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Isolation and Characterization of Human Myeloid Progenitor Populations. - TpoR as Discriminator between Common Myeloid and Megakaryocyte/Erythroid Progenitors.

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ABSTRACT

OBJECTIVE: The common myeloid progenitor (CMP) and its progeny the granulocyte/monocyte progenitor (GMP) and megakaryocyte/erythrocyte progenitor (MEP), have been isolated based on the surface expression of CD34, IL-3R α and CD45RA. However, high resolution of IL-3R α^{neg} and IL-3R α^{lo} cells required to adequately separate the CMP and MEP populations is difficult to achieve. The aim of this study was to find a complementary surface marker to obtain a better separation of these two populations and to further characterize the acquired progenitor populations.

MATERIALS AND METHODS: To evaluate the thrombopoietin receptor (TpoR) as a candidate marker, CD19 $^-$ /CD34 $^+$ /IL-3R $\alpha^{\text{lo/-}}$ /CD45RA $^-$ /TpoR $^-$ (CMP), CD19 $^-$ /CD34 $^+$ /IL-3R α^{lo} /CD45RA $^+$ /TpoR $^-$ (GMP) and CD19 $^-$ /CD34 $^+$ /IL-3R $\alpha^{\text{lo/-}}$ /CD45RA $^-$ /TpoR $^+$ (MEP) cells from human bone marrow were sorted to semisolid cultures for colony assays, and in addition analyzed for their surface expression of other growth factor receptors (GFRs) and sorted to real-time RT-PCR for gene expression analysis.

RESULTS: The colony formation and gene expression assays showed that inclusion of TpoR as a marker gave a distinct and reproducible separation of the myeloid progenitors. Furthermore, most GFR surface expression correlated to gene expression, but there were also striking discrepancies, in particular for the common β -chain of the IL-3R, GM-CSFR and IL-5R and for TpoR.

CONCLUSION: Our data establish the TpoR as an important tool for isolation of the myeloid progenitors and demonstrate that the surface expression of GFRs cannot be predicted by their gene expression. Importantly, the refined isolation of CMPs, will allow more detailed studies of regulatory mechanisms steering CMPs towards erythropoiesis versus granulopoiesis in steady state and response to peripheral demands.

Word count: 250

INTRODUCTION

There are several models for differentiation of hematopoietic stem cells (HSCs) into different hematopoietic lineages, primarily based on *in vitro* clonogenic assays. All models so far seem to agree on an initial separation of the megakaryocyte/erythrocyte (Meg/E) development from the lymphoid development, but disagree on whether the granulocyte/monocyte (G/M) branch co-localize with the former or latter, or both (reviewed by Katsura Y [1]). The most commonly accepted hypothesis proposes that HSCs first commit to either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP), the latter further committing to granulocyte/monocyte progenitors (GMPs) or megakaryocyte/erythrocyte progenitors (MEPs) [2]. This model is supported by the characterization of candidate murine CLP [3] and CMP [4], but was also recently challenged by findings of a murine co-existing lympho-myeloid progenitor separated from cells with Meg/E-potential by their surface expression of flt3 (CD135) [5]. However, in humans the classical model with a CLP and CMP still has the strongest experimental support, in the isolation of a human CLP [6,7] and CMP [8]. The phenotypic definition of the human CMP (Lin⁻/CD34⁺/CD38⁺/IL-3R α ^{lo}/CD45RA⁻) proposed by Manz *et al.* [8], also included definitions of the GMP (Lin⁻/CD34⁺/CD38⁺/IL-3R α ^{lo}/CD45RA⁺) and MEP (Lin⁻/CD34⁺/CD38⁺/IL-3R α ⁻/CD45RA⁻).

Characterization and isolation of the hematopoietic progenitor stages are essential to facilitate studies on the mechanisms that regulate lineage commitment and differentiation. Hence, it is important to establish generally applicable and reproducible progenitor definitions. However, we found that separation of the CMP- and MEP-populations based on their different IL-3R α (CD123) expression is difficult to achieve and therefore investigated the applicability of other surface markers resulting in a better separation of the progenitor populations. In this work we describe a modified, easily reproducible version of the myeloid progenitor definition presented by Manz *et al.*, introducing surface thrombopoietin receptor (TpoR; CD110) as a marker for the MEP-population. Comparative analysis of the clonogenic potential in *in vitro* colony assays showed that inclusion of TpoR resulted in a pure MEP-population,

and also allowed for separation between bipotent megakaryocyte/erythrocyte (Meg/E) progenitors and early erythroid and megakaryocyte progenitors (TpoR^{lo}) versus more mature erythroid and megakaryocyte progenitors (TpoR^{hi}). Additionally, with further characterization of the surface antigen and gene expression profiles of the CMP, GMP and MEP, using flow cytometry and real-time RT-PCR, we found discrepancies between surface and gene expression of some hematopoietic GFRs, indicating that simple gene expression profiling is insufficient to predict the distribution and role of these receptors. Finally, we found that increasing and decreasing expression of flt3 within the CMP-population might signal pre-commitment towards CMP and MEP, respectively.

MATERIAL AND METHODS

Isolation of CD34⁺ CD19⁻ cells from bone marrow

Human adult bone marrow was obtained from healthy volunteers after informed consent. Mononuclear cells (MNCs) were isolated by separation on Lymphoprep (Nycomed Pharma, Oslo, Norway) and CD19⁺ cells depleted through positive selection with magnetic bead labeling according to manufacturer's instructions (CD19 MicroBeads, Miltenyi Biotec, Bergisch-Gladbach, Germany), after which CD34⁺ cells were enriched from the CD19⁻ fraction by labeling with magnetic beads (CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec). The purity regarding CD34 was regularly above 90% and was further increased to above 99% during cell sorting (see below).

