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1,25-DIHYDROXYVITAMIN D3 INDUCES MONOCYTIC DIFFERENTIATION OF HUMAN MYELOID LEUKEMIA CELLS BY REGULATING C/EBP β EXPRESSION THROUGH MEF2C

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Abstract

Myogenic enhancer factor2 (Mef2) consists of a family of transcription factors involved in morphogenesis of skeletal, cardiac and smooth muscle cells. Among the four isoforms (Mef2A, 2B, 2C, and 2D), Mef2C was also found to play important roles in hematopoiesis. At myeloid progenitor level, Mef2C expression favors monocytic differentiation. Previous studies from our laboratory demonstrated that ERK5 was activated in 1,25-dihydroxyvitamin D3 (1,25D)-induced monocytic differentiation in AML cells and ERK5 activation was accompanied by increased Mef2C phosphorylation. We therefore examined the role of Mef2C in 1,25D-induced monocytic differentiation in AML cell lines (HL60, U937 and THP1) and found that knockdown of Mef2C with small interfering RNA (siRNA) significantly decreases the expression of the monocytic marker, CD14, without affecting the expression of the general myeloid marker, CD11b. CCAAT/Enhancer-binding protein (C/EBP) β , which can bind to CD14 promoter and increase its transcription, has been shown to be the downstream effector of 1,25D-induced monocytic differentiation in AML cells. When Mef2C was knocked down, expression of C/EBP β was reduced at both mRNA and protein levels. The protein expression levels of cell cycle regulators, p27^{Kip1} and cyclin D1, were not affected by Mef2C knockdown, nor the monopoiesis related transcription factor, ATF2 (Activating Transcription Factor 2). Thus, we conclude that 1,25D-induced monocytic differentiation, and CD14 expression in particular, is mediated through activation of ERK5-Mef2C-C/EBP β signaling pathway, and that Mef2C does not seem to modulate cell cycle progression.

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Keywords

Mef2C; vitamin D; AML; monocytic differentiation; C/EBB β ; CD14

INTRODUCTION

Mef2C is a member of the Mef2 family of transcription factors characterized by the MADS (MCM1-agamous-deficiens-serum)-box (1, 2). The four vertebrate isoforms, Mef2A, 2B, 2C and 2D, which can work in concert with each other as well as with transcription factors other than the Mef2 family to regulate specialized differentiation programs, were first described for myogenesis of skeletal, cardiac and smooth muscles (2, 3). Mef2C was also found to be involved in cell survival, neuronal differentiation, normal hematopoiesis and pathogenesis of AML (Acute Myeloid Leukemia) and ALL (Acute Lymphoblastic Leukemia) (4 – 9).

Mef2C expression level varies at different stages of hematopoiesis. At lineage progenitor level, Mef2C was found abundant in hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs) and common lymphoid precursors (CLP) (6, 7, 9). As these progenitor cells mature to more committed lineages: CMP to GMP (granulocyte-monocyte progenitor) and MEP (megakaryocyte-erythrocyte progenitor); CLP to B-cell and T-cell lineage, Mef2C expression decreases (6, 7, 9). Mef2C also peaks during the maturation of CMP to granulocytes and monocytes, and at this level Mef2C favors monocytic differentiation (6). Bone marrow cells from Mef2C knockout mice showed decreased monocytic differentiation in response to cytokines and colony stimulating factors, although the establishment of myeloid lineage was not affected (6). Structurally, all Mef2 family members share the same DNA binding domain in the N-terminus, but the C-terminus transactivation domain varies among different isoforms (2, 10). Importantly, Mef2C protein has been shown to be a target of MAPK (mitogen-activated protein kinase) signaling; a p38MAPK recombinant protein and ERK5 (Extracellular-signal-regulated kinase 5) activate Mef2C by phosphorylation of serine and threonine residues in the transactivation domain - p38MAPK phosphorylates Thr293, Thr300 and Ser387; ERK5 phosphorylates Ser387 (11, 12).

