sp3, various genes with hematopoietically restricted activity also require Sp1 activity in addition to lineage-restricted factors [13, 38].
While a slight intercellular variation was detected with Sp3 (a difference of less than twofold), Sp1 behaved like a typical housekeeping gene that was expressed at the same level by all cells tested (fig. 1, table 1). Thus, Sp1 served as a further control in addition to G6PDH.

Discussion

Over the past few years, substantial efforts have been made to understand the molecular processes in the nucleus that determine cell fate decision and maintenance in the hematopoietic system. However, there remain cellular subsets that have been poorly characterized up to now. One of these latter cell types is the human MC, which is difficult to obtain in sufficient amounts due to its exclusive maturation within tissues. Having established a technique to obtain high levels of pure primary MCs from human skin tissue, we have now analyzed various TFs with important functions in hematopoiesis. The expression was simultaneously monitored at the mRNA and nuclear protein levels. When adjusted to a house-keeping gene, transcript levels appeared comparable among primary and leukemic cells, since there was no clear-cut tendency for any system to produce overall lower or higher levels of TF transcripts. The situation was quite different when equivalent amounts of nuclear protein were used in parallel. On direct comparison with non-proliferating leukocytes, all leukemic cells showed substantially enhanced levels of TFs in their nuclear extracts (fig. 2).
Other differences between the mRNA and the protein level were also noted. For example, the MITF transcript was about the same in HMC-1 5C6 and skin MCs, but much higher protein expression was noted in the latter. In addition, the same or higher levels of C/EBPε mRNA were found in granulocytes compared to monocytes, while the protein was found in monocytes only. Moreover, NF-E2 mRNA was highest in granulocytes, while the protein was about the same as in monocytes. These latter data imply that the amount of TF protein per total nuclear protein is particularly low in end-stage differentiation cells such as granulocytes, establishing a further extension of the general theme of lower presence of TFs and/or a more rigid chromatin structure with ongoing maturation, and concomitant loss of proliferation or plasticity. Moreover, since there are various events lying between the transcription of a gene and the appearance and maintenance of the protein in the nucleus, it is not surprising that the data for these two levels of detection may not be completely alike, although with a variety of factors, the tendency was similar. The comparison between monocytes and skin MCs showed an especially good correlation between the mRNA and the protein level: there was higher (or much higher) expression of MITF, SCL, GATA-2 and Elf-1 transcript in skin MCs versus monocytes, but lower expression of PU.1, C/EBPε and c-fos, and this pattern was perfectly reproduced by immunoblotting (table 1, fig. 2). Since it is the protein that performs the function, its presence, absence and exact amount in the nucleus appears the stronger criterion to distinguish different lineages. However, the instability to detect a specific factor does not necessarily mean that it

is truly absent, but the number of copies may be too low to allow for detection, in particular with antibodies of low specific activity. An example is c-jun, whose expression could not be detected in nuclear extracts of primary cells, although it is undoubtedly highly expressed by monocytes [13], the latter perfectly in line with our own mRNA data (table 1).

Of the factors previously linked to rodent MCs, both MITF and SCL were also found to be preferentially associated with human MCs. Conversely, the factor NF-E2 does not seem to be MC specific, in accordance with a previous report that suggested NF-E2 plays a repressive role at the histidine decarboxylase promoter [11]. GATA-1 and, less so, GATA-2 could be confirmed to have a highly restricted distribution in human hematopoietic cells also. However, in contrast to reports from the mouse [6, 7, 39], GATA-2 expression is maintained or even further elevated in the course of human MC maturation, as it is particularly pronounced in skin MCs (table 1). C/EBPα and ε are absent from primary human MCs or expressed at very low levels and can therefore be regarded as candidate factors to distinguish MCs from other myelocytes. However, C/EBPε displayed the most striking difference in expression level between skin MCs and HMC-1 5C6 cells (fig. 1, Table 1).

Following previous attempts to discriminate myeloid cell types by TF patterns [13–16], the mature human MCs may be defined by the following pattern of the most extensively studied hematopoietic TFs: C/EBPα^{low}, C/EBPε^{-}, PU.1^{low}, GATA-1^{low}, Elf-1^{+}, GATA-2^{+}, SCL^{+}, MITF^{high}, where the term 'low' refers to detectable transcripts in the low to intermediate range, but the absence of protein signals. The fact that, with the exception of C/EBPα, the factors designated 'low' can be found in the immature MC line, implies that protein expression in the nucleus of skin MCs may be present but too low for detection. On the other hand, '–', '+', and 'high' refer to those factors where protein and transcript expression are in accord.

