PU.1 Promotes Monocyte Development in Part by Transcriptionally Activating c-Fos, Egr-1 and Egr-2

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INTRODUCTION

PU.1 is a transcription factor with multiple roles in hematopoiesis. PU.1 has been shown to regulate myeloid and lymphoid development in a graded manner with high levels of PU.1 favoring monocyte development and low expression levels inducing B cell fate[1,2]. However underlying molecular mechanisms remain incompletely understood[3]

AIMS

- To investigate if c-Fos, Egr-1, and Egr-2 are involved in monocyte development induced by PU.1.
- To examine the molecular mechanisms through which PU.1 regulates c-Fos, Egr-1, and Egr-2 expression in monocyte development.

MATERIAL & METHODS

- **Cells**: Murine myeloid 32D and PUER cells were used. Lin⁻ cells were purified using the mouse Lineage Cell Depletion kit (Miltenyi Biotec).
- **qRT-PCR**: Total RNA was extracted using TRIzol reagent and reverse transcribed into cDNA,Prior to qPCR.
- **Western blot**: Cells were lysed in SDS lysis buffer, followed by SDS-PAGE and then transferred onto (PVDF) membranes. The membranes were incubated with relevant antibodies, followed by ECL.
- **Chromatin immunoprecipitation (ChIP)**: DNA-protein complexes were fixed with formaldehyde and precipitated with anti-PU.1 antibody and precipitated DNA was analyzed by qPCR.
- **Luciferase Reporter Assay**: Cells were electroporated with the reporter constructs. Luciferase activities were measured after 30H.
- **Flow cytometry**: Cells were incubated with antibody against Mac-1 and analyzed using LSR Fortessa and FlowJo software.
- **Gene knockdown**: Cells were transduced with lentiviral constructs containing shRNAs against PU.1, c-Fos, and Egr-1, and selected in puromycin.

RESULTS

PU.1 increases c-Fos, Egr-1 and Egr-2 expression

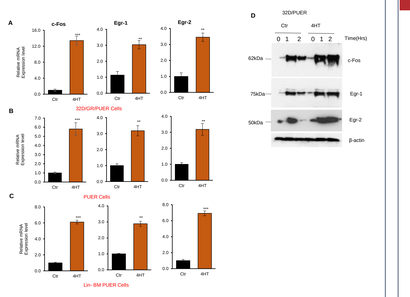

Figure 1: PU.1 increases the expression of c-Fos, Egr-1 and Egr-2: 32D/GR/PUER (A) ,PUER (B) and Lin –ve BM PUER cells were cultured in the absence(Ctr) and presence of 4HT for 24. The levels of c-Fos,Egr-1 and Egr-2 mRNAs were evaluated by qRT-PCR. 32D/GR/PUER cells were cultured in the absence (Ctr) or presence of 4HT for 24 hours, Cells were then starved for 2 hours and stimulated with G-CSF for the indicated times prior to evaluation of c-Fos, Egr-1 and Egr-2 Protein levels (D).

Knockdown of PU.1 reduces c-Fos, Egr-1 and Egr-2 expression

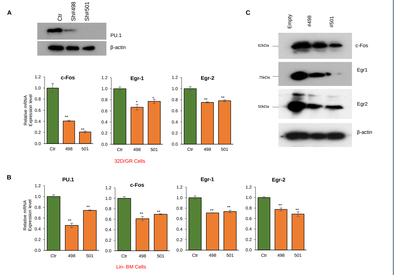

Figure 2:Knockdown of PU.1 reduces expression of c-Fos, Egr-1 & Egr-2: cells were transduced with empty lentiviral vector (Ctr) or two different PU.1 shRNAs (498 and 501) and examined for PU.1 protein level(A). After confirmation of knockdown, Cells were further evaluated for mRNA expression level of c-Fos, Egr-1 & Egr-2 (B) and Protein Level (C). Lin- cells were purified from mouse bone marrow and transduced with empty lentiviral vector (Ctr) and PU.1 shRNA(498 or 501), after confirmation of KD by mRNA expression level, cells were further evaluated for mRNA expression level of c-Fos, Egr-1 & Egr-2(D).