Transcription factors play pivotal roles in hematopoiesis. The lineage determining transcription factors, such as GATA1, GATA2, PU.1, C/EBP α , C/EBP β , etc., coordinate temporally and spatially to control the cell fate (reviewed in reference 13). C/EBP β is well known to be up-regulated and required for monopoiesis and induced monocytic differentiation (13 – 15). CD14 is the hallmark of monocytic differentiation with a potential C/EBP β binding sequence in its promoter region and C/EBP β over-expression leads to increased transcription of CD14 in luciferase reporter system (15). C/EBP α is generally believed to be involved in granulocytic differentiation, however, there is also evidence suggesting that C/EBP α can regulate CD14 expression by forming heterodimers with C/EBP β in 1,25D and TGF β -induced monocytic differentiation of leukemia cells (13, 16, 17).

Work from our laboratory and others has demonstrated that 1,25D and its analogs are able to induce monocytic differentiation of AML cells by activating MAP kinase signaling pathways including ERK1/2, p38MAPK and JNK (c-Jun N-terminal kinases) (17 – 25). Recently, ERK5, which signals in an overlapping fashion with ERK1/2 but can also transmit

different signals, has also been reported to be required in 1, 25D-induced monocytic differentiation, and in this process, monopoiesis-related transcription factors, Mef2C, ATF2 (Activating Transcription Factor 2), C/EBP α and β , were shown to be regulated by ERK5 activation (26, 27). Further, the pharmacological inhibitors of MEK5 (mitogen-activated protein kinase kinase 5) and ERK5 were able to attenuate/abolish CD14, a monocytic marker, expression induced by 1,25D, but in contrast, the expression of CD11b, a general myeloid marker, increased when MEK5/ERK5 were inhibited (27). The decreased CD14 expression correlated with decreased phosphorylation of Mef2C and decreased C/EBP β expression resulting from MEK5/ERK5 inhibition (27, 28). Based on the above findings, we postulated that 1,25D-induced monocytic differentiation, and CD14 expression in particular, are mediated through ERK5-Mef2C-C/EBP β activation. Here we demonstrate that Mef2C lies downstream of ERK5 and contributes to CD14 expression by activating C/EBP β expression.

MATERIALS AND METHODS

Reagents

1,25D was a kind gift from Dr. Milan Uskokovic (BioExell, Nutley, NJ). Mef2C rabbit polyclonal antibody was purchased from Cell Signaling Technologies (Danvers, MA). C/EBP β , C/EBP α , ATF2, Cylin D1, p27^{Kip1}, Crk-L and Calregulin antibodies, siMef2C, siControl and Lentiviruses containing shMef2C (short hairpin RNA), shControl and siRNA transfection reagent were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-CD11b MO-FITC and Anti-CD14 My4-RD-1 for flow cytometry were from Beckman Coulter Inc. (Brea, CA). cDNA Archive Kit and SYBR Green Master Kit were obtained from Applied Biosystems of Life Technologies (Grand Island, NY). HL60, U937 and THP1 cells were from American Type Culture Collection (Manassas, VA).

Cell culture and differentiation assay

HL60 (cultured from an acute promyeloblastic leukemia patient), U937 (monocytes from histiocytic lymphoma) and THP1 (monocytes from monocytic leukemia) were cultured in suspension under standard conditions (19, 29 – 31). To induce monocytic differentiation, 1,25D was added for 72 hrs to either non-transfected or siRNA/shRNA-transfected cells to a final concentration of 10 nM.

Flow cytometry

The harvested cells were incubated with 1 ml of PBS containing 0.5 μ g Anti-CD11b MO-FITC and 0.5 μ g Anti-CD14 My4-RD-1 antibodies and the labelled cells were analyzed by flow cytometry as we described before (25).

Western blotting

Protein expression levels were measured by Western blots using whole cell lysates according to standard procedures (19). Optical densities (ODs) of the bands were analyzed with ImageJ (NIH) and ODs were normalized to the band of the untreated control group and then normalized to the loading controls.

RT-PCR and real time PCR

mRNA levels were measured with extracts of total RNA by RT-PCR followed by real time PCR as described before (27). The following primers were used: Mef2C primers: forward 5'-CGAGATGCCAGTCTCCATCC-3', reverse 5'-AGCAGACCTGGTGAGTTTCG-3'; C/EBP β primers: forward 5'-GTTCTTGACGTTCTTCGGCCG-3', reverse 5'-TGGACAAGCACAGCGACGAGT-3'; RPII (RNA Pol II) primers: forward 5'-GCACCACGTCCAATGACAT-3, reverse 5'-GTGCGGCTGCTTCCATAA-3'.

siRNA and shRNA transfection

siRNA transfection and shRNA-containing Lentivirus infection were performed according to the manufacturer's protocols (Santa Cruz Biotechnology, Dallas, TX).