Flow cytometric analysis and cell sorting

CD34⁺ cells were labeled with FITC-conjugated monoclonal antibodies (BD Biosciences, San Jose, CA, USA) CD45RA (clone HI100), CD116 (M5D12); PE-conjugated anti-CD110 (BAH-1), -CD114 (LMM741), -CD117 (104D2), -CD123 (9F5) and -CD135 (4G8); PerCP-Cy5.5-conjugated anti-CD34 (8G12) and -CD38 (HIT2); APC-conjugated anti-CD45RA (HI100), -CD110 (BAH-1) and -CD117 (104D2); and biotin-conjugated anti-CD123 (9F5) and -CD131 (3D7). The biotin-conjugated antibodies were labeled in a second step with streptavidin-FITC or -PE

(BD Biosciences). In some experiments CD34-PE-Texas Red was used (clone 581; Coulter-Immunotech, Marseille, France). Cells were analyzed and sorted on a FACS Aria flow cytometer equipped with an automatic cell deposition unit (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Cells were sorted (purity mode) and resorted (single cell mode) directly into 100 μ L methylcellulose culture medium in 96-well plates for clonogenic assay, tubes containing MegaCult™ -C medium for megakaryocyte culture, or PCR-tubes containing lysis buffer for real-time RT-PCR assay. DAPI (3 μ M; Molecular Probes, Leiden, The Netherlands) was included to discriminate between live and dead cells and excited by the violet laser (405 nm; FACS Aria). Details of the gating strategy for cell sorting are given in Fig.1, 3, 6 and 9.

***In vitro* colony assay in methylcellulose**

Sorted cells (10 cells/well, 5-24 wells per population or 1 cell/well, 96 wells per population) were cultured for 14 days in Iscove's modified Dulbeccos's medium (IMDM)-based methylcellulose medium containing 30% FCS, 1% BSA, 100 μ m 2-mercaptoethanol, 2 mM L-glutamine, rhSCF 50 ng/mL, rhGM-CSF 20 ng/mL, rhIL-3 20 ng/mL, rhIL-6 20 ng/mL, and rhG-CSF 20 ng/mL (Methocult GF H4535, StemCell Technologies, Vancouver, BC, Canada) +/- Epo 3 U/mL (Eprex®, Janssen-Cilag, Wycombe, Buckinghamshire, UK) to evaluate clonogenic growth of CFU-GEMM, CFU-GM, CFU-M, CFU-G and BFU-E.

Quantification of megakaryocytic and erythroid progenitors

CFU-Meg and BFU-E colonies were assayed using the MegaCult-C system (StemCell Technologies) according to manufacturer's instructions. Briefly, 250-5500 cells were sorted to tubes containing 1 mL MegaCult-C serum-free medium with cytokines; IMDM containing 1% bovine serum albumin, rh insulin (10 μ g/mL), human transferrin (200 μ g/mL), L-glutamin (2mM), 2-mercaptoethanol (0.1 mM), rhTpo (50 ng/mL), rhIL-6 (10 ng/mL), rhIL-3 (10 ng/mL), supplemented with low density lipoprotein (40 μ g/mL, ICN Biomedicals, Irvine, CA, USA), 0.5% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and +/- Epo (3 U/mL,

Eprex®, Janssen-Cilag). Subsequently, 0.1 mL IMDM and collagen (1.1 mg/mL, final concentration) were added and 1.5 mL transferred to chamber slides. After 13-14 days of culture, clonogenic growth of BFU-E was evaluated microscopically before the cultures were fixed and stained with mouse anti-human GPIIb/IIIa (or mouse anti-TNP, isotype control), biotin conjugated goat anti-mouse IgG and avidin-alkaline phosphatase conjugate/substrate, and counterstained with Evans Blue.

Sample processing and Reverse Transcription (RT)

500 cells of selected populations were sorted to cell lysis buffer. The cell lysate was subjected to heat and reverse transcribed using Sensiscript RT kit (Qiagen, Hilden, Germany) as previously described [9]. The cDNA to be used in standard curves was obtained from fresh marrow MNCs, fresh CD34+ cells and cells cultured in neutrophil and erythroid differentiation culture, using total RNA-isolation (RNeasy mini kit, Qiagen) and subsequent RT (TaqMan RT Reagents, Applied Biosystems) as described previously [9]. The different cDNAs were pooled together and used in standard curves for all targets.

Real-time RT-PCR and data analysis

Gene expression in the obtained cDNA was analyzed by real-time RT-PCR as previously described [9]. Briefly, sample triplicates were run on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with the TaqMan® Gene Expression Assays (Applied Biosystems) or Primer Express (Applied Biosystems)-designed primers/probe listed in Table 1 and subsequently analyzed by the relative standard curve method. Target quantities were normalized to 18S ribosomal RNA and calibrated using values from the CMP-population defined as 1.0. All other quantities were expressed as an n-fold difference relative to the calibrator.

RESULTS

Validation of IL-3R α and CD45RA for separation of CMPs, MEPs and GMPs

Since CD19⁺/CD10⁺ pre-B-cells is a considerable fraction of CD34⁺ human marrow cells (mean 18.7% \pm 7.9 SD; n=10) CD19⁺ cells were removed by immunomagnetic

Table 1. TaqMan® Gene Expression Assays and Primer Express-designed primers/probe utilized for real time RT-PCR analysis.

Gene	Gene product/Protein	Assay ID
<i>CSF2RB</i>	β_c -chain	Hs 00166144_m1
<i>KIT</i>	c-kit	Hs 00174029_m1
<i>EPOR</i>	EpoR	Hs 00181092_m1
<i>FLT3</i>	FIt3	Hs00174690_m1
<i>GATA1</i>	GATA-1	Hs 00231112_m1
<i>GATA2</i>	GATA-2	Hs 00231119_m1
<i>CSF3R</i>	G-CSFR	Hs 01114427_m1
<i>CSF2RA</i>	GM-CSFR α	Hs 00538896_m1
<i>GPIIB</i>	GPIIb	Hs 00166246_m1
<i>IL3RA</i>	IL-3R α	Hs 00174356_m1
<i>NFE2</i>	NF-E2	Hs 00232351_m1
<i>PRTN3</i>	PR3	Hs 00160521_m1
<i>SPI1</i>	PU.1	Hs 00231368_m1
<i>TAL1</i>	SCL	Hs 00268434_m1
<i>MPL</i>	TpoR	Hs 00180489_m1
	18S rRNA	Hs 99999901_s1
Primer/probe sequence		
<i>HBB</i>	β -globin	
	Forward primer	5'-CACCTTTGCCCACTGAGTGA-3'
	Reverse primer	5'-GTGATGGGCCAGCACACA-3'
	Probe	5'-FAM-TGAGAACTTCAGGCTCCT-MGB-3'