A surprising finding was that with the exception of C/EBPε and c-fos, the differences between HMC-1 5C6 and skin MCs were fairly small and much less than those between skin MCs and the other two myelocytes. With high expression of c-kit and MC tryptase, intermediate expression of histidine decarboxylase and its product histamine, and low, but detectable FcεRIα and γ transcripts as well as FcεRI protein expression [26, 68, and further unpublished data], HMC-1 5C6 clearly resemple MCs which is in line with their origin from a patient with MC leukemia [25]. Nevertheless, HMC-1 5C6 are malignantly transformed cells (and as such, proliferating in culture) and of substantially lower maturation than skin MCs (lower expression of FcεRIα, β, and γ, lower presence of tryptase and histamine, no chymase expression, poor staining with acidic toluidine blue, immature granules and expression of distinct markers of other lineages [26, 69 and own unpublished data]). The striking degree of similarity in the repertoire of TFs between HMC-1 5C6 and skin MCs may imply that cells gain this pattern early in their differentiation process. On the other hand, the obviously aberrant expression of C/EBPε may be one reason for HMC-1 immaturity, especially given that dysregulated TF expression is a frequent hallmark of leukemogenesis [70]. Studies to clarify if C/EBPε has an adverse or unfavorable impact on MC differentiation are underway.

Conversely, on direct comparison with monocytes, to which MCs seem highly related [22, 27, 69], MCs express a considerably distinct TF pattern with exclusive or higher presence of GATA-1, GATA-2, SCL, MITF and Elf-1 in skin MCs, but lower levels of C/EBPα, C/EBPε, PU.1, c-fos and c-jun. Another cell type with which MCs shared distinct TFs are hematopoietic stem cells which are characterized by high levels of SCL, GATA-2 and Elf-1 [71, 72], all three of which were highly displayed by skin MCs in the present study (table 1, fig. 2). Of all their progeny, it is in fact exclusively MCs that express high levels of c-kit, while certain MC subtypes can even express the progenitor-specific marker CD34 [73].

Our present results, combined with previous reports for other cells [13–16], make the presence of one particular factor or a group of factors that are purely MC specific rather unlikely. Combinatorial regulation by different factors that intersect to orchestrate expression of lineage-specific genes will likely endow the cell with the required flexibility regarding the panel of markers expressed at a certain time. Also, this scenario will allow that markers highly (and rather specifically) expressed by MCs, are also found in a limited spectrum of other cells.

One possibility for a cell to develop and maintain a certain kind and state of differentiation may be through combination of TFs from different lineages. This seems quite relevant to MCs, which express megakaryocytic/erythroid, stem cell and myeloid TFs at predetermined concentrations, together with high levels of MITF. This is in accord with the fact that the large majority of promoters requires regulation by more than one TF, and that the few known examples of genes with a (more or less) MC-specific activity pattern suggest the same is true for the MC lineage [38, 41, 46]. One needs to point out that the precise dose of a factor is likely more decisive as a lineage property than its mere presence or absence, at least in the case of certain factors, and this is highlighted by a recent report: for the majority of cases, PU.1 and GATA factors act as antagonists and block each others' function [47]; however, they can also act in a synergistic fashion at selected promoters. This cooperation depends primarily on the precise levels and ratio of the factors [55]. Cooperation between PU.1 and GATA-2 also seems critical to murine mastopoiesis, while higher levels of PU.1 inhibit

mastopoeisis and favor monopoeisis instead [8, 74]. With other hematopoietic lineages also, the importance of dose has been documented extensively for PU.1 [summarized in ref. 47], where dendritic or Langerhans cell maturation in particular appears to require high levels of this factor [48, 49]. The low level of PU.1 found in mature human MCs (much lower than a fifth for protein and lower than 10% for transcript; fig. 2, table 1) is consistent with the findings for immature murine MC precursors [8, 74], and extends them to a fully differentiated MC subtype of the human system. In addition, the virtual absence of C/EBPα, a factor that can antagonize PU.1 function [48], in skin MCs implies that despite the low level, PU.1 may be functionally active in mature MCs.

Although our study attempted to cover those TFs that appeared most relevant based on the available literature, there are certainly other factors whose investigation will be crucial to complete the picture of MC-specific TF patterns. Further studies will need to establish how TF genes themselves are being switched on and off and to disclose the composition of unique TF complexes that control transcription from promoters with MC-specific activity. Answers to these questions will undoubtedly require numerous efforts but ultimately will elucidate how MC differentiation is accomplished at a molecular level and what actually defines a MC by molecular criteria. Our study should be viewed as one first fundamental contribution to this field.

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