Statistical Analysis

Each experiment was repeated at least three times and results were presented as mean $\pm$ SE. Significance of the differences between mean values (knockdown group vs control group) was assessed by two-tailed paired *t*-test using Microsoft EXCEL.

RESULTS

Mef2C knock-down attenuates CD14 expression in AML cells

As stated above, this laboratory has demonstrated that treatment of human AML cells with ERK5 pharmacological inhibitors results in decreased monocytic differentiation which is accompanied by decreased expression of phospho-Mef2C (27). To investigate whether Mef2C is required for 1,25D-induced monocytic differentiation, we transfected HL60 cells, a cell line comparable to the M2 subtype in the FAB (French-American-British) classification of AML, with siMef2C and induced differentiation with 1,25D. After 72 hrs incubation of 1,25D, differentiation was evaluated by flow cytometry using two cell surface markers, CD14, a monocytic specific marker, and CD11b, a general myeloid marker (32, 33). As shown in Figure 1A and B, the percentage of CD14 positive cells decreased by ~25% in the silencing Mef2C experimental groups ($p < 0.05$ compared to the control groups). By contrast, CD11b expression levels in the Mef2C knockdown groups and the control groups were comparable (21% for siMef2C groups vs 20.7% for control groups). As shown in Figure 1C, protein expression of Mef2C decreased significantly in the knock-down groups compared to the silencing control groups. To exclude the possibility that the requirement of Mef2C in 1,25D-induced monocytic differentiation is limited to the M2 AML cell subtype, we performed similar experiments with two other cell lines, THP1 and U937. The differentiation state of both cell lines is equivalent to M4 (U937) and M5 (THP1) subtypes in FAB classification of AML (34). Mef2C knockdown was shown to reduce CD14 expression markedly in both THP1 cells (~18%) and U937 cells (~25%), but leaving CD11b expression unaffected. The above data suggest that Mef2C is necessary for 1,25D-induced CD14 expression, but not for CD11b expression in AML cells. Though the decreased CD14 expression after Mef2C knockdown is not dramatic, we observed the same trend in all three cell lines blocked at various differentiation stages, and the unaffected

CD11b expression levels also makes nonspecific changes caused by transfection very unlikely.

Mef2C knockdown leads to decreased C/EBP β protein expression

MAPK family signaling is one of the major signaling pathways mediating 1,25D-induced monocytic differentiation of AML cells. ERK1/2, isoforms of p38MAPK as well as JNK, are all activated by 1,25D, and their regulation of CD14 expression converges on C/EBP β (23 – 25). Mef2C, which lies downstream of ERK5 and is required, at least partially, for CD14 expression, can perhaps also regulate CD14 expression by activating C/EBP β . To explore this possibility, we examined the expression levels of C/EBP β after Mef2C knockdown. As shown in Figure 2A, exposure to 1,25D resulted in increased protein expression of C/EBP β , as we have shown before (35), and C/EBP β protein expression was reduced in the Mef2C knock-down groups compared to the silencing control groups in all the cells lines (HL60, U937 and THP1) we tested (Figure 2B) (data not shown for U937). Since protein expression levels can be controlled either at transcriptional or post transcriptional levels, we further determined at which level that C/EBP β is regulated by Mef2C by measuring the mRNA levels by quantitative PCR. Significant decrease of C/EBP β mRNA levels was observed compared to the corresponding controls in HL60 and THP1 cells when Mef2C was knocked down (Figure 2C). The decreased C/EBP β expression upon Mef2C knockdown, together with the attenuated CD14 expression, support the idea that 1,25D regulates monocytic marker expression through the ERK5-Mef2C-C/EBP β -CD14 axis. Thus, we conclude that C/EBP β is a downstream effector of Mef2C in regulating CD14 expression, and that the regulation of C/EBP β by Mef2C takes place at the transcriptional level.