beads. Control experiments showed that the CD19-depleted CD34⁺ population gated for further cell sorting contained <0.5% CD38⁻ cells , <2.2% CD7⁺ plus CD10⁺ cells (2.2% \pm 0.7; n=4)), indicating that the sorted populations contained negligible numbers of early stem cells (CD34⁺/CD38⁻) and lymphoid progenitors (CD34⁺/CD38⁻/CD7⁺). The low numbers of neutrophils and occasional monocytes were gated out by their scatter properties during cell sorting. Thus these procedures result in a CD34⁺ cell purity comparable with that of depletion by lineage markers as part of the cell sorting. To investigate the practical utility of the definition of CMP, GMP and MEP presented by Manz *et al.* [8], CD34⁺CD19⁻ cells were isolated from human bone marrow and

labeled for their expression of CD34, IL-3R α and CD45RA. The CD45RA⁻ cells were clearly separated from the CD45RA⁺ cells, but in our hands the difference between the IL-3R α ^{lo}/CD45RA⁻ and the IL-3R α ^{hi}/CD45RA⁻ populations was much less distinct (Fig.1C). Accordingly, when sorted, resorted and reanalyzed, the IL-3R α ^{lo}/CD45RA⁺ population showed great purity (Fig.1E) and gave rise exclusively to G/M colonies (Fig.2), whereas the cells sorted as IL-3R α ^{lo}/CD45RA⁻ and as IL-3R α ^{hi}/CD45RA⁻ were poorly separated (Fig.1D and 1F), and the latter population, although dominated by erythroid colony-forming cells, gave rise also to G/M colonies (Fig.2). These results demonstrate that the IL-3R α is not an ideal marker for discrimination between CMP and MEP.

TpoR as a complement in the isolation of MEPs

In a search for additional markers to achieve a better separation of the erythroid progenitors we found that cells expressing the receptor for Tpo gave rise solely to erythroid colonies when sorted to methylcellulose culture (data not shown). Therefore,

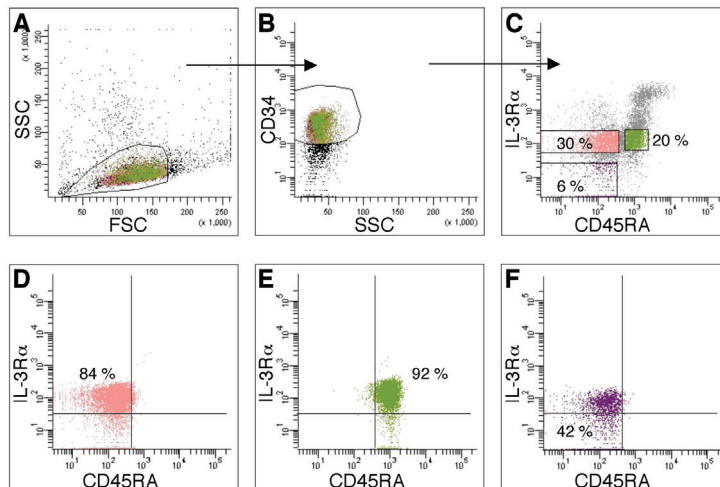


Figure 1. Isolation of proposed myeloid progenitors by means of the expression of IL-3R α and CD45RA. Enriched CD34⁺CD19⁻ cells from human adult bone marrow were gated for forward (FSC) and side scatter (SSC) properties (A), high CD34-expression (B) and subdivided according to their expression of IL-3R α and CD45RA (C). Subsequently, the sorted populations were resorted and analyzed (D-F). Cells within the FSC/SSC region contained <1% dead cells by DAPI. The percentage of each population within a region or quadrant is shown.

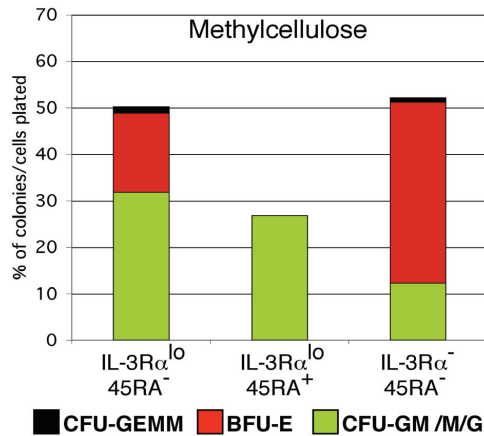


Figure 2. Colony forming potential of populations differing in IL-3R α - and CD45RA-expression. Cell populations isolated as described in Fig.1D-F were resorted to methylcellulose culture (10 cells/well) and scored for colony formation after 14 days. A total of 1320 CD34 $^{+}$ /CD19 $^{-}$ /IL-3R α^{lo} /CD45RA $^{-}$ cells, 1290 CD34 $^{+}$ /CD19 $^{-}$ /IL-3R α^{lo} /CD45RA $^{+}$ cells and 1354 CD34 $^{+}$ /CD19 $^{-}$ /IL-3R α^{-} /CD45RA $^{-}$ cells from 10 donors were sorted. CFU-GEMM, colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte; BFU-E, burst-forming units-erythroid; CFU-GM/M/G, CFU-granulocyte/macrophage, CFU-macrophage and CFU-granulocyte.

we added anti-TpoR to the previously used antibody-combination. Through serial gating (Fig. 3A-D), three populations defined as CD19 $^{-}$ /CD34 $^{+}$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^{-}$ /TpoR $^{-}$, CD19 $^{-}$ /CD34 $^{+}$ /IL-R α^{lo} /CD45RA $^{+}$ /TpoR $^{-}$ or CD19 $^{-}$ /CD34 $^{+}$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^{-}$ /TpoR $^{+}$ were sorted, resorted and reanalyzed, all three with more than 95% purity (Fig. 3E-G). As anticipated by our initial data the IL-3R $\alpha^{lo/-}$ /CD45RA $^{-}$ /TpoR $^{-}$ cells displayed a mixed colony-forming potential with multi-, bi- and unilineage colonies in methylcellulose culture, whereas the IL-3R α^{lo} /CD45RA $^{+}$ /TpoR $^{-}$ cells only contained G/M colony-forming potential and the clonogenic cells in the IL-3R $\alpha^{lo/-}$ /CD45RA $^{-}$ /TpoR $^{+}$ population were almost exclusively of erythroid lineage, with only a rare addition of small CFU-G (0.4 % or six colonies) (Fig. 4A). When single IL-3R $\alpha^{lo/-}$ /CD45RA $^{-}$ /TpoR $^{-}$ cells were sorted to methylcellulose with Epo, some wells contained colonies with cells of both erythroid and G/M-morphology and with the exclusion of Epo from the medium, the absent erythroid colonies were partly replaced by an increasing percentage of G/M colonies, confirming the presence of multipotency in this population (Fig. 4B). When the sorted populations were assayed for megakaryocytic potential in MegaCult (with the addition of Epo) the IL-3R $\alpha^{lo/-}$ /

