

Role of Transcription Factors in Differentiation and Reprogramming of Hematopoietic Cells

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(Received for publication on September 29, 2010)

(Revised for publication on November 22, 2010)

(Accepted for publication on January 20, 2011)

Differentiation of hematopoietic cells is a sequential process of cell fate decision originating from hematopoietic stem cells (HSCs), allowing multi- or oligopotent progenitors to commit to certain lineages. HSCs are cells that are able to self-renew and repopulate the marrow for the long term. They first differentiate into multipotent progenitors (MPPs), which give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CMPs then differentiate into granulocyte monocyte progenitors (GMPs) and megakaryocyte erythroid progenitors (MEPs), which are the precursors of granulocytes/monocytes and erythrocytes/megakaryocytes, respectively. Lineage specification at differentiation branch points is dictated by the activation of lineage-specific transcription factors such as C/EBP α , PU.1, and GATA-1. The role of these transcription factors is generally instructive, and the expression of a single factor can often determine cell fate. Differentiation was long regarded as an irreversible process, and it was believed that somatic cells would not change their fate once they were differentiated. This paradigm was first challenged by the finding that ectopic cytokine signals could change the fate of differentiation, probably through modulating internal transcription networks. Subsequently, we and others showed that virtually all progenitors, including CLPs, CMPs, GMPs, and MEPs, still retain differentiation plasticity, and they can be converted into lineages other than their own by ectopic activation of only a single lineage-specific transcription factor. These findings established a novel paradigm for cellular differentiation and opened up an avenue for artificially manipulating cell fate for clinical use. (Keio J Med 60 (2) : 47–55, June 2011)

Keywords: transcription factor, hematopoiesis, differentiation, reprogramming, plasticity

Introduction

It has been only 5 years since the first report of successful generation of induced pluripotent stem (iPS) cells by defined factors.¹ Now we are in the decade of cellular reprogramming,^{2–4} and people take it for granted that somatic cells of any type can be turned into an immature state by manipulation of transcription networks. Differentiation plasticity of somatic cells was actually proven in a hematopoietic system well before the generation of iPS cells. We and others have reported lineage conversion of hematopoietic progenitors as well as mature hematopoietic cells in response to certain stimuli such as ectopic cytokine signals or activation of transcription factors.^{5–8}

The hematopoietic system is undoubtedly the best-studied differentiation system throughout the body. Differentiation of hematopoietic cells originates from hematopoietic stem cells (HSCs), followed by a hierarchical process of cell fate decision and lineage commitment leading to terminally differentiated mature blood cells.⁹ These processes are often dictated by the instructive action of hematopoietic transcription factors, which ultimately restrict the fate of differentiation.¹⁰ It was long thought that commitment was an irreversible process, and that cells differentiated into a certain lineage could not change their fate. However, this paradigm was first challenged by the seminal work of Kondo et al. just over 10 years ago, showing that ectopic cytokine signals could change

the fate of differentiation.⁶ Since then, experimental evidence has accumulated supporting enormous plasticity of hematopoietic cells during differentiation, and a novel paradigm has been reached that differentiation can be controlled by modulation of transcription networks.

This review will focus on the differentiation pathways of hematopoiesis, the role of transcription factors in hematopoietic differentiation, and the amazing plasticity retained by hematopoietic progenitors and mature blood cells.

Differentiation Program of Hematopoietic Cells

Outstanding efforts by many groups over a decade have now arrived at a comprehensive road map of hematopoietic differentiation from hematopoietic stem cells (HSCs) to terminally differentiated cells.^{9,11,12} This was mostly done by prospective isolation of HSCs and differentiation intermediates including hematopoietic progenitor cells (HPCs) using flow cytometry (FACS) (**Fig. 1**).

HSCs are cells that can self-renew and can produce all types of blood cells throughout life. The two critical features of HSCs that distinguish them from other hematopoietic cells are their self-renewal capacity and multilineage differentiation potential. In murine hematopoiesis, HSCs make up a very small fraction of cells in the bone marrow (BM) and lack lineage-affiliated cell surface markers (Lin) but express high levels of c-Kit and Sca-1 (KSL cells). The most primitive HSCs with self-renewal and long-term repopulating (LTR) capacities (long-term HSCs; LT-HSCs) are enriched in Thy1.1^{low/-}, CD34^{low/-} or CD150⁺ Flt3/Flk2⁻ KSL.¹³⁻¹⁵ CD150⁺CD244⁻CD48⁻ cells have also been described as nearly pure HSCs.¹⁵ Wilson et al. have further shown that CD150⁺CD41⁻CD48⁻CD34⁻ KSL cells are more immature HSCs, a small subset of which (about 20%) is in a dormant state.¹⁶ These populations of cells constitute about 0.01% of total BM cells, and of this 0.01%, 1 in 3 to 1 in 5 cells were found to have LTR capacity in a competitive repopulation assay.

