Synergistic Induction of Galectin-1 by CCAAT/Enhancer Binding Protein α and Hypoxia-inducible Factor 1α and Its Role in Differentiation of Acute Myeloid Leukemic Cells

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Xu-Yun Zhao, Ke-Wen Zhao, Yi Jiang, Meng Zhao, and Guo-Qiang Chen

From the Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis of the Ministry of Education of China and the Chemical Biology Division of Shanghai Universities E-Institutes, Shanghai Jiao Tong University School of Medicine (SJTU-SM) and the Institute of Health Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China.

Background: Galectin-1 is a widely investigated cell surface glycoprotein-binding protein that mediates multiple cellular activities.

Results: Galectin-1 is synergistically induced by C/EBPα and HIF-1α and facilitates the differentiation of leukemic cells.

Conclusion: Galectin-1 is an important mediator for leukemic cell differentiation.

Significance: Learning how galectin-1 expression is regulated by hematopoietic cell differentiation-associated transcription factors is of significance in understanding leukemic cell differentiation.

Galectin-1 is a member of the galectin family and has a high affinity for galactose and N-acetylgalactosamine moieties of glycoproteins. It mediates multiple signal transduction pathways to modulate cellular proliferation, survival, differentiation, and migration. However, the mechanisms for the regulation of its expression remain greatly elusive. We reported previously that galectin-1 is a direct target of the hypoxia-inducible factor 1 (HIF-1), a key heterodimeric transcriptional factor for the cellular response to hypoxia. Here we show that CCAAT enhancer binding protein (C/EBP)α, a critical transcriptional factor for hematopoietic cell differentiation, can directly activate galectin-1 through binding to the −48 to −42 bp region of its promoter. Based on the physical interaction of C/EBPα and HIF-1α, the synergistic transcriptional activity of C/EBPα and HIF-1α on the promoter of the galectin-1 gene is also found by chromatin immunoprecipitation (ChIP), ChIP followed by ChIP (ChIP-recH1P), and luciferase assay. Moreover, knockdown or chemical inhibition of galectin-1 partially blocks the differentiation induced by HIF-1α or C/EBPα, which can be rescued by recombinant galectin-1. These discoveries would shed new insights on the mechanisms for galectin-1 expression regulation and HIF-1α and C/EBPα-induced leukemic cell differentiation.

Galectins are an evolutionally conserved family of carbohydrate-binding lectins with a high affinity for β-galactosides (1). They are intra- and extracellularly distributed. The extracellular galectins exert their effects by binding to the cell surface oligosaccharides. To date, at least 15 members of the family have been identified. All of them carry the conserved carbohydrate recognition domain (CRD), which is responsible for their binding to the galactose and N-acetylgalactosamine moieties of glycoproteins (2). Galectin-1 has been found to be differentially expressed in various normal and pathological tissues and to mediate a series of signal pathways involving cellular adhesion, migration, proliferation, differentiation, and death (3–6). For example, the mesenchymal stem cells of the bone marrow highly express galectin-1 protein (7, 8), and galectin-1-expressing stromal cells constitute a specific niche for pre-BII cell development in mice (9). Upon erythroid differentiation of leukemic K562 cells, the galectin-1 is externalized and bound to the cell surface (10). Also, recombinant galectin-1 can mediate the growth and death of murine and human hematopoietic stem and progenitor cells (11). However, the mechanisms for the molecular regulation of galectin-1 expression in normal and malignant hematopoietic cells remain largely unknown.

Hypoxia-inducible factor-1 (HIF-1) is a key heterodimeric transcriptional factor for cellular response to hypoxia. It consists of an oxygen-sensitive α subunit (HIF-1α) and constitutively expressed β subunit (HIF-1β, also designated as aryl hydrocarbon receptor nuclear translocator) (12). We showed previously that galectin-1 is a direct target of the HIF-1 protein (13) and that HIF-1α can interact with and enhance the transcriptional activity of CCAAT enhancer binding protein α.

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§ Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Key Laboratory of Cell Differentiation and Apoptosis of Ministry of Education of China, Shanghai Jiao Tong University School of Medicine (SJTU-SM), No. 280, Chong-Qing South Rd., Shanghai 200025, China Tel.: 86-21-64154900; Fax: 86-21-64154900; E-mail: chengq@shsmu.edu.cn.