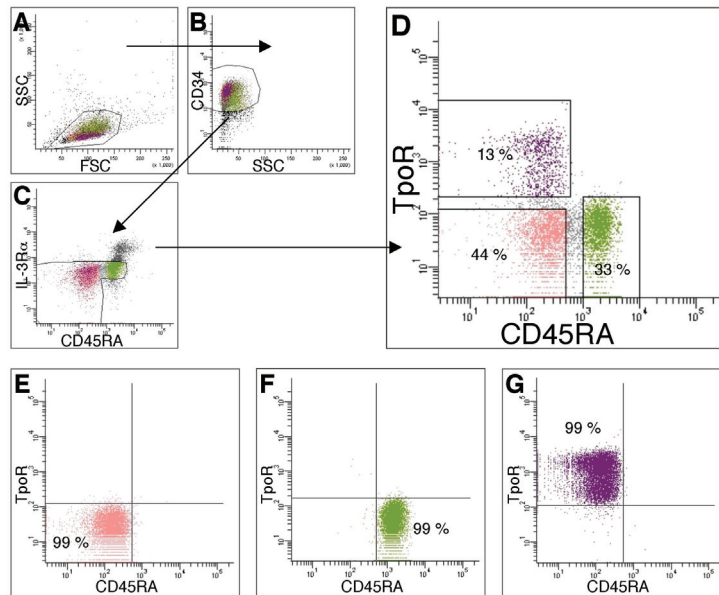


Figure 3. Isolation of putative myeloid progenitors by means of the expression of IL-3R α , CD45RA and TpoR. Enriched CD34⁺CD19⁻ cells from human adult bone marrow were sorted through serial gating as indicated in panels (A)-(C), excluding IL-3R α ⁺/CD45RA⁺ cells (dendritic and late monocyte progenitors) and IL-3R α ⁺/CD45RA⁺ cells, and further subdivided according to their expression of CD45RA and TpoR as shown in panel (D). Subsequently, the sorted populations were resorted and analyzed (E-G). The purity of each resorted population is shown as percentage within the respective quadrant.

CD45RA⁻/TpoR⁻ cells produced colonies of all lineages, whereas the IL-3R α ^{lo/-}/CD45RA⁻/TpoR⁺ cells produced colonies of megakaryocytic and erythroid origin, most of them smaller than in the CMP-population, but no G/M containing colonies (Fig.4C). The IL-3R α ^{lo/-}/CD45RA⁺/TpoR⁻ cells had no megakaryocytic or erythroid potential. The addition of Epo to the MegaCult assay did not hamper the megakaryocytic potential (if anything slightly more colonies with megakaryocyte potential appeared in cultures with Epo), but allowed simultaneous scoring of erythroid colonies and formation of an equal number of erythroid colonies as when cultured in methylcellulose (data not shown). The results suggest that TpoR is a better marker for the MEP-population than intensity of IL-3R α expression.

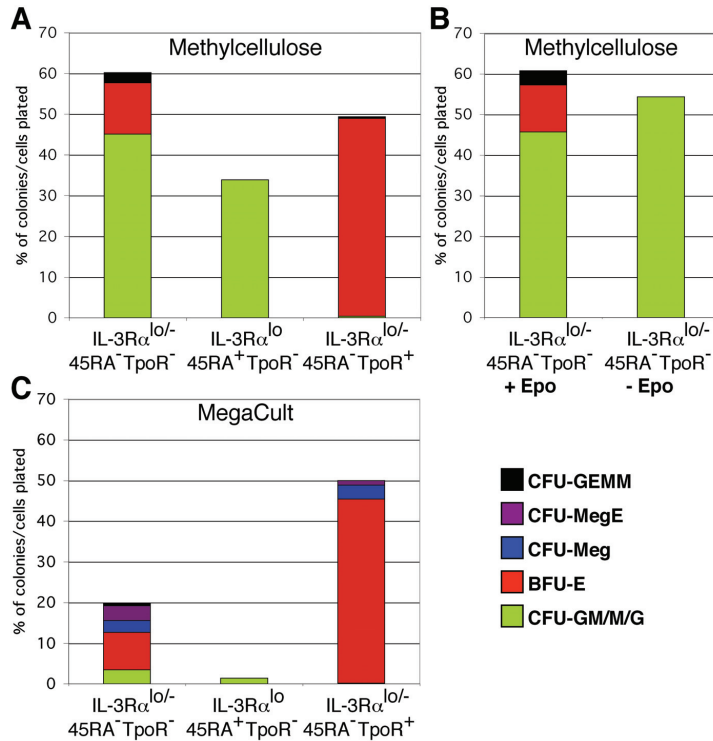


Figure 4. Colony-forming potential of populations differing in CD45RA- and TpoR-expression. Cell populations isolated as described in Fig.3E-G were resorted to methylcellulose culture or MegaCult (with Epo). (A) 1480 CD34 $^+$ /CD19 $^-$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^-$ cells, 1670 CD34 $^+$ /CD19 $^-$ /IL-3R α^{lo} /CD45RA $^+$ /TpoR $^-$ cells and 1689 CD34 $^+$ /CD19 $^-$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^+$ cells (5 donors) were sorted at 10 cells/well. (B) 576 CD34 $^+$ /CD19 $^-$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^-$ cells (6 donors) each were sorted at 1 cell/well to methylcellulose culture with or without Epo. After 14 days the cultures were scored for colony formation. (C) 21000 CD34 $^+$ /CD19 $^-$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^-$ cells (6 donors), 20761 CD34 $^+$ /CD19 $^-$ /IL-3R α^{lo} /CD45RA $^+$ /TpoR $^-$ cells (6 donors) and 14504 CD34 $^+$ /CD19 $^-$ /IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^+$ cells (6 donors) were sorted to MegaCult. After 13-14 days erythroid colonies were counted in an inverted microscope whereupon the cultures were fixed, stained for the megakaryocytic marker GPIIb/IIIa and scored for CFU-MegE, CFU-Meg and non-erythroid colonies. CFU-MegE, colony-forming units-megakaryocyte/erythroid; CFU-Meg, CFU-megakaryocyte.

