Stem Cell Reports
Article

OPEN ACCESS

C/EBPα Activates Pre-existing and De Novo Macrophage Enhancers during Induced Pre-B Cell Transdifferentiation and Myelopoiesis

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http://dx.doi.org/10.1016/j.stemcr.2015.06.007

SUMMARY

Transcription-factor-induced somatic cell conversions are highly relevant for both basic and clinical research yet their mechanism is not fully understood and it is unclear whether they reflect normal differentiation processes. Here we show that during pre-B-cell-to-macrophage transdifferentiation, C/EBPα binds to two types of myeloid enhancers in B cells: pre-existing enhancers that are bound by PU.1, providing a platform for incoming C/EBPα; and de novo enhancers that are targeted by C/EBPα, acting as a pioneer factor for subsequent binding by PU.1. The order of factor binding dictates the upregulation kinetics of nearby genes. Pre-existing enhancers are broadly active throughout the hematopoietic lineage tree, including B cells. In contrast, de novo enhancers are silent in most cell types except in myeloid cells where they become activated by C/EBP factors. Our data suggest that C/EBPα recapitulates physiological developmental processes by short-circuiting two macrophage enhancer pathways in pre-B cells.

INTRODUCTION

The discovery that transcription factors (TFs) can convert somatic cells into both specialized and induced pluripotent stem cells (iPSCs) has revolutionized stem cell research and promises to have major clinical applications (Graf and Enver, 2009; Yamanaka and Blau, 2010). Lineage-instructive TFs activate and repress tissue-specific genes by recognizing sequence-specific DNA consensus motifs contained within enhancers and promoters (Ptashne, 2007). They establish gene regulatory networks (GRNs) of the novel gene expression program while dismantling those of the old program, involving the formation of feedforward, cross-inhibitory, and auto-regulatory loops (Bertrand and Hobert, 2010; Davidson, 2010; Graf and Enver, 2009; Holmberg and Perlmann, 2012). However, how these processes are coordinated and whether they recapitulate normal development remain unclear (Vierbuchen and Wernig, 2011), especially as neither TF-induced lineage conversions nor iPSC reprogramming appear to retrace normal developmental pathways (Apostolou and Hochedlinger, 2013; Di Tullio et al., 2011; Ladewig et al., 2013; Vierbuchen and Wernig, 2011).

Lineage-instructive TFs act through synergistic and cross-antagonistic interactions, are typically able to access closed chromatin (Zaret and Carroll, 2011), preferentially target sites with specific histone mark combinations, and bind to either nucleosome-depleted or nucleosome-dense regions (Soufi et al., 2012; Taberlay et al., 2011; Wapinski et al., 2013). However, what establishes these chromatin configurations in the first place and what proportion of the incoming reprogramming factors interacts with pre-existing TF complexes are largely unknown. A major reason for these gaps in our knowledge is that cell conversion frequencies in most cell systems are low, complicating efforts to study early events in a time-resolved fashion.

An exception is the transdifferentiation of pre-B/B cells into macrophages induced by the leucine zipper-type TF C/EBPα, which is arguably the most efficient and rapid system described so far (Bussmann et al., 2009; Di Tullio and Graf, 2012; Xie et al., 2004). C/EBPβ, like C/EBPα, can likewise induce B cell transdifferentiation into macrophages (Bussmann et al., 2009; Xie et al., 2004), but the two factors also have non-redundant functions. Mice ablated for C/EBPα die shortly after birth because they lack granulocyte/macrophage progenitors (GMPs, precursors of neutrophil granulocytes and macrophages, two closely related myeloid cell types) as well as granulocytes, while C/EBPβ-knockout animals are fully viable but contain macrophages and B cells with functional defects (Chen et al., 1997; Tanaka et al., 1995). C/EBPα cooperates with PU.1 (Spi1) to regulate myeloid gene expression (Friedman, 2007), the two factors interact physically (Reddy et al., 2002), and a combination of C/EBPα and PU.1 converts fibroblast into macrophage-like cells (Feng et al., 2008). The *Pu.1* gene encodes an Ets family TF specifically expressed in the early

stages of hematopoiesis and its knockout generates mice that lack both myeloid and lymphoid cells (Scott et al., 1994). Low-level expression of PU.1 in hematopoietic precursors induces B cell differentiation, whereas high levels favor myeloid differentiation (DeKoter and Singh, 2000).

Here we have analyzed, in a time-resolved manner, how C/EBPα establishes a myeloid expression program in pre-B cells, and we found that it binds to both pre-existing enhancers occupied by PU.1 and de novo enhancers where it acts as a pioneer factor. Strikingly, the combined activation of these enhancer types, regulating the expression of nearby macrophage genes, recapitulates the activation of myeloid enhancers and associated genes during normal hematopoiesis.