Mef2C knockdown does not affect p27^{Kip1}, cyclin D1 and ATF2 expression

The cell cycle regulators, p27^{Kip1} and cyclin D1, are involved in G1/S arrest and can be regulated by ERK5 (28, 34, 36, 37). Since Mef2C is downstream of ERK5, we determined whether the signals regulating p27^{Kip1} and cyclinD1 are transmitted through Mef2C. Though the protein expression of p27^{Kip1} and cyclin D1 was up regulated by 1,25D, no credible changes were seen in the siMef2C experimental groups compared to the siControl groups in either HL60 or U937 cells (data not shown). ATF2 is a component of AP-1 (Activator Protein 1) and AP-1 has been reported to be involved in 1,25D-induced monocytic differentiation (26). Therefore, we also examined whether Mef2C affects ATF2 expression. However, Mef2C knockdown did not show any apparent effect on ATF2 expression. These data imply that the previously reported effects of ERK5 associated with cell cycle progression may not be transmitted through Mef2C, though further studies are warranted (28).

DISCUSSION

We report here for the first time that Mef2C, a downstream target of ERK5, is involved in 1,25D-induced monocytic differentiation of human AML cells, and that it controls CD14 expression by up-regulating C/EBP β levels. We also demonstrate that the effects of ERK5 on cell cycle and CD11b expression are not mediated by Mef2C. Transcriptional control of

Mef2C has been extensively studied in myogenesis, and several enhancer elements have been described which control cardiac and somatic muscle development in *Drosophila* (2). However, to our knowledge, no validated VDRE (vitamin D receptor element) has been reported in the promoter/enhancer region of Mef2C, thus direct binding of VDR to Mef2C is unlikely, and its up-regulation by 1,25D appears to be the result of indirect effects of 1,25D.

We propose that Mef2C lies immediately downstream of ERK5 since there is a demonstrated ERK5 phosphorylation site in the transactivation domain of Mef2C (Figure 3). But although Mef2C can be regulated directly by ERK5, Mef2C does not seem to transmit all the signals from ERK5 required to control CD14 expression. As we reported before, inhibition of ERK5 by a pharmacological inhibitor XMD8-92 resulted in over 90% decrease of CD14 expression (27), yet when we knocked down Mef2C expression with shRNA by approximately 90%, we observed only ~50% decrease in CD14 expression (data not shown). So it is likely that mechanisms other than Mef2C activation also participate in the regulation of the expression of monocytic markers by ERK5.

C/EBP transcription factors play important roles in hematopoiesis, and their functions are highly regulated by various signals (38 – 41). Our results show that Mef2C knockdown decreased C/EBP β mRNA levels, which supports its transcriptional regulation by Mef2C. Though a potential binding sequence of Mef2C was found at approximately 2Kb upstream from the transcription starting site of C/EBP β , there is no documented evidence showing functional binding of Mef2C to this region, and further investigations are needed to determine its function. Regulation of C/EBP β at translational as well as post-translational levels has also been reported (42 – 44), so post-transcriptional regulation of C/EBP β by Mef2C cannot be excluded.

The DNA binding domains of C/EBP isoforms are highly conserved and it has been reported that several C/EBPs (α , β and δ) can bind to the CD14 promoter and activate its transcription in monocyte development (15). This may be linked to Mef2C as it has been shown that Mef2C expression is required for monopoiesis in a mouse knockdown model (6). Our results demonstrate that protein expression of C/EBP β robustly increases following exposure to 1,25D, and is reduced by Mef2C knockdown in all the cell lines we tested. C/EBP α expression is more cell type specific, as in HL60 cells no apparent changes are seen in the presence 1,25D following Mef2C knockdown (data not shown). However, in THP1 cells we saw a modest increase of C/EBP α protein expression when these cells are treated with 1,25D, but a decrease of C/EBP α upon Mef2C knockdown. The above observations imply that although multiple C/EBPs can be activated by 1,25D and may contribute to CD14 expression, C/EBP β is likely to be the principal transcription factor that is indispensable for 1,25D-induced monocytic differentiation.

Together with previous studies, our results suggest that 1,25D-induced expression of the monocytic marker CD14 results from the activation of VDR, which in concert with growth factors and cytokines initiates a sequence of steps that include Cot1, MEK5, ERK5, Mef2C, and C/EBPs (Figure 4). However, it appears that the various aspects of the differentiated phenotype can be dissociated, since unlike the expression of CD14, neither the expression of CD11b, another monocytic marker, nor the cell cycle regulators are controlled by Mef2C.