TpoR expression discriminates between CMP and MEP and between maturing Meg/E progenitors

To further substantiate the notion that TpoR truly discriminates between MEP and CMP, these two populations in accordance with the two alternative definitions, were sorted and resorted from the same bone marrows to methylcellulose and MegaCult (the GMP was the same irrespective of the definition used). As shown in Fig. 5 both CMPs had mixed potentials with colonies of all lineages including multi- and bipotent

progenitors, in similar proportions. However, the $IL-3R\alpha^{lo/-}/CD45RA^{-}/TpoR^{+}$ population gave rise exclusively to Meg/E cells and, as previously noted, these were generally of more mature origin than the CMP-derived colonies. The $IL-3R\alpha^{-}/CD45RA^{-}$ population on the other hand did contain more CFU-MegE and more immature BFU-E and CFU-Meg than the $TpoR^{+}$ population, but also displayed a significant presence of cells with mixed and G/M potential in both methylcellulose and MegaCult cultures, resembling a CMP-population enriched for the Meg/E lineages.

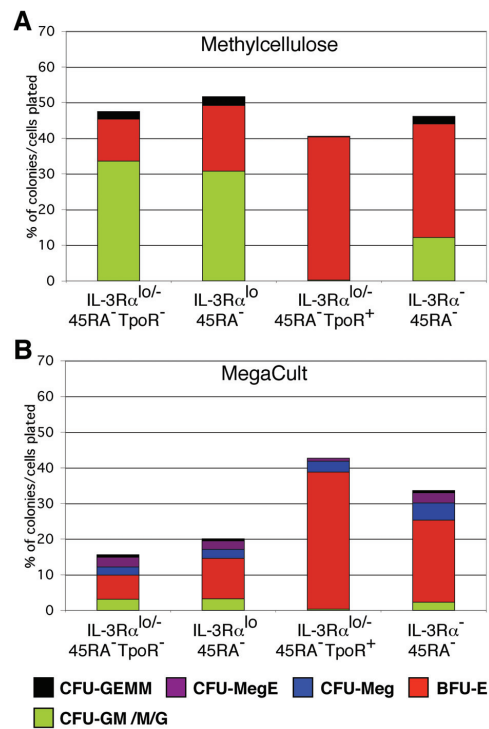


Figure 5. The use of TpoR expression improves discrimination between CMP and MEP. Cell populations from the same donor were isolated according to the original definition without TpoR (as in Fig.1) and the alternative definition with TpoR (as in Fig.3) and compared for their respective clonogenic capacity in methylcellulose (A) and MegaCult (B). Cells were sorted to methylcellulose and MegaCult with Epo and scored for colony formation after 13-14 days. The $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo/-}/CD45RA^{-}/TpoR^{+}$ populations were virtually free from G/M progenitors in both methylcellulose and MegaCult in contrast to the $CD34^{+}/CD19^{-}/IL-3R\alpha^{-}/CD45RA^{-}$ cells. The $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo/-}/CD45RA^{-}/TpoR^{-}$ and the $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo}/CD45RA^{-}$ populations showed similar mixtures of all colony types. $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo/-}/CD45RA^{-}/TpoR^{-}$, $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo}/CD45RA^{-}$, $CD34^{+}/CD19^{-}/IL-3R\alpha^{lo/-}/CD45RA^{-}/TpoR^{+}$ and $CD34^{+}/CD19^{-}/IL-3R\alpha^{-}/CD45RA^{-}$ cells were sorted from seven donors to methylcellulose (864, 920, 879, and 948 cells, respectively), and from three donors to MegaCult with Epo (10500, 9282, 2504 and 2252 cells, respectively).

These results clearly demonstrate that the addition of TpoR to the earlier definition gives a superior resolution and separation of the three progenitor populations. Accordingly, the modified definition was used in the following experiments and the terms CMP, GMP and MEP used from now on refer to the populations resulting from this version.

An additional advantage of the modified definition was discovered when the TpoR⁺ population was subdivided into high- (TpoR^{hi}) and low-expressing (TpoR^{lo}) cells as illustrated in Fig. 6. Sorting to methylcellulose showed that BFU-E accumulated in the TpoR^{lo}-population while cells with high expression primarily contained more mature CFU-E (data not shown). In accordance with this, when sorted to MegaCult, the TpoR^{lo}-cells gave rise to CFU-MegE and a relatively large proportion of medium-sized CFU-Meg colonies, whereas the TpoR^{hi}-cells had lower overall clonogenicity and primarily contained CFU-E, small CFU-Meg and no CFU-MegE. Notably, almost all cells with megakaryocytic clonogenic potential were located in the TpoR^{lo} population. These data indicate that labeling of the TpoR offers a way to separate early and late progenitors of the Meg/E lineages.