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(C/EBPα) (14), one of the most transcriptional factors involved in the differentiation of hematopoietic cells (15, 16). Here we report that C/EBPα directly up-regulates the transcriptional expression of galectin-1 and presents a synergistic effect with HIF-1α for the induction of galectin-1 expression. More intriguingly, suppression of galectin-1 by shRNA or the classical galectin inhibitor lactose antagonizes HIF-1α- and C/EBPα-induced cell differentiation of acute myeloid leukemia (AML), which can be restored by recombinant galectin-1. Understanding the mechanisms of the regulation of galectin-1 expression provides new insights into the roles of HIF-1α and C/EBPα in leukemic cell differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Leukemic cell lines, U937, NB4, and Jurkat cells were cultured in RPMI 1640, and human embryonic kidney 293T cells and gastric cancer SGC7901 cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO). Both embryonic kidney 293T cells and gastric cancer SGC7901 cells were cultured in RPMI 1640 with 10% FBS, which were kindly provided by Daniel G. Tenen of Harvard Medical School, were cultured in RPMI 1640 with 10% FBS, 1 μg/ml of tetracycline (Sigma-Aldrich), and 0.5 μg/ml of puromycin (Sigma-Aldrich). U937T stable transformants with inducible HIF-1α, C/EBPα, galectin-1, and empty vector, which were designated respectively as U937THIF-1α, U937T/C/EBPα, U937TGal-1, and U937TPTR3 cells, were established as described previously (17, 18). For the inducible expression of HIF-1α, C/EBPα, or galectin-1, these cells were cultured in tetracycline-free medium for the indicated number of days. All cell lines were cultured in 5% CO2 and 95% air in a humidified atmosphere at 37 °C. For hypoxic treatment, cells were cultured in a specially designed hypoxia incubator (Thermo Electron, Forma, MA) in an atmosphere that consists of 94% N2, 5% CO2, and 1% O2.

Quantitative Real-time RT PCR (Q-PCR)—Total RNA was isolated by TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Promega, Madison, WI). Reverse transcription was performed with the TaKaRa RNA PCR kit (TaKaRa, Dalian, China), and double-stranded DNA dye SYBR Green PCR Master Mix reagent (Applied Biosystems, Warrington, UK) was used for Q-PCR analysis as described previously (19). Specific primers were used: Galectin 1, 5'-CGCTAAGAGCTCTGTCGCTGAC-3' (forward) and 5'-CCACACCTCTGCAACACCTTCAGC-3' (reverse); macrophage colony-stimulating factor receptor (M-CSFR), 5'-CAATGGCAGCCTGGAATG-3' (forward) and 5'-GCAGTATGCTCCTGCTCCTC-3' (reverse); granulocyte colony-stimulating factor receptor (G-CSFR), 5'-ACATGGGACGAGCTGTCTTTCCATGC-3' (forward) and 5'-AGACTGGGAGCAGAGCTTCCTTC-3' (reverse); and β-actin as control, 5'-CATCCTTCCACTGGAATGCC-3' (forward) and 5'-AGCCCTGATGACCATGC-3' (reverse). The folds of changes were shown as mean ± S.D. in three independent experiments, each with triplicate samples.

Luciferase Assay—The indicated sequence (-569 bp ~ +194 bp) in the promoter of galectin-1 was obtained from the NCBI, amplified by PCR from genomic DNA, and subcloned into the pGL3-Basic vector (Promega) to construct a luciferase reporter plasmid (pGL3-Gal-1). A site-directed mutagenesis kit was used to make the point mutations of the sequence of pGL3-Gal-1 reporter plasmids according to the manufacturer’s instructions (Stratagene, La Jolla, CA). For the luciferase assay, 293T cells were seeded in a 12-well plate (BD Biosciences) and cotransfected with pEF-BOS-HIF-1α (kind gift from Dr. K. Sogawa, Tohoku University, Japan) and/or pcDNA3.1(-)C/EBPα, pGL3-Gal-1, and pRLSV40-Renilla. Thirty-six hours after transfection, cells were lysed and analyzed by the dual luciferase assay system according to the manufacturer’s instructions (Promega).

