C/EBP-Induced Transdifferentiation Reveals Granulocyte-Macrophage Precursor-like Plasticity of B Cells

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http://dx.doi.org/10.1016/j.stemcr.2016.12.015

SUMMARY

The lymphoid-myeloid transdifferentiation potentials of members of the C/EBP family (C/EBPa, β, δ, and ε) were compared in v-Abl immortalized primary B cells. Conversion of B cells to macrophages was readily induced by the ectopic expression of any C/EBP, and enhanced by endogenous C/EBPa and β activation. High transgene expression of C/EBPβ or C/EBPe, but not of C/EBPa or C/EBPe, also induced the formation of granulocytes. Granulocytes and macrophages emerged in a mutually exclusive manner. C/EBPa-expressing B cells produced granulocyte-macrophage progenitor (GMP)-like progenitors when subjected to selective pressure to eliminate lymphoid cells. The GMP-like progenitors remained self-renewing and cytokine-independent, and continuously produced macrophages and granulocytes. In addition to their suitability to study myelomonocytic lineage bifurcation, lineage-switched GMP-like progenitors could reflect the features of the lympho-myeloid lineage switch observed in leukemic progression.

INTRODUCTION

Hematopoiesis is thought to begin from stem cells that progress through consecutive precursor cell stages in a hierarchical fashion whereby lineage commitment precludes alternative cell differentiation. However, there has been increasing evidence of hematopoietic plasticity and cell lineage conversion, particularly during leukemogenesis (Cobaleda and Busslinger, 2008; Graf, 2008; Greaves et al., 1986; Regalo and Leutz, 2013). The transcription factors C/EBPa and C/EBPβ are potent inducers of myelomonocytic genes in heterologous cell types (Ness et al., 1993), and the experimental conversion of lymphoid cells to myeloid cells modulated by both C/EBPs has highlighted their lympho-myeloid transdifferentiation potential (Graf and Enver, 2009).

The C/EBP family members C/EBPa, C/EBPβ, C/EBPe, and C/EBPs are expressed in myeloid cells (Cloutier et al., 2009; Scott et al., 1992). Loss-of-function studies in genetically modified mice suggested combinatorial and partially redundant functions of C/EBPs in myelopoiesis (Tsukada et al., 2011). Knockout studies showed that deletion of Cebpa has the strongest impact on myelopoiesis, resulting in an almost complete loss of neutrophils and impaired development of granulocyte-macrophage progenitor (GMP) cells (Zhang et al., 1997, 2004). However, cytokines could compensate for the lack of Cebpa by the concomitant activation of Cebpb, and genetic replacement of Cebpa with Cebpb in the Cebpa locus compensates for the Cebpa requirement in hematopoiesis and liver functions (Chen et al., 2000; Hirai et al., 2006; Jones et al., 2002). Individual deletions of C/EBPβ, δ, and ε evoke milder and gene-specific phenotypes, such as susceptibility to infections, failure of emergency granulopoiesis, impaired cytokine production, and partial granulocyte deficiency that is intensified by compound C/EBP gene deletions. For example, compound Cebpb/Cebpe deletion mutants display impaired granulopoiesis, defective macrophage functions, and a disrupted innate immune regulatory gene expression network, confirming the compensatory and redundant functions of the C/EBPs (Akagi et al., 2010; Hirai et al., 2006; Litvak et al., 2009; Tanaka et al., 1995; Yamanaka et al., 1997).

C/EBPa can stimulate the transdifferentiation of B and T cells and, together with PU.1, even fibroblasts into macrophages (Bussmann et al., 2009; Feng et al., 2008; Ness et al., 1993; Xie et al., 2004). Conversion of B cells into inflammatory-type macrophages occurs rapidly after C/EBP expression, with high efficiency and through a direct route (Bussmann et al., 2009; Di Tullio et al., 2011; Xie et al., 2004). An experimental transdifferentiation system based on an estrogen-responsive, conditional C/EBPa protein in the v-H-ras-transformed pre-B cell line HAFTL1 (Holmes et al., 1986) has served as a tool to examine the mechanistic aspects of lympho-myeloid lineage conversion, including alterations of chromatin occupancy, gene expression kinetics, non-coding RNA expression, and DNA methylation (Barneda-Zahonero et al., 2013; Di Tullio et al., 2011; Källin et al., 2012; Krüger et al., 2016; Rodriguez-Ubreva et al., 2012, 2014; van Oevelen et al., 2015).

We recently found that structural alterations and post-translational modification sites of C/EBPβ may determine the path of transdifferentiation of primary progenitor B cells toward distinct myeloid cell fates (including...
granulocytes and dendritic cells), suggesting that epigenetic instructions beyond the inflammatory macrophage cell fate are encoded in the C/EBP structure and could account for cell-type specification (Stoilova et al., 2013). This observation has prompted us to compare the lineage conversion capacity of all transactivator C/EBP family members and to develop a lympho-myeloid transdifferentiation system that is amenable to targeted mouse genetics and cell-culture manipulation.