PU.1 Q202L mutant is less capable of upregulating c-Fos, Egr-1 and Egr-2 expression

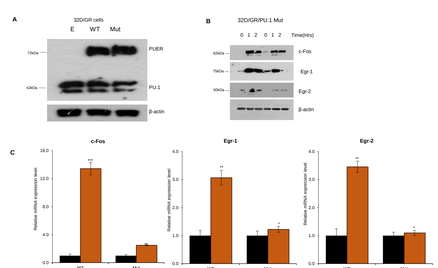

Figure 3: PU.1 Q202L mutant(defective in c-Jun binding) is less capable of upregulating c-Fos, Egr-1 and Egr-2 expression: Western blot shows that mut PU.1 does not affect the expression of PU.1 compared to WT(A). 32D/GR/PU.1 Mut cells were cultured in the absence (Ctr) or presence of 4HT for 24 hours, Cells were then starved for 2hours and stimulated with G-CSF for the indicated times prior to evaluation of c-Fos, Egr-1 and Egr-2 Protein levels (B). 32D/GR/PU.1Q202L mutant cells were cultured in GCSF media without(Ctr) and with 4HT for 24H prior to evaluating mRNA expression level of c-Fos, Egr-1 and Egr-2(C).

PU.1 binds to & activates c-Fos, Egr-1 and Egr-2 promoters

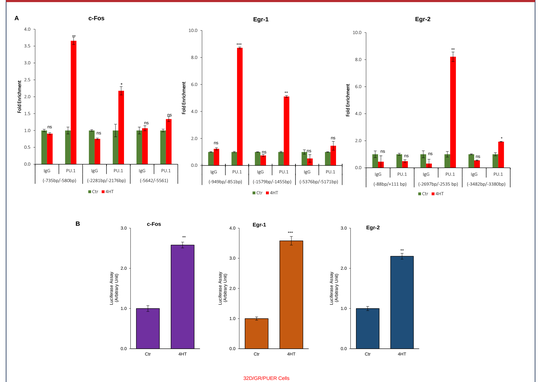

Figure 4: PU.1 binds to and activates c-Fos, Egr-1 and Egr-2 promoters: 32/GR/PUER cells were cultured with or without 4HT for 24 hours. Binding of PU.1 to c-Fos, Egr-1 and Egr-2 promoters were evaluated by ChIP-qPCR(A). 32D/GR/PUER cells were transfected via electroporation with pGL3-basic vector containing c-Fos, Egr-1 or Egr-2 promoter fragment and then cultured with or without 4HT. Luciferase activity was measured 24hours later(B).

KD of c-Fos partially abolishes PU.1 induced monocyte development

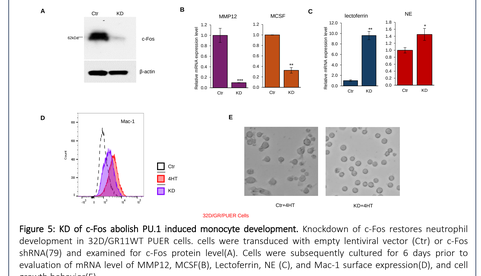

Figure 5: KD of c-Fos abolish PU.1 induced monocyte development. Knockdown of c-Fos restores neutrophil development in 32D/GR11WT PUER cells. cells were transduced with empty lentiviral vector (Ctr) or c-Fos shRNA(79) and examined for c-Fos protein level(A). Cells were subsequently cultured for 6 days prior to evaluation of mRNA level of MMP12, MCSF(B), Lectoferrin, NE (C), and Mac-1 surface expression(D), and cell growth behavior(E).

KD of Egr-1 partially abolishes PU.1 induced monocyte development

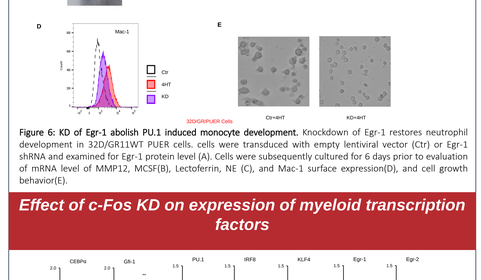

Figure 6: KD of Egr-1 abolish PU.1 induced monocyte development. Knockdown of Egr-1 restores neutrophil development in 32D/GR11WT PUER cells. cells were transduced with empty lentiviral vector (Ctr) or Egr-1 shRNA and examined for Egr-1 protein level (A). Cells were subsequently cultured for 6 days prior to evaluation of mRNA level of MMP12, MCSF(B), Lectoferrin, NE (C), and Mac-1 surface expression(D), and cell growth behavior(E).