Surface and gene expression profiles of CMP, GMP and MEP

To investigate whether inclusion of other surface markers could further improve separation of the progenitor populations, we examined the surface and gene expression of other hematopoietic GFRs (Fig.7). Per definition TpoR was only expressed on MEPs, whereas the TpoR gene was expressed in all three populations and with the highest values in CMPs and a surprisingly small difference between MEP and GMP. C-kit (SCF receptor/CD117) appeared on more than 85% of cells in all three populations and with similar gene expression in all three although somewhat higher in MEPs. Flt3, the receptor of the early-acting flt3-ligand (FL), was on average expressed on close to 50% of CMPs, 75% of GMPs and at most 10% of MEPs, with the highest intensity on GMPs, and the gene expression of flt3 showed the same pattern. The common β -chain (β_c) of the IL-3, IL-5 and GM-CSF receptors (CD131) was expressed on about half of the CMPs, and 60-70% of GMPs and MEPs, but the gene expression

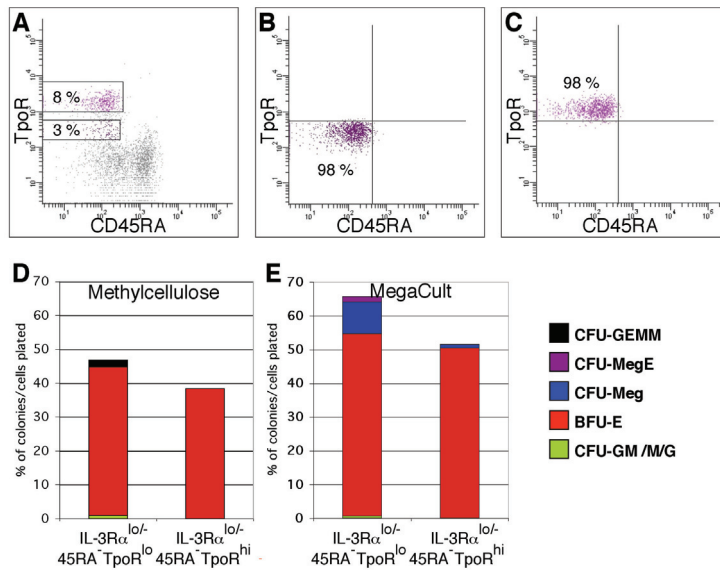


Figure 6. The level of TpoR expression discriminates between early and later stages of Meg/E differentiation. Cells were isolated as described in Fig.3 and cells with low or high TpoR expression as shown in panel (A) were sorted. Subsequently, sorted populations were resorted (B-C) to methylcellulose (D) (10 cells/well) and MegaCult with Epo (E). 312 IL-3Rα^{lo/lo}/CD45RA^{lo}/TpoR^{lo} cells and 583 IL-3Rα^{lo/lo}/CD45RA^{lo}/TpoR^{hi} cells (3 donors) were sorted to methylcellulose and 1226 and 3204 cells, respectively, (2 donors) were sorted to MegaCult. The megakaryocytic potential is higher in the TpoR^{lo} fraction, which contains all bipotent CFU-MegE. The purity of the resorted populations is shown in percentage within the respective quadrant.

differed considerably, with a notable increase in MEPs and very low levels in GMPs. The IL-3Rα was part of the original progenitor definition [8], where high-expressing primarily dendritic [10,11] and more differentiated G/M cells [12] as well as CD45RA⁺ cells without IL-3Rα on the surface were excluded (see Fig 3B). Thus, the majority of the GMPs had a low intensity expression of this receptor, while it appeared on about 60% of both CMPs and MEPs. The IL-3Rα gene expression was comparable in CMPs and GMPs, but very low in MEPs. Similarly, the GM-CSFRα (CD116) appeared on all three populations with the highest proportion of positive cells on GMPs, a pattern that essentially reflected the corresponding gene expression. As for more lineage-associated receptors, G-CSFR (CD114) was present on 40 and 50% of CMPs and GMPs, respectively, whereas MEPs were essentially negative and this pattern directly reflected the gene expression of G-CSFR. Due to the lack of a

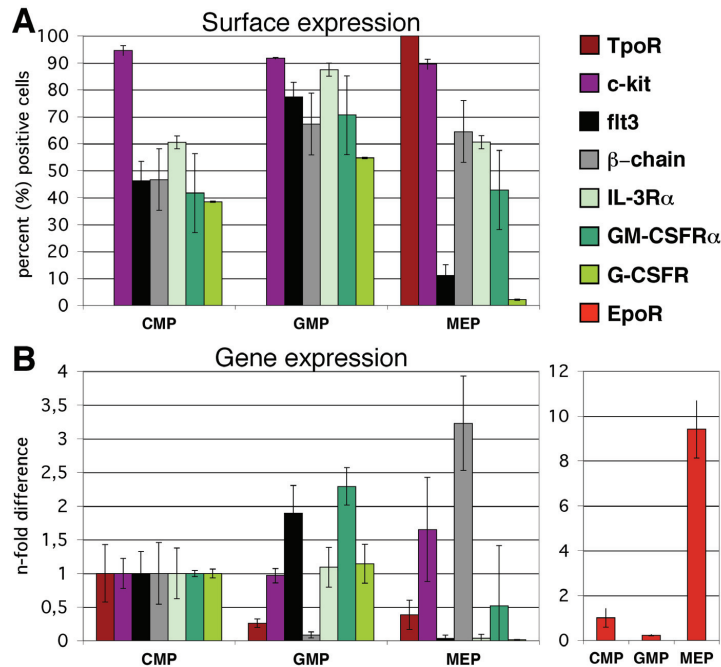


Figure 7. Surface and gene expression of GFRs in myeloid progenitors. (A) The myeloid progenitors CMP, GMP and MEP (defined as in Fig. 3) were analyzed for their surface expression of a selection of GFRs by flow cytometry. The graph shows the percentage of cells in the populations expressing the respective receptors and represents the mean values and standard deviation of three bone marrow samples. (B) The populations were sorted and resorted to PCR-tubes (500 cells/tube) containing lysis buffer and subsequently taken to RT and real-time PCR for gene expression analysis. Target quantities were normalized to 18S ribosomal RNA. The graph shows n-fold difference in gene expression relative to the CMP-population and represents the mean value and standard deviation of three bone marrows. Regarding the EpoR only the gene expression could be analyzed.

commercially available EpoR-antibody FACS-analysis was not possible, but EpoR gene expression showed a ten-fold upregulation between CMPs and MEPs and a downregulation between CMPs and GMPs of a similar magnitude.