In this study, we generated murine v-Abl-immortalized B cells from wild-type and genetically altered mice to compare the lympho-myeloid transdifferentiation potential of the C/EBP family members C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε. Our data showed that C/EBPβ and C/EBPε readily induce a granulocytic fate in addition to macrophage formation. Granulocytic conversion largely depended on transgene dosage. In addition, efficient transdifferentiation required endogenous Cebpa/Cebpb. Importantly, applying selective pressure on immortalized B cells expressing C/EBPβ by depriving β-mercaptoethanol resulted in the rapid extinction of B cells and massive expansion of stable myeloid cells. These myeloid progenitors displayed bipotential GMP-like properties and continuously produced macrophages and granulocytes. This process could suggest a link between C/EBP-induced lympho-myeloid lineage switch and a B cell-derived leukemic myelomonocytic GMP-like phenotype (Slamova et al., 2013).

RESULTS

Transdifferentiation Potential of Various C/EBP Family Members

v-Abl-immortalized pre-B cells derived from primary mouse bone marrow cells (hereafter termed “B cells”) were used to assess the ability of individual C/EBP family members to induce B-lymphoid to myeloid transdifferentiation (Rosenberg et al., 1975; Shore et al., 2002). pMSCV-based retroviral expression vectors (MIG) encoding full-length C/EBPα, C/EBPβ, C/EBPδ, or C/EBPε-IRES-EGFP were constructed (Figure 1A). Transgene expression was confirmed in the retrovirus-packaging cell line (Figure S1A). As shown in Figure 1B, each C/EBP family member could induce expression of the myeloid marker CD11b (Mac-1) and cause the transdifferentiation of B cells into CD11b⁺CD19dim

Figure 1. C/EBPα, β, δ, and ε Transdifferentiate B Cells to Myeloid Cells

(A) pMSCV-based vectors containing an internal ribosomal entry site (IRES) and EGFP (MIG) marker used for retroviral transduction. Full-length open reading frames of Cebpα, Cebpβ, Cebpδ, or Cebpε were inserted upstream of the IRES element. Empty vector (MIG) served as control.

(B) Flow cytometric analysis of GFP⁺-infected B cells 4 days after transduction with individual C/EBPs.

(C) Analysis of granulocyte (Ly-6G) and macrophage (CD115) cell-surface markers in GFP⁺CD11b⁺ transdifferentiated cells 6 days after transduction.

(D) May-Grünwald staining of GFP⁺-sorted B cells 4 days after transduction. Arrows indicate cells with typical macrophage morphology and arrowheads mark granulocyte-like cells. Primary bone marrow (BM) and sorted granulocytes (Gr) were used as references (far right).

See also Figure S1.
myeloid cells. Additional myeloid surface markers indicative of dendritic cells (CD11c+), macrophages (CD115+), or granulocytes (Ly-6G+), were included to assess myeloid subsets in the CD11b+ population (Figure S1B). While no CD11c+ cells were found, a fraction of transdifferentiated cells showed upregulated CD115 (MCSF-R), a macrophage marker and C/EBP target gene. A CD11b+Ly-6G+ population, characteristic of granulocytes, was detected with C/EBP protein expression, consistent with observations of strong mRNA expression of C/EBP in bone marrow-derived granulocytes (Figures S1B and S1C). C/EBP expression also caused the formation of a CD11b+Ly-6G+ population after prolonged transdifferentiation for 6 days, whereas C/EBPα and C/EBPβ expression did not (Figures 1C and S1B). Interestingly, the expression of Ly-6G and CD115 appeared to be mutually exclusive, as no double-positive cells could be detected, indicating a stringent mechanism of cell-fate decision (Figure 1C). Cytospin preparations of GFP+ cells confirmed that all C/EBPs stimulated the formation of myeloid cells with monocyte/macrophage features (Figure 1D). The C/EBPβ- and C/EBPε-transdifferentiated cell population contained small cells with segmented or indented nuclei, strongly resembling bone marrow-derived granulocytes and suggesting that C/EBPε or C/EBPβ may induce granulocyte/macrophage (G/M) cell fates. Transdifferentiation into granulocytes by C/EBPβ and C/EBPε was not restricted to the v-Abl B cell system, but also occurred in the fetal liver-derived H-ras-transformed HAFTL1 pre-B cell line (Figure S1D) (Bussmann et al., 2009). These findings suggest that all the tested C/EBP transcription factors could cause the conversion of B cells to myeloid cells and that C/EBPβ and C/EBPε could stimulate macrophage and granocyte transdifferentiation of B cells.