Chromatin Immunoprecipitation (ChIP) and ChIP Followed by ChIP (ChiP-ChiP) Assay—A ChiP assay was performed according to our previous report (13). Briefly, U937 cells were grown under normoxia or hypoxia for 24 h and then cross-linked with 1% formaldehyde at room temperature for 10 min. Then, cells were pelleted and resuspended in 400 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0)). Cellular DNA was sonicated and sheared to small fragments of 500–1000 bp with a Sonicator ultrasonic processor (Misonix, Farmingdale, NY). Subsequently, the supernatant of the sonicated cells was collected, diluted, and precleared by protein A-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-human HIF-1α antibody (BD Transduction Laboratories) or anti-human C/EBPα antibody (Santa Cruz Biotechnology, Inc.) was added to the supernatant for immunoprecipitation with normal preimmuned IgG (Santa Cruz Biotechnology, Inc.) as a normal control. After overnight incubation, the protein G-agarose for HIF-1α or protein A-agarose for C/EBPα were added and incubated for 3 h. For the ChiP-ChIP assay, the primary immunoprecipitated was eluted by elution buffer (10 mM Tris-HCL, 1 mM EDTA, 10 mM DTT) with rotation at 37 °C for 30 min, and its supernatant was immunoprecipitated with the secondary antibody. The immunoprecipitated DNA was retrieved by 5 M NaCl at 65 °C for 4 h and purified with a PCR purification kit (Qiagen, Hilden, Germany) (20). PCR for the HIF-1α or C/EBPα binding site as well as negative control in the promoter of galectin-1 was performed with specific primers: 5'-GCAGTGGCGGCTTTCGC-3' (forward, P1) and 5'-CGGAGGAAGTGCTGGCTTT-3' (reverse, P2) for negative control, 5'-AATTTTTCCACTGGCTTCC-3' (forward, P3) and 5'-CTGGAAGTTCGCAATTCGA-3' (reverse, P4) for the C/EBPα binding site, and 5'-CCCGCCCTTCTTTAGCGCTTC-3' (forward, P5) and 5'-GATGATGAGCTAGGCCCAACAG-3' (reverse, P6) for the hypoxia responsive element (HRE).

Design and Transfection of shRNA—The target shRNA sequences for galectin-1, HIF-1α, and C/EBPα were synthesized, annealed, and ligated into pSilencer 3.1-H1-neo according to the manufacturer’s instructions (Ambion, Austin, TX) as described previously (13, 17, 18). Then, these targets and negative control shRNA-containing plasmids were transfected into U937T cells using the Bio-Rad Gene-Pulser II for electroporation. After 48 h, 1500 μg/ml G418 (Calbiochem, San Diego, CA) was added to the medium to select stable transformants.

Western Blot Analysis—Twenty micrograms of the indicated cell lysates was separated by 10 or 15% SDS-polyacrylamide gel,
transferred to a nitrocellulose membrane (Bio-Rad), blocked by 5% nonfat milk in TBS, and immunoblotted with antibodies against HIF-1α, HIF-1β (BD Transduction Laboratories), galectin-1, and C/EBPα (Santa Cruz Biotechnology, Inc.) together with β-tubulin (Sigma-Aldrich) as the internal control. Followed by incubation with HRP-linked second antibody (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature, detection was performed with a SuperSignal West Pico chemiluminescent substrate kit (Pierce) according to the manufacturer’s instructions.

**Cell Differentiation Assay and Flow Cytometric Analysis**—Cell differentiation was evaluated by morphological feature, myeloid differentiation-related surface antigens CD11b/c, and nitro blue tetrazolium (NBT) reduction. For morphological observation, cells were collected onto slides by cytospin (Shandon, Runcorn, UK) and then stained with Wright-Giemsa staining. The morphology was examined under an Olympus BX51 microscope, and the images were photographed with a SPOT digital camera. The nuclear and cellular areas of each cell were measured by NIS-Elements software (Nikon, Tokyo, Japan), and the nucleus/cytoplasm (N/C) ratio was calculated according to the formula N/C ratio = Nuclear area/(Cellular area - Nuclear area) (21). The percentage of differentiated cells with typical morphological changes such as condensed chromatin, smaller nuclei, and more abundant cytoplasm was calculated by randomly counting 200 cells on each slide. The percentage of CD11b- and CD11c-positive cells was measured by using FITC- or phycoerythrin (PE)-labeled antibodies on a flow cytometer (BD Biosciences) as in our previous report (18). The percentage of CD11b- and CD11c-positive cells was measured by using FITC- or phycoerythrin (PE)-labeled antibodies on a flow cytometer (BD Biosciences) as in our previous report (18).