RESULTS

C/EBPα Induces High-Level Expression of *Pu.1* and *Cebpb*

To study how C/EBPα induces transdifferentiation, we used two pre-B cell lines that express an inducible C/EBPαER fusion protein tagged with either human CD4 (hCD4; C11 cells) or GFP (C10 cells). In both lines, treatment with 17 beta-estradiol (β-Est) shuttles C/EBPαER into the nucleus and induces the formation of macrophage-like cells within 2 to 3 days (Bussmann et al., 2009). Importantly, C/EBPα mRNA levels in C10 cells at 0 hr post-induction (hpi) or 24 hpi did not exceed C/EBPα levels observed in primary macrophages (MΦ) (Figure S1A). To monitor two important myeloid regulators known to cooperate with C/EBPα, we tested the expression levels of *Cebpb* and *Pu.1*. These genes were expressed at low to intermediate levels in pre-B cells (Figure S1B) and became upregulated within 3–12 hpi (Figure 1A). As C10 cells become transgene independent 24 hpi (Bussmann et al., 2009), i.e., before the expression of endogenous C/EBPα (Figure 1A), we determined whether the rapid activation of C/EBPβ and PU.1 is necessary for transdifferentiation. We generated C11 cells stably expressing a short hairpin RNA (shRNA) against C/EBPβ, PU.1, or both. Cells were induced with β-Est and analyzed by fluorescence-activated cell sorting (FACS) for the presence of Cd19 and Mac-1 (CD11b) at different days thereafter. At 3 days post-induction (dpi), the knockdown of C/EBPβ and of PU.1 resulted in a 35% and 50% reduction in the formation of Mac-1^{+}Cd19^{-} cells, respectively, while deleting both factors further enhanced the effect. At 7 dpi, Mac-1 expression in shC/EBPβ cells caught up with wild-type levels, whereas cells expressing shPU.1 exhibited extensive cell death (Figures 1B and S1C). These data show that C/EBPα rapidly upregulates *Pu.1* and *Cebpb,* that PU.1 is necessary to establish the myeloid GRN, and that C/EBPβ plays a more minor role.

A Limited Set of Sites Stably Bound by C/EBPα Correlates with the Upregulation of Macrophage Genes

To explore the mechanism by which C/EBPα turns on the myeloid program in pre-B cells, we treated C10 cells for different times with β-Est and performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) experiments, using antibodies to C/EBPα, C/EBPβ, and PU.1 (Table S1 gives a summary of ChIP-seq results and peak calling). A total of 54,198 non-redundant C/EBPα-enriched regions could be detected during the time course of which 10,849 sites were stably bound (i.e., up to 48 hpi, Table S2), whereas the remaining sites were transiently bound. Genes nearest stable binding sites, but not transient sites, were enriched for upregulated genes (Figure S1D). In addition, using a sliding-window approach, we observed that 70% of upregulated genes were localized within 100 kb of a stable C/EBPα-binding site, whereas no such enrichment was seen for downregulated genes (Figure 1C). Motif analysis of the stable sites in 48-hpi cells (hereafter referred to as induced macrophages or iMΦ) showed strong enrichment for consensus motifs of C/EBP and PU.1. The same sites also were enriched for AP-1 (Jun and Fos) and RUNX motifs, as previously reported (Figure 1D; Heinz et al., 2010) and more weakly enriched for EBF1 (Figure 1D; also see Figure 3). The majority of stable C/EBPα sites were co-occupied by C/EBPβ and PU.1 in iMΦ, and ~40% of these were pre-bound by PU.1 in pre-B cells, however, showing lower intensity signals (Figure 1E). Low-intensity signals in pre-B cells also were detectable for C/EBPβ, reflecting its low-level expression, as well as for C/EBPα (Figure S1E), suggesting some leakiness of the transgene.

A total of 10,849 C/EBPα sites were detected in iMΦ and 62,814 in bone-marrow-derived macrophages (MΦ) (Zhang et al., 2013), showing 9,288 common sites (Figure 1F). The larger number of sites in MΦ compared to iMΦ cannot be explained by a higher sequencing depth (Table S1). However, these differences became smaller when the numbers of associated genes were compared as follows: C/EBPα sites combined with 5,849 and 14,078 genes in iMΦ and MΦ, respectively, and shared 5,252 genes (Figure S1F). The shared gene set was enriched for genes that became upregulated during transdifferentiation of primary B cells into macrophages (Di Tullio et al., 2011), whereas the gene set unique for MΦ (8,826) was actually depleted (Figure S1G). In addition, shared upregulated genes were enriched for gene ontology (GO) terms associated with myeloid function, while upregulated genes unique for MΦ were not (Figure S1H). The induced rapid and efficient conversion of pre-B cells into highly motile, aggregating, and phagocytic macrophages within 51 hr (Movie S1; Bussmann et al., 2009) further supports the interpretation that C/EBPα