Quantitative gene expression analysis was performed by NanoString hybridization 1 day after transduction with individual C/EBPs, at the earliest emergence of GFP-positive cells (Figures 2A and 2B; Table S1). A predefined mouse immunology code set covering 547 probes, including key transcription factors, was used. Genes with 2-fold altered expression levels, compared with empty vector-transduced B cell controls, were considered. As shown in Figures 2A and 2B, all C/EBPs upregulated and downregulated a core set of 22 and 12 genes, respectively. Each C/EBP family member also displayed additional and partial overlapping regulatory specificity. The core transdifferentiation signature of 22 upregulated genes included myeloid factors, such as Csf1r, Csf2rb, Fcgr1, and Gfi1, and several chemokine CC-family members (Figure 2C). The 12 downregulated genes included lymphocyte genes, such as Il7r, Cd2, and the Rag genes (Figure 2D). These data suggest that C/EBPα, β, δ, and ε likely suppress the B cell program and induce lympho-myeloid conversion.

**Deletion of Endogenous Cebpa and Cebpb Impairs Transdifferentiation but Has No Impact on Cell-Type Outcome**

As previously reported by Bussmann et al. (2009) and shown in Figure S2A, activation of conditional C/EBPα-ER induced endogenous Cebpb and Cebpa gene expression in HAFTL1 B cells. We induced myeloid transdifferentiation via C/EBP's expression in HAFTL1 cells and analyzed endogenous C/EBPα and C/EBPβ protein expression. Interestingly, the expression of C/EBPε also led to a marked upregulation of endogenous C/EBPα after 16 hr, while only low levels of C/EBPβ were detected. However, after 24 hr, C/EBPβ protein expression increased while C/EBPα expression was diminished. A surge of C/EBPα and C/EBPβ expression was observed after 120 hr, indicating crosstalk between transgenic and endogenous C/EBPs during transdifferentiation (Figure S2B).

Synergistic collaboration between C/EBP family members was previously described as a key element to specify myeloid differentiation (Akagi et al., 2010). To examine the contribution of endogenous C/EBPs to lympho-myeloid transdifferentiation, we treated B cells generated from a homozygous Cebpa+/−,Cebpb−/− mouse with cell-permeable Cre-recombinase to generate double-knockout derivatives (Figure S2C). Verified Cebpa−/−;Cebpb−/− double-knockout B cell clones were designated as “B-DKO” (Figure S2D).

B-DKO cells or isogenic controls (not treated with Cre-recombinase) were infected with the G/M-transdifferentiation proficient retroviral constructs that co-expressed C/EBPβ and GFP (MIG) or C/EBPs and BFP (MIB) (Figures 3A and 3B). GFP*BFP+ gated cell populations, indicative of C/EBPβ and C/EBPε expression, were analyzed (Figure 3C). Deletion of endogenous Cebpa and Cebpb strongly impaired transdifferentiation induced by C/EBPα or C/EBPβ after 4 days, in comparison with isogenic controls (Figures 3C and S2E). Transdifferentiation efficacy in C/EBPα-expressing B-DKO cells could be partially rescued by retroviral co-expression of C/EBPβ. These data suggest synergistic effects between endogenous and exogenous C/EBPs during transdifferentiation (Figures 3C and S2E).

Comparison of gene expression in C/EBPs-transduced wild-type or B-DKO cells revealed genes that were dependent on endogenous Cebpa and Cebpb (Figure S2F and Table S1). The list of refractory genes included Csfr1, Fcgr1, Itgam (CD11b), and the transcription factor Gfi1, known to promote granulocyte differentiation. These genes represent important myeloid cell factors, and failure of induction may explain the impaired transdifferentiation (Figures 3C and S2E). In addition, Pax5 or Pou2f2 (Oct-2) were refractory to downregulation, which could contribute to the persistence of the B cell phenotype (Figure S2G and Table S1).
To determine whether the G/M cell-type outcome was affected by deletion of endogenous *Cebpa* and *Cebpb*, we analyzed C/EBP*ε*-transdifferentiated GFP+CD11b+ cells for Ly-6G or CD115 surface marker expression after 7 days of culture. Comparison of transdifferentiated B-DKO and control cells did not reveal differences in the frequency of Ly-6G+ versus CD115+ cells in the CD11b+ gated cell population (Figure 3D), suggesting that the continuously expressed transgene product but not endogenous *Cebpa* and *Cebpb* predominantly affected myeloid cell lineage choice.