Cells with multilineage differentiation potential that can reconstitute the marrow for a limited period are termed short-term HSCs (ST-HSCs), and are the immediate downstream progenies of LT-HSCs. ST-HSCs give rise to multipotent progenitors (MPPs), cells with multilineage potential and a shorter reconstitution capability than ST-HSCs. ST-HSCs and MPPs are considered to reside in the CD34⁺ Flt3⁻ KSL and CD34⁺ Flt3⁺ KSL fractions, respectively. However, other reports have stated that there is no clear-cut separation between these two populations. In fact, expression of Flt3 was reported recently to couple with a loss of MegE potential, as described below.

Diversification of myeloid and lymphoid pathways follows after the MPP stage (**Fig. 1A**). Common lymphoid progenitors (CLPs) are the most primitive common precursor for T and B lymphocytes and have a Lin⁻ IL-7Ra⁺

Thy-1⁻ Sca-1^{lo/-} c-Kit^{lo} phenotype.¹⁷ The hallmark of this population is the expression of interleukin (IL)-7 receptor, which is critical for the expansion of the early lymphoid population. Indeed, abrogation of IL-7R leads to severe combined immunodeficiency (SCID).

Common myeloid progenitors (CMPs), isolated as a Lin⁻ IL-7Ra⁻ Sca-1⁻ c-Kit⁺ FcgRII/III^{lo} CD34⁺ population, are the earliest precursors for non-lymphoid cells that have the potential for erythroid, megakaryocyte, granulocyte and monocyte development¹⁸ (**Fig. 1A**). CMPs further generate granulocyte-monocyte progenitors (GMPs; Lin⁻ IL-7Ra⁻ Sca-1⁻ c-Kit⁺ FcgRII/III^{hi} CD34⁺) and megakaryocyte-erythroid progenitors (MEPs; Lin⁻ IL-7Ra⁻ Sca-1⁻ c-Kit⁺ FcgRII/III^{lo} CD34⁻), which give rise to granulocytes/monocytes and erythrocytes/megakaryocytes, respectively¹⁸ (**Fig. 1A**). These progenitors were shown to generate respective progenies in colony assays and in *in vivo* transplantation experiments.

It was initially thought that bifurcation of the myeloid vs. lymphoid lineage occurred right after the MPP stage, but the story later turned out to be not as simple as that. Recent evidence showing the existence of MPPs lacking megakaryocyte/erythroid (MegE) potential suggests that MPPs are heterogeneous.^{9,11,12} A current model of myeloid/lymphoid commitment predicts that some MPPs selectively lose MegE potential, leaving the lymphoid and granulocyte/monocyte potential intact, on their way to becoming newly identified lymphoid primed multipotent progenitors (LMPPs) or granulocyte-monocyte-lymphoid progenitors (GMLPs) (**Fig. 1B**). LMPPs are described as CD34⁺MPPs expressing Flt3, and GMLPs are CD34⁺MPPs marked by PU.1 expression. Of note, CMPs are marked by the expression of GATA-1 as described below. Please refer to the selected readings for a detailed scheme of lineage commitment from HSCs.¹²

Transcriptional Control of Cell Fate Decision

It is widely known that lineage commitment of hematopoietic cells requires precise regulation of transcription factors. In the past few decades, disruption of various transcription factors in the mouse genome has uncovered a fine picture of transcriptional networks regulating hematopoietic differentiation (**Fig. 2**).

Emergence of CMPs from MPPs is considered to depend on the simultaneous expression of PU.1 and GATA-1. PU.1 and GATA-1 play critical, instructive roles for granulocyte/monocyte (GM) and MegE lineage commitment, respectively.¹⁹⁻²¹ PU.1 transactivates a number of GM and lymphoid genes, and PU.1-deficient mice display profound defects in the development of B cells, monocytes and granulocytes.²² On the other hand, GATA-1 induces MegE-related genes, and is critical for the development of megakaryocytes and erythroid cells.²³⁻²⁷ A counter-regulatory mechanism of PU.1 and GATA-1 activities has been reported in which either one of the two