In addition to GFRs, the gene expression of a number of transcription factors and other lineage-associated proteins was investigated. The obtained expression patterns (Fig. 8) supported the results of the clonogenic assays. GATA-2 had comparable expression in CMPs and MEPs, but considerably lower levels in GMPs. SCL and GATA-1 were most highly expressed in MEPs, to a lesser extent in CMPs and had minor or no

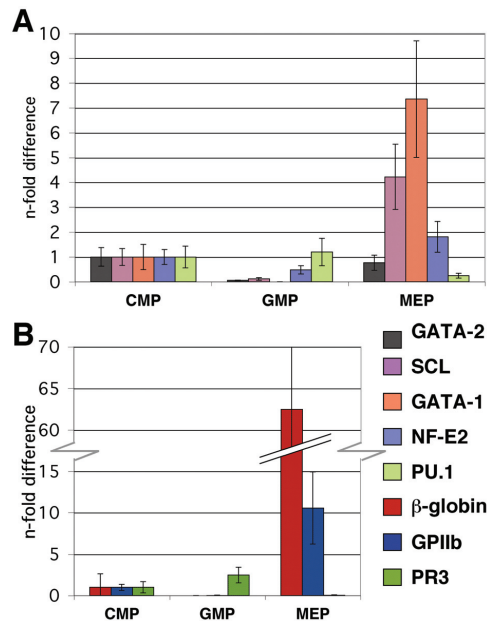


Figure 8. Gene expression of hematopoietic transcription factors and lineage markers in myeloid progenitors. The myeloid progenitors CMP, GMP and MEP (defined as in Fig. 3) were sorted and resorted directly to PCR-tubes (500 cells/tube) containing lysis buffer and subsequently taken to RT and real-time PCR for gene expression analysis of transcription factors (A) and other lineage markers (B). Target quantities were normalized to 18S ribosomal RNA. The graphs show n-fold difference in gene expression relative to the CMP-population and represent the mean value and standard deviation of three bone marrows.

expression in GMPs. NF-E2 increased slightly in MEPs and decreased in GMPs, in relation to the CMP-expression. Similar, but more pronounced, patterns were seen for β -globin and GPIIb, with undetectable levels in the GMP-population, low levels in CMPs, but a considerable increase in MEPs. The G/M-associated transcription factor PU.1 was expressed at significantly higher levels in CMPs and GMPs than in MEPs and neutrophil proteinase 3 (PR3) was higher expressed in GMPs than CMPs, while disappearing in MEPs.

Flt3 expression discloses heterogeneity within the CMP-population

As demonstrated above there were distinct differences in surface expression of flt3 between the GMP and MEP populations and even more so at gene expression level. This made us suspect that surface flt3 expression among CMPs might reflect

heterogeneity within the population. Thus CMPs (IL-3R α ^{low}/CD45RA⁻/TpoR⁻) were sorted into flt3 positive and negative subpopulations (Fig. 9A) and assayed for colony-formation in methylcellulose. As shown in Fig.9D flt3-negative CMPs contained a significantly higher erythroid potential than flt3-positive CMPs.

DISCUSSION

Many attempts have been made at isolating the early myeloid progenitors, but a generally applicable and reproducible definition has proven allusive. It is generally believed that a CMP separating from the CLP and subsequently giving rise to GMPs and MEPs, constitutes the major developmental pathway. This assumption was supported when Manz *et al.* presented a way to isolate three populations representing the CMP, GMP and MEP, based on differences in the surface expression of IL-3R α and CD45RA. The almost complete absence of IL-3R α gene expression in our MEPs (TpoR⁺ cells) indicates that this definition was correct, but due to the low resolution between weakly positive and negative IL-3R α expression their results are difficult to repeat.

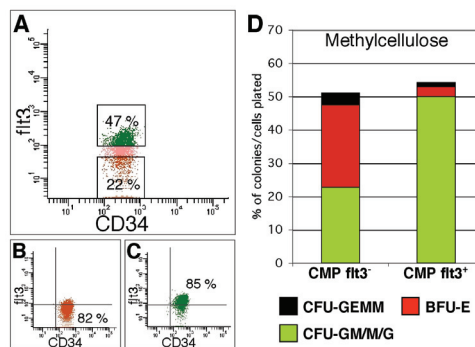


Figure 9. Clonogenic capacity of flt3⁻ and flt3⁺ CMPs. CD34⁺/CD19⁻/IL-3R α ^{low}/CD45RA⁻/TpoR⁻ cells (as shown in Fig.3E) were sorted into flt3 positive and negative subpopulations (A). 530 IL-3R α ^{low}/CD45RA⁻/TpoR⁻/flt3⁻ cells and 530 IL-3R α ^{low}/CD45RA⁻/TpoR⁻/flt3⁺ cells (3 donors) were resorted (B-C) to methylcellulose (D) (10 cells/well). After 14 days the cultures were scored for colony formation. Both populations contained multipotent (CFU-GEMM), erythroid (BFU-E) and granulocyte/macrophage (CFU-GM/M/G) colonies but GM-derived colonies were enriched in the flt3⁺ subpopulation. The purity of the resorted populations is shown in percentage within the respective quadrant.

Therefore, we looked for a complementing surface antigen to achieve a more reproducible separation of the myeloid progenitors and found that TpoR was a candidate marker. When combining TpoR with the markers previously used the IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^-$ population, representing putative CMPs, showed a mixed early myeloid potential with the whole spectrum of colony-forming cells. Moreover, the increased number of G/M-colonies appearing in single cell-cultures in the absence of Epo implies that many of the cells giving rise to erythroid colonies in standard culture were not restricted to this fate, thus reinforcing the CMP-definition. As expected of a GMP-population the IL-3R α^{lo} /CD45RA $^+$ /TpoR $^-$ cells formed only G/M colonies irrespective of culture system and the IL-3R $\alpha^{lo/-}$ /CD45RA $^-$ /TpoR $^+$ cells, the putative MEPs, displayed a purely Meg/E clonogenicity primarily of a somewhat more mature origin than colonies from the CMP-population, but clearly including bipotent CFU-MegE. Notably, rather than hampering growth of the separate lineages through simultaneous induction of competing megakaryocyte and erythroid developments, the presence of both Epo and Tpo seemed to slightly increase the number of colonies containing megakaryocyte potential and erythroid potential for both putative CMPs and MEPs. This is consistent with the synergistic effect that has been suggested for these two cytokines in both megakaryopoiesis and erythropoiesis [13-18] and further supports the notion that TpoR is expressed on bipotent and unipotent progenitors of the Meg/E pathway.