C/EBP Dosage and Granulocyte-Macrophage Lineage Choice

Transcription factor dosage is an important determinant in hematopoietic lineage choice (Dahl et al., 2003; DeKoter et al., 2007; DeKoter and Singh, 2000; Di Tullio and Graf, 2012; Ma et al., 2014; Rosenbauer et al., 2004; Simmons et al., 2012). We therefore examined how the level of C/EBP transgene expression would alter lympho-myeloid cell fate. As shown in Figures S3A and S3B, GFP intensity directly correlated with retroviral C/EBP protein expression and enabled us to examine transcription factor dosage effects on lympho-myeloid transdifferentiation. Based on increasing GFP intensity, C/EBP*ε*-transduced B cells were divided into six fractions, GFP1–6 (Figure 4A). Macrophage and granulocyte surface marker analysis of the CD11b+ cells revealed a bipartite profile when all GFP+ cells were included in the analysis (as in Figure 1C). As shown in Figures 4B and 4C, the distribution of CD11b+Ly-6G+ versus CD11b+CD115+ shifted according to the GFP intensity. GFPdim fractions were preferentially CD11b+CD115−/− and Ly-6G+. The CD11b+Ly-6G+ cell population expanded with increasing GFP intensity, while the CD11b+CD115+ cell fraction diminished. The fraction with the highest GFP intensity (GFP6) consisted mostly of CD11b+Ly-6G+ cells (Figures 4B and 4C). Cytospin preparations of GFPdim and GFPhigh-sorted cells showed that the GFPdim fraction consisted of large cells, characteristic of monocyte/macrophages, while the GFPhigh fraction contained cells with indented and segmented nuclei—typical features of mature granulocytes (Figure 4D). Finally, B cells transduced with C/EBP*ε* were separated by flow cytometry according to their GFP intensity 24 hr post infection. Six days later, cell-surface marker expression analysis indicated that the GFPhigh cells were enriched for Ly-6G+ versus CD115+ cells when compared with the GFP+ culture. These data indicate a prospective cell-type outcome according to the initial C/EBP dosage (Figure 4E). To further exclude artifacts produced by the bicistronic C/EBP-IRES-EGFP MIG constructs, we examined the dosage effect using retroviral vectors with direct C/EBP*ε*-GFP fusion. As shown in Figure S3C, higher GFP intensity correlated with the G/M surface marker profile. Taken together, these findings show that the dosage of C/EBP*ε* or C/EBP transgenes determines granulocyte versus macrophage cell fate outcome during lympho-myeloid transdifferentiation.

Long-Term Proliferating C/EBP*β*-Lympho-Myeloid Progeny Display a GMP-like State and Spontaneously Differentiate into Granulocytes and Macrophages

To assess the proliferation and lineage-forming capacity of C/EBP*β*-transdifferentiated B cells, we aimed to establish a stably converted B cell line. After the removal of β-mercaptoethanol, an essential culture supplement for B cell maintenance, all B cells perished within a few days in C/EBP*β*-transduced and empty vector-transduced cells. In contrast to the controls, however, proliferating GFP*+ cells emerged from C/EBP*β*-transduced B cells within 2 weeks after infection. Comparison of surface marker expression after 8 days and 6 weeks of cell culture showed that the long-term proliferating cells maintained both macrophage and granulocyte differentiation potential (Figures 5A and 5C). Cytospin preparations from CD11b+Ly-6G+ and CD11b+CD115+ sorted cells resembled typical granulocytes and macrophages, respectively (Figures 5B and 5D). Genotyping of rearranged immunoglobulin heavy chain (IgH) locus of C/EBP*β*-transduced cells ruled out the possibility of a contamination with myeloid remnants from initiating cultures (Figure 5E). Immunoblot analysis showed that transgenic C/EBP*β* protein is present and that endogenous C/EBP*β* protein isoforms are also upregulated in transdifferentiated cells (Figure 5F). In addition, C/EBP*β*-transduced cells grew in a semi-adherent fashion and showed irregular morphological features typical of myeloid progenitor cells, in contrast to round, refractile B cells. Upon treatment with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) the proliferation rate decreased and cells became adherent, large, and motile, resembling activated macrophages and suggesting that the transdifferentiated cells respond to myeloid signaling pathway activation (Figures S4A and S4B).

The G/M lineage phenotype of the transdifferentiated cells was maintained during more than 6 months of continuous cell culture. Nevertheless, C/EBP*β* may

Figure 2. Transdifferentiation Core Gene Signatures of B Cells Induced with C/EBP Family Members

Quantification of mRNA from GFP*+* sorted B cells 24 hr after transduction with individual C/EBPs. Venn diagram of (A) upregulated and (B) downregulated genes, compared with empty vector (MIG) control. Overlapping core signatures of (C) 22 upregulated or (D) 12 downregulated genes. Key myeloid (in C) and lymphoid (in D) genes are shown in bold. The fold change (FC) relative to empty vector (MIG)-transduced B cells is shown. See also Table S1.
Figure 3. Endogenous Cebp and Cebpb Promote Transdifferentiation but Do Not Affect Lineage Outcome