**RESULTS**

**C/EBPα Transcriptionally Up-regulates the Expression of Galectin-1 in AML Cells**—In the engineered U937T/C/EBPα cells, as described previously (17), C/EBPα protein was induced significantly after tetracycline withdrawal (Fig. 1A). Upon the conditional induction of C/EBPα, galectin-1 was significantly up-regulated in terms of both mRNA and protein levels (Fig. 1A, right panel). Of note, tetracycline removal did not alter the expression of galectin-1 in empty vector-transfected U937T/PTRE cells (Fig. 1A, left panel). As documented (25, 26), the C/EBPα prefers to bind to the consensus sequence GCAAT/CAT in the promoters of its target genes. Bioinformatic analysis showed that there was such a potential C/EBPα binding site between −48 to −42 bp of the galectin-1 gene (Fig. 1B). Hence, we subcloned the fragment between −569 to +194 bp of the galectin-1 gene into the luciferase reporter vector pGL3-Basic to construct the pGL3-Gal-1 plasmid (Fig. 1B). The plasmid was cotransfected with different doses of C/EBPα-expressing plasmids as well as Renilla as a normalizing control vector into 293T cells. As depicted in Fig. 1C, C/EBPα expression increased luciferase activities driven by the galectin-1 promoter in a dose-dependent manner. Mutating −48TTGCAAT−42 to −48TTAAGGT−42 significantly reduced the promoter activity (Fig. 1D). Furthermore, anti-C/EBPα antibody but not normal IgG could precipitate the C/EBPα-binding consensus sequence between −48 to −42 bp but not that between −2021 to −2017 bp in the ChIP assay of U937 cells (Fig. 1E). All of these results suggest that galectin-1 is a direct target of C/EBPα protein.

**C/EBPα and HIF-1α Synergistically Regulate Galectin-1 Expression**—Hypoxia or treatment with hypoxia-mimetic agents, such as cobalt chloride (CoCl2) or the iron chelator desferrioxamine (DFO), can stabilize and accumulate HIF-1α protein. The stabilized HIF-1α protein translocates into nuclei where it forms a heterodimer with HIF-1β and recognizes the HREs at promoters of its target genes (12). When HIF-1α was

**Statistical Analysis**—All experiments were repeated at least three times with similar results. The values were expressed as mean ± S.D. A paired t test was used for statistical analysis between two groups. The significant difference was set at p < 0.05.
induced in U937THIF-1 cells after tetracycline removal as described previously (18), the increased galectin-1 mRNA and protein levels could be seen by Q-PCR (supplemental Fig. S1), differential in-gel electrophoresis (supplemental Fig. S1A), and Western blot analysis (supplemental Fig. S1B). On the contrary, galectin-1 expression was inhibited in U937 cells with knock-down of HIF-1α and H9251 by its specific shRNAs (named 14 and 16) under hypoxia (supplemental Fig. S2A). Moreover, the ChIP assay showed that hypoxia-stabilized HIF-1α could bind to HREs in the promoter of galectin-1 in U937 cells (supplemental Fig. S2B). These results suggest that HIF-1α directly targets the promoter of galectin-1, like that found in colorectal cancer cell lines (13). Considering that C/EBPα can interact with HIF-1α protein (27) and that galectin-1 is also a direct target of C/EBPα, we addressed the potential relationship between C/EBPα and HIF-1α on the expression of galectin-1. For this, U937THIF-1α transforms were transfected by shRNA specifically against C/EBPα (sh-C2) or a scrambled negative control (NC). The sh-C2, but not NC shRNA, significantly suppressed C/EBPα expression in U937THIF-1α cells, regardless of HIF-1α expression (Fig. 2A). The silencing of C/EBPα expression remarkably suppressed HIF-1α-induced galectin-1 expression in terms of both its mRNA and protein levels (Fig. 2A). This was the case in the U937 cells under hypoxia-induced HIF-1α protein accumulation (Fig. 2B). On the other hand, different doses of C/EBPα were cotransfected into 293T cells with pGL3-Gal-1 luciferase and the Renilla-expressing plasmid in the presence or absence of the HIF-1α-expressing plasmid. The results showed that C/EBPα could increase the galectin-1 promoter-driven luciferase activity, which could be remarkably enhanced by the ectopic expression of HIF-1α (Fig. 2C). Such a synergistic effect of C/EBPα and HIF-1α on the endogenous expression of galec-
tin-1 could also be found in the gastric cancer cell line SGC7901 (Fig. 2D). Two HREs were also found in the promoter of the galectin-1 gene (Figs. 1B and 2E). In the ChIP-reChIP assay, both the HREs between −441 to −423 bp and the C/EBPα-binding site between −49 to −42 bp, but not −2021 to −2017 bp, in the promoter region of galectin-1 could be precipitated by anti-HIF-1α followed by anti-C/EBPα antibody or anti-C/EBPα followed by HIF-1α antibody (Fig. 2E). Furthermore, the sh-C2 partially suppressed the binding activity of HIF-1α to HREs in the galectin-1 promoter (Fig. 2F), whereas sh-α14/α16 also partially inhibited the binding of C/EBPα to the C/EBPα-binding site in the promoter of galectin-1 (Fig. 2G). Collectively, C/EBPα and HIF-1α proteins synergistically regulate galectin-1 expression.