Effect of c-Fos KD on expression of myeloid transcription factors

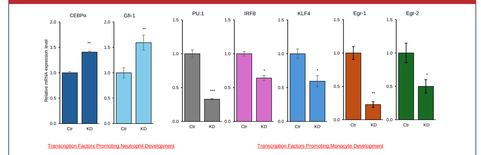

Figure 7: KD of c-Fos affects the expression of other myeloid specific transcription factors: The mRNA expression levels of indicated transcription factors were evaluated after c-Fos KD.

Effect of Egr-1 KD on expression of myeloid transcription factors

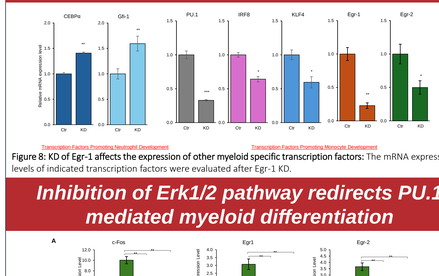

Figure 8: KD of Egr-1 affects the expression of other myeloid specific transcription factors: The mRNA expression levels of indicated transcription factors were evaluated after Egr-1 KD.

Inhibition of Erk1/2 pathway redirects PU.1 mediated myeloid differentiation

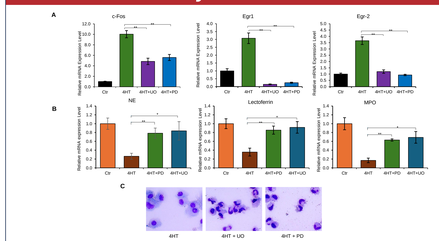

Figure 9: Inhibition of EKR1/2 pathway by Mek1/2 Inhibitor partially rescues neutrophils development in PUER cells. Inhibition of Erk1/2 pathway restored neutrophils development in PUER cells. PUER cells cultured in WEHI media in the absence(Ctr) or presence of 4HT, and Mek1/2 inhibitor U0126 (UO) or PD0325901 (PD) for 24 hours prior to evaluation of c-Fos ,Egr-1 AND Egr-2 mRNA (A). Cells were subsequently cultured for 7 days prior to evaluation of m RNA expression level of NE, MPO and Lectoferrin (B) and morphology(C).

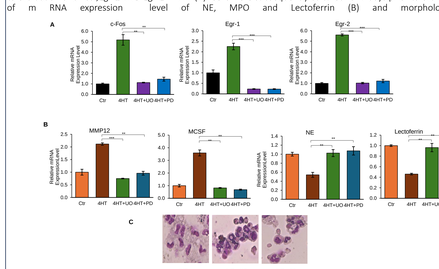

Figure 10: Inhibition of EKR1/2 pathway by Mek1/2 Inhibitor partially rescues neutrophils development in Lin-ve BM PUER cells. Inhibition of Erk1/2 pathway restored neutrophils development in lin-ve BM PUER cells. Cells were cultured in media in the absence(Ctr) or presence of 4HT, and Mek1/2 inhibitor U0126 (UO) or PD0325901 (PD) for 24 hours prior to evaluation of c-Fos ,Egr-1 AND Egr-2 mRNA (A). Cells were subsequently cultured for 6 days prior to evalution of m RNA expression level of MMP12, MCSF NE, Lectoferrin (B) and morphology(C).

CONCLUSIONS

- PU.1 directly binds to the promoters of c-Fos, Egr-1, and Egr-2, activating their transcription and driving monocyte development.
- PU.1 mutant defective in inducing monocyte development is partially impaired in the ability to upregulate c-Fos, Egr-1 and Egr-2 expression
- Suppression of c-Fos or Egr-1 expression through shRNAs, or inhibition of Mek1/2, shifts PU.1-induced differentiation to favor neutrophil development over monocytes.

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ACKNOWLEDGEMENTS

We thank Dr Richard Dahl (Indiana University School of Medicine, South Bend, IN) for providing the PUER cells and the PUER retroviral expression construct.

This work was supported by grant R15HL135695 (F.D) from the National Heart, Lung, and Blood Institute, National Institutes of Health.

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