The direct comparison of the two aforementioned isolation methods provided the final confirmation of the superiority of our modified strategy. Moreover, the addition of TpoR offers another advantage as its low versus high expression allows for separation of cells within the MEP-population according to their developmental potential. All Meg/E progenitors with high differentiation and proliferative potential (e.g. the bipotent progenitors) seem to have low expression of TpoR, whereas more differentiated cells express higher levels. The near lack of megakaryocytic clonogenicity in the TpoR hi -population can be explained by maturing cells of this lineage losing their clonogenic potential, before they reach full TpoR expression.

There are very few previous reports on the surface expression of TpoR on hematopoietic stem and progenitor cells demonstrated by FACS analysis. Solar *et al.* [19] used a biotinylated hamster-anti-murine c-mpl monoclonal antibody and found that approximately 70% of both murine Lin^{lo}Sca⁺c-kit⁺ and human CD34⁺CD38⁻ hematopoietic stem cells showed TpoR surface expression and that cells with repopulating capacity primarily resided within the TpoR-positive fraction. This is in contrast with the murine-anti-human-TpoR monoclonal antibody used in the present report (PE- or APC-conjugated CD110, clone BAH-1) [20], which does not stain human CD34⁺/CD38^{lo/-} cells. Furthermore, the TpoR-positive cells are all concentrated within the CD34⁺/CD38^{high} population (Fig.10). Taking into consideration that Tpo alone or together with other cytokines stimulates survival and proliferation of early hematopoietic cells [21, 22] it is reasonable to assume that these cells have receptors for Tpo, but at too low a concentration to be detected by the current antibody. Thus the CD34⁺CD45RA⁻TpoR⁺ cell population characterized as MEP does not harbor any

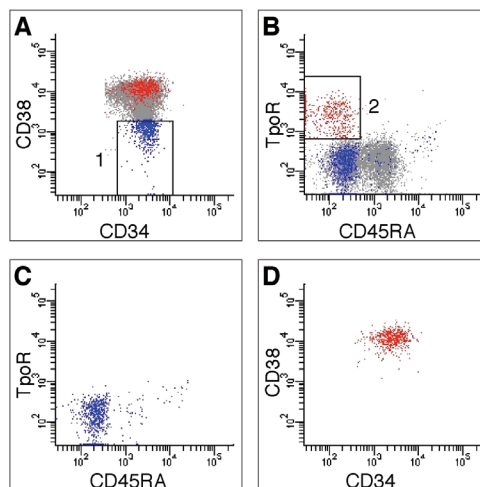


Figure 10. CD34⁺CD38^{lo/-} cells do not express TpoR. CD34⁺ human marrow cells were analyzed for their surface expression of CD38, TpoR (CD110), and CD45RA. Panel (A) shows the CD38 expression of CD34⁺ cells. Panel (B) shows CD45RA and TpoR expression of all CD34⁺ cells in panel (A). Panel (C) shows the TpoR expression of CD34⁺/CD38^{lo/-} cells in region #1 in panel (A). Panel (D) shows the CD38 expression of the TpoR-positive cells in region #2 in panel (B). CD34⁺CD38^{lo/-} cells are TpoR-negative and all TpoR-positive cells are CD38^{high}. Identical results were reached with four other marrow samples.

early stem cells, and the CD34⁺CD38^{lo/-} cells remaining within the sorted populations in this report will all be confined to the CMP-population.

Unlike the well-defined expression of the TpoR most of the other investigated GFRs did not display marked differences in expression between the three populations and will most likely not contribute significantly to further separation of the progenitor populations with one possible exception, i.e. flt3. The enrichment of G/M colonies within the flt3⁺ part of the CMP-population, as demonstrated here, could be a sign of pre-commitment towards GMPs. Flt3 has been shown to be expressed on the majority of human *in vivo* repopulating CD34⁺ HSCs [23,24] and the low levels seen on multipotent cells to be maintained or upregulated initially in the G/M-pathway, but shut down immediately in erythroid and megakaryocytic differentiation [25-30], in agreement with our findings here. A relationship between expression of flt3 and GM-potential within the CMP population has also been observed in mice [31].

Furthermore, our GFR studies clearly demonstrate that the gene expression pattern of any GFR does not necessarily predict or correlate with the surface expression of that specific receptor. The apparent surface expression of IL-3R α on MEPs, without a corresponding gene expression, can very likely be ascribed to the previously mentioned difficulty of obtaining adequate resolution of surface IL-3R α . However, no such explanation is available for the far greater discrepancy between surface and gene expression of the β_c and TpoR, indicating that post-transcriptional mechanisms have a major role in regulating surface expression of these, and probably other, GFRs. Hence, gene expression for the EpoR cannot be used for a definite prediction of the surface expression. Nevertheless, the marked increase in the MEP-population concurs with the main function of Epo as a stimulator of erythroid, and to some extent megakaryocytic, differentiation [16,32] and the obtained gene expression pattern, probably gives a true estimate of the surface expression, just as for the equally lineage-associated G-CSFR.

The gene expression of the investigated transcription factors gave further support to the progenitor classification. The primarily early acting GATA-2 [33,34] was

expressed in accordance with previous reports [9,35-37] and dramatically downregulated in GMPs together with GATA-1 and SCL, whereas GATA-2 was maintained in MEPs concomitantly with a significant upregulation of GATA-1 and SCL, known regulators of Meg/E differentiation [9,32,38,39]. NF-E2, regarded as a Meg/E-associated transcription factor [40,41], did appear in the GMPs, but since we have previously shown that NF-E2 is expressed during G/M differentiation [9], this suggests an unexplored role for NF-E2, rather than disputing the purity of the GMP-population. The differences in PU.1 in GMPs and MEPs further dissociate these populations, as well as the expression patterns of the lineage-restricted proteins β -globin (erythroid), GPIIb (megakaryocyte) and PR3 (G/M). The fact that β -globin, GPIIb and PR3 showed some expression even in CMPs, is most likely a sign of a pre-commitment activation of these genes, as have been suggested for the lineage-associated transcription factors [8,37].

The use of TpoR, as shown here, to obtain a more defined common myeloid population as well as purified Meg/E progenitors at different stages of commitment, should greatly facilitate studies of the mechanisms of myeloid lineage choice.

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