Suppression of Galectin-1 by Its shRNA or a Chemical Inhibitor Antagonizes HIF-1α- and C/EBPα-induced U937T Cell Differentiation—Several reports propose that hypoxia, hypoxia-mimetic agents, and conditional induction of HIF-1α can induce differentiation of AML cells through its interaction with the hematopoietic transcriptional factor C/EBPα (14, 18).

**FIGURE 2.** C/EBPα and HIF-1α synergistically regulate galectin-1 expression. A and B, U937T<sup>HIF-1α</sup>- or U937 cells were stably transfected with NC or C/EBPα shRNA (sh-C2). Then, they were cultured for days as indicated after tetracycline removal (Tet R) and under normoxia or hypoxia for 24 h, respectively. The assays of the galectin-1 mRNA and the indicated proteins followed. C, the wild-type galectin-1 promoter-driven luciferase reporter was cotransfected with HIF-1α or empty vector (EV) and indicated doses of C/EBPα. Fold of relative luciferase activity against cells transfected with EV and without C/EBPα are shown. D, HIF-1α- and/or C/EBPα-expressing plasmids were transfected into SGC7901 cells. Thirty-six hours later, galectin-1 mRNA and the indicated proteins were detected. All values represent mean ± S.D. of three independent experiments with triplicate samples each. *, p values for comparison between line-linked groups for A, B, and D or cells with HIF-1α and EV in the presence of the same doses of C/EBPα for C, E–G. ChiP-reChIP assays for U937 cells (E) and ChIP assay for U937 cells with stable transfection of NC and sh-C2 (F) and U937 cells with stable transfection of HIF-1α shRNA, α14, α16, and NC (G) were performed under hypoxia for 24 h. The white and black ovals and ○ represent the C/EBPα binding site, HREs, and the transcriptional starting site of galectin-1, respectively.

**FIGURE 3.** Galectin-1 involves in HIF-1α or C/EBPα-induced U937T cell differentiation. A and B, the NC or shRNA against galectin-1 (sh-G8)-transfected U937T<sup>HIF-1α</sup> and U937T<sup>C/EBPα</sup> cells were grown for days as indicated after tetracycline removal (Tet R) and Western blot analysis for proteins as indicated followed. C–H, the NC or sh-G8-transfected U937T<sup>HIF-1α</sup> or U937T<sup>C/EBPα</sup> (C, E, and G) and U937T<sup>HIF-1α</sup>-/C/EBPα− (D, F, and H) cells were grown for 4 days and 8 days, respectively, in tetracycline-free medium. The N/C ratio (C and D), the percentage of CD11b<sup>−</sup>/CD11c<sup>+</sup> cells (E and F), and the mRNA levels of M-CSFR, GM-CSFR, and GM-CSFR (G and H) were detected. All values represent mean ± S.D. of three independent experiments with triplicate samples each. *, p values for comparison between line-linked groups for A, B, and D or cells with HIF-1α and EV in the presence of the same doses of C/EBPα for C, E–G. ChiP-reChIP assays for U937 cells (E) and ChIP assay for U937 cells with stable transfection of NC and sh-C2 (F) and U937 cells with stable transfection of HIF-1α shRNA, α14, α16, and NC (G) were performed under hypoxia for 24 h. The white and black ovals and ○ represent the C/EBPα binding site, HREs, and the transcriptional starting site of galectin-1, respectively.
28–31). Hence, we addressed whether the increased galectin-1 expression was involved in the differentiation of U937 induced by HIF-1α and/or C/EBPα. For this purpose, a shRNA specifically targeting galectin-1 (sh-G8) was transfected into U937THIF-1α or U937TC/EBPα cells together with a scrambled NC shRNA plasmid as a control. We found that sh-G8 signifi-