(A) pMSCV-based vectors for the expression of C/EBPβ linked to EGFP marker or C/EBPε linked to BFP. Empty vectors (MIG/MIB) were used as controls.
stimulate B cells to transdifferentiate into distinct G and M progenitors either directly or through a common GMP cell type. To distinguish between these possibilities, we seeded 4,500 of the stably transdifferentiated cells into semi-solid medium to derive colonies from single cells. Approximately 40–70 colonies were obtained in a single experiment. Individual GFP+ colonies were inspected from day 6 to day 18 and displayed a compact core and a periphery of larger motile, oval cells, reminiscent of colony-forming unit GM colonies (Figure 5G). Two colonies were isolated

Figure 4. C/EBPβ Transgene Dosage Affects Macrophage versus Granulocyte Lineage Outcome
(A) Data binning of C/EBPβ-transduced B cells according to GFP intensity (GFP1–6).
(B) Flow cytometric analysis of GFP1–6 bins (CD11b+ gated) 6 days after transduction.
(C) Quantification from five independent experiments. Data are presented as mean ± SEM.
(D) Cytospin preparation and May-Grünwald staining of GFPdim- and GFPhigh-sorted C/EBPβ-transduced B cells at day 7. Arrows indicate macrophage and arrowheads granulocyte-like cells.
(E) B cells were transduced with C/EBPε, sorted after 24 hr as GFP+ or GFPhigh, and recultured. Flow cytometric analysis of granulocyte (Ly-6G+) versus macrophage (CD115+) markers in GFP+CD11b+-gated populations 6 days post sorting.

See also Figure S2.
and propagated in liquid culture and both displayed a Ly-6G+ and CD115+ phenotype indicative of GMP-like potential (Figure 5H, result for one clone is shown). Subsequent recloning of one of the isolates in semi-solid medium resulted in stably growing secondary subclones (cloning efficiency between ~4% and 6%) and persistence of the GMP-like phenotype of all 17 subclones examined, as shown in Figure S5.

We conclude that C/EBPβ-induced lympho-myeloid conversion confers a selective advantage by abrogating the dependence on B cell survival conditions. These C/EBPβ-converted cells acquire cytokine-independent long-term proliferation capacity and a robust GMP-like potential.

DISCUSSION

Transcription factor-mediated conversion of one cell type into another is an important avenue by which we can advance our understanding of instructive mechanisms of cellular lineage commitment and differentiation. The primary B cells immortalized by v-Abl provide an experimental system to combine B cell mouse genetics and ectopic gene expression for the exploration of progenitor biology, lympho-myeloid lineage plasticity, maintenance of cell identity, and regulatory mechanisms of myeloid lineage commitment.

Experimental transdifferentiation of B cells by ectopic expression of conditional C/EBPα has shed light on the molecular dynamics of the conversion of lymphoid cells into inflammatory macrophages (Bussmann et al., 2009; Di Tullio et al., 2011; Krijger et al., 2016; Laiosa et al., 2006; Rodríguez-Ubreva et al., 2012, 2014; van Oevelen et al., 2015; Xie et al., 2004). However, lympho-myeloid transdifferentiation may not be restricted to activated macrophages but may yield other myeloid cell types depending on the C/EBP structure and post-translational modification pattern (Stoilova et al., 2013). Here, we assessed the lympho-myeloid transdifferentiation potential of C/EBPα, β, δ, and ε in primary v-Abl-immortalized B cells. Our data show that v-Abl B cells represent a robust in vitro system for experimental cell lineage switch and may also be used for targeted murine genetics and in vitro genetic manipulation at the B cell stage.

Lineage conversion could be achieved using any of the four C/EBP members, revealing a common gene signature, and their common potential to extinguish B cell identity and induce the myeloid cell fate. C/EBPβ and C/EBPα induced granulocytic differentiation in addition to monocytic transdifferentiation. Granulopoietic capacity of C/EBPβ has previously been documented in emergency granulopoiesis (Hirai et al., 2006) and by restoring Cebpa deficiency (Jones et al., 2002). The lack of granulopoietic transdifferentiation potential of C/EBPα was surprising and has been discussed previously (Xie et al., 2004), because C/EBPα induced granulocyte differentiation in a myeloid progenitor cell line (Radomska et al., 1998; Suh et al., 2006). Although we do not know why C/EBPα and C/EBPβ fail to induce granulocyte formation, the strong proliferation arrest elicited by C/EBPα could account for the failure of granulocytic conversion. On the other hand, our data may also raise the possibility that some of the functions of C/EBPα reported in established progenitor cell lines may depend on the activation of endogenous C/EBPβ. Nevertheless, the v-Abl B cell system could be useful to resolve quantitative and qualitative C/EBP functions and help uncover mechanisms of lineage specification, e.g., by C/EBP domain-swap experiments and mutational analysis.