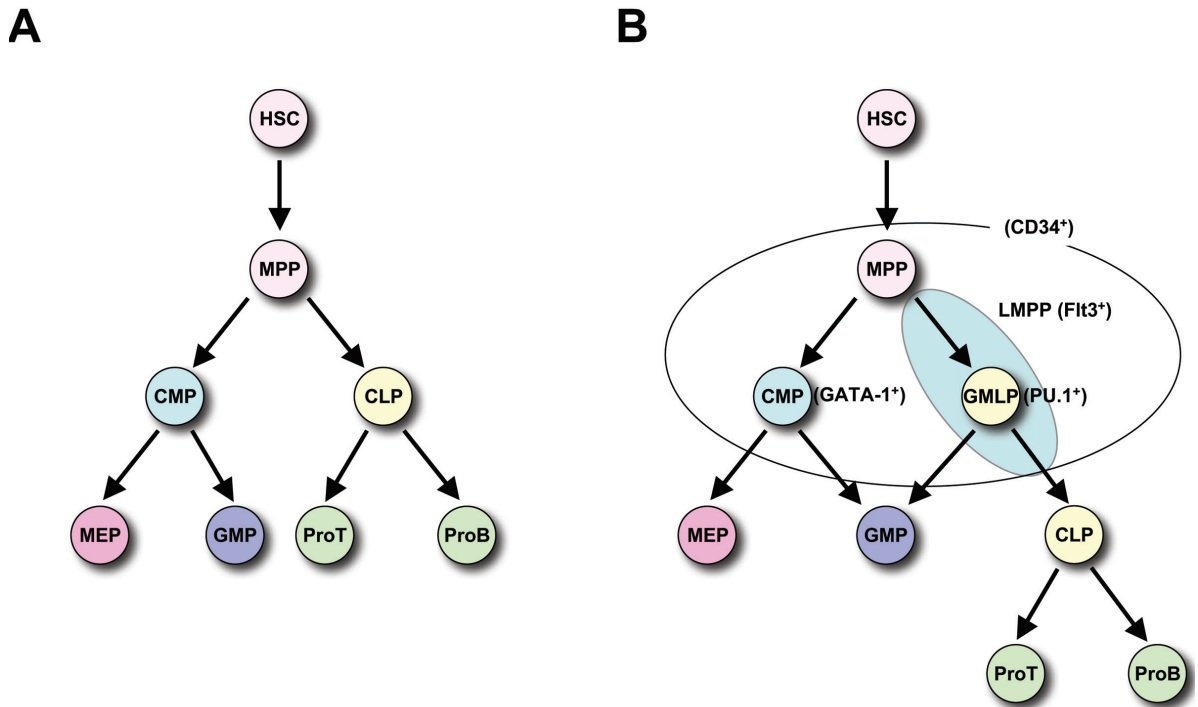


Fig. 1 Cellular pathways of hematopoietic differentiation.

(A) Classical myeloid versus lymphoid differentiation pathway. Myeloid and lymphoid pathways diverge right after the multipotent progenitor (MPP) stage. (B) The new developmental pathways of myeloid versus lymphoid differentiation. This model integrates newly identified lymphoid-primed multipotent progenitors (LMPPs) and granulocyte-monocyte-lymphoid progenitors (GMLPs), multipotent progenitors that selectively lost MegE potential. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor.

factors represses the other's expression or transcriptional activity.^{28–31} A current model predicts that low-level expression of both PU.1 and GATA-1 in CMPs makes them competent for both GM and MegE lineages.³² Upregulation of PU.1 activity is considered to be a critical step for CMPs to commit to the GM lineage (and lose MegE activity), whereas activation of GATA-1 skews CMPs to follow the MegE pathway.^{33,34} The molecular mechanism for this reciprocal regulation of PU.1 and GATA-1 is currently not well understood.

Differentiation of GMPs from CMPs depends on the expression of CCAAT/enhancer binding protein (C/EBP) α . C/EBP α -deficient mice lack mature neutrophils and eosinophils,³⁵ and conditional disruption of C/EBP α in adult mice leads to the block of CMP-GMP transition.³⁶ C/EBP α activates a number of myeloid genes, such as granulocyte-colony stimulating factor receptor (G-CSF R), macrophage colony stimulating factor receptor (M-CSF R), myeloperoxidase (MPO), and neutrophil elastase.^{37–40} The early stage of neutrophil differentiation is also regulated by Gfi-1.^{41–44} Gfi-1^{-/-} mice have defects in neutrophil differentiation subsequent to the promyelocyte stage. The terminal stage of neutrophil differentiation critically depends on another member of the C/EBP fam-

ily, C/EBP ϵ . C/EBP ϵ plays a critical role in the expression of secondary and tertiary granule proteins, since lack of C/EBP ϵ leads to the neutrophil-specific granule deficiency phenotype.⁴⁵

As discussed above, GATA-1 is critical for commitment and further differentiation of the MegE lineage. The stem cell leukemia (SCL) gene and EKLf are also essential for erythropoiesis, since lack of either protein leads to severe anemia.^{46,47} GATA-1 collaborates with FOG-1 to induce erythroid differentiation, whereas GATA-1 together with AML-1 induces megakaryocytic differentiation.