Additional studies are warranted to establish how cancer therapeutic drugs can be made more effective by the modulation of Mef2C expression.

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References

1. Molken JD, Li L, Olson EN. Phosphorylation of the MADS-Box transcription factor MEF2C enhances its DNA binding activity. *J Biol Chem.* 1996; 271:17199–17204. [PubMed: 8663403]
2. Black BL, Olson EN. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol.* 1998; 14:167–196. [PubMed: 9891782]
3. Olson EN, Perry M, Schulz RA. Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev Biol.* 1995; 172:2–14. [PubMed: 7589800]
4. Suzaki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, Tamaki T. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. *J Biol Chem.* 2002; 277:9614–9621. [PubMed: 11782488]
5. Wilker PR, Kohyama M, Sandau MM, Albring JC, Nakagawa O, Schwarz JJ, Murphy KM. Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. *Nat Immunol.* 2008; 9:603–612. [PubMed: 18438409]
6. Schuler A, Schwieger M, Engelmann A, Weber K, Horn S, Muller U, et al. The MADS transcription factor Mef2c is a pivotal modulator of myeloid cell fate. *Blood.* 2008; 111:4532–4541. [PubMed: 18326819]
7. Stehling-Sun S, Dade J, Nutt SL, DeKoter RP, Camargo FD. Regulation of lymphoid versus myeloid fate ‘choice’ by the transcription factor Mef2c. *Nat Immunol.* 2009; 10:289–296. [PubMed: 19169261]
8. Cho EG, Zaremba JD, McKercher SR, Talantova M, Tu S, Masliah E, Chan SF, Nakanishi N, Terskikh A, Lipton SA. MEF2C enhances dopaminergic neuron differentiation of human embryonic stem cells in a parkinsonian rat model. *PLoS One.* 2011; 6:e24027. [PubMed: 21901155]
9. Canté-Barrett K, Pieters R, Meijerink JP. Myocyte enhancer factor 2C in hematopoiesis and leukemia. *Oncogene.* 2014; 33:403–410. [PubMed: 23435431]
10. Molken JD, Black BL, Martin JF, Olson EN. Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. *Mol Cell Biol.* 1996; 16:2627–2636. [PubMed: 8649370]
11. Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* 1997; 16:7054–7066. [PubMed: 9384584]
12. Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature.* 1997; 386:296–299. [PubMed: 9069290]
13. Brown, G.; Ceredig, R. *Cell Determination During Hematopoiesis.* New York: Nova Science Publishers, Inc.; 2009. Chapter 3.
14. Ji Y, Studzinski GP. Retinoblastoma protein and CCAAT/enhancer-binding protein beta are required for 1,25-dihydroxyvitamin D₃-induced monocytic differentiation of HL60 cells. *Cancer Res.* 2004; 64:370–377. [PubMed: 14729647]
15. Pan Z, Hetherington CJ, Zhang DE. CCAAT/enhancer-binding protein activates the CD14 promoter and mediates transforming growth factor beta signaling in monocyte development. *J Biol Chem.* 1999; 274:23242–23248. [PubMed: 10438498]
16. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, Shigematsu H, Levantini E, Huettner CS, Lektrom-Himes JA, Akashi K, Tenen DG. Enhancement of

hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 2004; 21:853–863. [PubMed: 15589173]