Our findings support the concept that subtle variations in transcription factor levels have an impact on hematopoietic cell lineage decisions. In addition to transcription factor dosage, specificity and functional redundancy play major roles; high levels of C/EBPα or C/EBPε consistently increased the granulocyte over macrophage ratio. The exact interplay between C/EBP quantity and qualitative...
specificity remains to be explored, but is likely similar to graded PU.1 expression that has an impact on lymphomyeloid specification by occupancy of low-affinity binding sites only at high transcription factor concentrations (DeKoter and Singh, 2000; Pham et al., 2013). Relative expression levels of PU.1 and C/EBPα are also key determinants in granulocyte versus macrophage specification. Higher PU.1 levels promote macrophage differentiation, while higher C/EBPα levels lead to granulopoiesis (Dahl et al., 2003; Ma et al., 2014). Therefore, less accessible “granulocytic genes” may require higher C/EBP concentrations in addition to other features of C/EBPα and C/EBPε proteins.

Activation of endogenous C/EBP genes and impaired lineage conversion after their removal suggests that autoregulatory loops are involved in the kinetics of lineage conversion (Bussmann et al., 2009; Lu et al., 2009). However, deletion of endogenous C/EBPα and C/EBPβ appears to affect the conversion efficiency but not the lineage outcome, suggesting a dominant and distinguished qualitative role for the constitutively expressed C/EBP transgene.

Leukemia with indifferent or promiscuous lineage association is frequently resistant to standard chemotherapy and may show changed lineages during relapse (Dorantes-Acosta and Pelayo, 2012; Golemovic et al., 2006; Matutes et al., 1997). Inter-myeloid and lympho-myeloid lineage ambiguity therefore emerges as an important pathological entity (Greaves et al., 1986; Smith et al., 1983). It is therefore conceivable that switching lineages reflect an epigenetic selection process in leukemogenesis associated with gain-of-function survival and proliferation capacity (Greaves et al., 1986; Janz et al., 2006; Regalo and Leutz, 2013). Our experimental data provide direct evidence that enhancing the selective pressure on B cells by removal of β-mercaptoethanol strongly promotes C/EBPβ-induced lympho-myeloid converts that remained immortalized and highly proliferative. We therefore conclude that cancerous B cells may escape from hostile conditions by lineage conversion through C/EBPβ activation. Furthermore, B cells could profit from lineage-foreign survival signals by aberrant expression of growth factor receptors, such as CSF1-R, a target gene of C/EBPα (Lamprecht et al., 2010). Various C/EBP translocations have been associated with B cell-derived leukemia, implying their cancer-supporting function. However, the role played by transdifferentiation remains unknown (Akasaka et al., 2007; Chapiro et al., 2006; Slamova et al., 2013; Wiemels et al., 2015). Strikingly, DNA demethylation and upregulation of C/EBPα was suspected to be involved in lineage switching during leukemia progression (Slamova et al., 2013). In addition to pre-B cell lymphoma, v-Abl retroviral infection of murine bone marrow cells also evoked a chronic myeloid leukemia-like disease that involved immunoglobulin rearrangements (Kellhier et al., 1990).

In a recent study, pulsed C/EBPα expression was shown to induce a GMP-like state according to gene expression and chromatin accessibility data. However, during prolonged exposure only a macrophage fate was consolidated (Di Stefano et al., 2016). Remarkably, the stable C/EBPβ B cell converts reported here resembled self-renewing bipotential GMP progenitors that were responsive to GM-CSF but not dependent on cytokine signaling, and stably retained G/M differentiation potential. In the absence of cytokines, expression of macrophage- and granulocyte-specific markers emerged in a mutually exclusive fashion. These data suggest the involvement of C/EBPβ in maintenance, commitment, or both at the GMP stage.

The cytokine-independent, self-renewing, spontaneously differentiating GMP-like cells present a valuable experimental tool for the study of determinants of granulocyte versus macrophage specification by genetic manipulation and screening approaches. In addition, maintenance of the transdifferentiation capacity of the v-Abl B cells establishes a sound basis for using targeted genetic mouse models to examine signaling pathways and other instructive cues of cell specification. The fact that v-Abl B cells are amenable to DNA transfection permits experimental alteration at the B cell stage, such as stable transfection, gene deletion, or chromosomal engineering, and is an important technical advance in overcoming the hurdle of difficult genetic manipulation of myelomonocytic cells.

In summary, we present a robust in vitro lymphomyeloid transdifferentiation model to study determinants of lineage promiscuity, GMP progenitor biology, and macrophage versus granulocyte cell-fate decisions. In addition, our findings lend experimental evidence to lymphomyeloid transdifferentiation processes as a trait of lineage ambiguity in leukemia progression.