Early lymphoid differentiation requires Ikaros.⁴⁸ Commitment and further differentiation of CLPs to the B lymphoid lineage critically depends on the expression of three transcription factors: E2A, EBF and Pax5.⁴⁹ Loss of E2A or EBF leads to differentiation block at the pre-pro-B cell stage.⁴⁹ In the case of T cells, GATA-3 is absolutely essential for early thymocyte differentiation.⁵⁰

Sequential Activation of Transcription Factors Specifies Eosinophil and Basophil Commitment

It has been shown that mast cells, eosinophils and basophils are progenies of GMPs. Eosinophil-committed

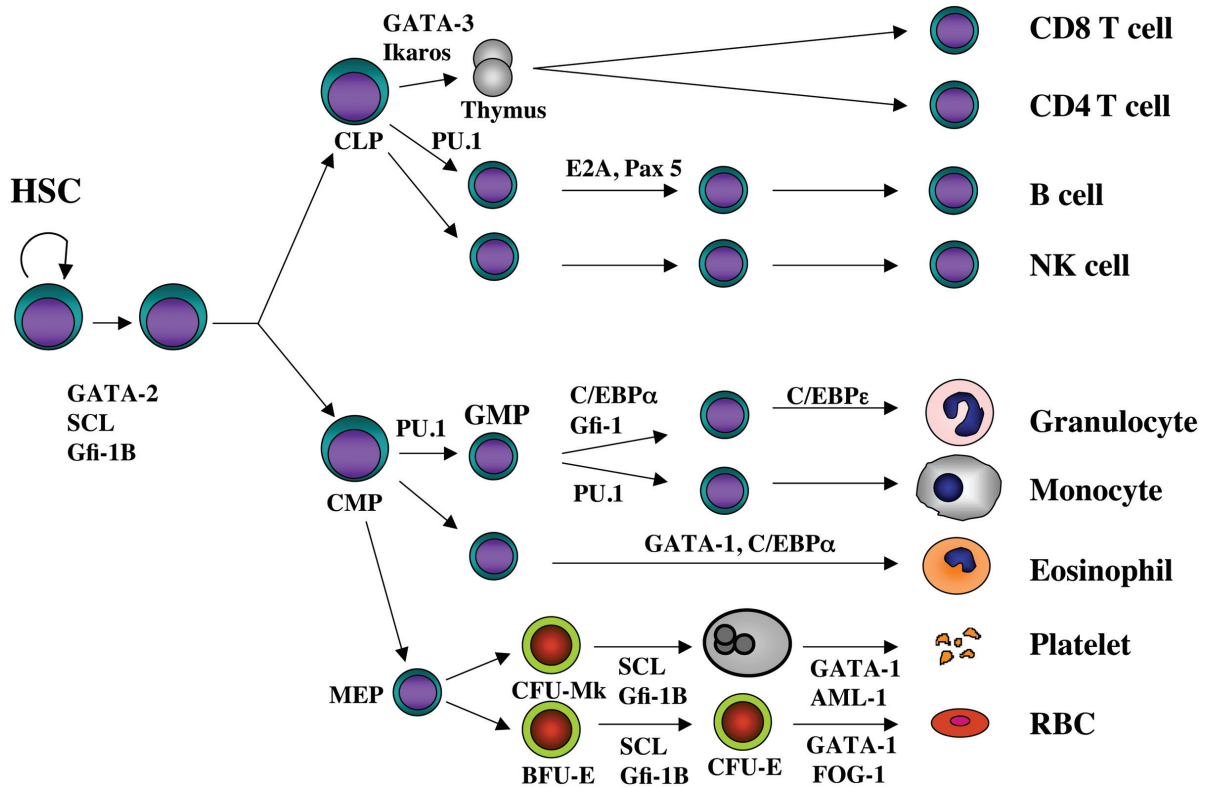


Fig. 2 Transcription factors regulating hematopoietic differentiation.

Transcription factors regulating lineage specification at various differentiation branch points or facilitating differentiation along specific lineage pathways are indicated.

progenitors (EoPs) can be isolated as cells with the IL-5Ra⁺ Lin⁻ Sca-1⁻ CD34⁺ c-Kit^{lo} phenotype.⁵¹ Mast cell progenitors (MCPs) and basophil progenitors (BaPs) have the Lin⁻ CD34⁺ β 7^{hi} Fc ϵ RI α ^{lo} Fc γ RII/III⁺ c-Kit^{lo} Thy-1⁻ and Lin⁻ CD34⁺ Fc ϵ RI α ^{hi} c-Kit⁻ phenotypes, respectively.⁵² It has been reported that a common precursor for mast cells and basophils, the basophil-mast cell bipotent progenitor (BMCP), exists in spleen, and has the Lin⁻CD13^{lo}CD34⁺ β 7^{hi}Fc ϵ RI α ⁻Fc γ RII/III⁺c-Kit⁺Thy-1⁺ phenotype.⁵³