17. Studzinski GP, Wang X, Ji Y, Wang Q, Zhang Y, Kutner A, Harrison JS. The rationale for deltanoids in therapy for myeloid leukemia: Role of KSR-MAPK-C/EBP pathway. *J Steroid Biochem Mol Biol*. 2005; 97:47–55. [PubMed: 16046262]
18. Marcinkowska E, Wiedłocha A, Radzikowski C. 1,25-Dihydroxyvitamin D3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. *Biochem Biophys Res Commun*. 1997; 241:419–426. [PubMed: 9425286]
19. Wang X, Rao J, Studzinski GP. Inhibition of p38 MAP kinase activity up-regulates multiple MAP kinase pathways and potentiates 1,25-dihydroxyvitamin D(3)-induced differentiation of human leukemia HL60 cells. *Exp Cell Res*. 2000; 258:425–437. [PubMed: 10896794]
20. Wang X, Studzinski GP. Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cell. *J Cell Biochem*. 2001; 82:68–77. [PubMed: 11400164]
21. Wang X, Studzinski GP. J Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D3-induced differentiation of HL60 cells. *Cell Biochem*. 2001; 80:471–482.
22. Ji Y, Kutner A, Verstuyf A, Verlinden L, Studzinski GP. Derivatives of vitamins D2 and D3 activate three MAPK pathways and upregulate pRb expression in differentiating HL60 cells. *Cell Cycle*. 2002; 1:410–415. [PubMed: 12548017]
23. Studzinski GP, Garay E, Patel R, Zhang J, Wang X. Vitamin D receptor signaling of monocytic differentiation in human leukemia cells: role of MAPK pathways in transcription factor activation. *Curr Top Med Chem*. 2006; 6:1267–1271. [PubMed: 16848740]
24. Gocek E, Studzinski GP. Vitamin D and differentiation in cancer. *Crit Rev Clin Lab Sci*. 2009; 46:190–209. [PubMed: 19650715]
25. Zhang J, Harrison JS, Studzinski GP. Isoforms of p38MAPK gamma and delta contribute to differentiation of human AML cells induced by 1,25-dihydroxyvitamin D₃. *Exp Cell Res*. 2011; 317:117–130. [PubMed: 20804750]
26. Wang X, Studzinski GP. The requirement for and changing composition of the activating protein-1 transcription factor during differentiation of human leukemia HL60 cells induced by 1,25-dihydroxyvitamin D3. *Cancer Res*. 2006; 66:4402–4409. [PubMed: 16618766]
27. Wang X, Pesakhov S, Harrison JS, Danilenko M, Studzinski GP. ERK5 pathway regulates transcription factors important for monocytic differentiation of human myeloid leukemia cells. *J Cell Physiol*. 2014; 229:856–867. [PubMed: 24264602]
28. Wang X, Pesakhov S, Weng A, Kafka M, Gocek E, Nguyen M, Harrison JS, Danilenko M, Studzinski GP. ERK 5/MAPK pathway has a major role in 1 α ,25-(OH)₂ vitamin D3-induced terminal differentiation of myeloid leukemia cells. *J Steroid Biochem Mol Biol*. 2013; S0960-0760:00195–00197.
29. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, et al. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood*. 1979; 54:713–733. [PubMed: 288488]
30. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer*. 1976; 17:565–577. [PubMed: 178611]
31. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer*. 1980; 26:171–176. [PubMed: 6970727]
32. Todd RF 3rd, Nadler LM, Schlossman SF. Antigens on human monocytes identified by monoclonal antibodies. *J Immunol*. 1981; 126:1435–1442. [PubMed: 6937561]
33. Goyert SM, Ferrero E, Rettig WJ, Yenamandra AK, Obata F, Le Beau MM. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. *Science*. 1988; 239:497–500. [PubMed: 2448876]
34. Wang X, Gocek E, Novik V, Harrison JS, Danilenko M, Studzinski GP. Inhibition of Cot1/Tlp2 oncogene in AML cells reduces ERK5 activation and upregulates p27Kip1 concomitant with