EXPERIMENTAL PROCEDURES

Expression Constructs

C/EBP genes were inserted into pMSCV-IRES-EGFP (MIG) or pMSCV-IRES-BFP (MIB) retroviral vectors using XhoI/EcoRI restriction sites. C/EBPs lacking stop codons were cloned in-frame with the EGFP open reading frame and inserted into pMSCV for C/EBP-GFP fusion construct. Expression vectors encoding C/EBPα p42 isoform and C/EBPβ LAP* isoform were described previously (Stoilova et al., 2013). Murine C/EBPα was isolated from pMEC-mC/EBPα (a gift from Dr. E Sterneck). Codon-optimized human C/EBPβ p32 isoform was commercially synthesized (MWG-Biotech) and subcloned from pEX-A2 into MIG or MIB using EcoRI sites flanking C/EBPs. The v-Abl-expressing pMSCV vector was a gift from Dr. F Melchers (Ohnishi and Melchers, 2003). For production of infectious supernatants the retroviral packaging cell line
Plat-E was used. Infectious supernatants were collected 48 hr and 72 hr after transfection.

**Generation of v-Abl-Transformed B Cells**
Bone marrow cells were isolated from the femur and tibia of 8- to 9-week-old Cebpd<sup>j346–359</sup>-crossed (Zhang et al., 2004) and Cebpb<sup>j346</sup>-crossed (Sternick et al., 2006) mice and C57Bl/6j wild-type controls. These mice were maintained and handled in compliance with protocols approved by the institutional Animal Care and Use Committee. After erythrolysis, bone marrow cells were transduced with v-Abl-expressing retroviral supernatants and 8 μg/mL hexadimethrine bromide in complete DMEM (10% fetal calf serum (FCS), 10 mM HEPES, and penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol. Bone marrow cells were washed on the following day and medium was changed every other day. A stable cell line with a pre-B cell-like phenotype (CD19+, c-kit<sup>+</sup>) was induced with 200 nM hydroxytamoxifen (Sigma). 293T-Retro viral transgene was cultured in complete phenol red-free DMEM (10% fetal calf serum (FCS), 10 mM HEPES, and penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol. Bone marrow cells were washed and processed for flow cytometry or replated with 8 μg/mL hexadimethrine bromide in complete DMEM (10% fetal calf serum (FCS), 10 mM HEPES, and penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol. Bone marrow cells were washed in serum-free DMEM and incubated with purified TAT-Cre protein (50 μg/mL; a gift from Dr. K Rajewsky) in serum-free DMEM at 37°C for 45 min (Petz et al., 2002). The cells were then washed and cultured for 24 hr and seeded subsequently as single-cell clones (by fluorescence-activated cell sorting (FACS)) into 96-well plates. Deletion of the Cebp<sub>a</sub> and Cebp<sub>b</sub> genes was checked by PCR to identify double-knockout clones. Primer sequences used are shown in Table S2.

**Cell Lines**
The monocytic/macrophage Raw264.7 cell line was cultured in complete DMEM. The fetal liver-derived pre-B cell line HAFTL1 was cultured in complete RPMI-1640 (10% FCS, 10 mM HEPES, penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol (Pierce and Aaranson, 1982). The HAFTL1-derived C10 clone (a gift from Dr. T Graf) containing a C/EBPα-ER<sup>j346–359</sup>GFP transgene was cultured in complete phenol red-free RPMI-1640 (10% charcoal-stripped FCS (HyClone), 10 mM HEPES, penicillin/streptomycin (Gibco)) supplemented with 50 μM β-mercaptoethanol (Bussmann et al., 2009). Transdifferentiation of C10 cells was induced with 200 nM hydroxytamoxifen (Sigma). 293T-derived Plat-E line (Cell Biolabs) was used for packaging.

**Retroviral Transduction**
v-Abl-generated stable B cells or HAFTL1 cells (2 × 10<sup>7</sup>/12-well or 1 × 10<sup>8</sup>/6-well) were mixed with freshly prepared retroviral supernatants (1/3 of total volume) in complete medium supplemented with 8 μg/mL hexadimethrine bromide and 50 μM β-mercaptoethanol. Cells were centrifuged at 1,000 rpm without brakes for 10 min at room temperature and incubated overnight. Infected cells were washed and processed for flow cytometry or replated in 6-well plates (BD Falcon). For the generation of the long-term proliferating transdifferentiated cells, β-mercaptoethanol supplementation was omitted after 24 hr. Half of the medium was changed every other day.

**Flow Cytometry**
For flow cytometry or cell sorting, cells were transferred to 15-mL tubes and washed in cold buffer (2% FCS, 2 mM EDTA in PBS).