Interestingly, the order of expression of C/EBP α and GATA-2 specifies the eosinophil versus basophil lineage from uncommitted progenitors⁵⁴ (**Fig. 3**). If C/EBP α expression is followed by GATA-2, the cells become eosinophils; in turn, if the order of expression is reversed, they become basophils. The same uncommitted progenitors can also generate neutrophil-committed progenitors and MCPs. However, sustained C/EBP α expression is required for the cells to go into the neutrophil lineage through GMP. In contrast, sustained GATA-2 expression leads cells to become MCPs.

Conversion of Hematopoietic Lineages by Ectopic Cytokine Signals

It was long believed that differentiation was an irreversible process and cells could not change lineages once they were differentiated. However, this paradigm was recently challenged by the finding of lineage infidelity of committed hematopoietic progenitors.^{6,55,56} A seminal report from Kondo et al. showed that enforced signals from interleukin (IL)-2 or granulocyte macrophage-colony stimulating factor (GM-CSF) receptor could induce myeloid conversion in common lymphoid progenitor (CLP) and pro-T cells.⁶ When CLPs and pro T cells taken from transgenic mice expressing IL-2 receptor β chain were cultured in the presence of IL-2, cells converted to granulocytes and monocytes after several days. Irreversible commitment to myeloid lineage required at least 2 days of cytokine stimulation. Interestingly, pro B cells and committed DN3 thymocytes were resistant to this IL-2-induced lineage conversion.^{6,55} In addition, CLPs and pro T cells could only be directed to GM lineages even under erythroid culture conditions. These data clearly showed that at least CLPs and pro T cells possess latent myeloid differentiation potential, which can be activated by ectopic cytokine signals.

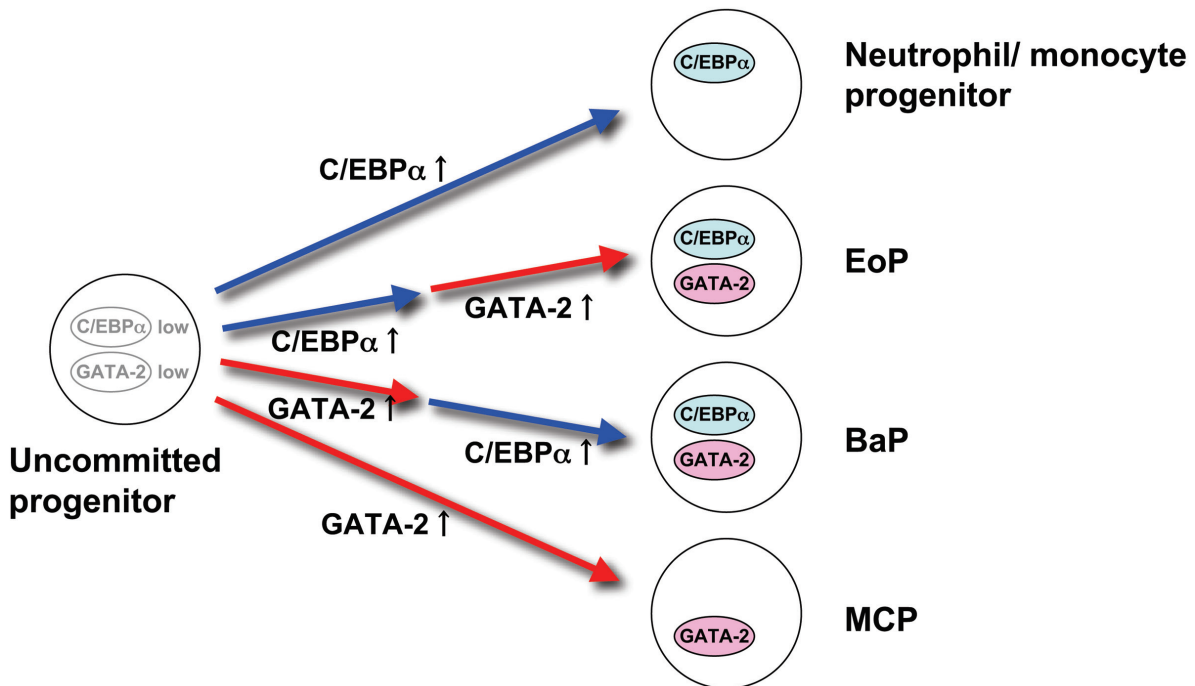


Fig. 3 Order of expression of C/EBPα and GATA-2 specifies eosinophil versus basophil lineage commitment.