- enhancement of differentiation and cell cycle arrest induced by silibinin and 1,25-dihydroxyvitamin D3. *Cell Cycle*. 2010; 9:4542–4551. [PubMed: 21084834]
35. Marcinkowska E, Garay E, Gocek E, Chrobak A, Wang X, Studzinski GP. Regulation of C/EBP β isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D3. *Exp Cell Res*. 2006; 312:2054–2065. [PubMed: 16624284]
36. Wang QM, Jones JB, Studzinski GP. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. *Cancer Res*. 1996; 56:264–267. [PubMed: 8542578]
37. Mulloy R, Salinas S, Philips A, Hipskind RA. Activation of cyclin D1 expression by the ERK5 cascade. *Oncogene*. 2003; 22:5387–5398. [PubMed: 12934098]
38. De Obaldia ME, Bell JJ, Wang X, Harly C, Yashiro-Ohtani Y, DeLong JH, Zlotoff DA, Sultana DA, Pear WS, Bhandoola A. T cell development requires constraint of the myeloid regulator C/EBP- α by the Notch target and transcriptional repressor Hes1. *Nat Immunol*. 2013; 14:1277–1284. [PubMed: 24185616]
39. Cain DW, O’Koren EG, Kan MJ, Womble M, Sempowski GD, Hopper K, Gunn MD, Kelsoe G. Identification of a tissue-specific, C/EBP β -dependent pathway of differentiation for murine peritoneal macrophages. *J Immunol*. 2013; 191:4665–4675. [PubMed: 24078688]
40. Larsen MT, Häger M, Glenthøj A, Asmar F, Clemmensen SN, Mora-Jensen H, Borregaard N, Cowland JB. miRNA-130a regulates C/EBP- ϵ expression during granulopoiesis. *Blood*. 2014; 123:1079–1089. [PubMed: 24398327]
41. Hasemann MS, Lauridsen FK, Waage J, Jakobsen JS, Frank AK, Schuster MB, Rapin N, Bagger FO, Hoppe PS, Schroeder T, Porse BT. C/EBP α is required for long-term self-renewal and lineage priming of hematopoietic stem cells and for the maintenance of epigenetic configurations in multipotent progenitors. *PLoS Genet*. 2014; 10:e1004079. [PubMed: 24415956]
42. Ramji DP1, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J*. 2002; 365:561–575. [PubMed: 12006103]
43. Tsukada J, Yoshida Y, Kominato Y, Auron PE. The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine*. 2011; 54:6–19. [PubMed: 21257317]
44. Serrat N, Sebastian C, Pereira-Lopes S, Valverde-Estrella L, Lloberas J, Celada A. The response of secondary genes to lipopolysaccharides in macrophages depends on histone deacetylase and phosphorylation of C/EBP β . *J Immunol*. 2014; 192:418–426. [PubMed: 24307736]

Highlights

- Mef2C is required for 1,25D-induced monocytic differentiation in AML cells.
- Mef2C regulates CD14 expression through C/EBP β activation at transcriptional level.
- Mef2C is not involved in p27^{Kip1} and cyclin D1 expression in 1,25D-treated AML cells.

Fig.1. Mef2C knockdown leads to decreased CD14 expression

(A) Flow cytometry results illustrating CD14 and CD11b expression in untreated, 10 nM 1,25D, 10 nM 1,25D + siMef2C, and 10 nM 1,25D + siControl groups. (B) Statistical summary of the flow cytometry results. Bars represent means $\pm$ SE, $n = 3 - 4$ for each cell line. Paired t - test was used for statistical analysis (siMef2C vs siControl). *, $p < 0.05$. (C) Western blots representative of three experiments with each cell line showing reduced Mef2C protein levels in the knockdown group. ODs were normalized to loading controls provided by reblotting for Crk-L or Calregulin. Samples were loaded in duplicate.

Fig.2. C/EBP β expression levels decrease after Mef2C knockdown

(A) Representative Western blots showing C/EBP β expression levels in different treatment groups of HL60 and U937 cells. C/EBP β showed two bands with ~ 46 kDa migration level, but only the upper bands were taken for OD measurement and subsequent statistical analysis shown here because of their more prominent changes. Loading controls were provided by reblotting for Crk-L or Calregulin. Samples were loaded in duplicate. (B) Statistical analysis of the upper band of C/EBP β at 46 kDa in Western blots. Bars represent mean $\pm$ SE, $n = 3 - 4$ for each cell line. Paired t - test was used for the analysis (siMef2C vs siControl). *, $p < 0.05$; **, $p < 0.01$. (C) Statistical summary of the relative mRNA levels in Mef2C knockdown groups and silencing control groups. Of note, shRNA was used in HL60 cells and siRNA in THP1 cells. Bars represent means $\pm$ SE, $n = 6$ for HL60 cells and $n = 3$ for THP1 cells. *, $p < 0.05$; **, $p < 0.01$.

Fig.3. The schematic structure of Mef2C

Mef2C protein consists of a DNA-binding domain and a transactivation domain. One constitutive phosphorylation site is located in the DNA-binding domain and three regulatory phosphorylation sites in the transactivation domain. (Modified Mef2C schematic from Ref 2)

Fig.4. Summary of MAPK signaling pathways in 1,25D-induced monocytic differentiation of AML cells

Mef2C lies downstream of ERK5, working in concert with other members in MAPK family and other signaling pathways to regulate 1,25D-induced monocytic differentiation in AML cells. Solid lines: direct regulation; dotted lines: suggested indirect regulation.