Before antibody labeling, cells were incubated with Fc-Block (1:200, anti-CD16/32 antibody; 2.4G2, BD Pharmingen) for 10 min at 4°C and washed. Cells were stained in buffer with (1:100) fluorescein labeled antibodies against CD11b (clone M1/70, BD Pharmingen), CD11c (clone N418, Biolegend), CD11b (clone AF598, eBioscience), CD19 (clone 1D3, BD Pharmingen), and Ly-6G (1A8, Biolegend) for 30 min at 4°C in the dark. 7-AAD (BD Pharmingen) was added before measurement to exclude dead cells. Analysis was performed on LSRFortessa and sorting on FACSAria II or III (BD Pharmingen). Unstained, empty vector-transduced cells or fluorescence-minus-one staining setups served as controls.

**PCR**
Total RNA was prepared using TriPure (Roche) following the manufacturer’s instructions. DNA contamination was removed by DNase I treatment (Roche). For standard cDNA synthesis the RevertAid First Strand Synthesis Kit (Thermo Scientific) was used. Ten nanograms of cDNA was applied to PCR according to the Manganese Taq kit protocol (Bioline). PCR reaction was subjected to 94°C for 3 min and 27 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min on a Mastercycler Pro (Eppendorf). For detection of Dis1-H IgH rearrangements, DNA was prepared as described by Truett et al. (2000) and subjected to PCR conditions as reported by Ehlich et al. (1994), using the combination of primers DQ52 FW1, DFS FW2, and JH4A RV. Cebp<sub>a</sub> and Cebp<sub>b</sub> genotypes were assessed using “+/−” or “Δ” primer pairs, respectively.

**Immunoblotting**
Total protein lysates were prepared by incubation of snap-frozen cell pellets with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% glycerol, and proteinase inhibitor cocktail [Roche] in H<sub>2</sub>O) for 30 min on ice. After centrifugation protein concentration was measured in supernatants using Pierce Reagent (Thermo Scientific). Protein lysates were subjected to electrophoresis and transferred to a nitrocellulose membrane on the Trans-Blot Turbo System (Bio-Rad). Specific protein signals were detected by incubation with antibodies against C/EBPα (14AA, Santa Cruz Biotechnology), C/EBPβ (C-19, Santa Cruz), C/EBPΔ (C-22, Santa Cruz), C/EBPε (C-22, Santa Cruz), GFP (7.1/13.1, Roche), and ACTB (AC-15, Sigma).

**NanoString Analysis**
Total RNA from freshly FACS-enriched cells or cell cultures was prepared and quality checked on a 2100 Bioanalyzer (Agilent Technologies). Detection of mouse protein-coding transcripts was performed using a predefined panel (GXA-MIM/12, nCounter), containing 547 probes against immunology-related mRNAs and 14 probes against housekeeping genes (G6pd, Hprt, Gapdh, Alas1, Oaz1, Sdha, Rpl19, Eef1g, Tbp, Ppia, Polr2a, Gusb, Tubb5, and Polr1b) for normalization. Primary data were processed using default settings of the nSolver software. Genes were defined as “up-regulated” or “downregulated” if normalized expression values were increased or decreased at least 2-fold compared with empty vector-transduced controls.
Statistical Analysis
Processed data were visualized using GraphPad Prism software (Version 5.0a). Error bars represent the SEM. Hypothesis testing was performed from at least three independent experiments using the t-test function (two-tailed) in Rstudio (R version 3.2.1, R Foundation). p Values of <0.05 were defined as significant and p < 0.01 highly significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.015.

AUTHOR CONTRIBUTIONS
B.C. conceived the methodology, performed the experiments, analyzed the data, and wrote the draft. V.B. provided Cebpα^{ΔF}; Cebpβ^{ΔF} mouse resources and experimental advice. E.K.-L. generated CEBP-EGFP fusion construct resources, and J.S. and C.K. validated experiments. V.B., J.I., N.P., J.S., and E.K.-L. were involved in dated experiments. B.C. and A.L. conceptualized the work and wrote the manuscript. A.L. was responsible for supervision, project administration, and funding acquisition.

ACKNOWLEDGMENTS
We thank Dr. B. Niesler and R. Roeth of the nCounter Core Facility, Heidelberg, for providing services; Dr. H.-P. Rahn and K. Rautenberg (MDC, Berlin, Germany) for flow cytometry support; Dr. T. Graf (CRG, Barcelona, Spain) for the C10 cell line; M. Hofstätter and J. Bergemann (MDC, Berlin, Germany) for technical assistance; and Dr. E. Sterneck (NCI, Frederick, MD), Dr. F. Melchers (MPI for Infection Biology, Berlin, Germany), and Dr. K. Rajewsky (MDC, Berlin, Germany) for reagents and mice.

Received: July 16, 2016
Revised: December 14, 2016
Accepted: December 15, 2016
Published: January 19, 2017

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Keywords: C/EBP; MYD88; B cell; myeloid differentiation; reprogramming system; iPSC
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