In uncommitted progenitors such as CMPs, the expression of C/EBPα and GATA-2 is kept at a low level. If C/EBPα or GATA-2 is upregulated and maintained, cells are committed to neutrophil/monocyte or mast cell pathways, respectively. If C/EBPα is upregulated first and GATA-2 follows, the cells become eosinophils. If the order of expression is reversed, they become basophils. EoP, eosinophil-committed progenitor; BaP, basophil-committed progenitor; MCP, mast cell progenitors.

Ablation of Lineage-specific Transcription Factors Unfixes Lineage Commitment

As described, commitment to a specific lineage critically depends on the activity of lineage-specific transcription factors. The process of commitment may require only transient action of transcription factors, but in some cases, their activity must be maintained throughout differentiation to preserve lineage identity.^{57–59} One such example is Pax5, an essential regulator for B cell differentiation (Fig. 4). Pax5 is critical for transition from pre-pro-B cells to pro-B cells, and its absence leads to differentiation arrest at the early pro-B cell stage. Interestingly, Pax5^{-/-} pro-B cells can be cultured indefinitely on stromal cells with IL-7. Furthermore, these cells are not actually committed to B cells, but instead retained multilineage differentiation potential.^{57,60} Conditional inactivation of Pax5 in pro-B cells canceled their commitment to the B cell lineage, and they regained their differentiation potential to macrophages and T cells.⁵⁸ Moreover, conditional ablation of Pax5 led to the dedifferentiation of mature B cells back to early uncommitted progenitors that were capable of differentiating into T cells *in vivo*.⁵⁹ These surprising observations underscore the critical role of Pax5 not only in commitment to the B cell lineage but also in

maintenance of B cell identity.

Another example came from the analysis of GATA-1 knockout mice (Fig. 4). GATA-1^{-/-} proerythroblasts proliferate indefinitely on OP9 stroma in the presence of erythropoietin (Epo) and display differentiation potential to myeloid and mast cells by the stimulation of granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-3, respectively.⁶¹ This observation also suggests that lack of lineage-specific transcription factors unfixes commitment to the respective lineage and allow cells to dedifferentiate back to an immature state that retains multilineage differentiation potential.

Lineage Conversion by Ectopic Expression of Lineage-specific Transcription Factors

Lineage infidelity of differentiated hematopoietic cells and strong lineage instructing capability of lineage-specific transcription factors suggested to us that such transcription factors may induce lineage conversion when expressed ectopically (Fig. 4).

Iwasaki et al. were the first to show that enforced expression of lineage-specific transcription factor induced transdifferentiation of hematopoietic progenitors.⁷ They showed that ectopic expression of GATA-1 in CLPs, pro-

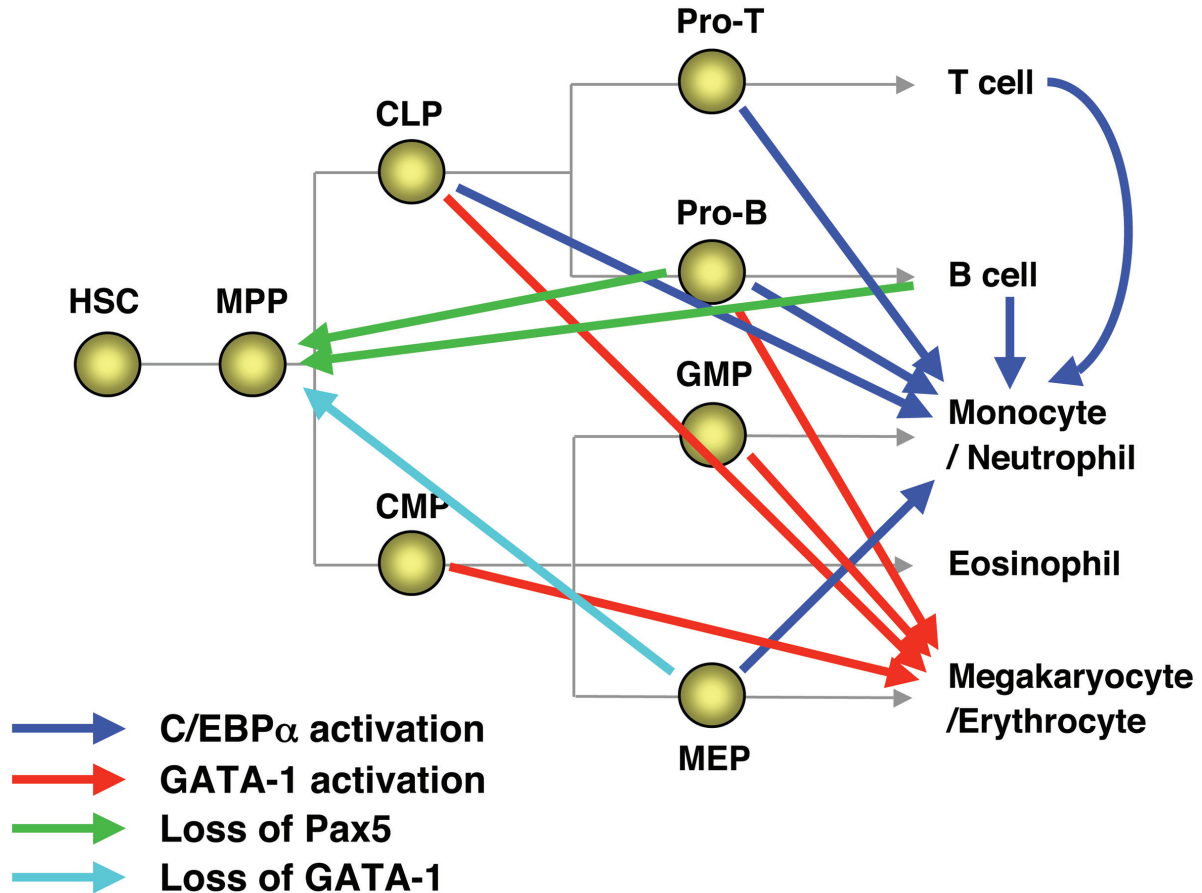


Fig. 4 Lineage conversion and dedifferentiation of hematopoietic cells by modulation of transcription factors.

Lineage conversion of hematopoietic cells by activation of C/EBP α and GATA-1, and dedifferentiation of B or MegE cells by loss of Pax5 or GATA-1. Reported pathways of conversion are shown by arrows. Loss of Pax5 or GATA-1 unfixes lineage commitment and leads to dedifferentiation back to multipotent progenitors.

B cells, CMPs and GMPs induced their transdifferentiation to the MegE lineage. Our group reported the conversion of CLPs, MEPs, early B cells and early T cells by induction of C/EBP α activity.⁵ Likewise, retroviral expression of C/EBP α efficiently converted mature B cells, CLPs and pro-T cells into the granulocyte/macrophage lineage.^{8,62}

To investigate the conversion process in more detail, we generated transgenic mice expressing a conditional form of C/EBP α (C/EBP α -ER) in all hematopoietic cells.⁵ Since activity of C/EBP α can be instantly induced in these mice by addition of 4-hydroxy tamoxifen (4-HT), this system allowed us to examine the detailed conversion process without cultivation of cells with cytokines *in vitro* and infection of retrovirus, both of which may artificially skew differentiation processes. When C/EBP α activity was induced in MEPs and CLPs by addition of 4-HT, these cells were efficiently reprogrammed to the GM lineage. Clonal analysis revealed that the conversion occurred at the single cell level. Interestingly, transient

activation of C/EBP α for 12 h for CLPs and 4 days for MEPs was sufficient to initiate the conversion process.⁵ We have also shown that conversion to the GM lineage could be induced not only *in vitro*, but also *in vivo* by systemic administration of 4-HT.

Mechanism of Lineage Conversion

Given that lineage conversion is induced by the activation of transcription factors, it raises the question whether the conversion process is accompanied by dedifferentiation of mature hematopoietic cells back to immature progenitors followed by tracing a differentiation path to other lineages, or whether cells are directly converted to other lineages through biphenotypic intermediate cells. Analysis of C/EBP α -ER transgenic mice indicated the latter possibility to be the case⁵ (**Fig. 5**). For example, we identified cells expressing both erythroid (CD71) and GM (Gr-1, CD11b) markers during C/EBP α -mediated conversion of MEPs to the GM lineage. Similarly, conversion of

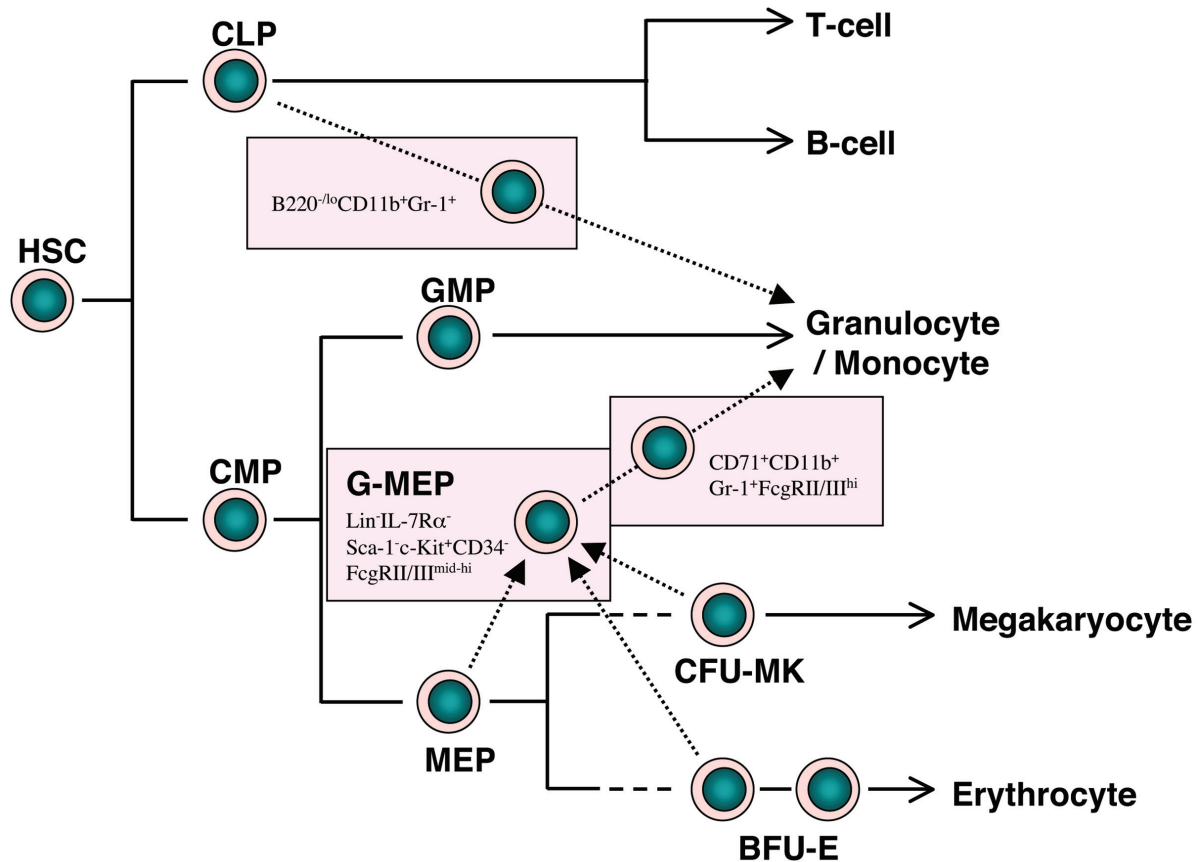


Fig. 5 Mechanism of lineage conversion.

Conversion of various progenitors by ectopic activation of C/EBP α . Lineage conversion occurs through biphenotypic intermediate cells, not by tracing back normal differentiation pathways. Cells in pink boxes are biphenotypic intermediate cells that have cell surface markers of original cells and myeloid lineage cells.

B cells to myeloid cells occurred through B220^{lo}CD11b⁺ or CD19⁺CD11b⁺ biphenotypic intermediate cells. It is interesting to note that these intermediate cells have rearranged immunoglobulin (Ig) genes, clearly indicating that these cells originated from B cells. These observations demonstrate that enforced, ectopic expression of transcription factors induces direct lineage conversion between lineages through biphenotypic intermediate cells.

In contrast, the dedifferentiation mechanism is responsible for the conversion of mature B cells to T cells *in vivo* by inactivation of Pax5⁵⁹ (**Fig. 4**). As discussed in the previous section, cells reacquire multipotentiality upon loss of Pax5, and these cells can follow the physiological differentiation path to T cells. This is supported by the fact that Pax5-deleted mature B cells can restore T lymphopoiesis in Rag2^{-/-} mice with a normal differentiation pattern.⁵⁹ Double-positive immature T cells taken from these mice retained the Ig rearrangement, indicating that the cells were derived from mature B cells.

Conclusions and Future Directions

It is now clear from *in vitro* studies that virtually all hematopoietic cells at any stage of differentiation sustain differentiation plasticity. The problem here is that most of the studies were done in artificial settings, such as overexpression of transcription factors or ectopic cytokine stimulation. This raises the question whether lineage conversion could occur in physiological settings *in vivo*. Our study using transgenic mice expressing an inducible form of C/EBP α revealed that lineage conversion could occur in the *in vivo* environment, at least by enforced activation of transcription factors. So the question is, are there any physiological conditions where lineage-specific transcription factors are activated ectopically *in vivo*? Currently, we cannot think of any possibility of such a condition existing *in vivo*, suggesting that lineage conversion is simply an observation indicating the latent plasticity of hematopoietic cells. However, if in future studies novel physiological pathways ectopically activating transcription factors were discovered, that development would

definitely add a novel and exciting twist to the current understanding of hematopoiesis.

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