![Morphologic evidences demonstrate the contribution of galectin-1 to HIF-1α- or C/EBPα-induced differentiation in AML cells.](image)

**FIGURE 4.** Morphologic evidences demonstrate the contribution of galectin-1 to HIF-1α- or C/EBPα-induced differentiation in AML cells. A and B, the NC or shRNA against galectin-1 (sh-G8)-transfected U937THIF-1α and U937TC/EBPα cells were grown for days as indicated after tetracycline removal (Tet R). C and D, U937 and NB4 cells, which stably expressed sh-G8 or NC, were grown for 6 days under CoCl₂ (50 μM) or for 8 days under hypoxia (HP, 1% O₂). E, U937THIF-1α and U937TC/EBPα cells were grown for 4 days and 8 days in the indicated treatments, respectively. F, U937THIF-1α-NC and -sh-G8 cells were grown for 4 days in tetracycline-free medium in the presence or absence of the recombinant galectin-1 (rGal-1, 10 μg/ml) or control buffer. All cells were stained by Wright-Giemsa staining, and morphologic images (×1000) were taken with an Olympus BX-51 light microscope. The values represent mean ± S.D. of the percentage of the nuclear differentiated cells of three independent experiments. *, p < 0.05 for comparison between NC and sh-G8 (A–D), lactose and sucrose treatment (E), and recombinant galectin-1 (rGal-1) and control buffer treatment groups (F).
significantly suppressed endogenous or the HIF-1α- or C/EBPα-induced galectin-1 expression after tetracycline removal in U937T cells (Fig. 3, A and B). Under HIF-1α or C/EBPα induction, as reported previously (15, 18), U937 cells presented morphological features of differentiated myeloid cells, such as condensed chromatin, smaller nuclei, and a decreased N/C ratio, although nucleoli were visible in some cells (Figs. 3, C and D, and 4, A and B). HIF-1α or C/EBPα-induced differentiation could also be confirmed further by the increase of the percentage of cells positive for the myeloid differentiation-associated antigens CD11b/CD11c cells (Figs. 3, E and F), and the mRNA levels of M-CSFR, G-CSFR, and GM-CSFR (Fig. 3, G and H). More intriguingly, the suppression of galectin-1 expression by sh-G8 partially inhibited granulocytic differentiation under the conditional induction of HIF-1α or C/EBPα in U937T cells (Figs. 3, C, E, and G, and 4A) and U937T cells (Figs. 3, D, E, and H, and 4B), respectively.

We also transfected NC or sh-G8 shRNAs into U937 or NB4 cell lines (named U937-NC/sh-G8 or NB4-NC/sh-G8, respectively). With CoCl2 or hypoxia treatment, the expression of galectin-1 was remarkably suppressed in U937-sh-G8 and NB4-sh-G8 cells when compared with that in U937-NC and NB4-NC cells (Fig. 5, A and B). Such inhibition also partially antagonized CoCl2- or hypoxia-induced differentiation in these two cell lines (Figs. 4, C and D, and 5, C–H).

As depicted in Fig. 6, A and B, the amount of secreted galectin-1 in the culture medium together with the percentage of galectin-1-binding U937T cells significantly increased upon HIF-1α or C/EBPα induction. To address whether the secreted galectin-1 contributed to HIF-1α- or C/EBPα-induced U937 cell differentiation, the classical galectin inhibitor lactose (32) was used to compete for the CRD activity of galectin-1 on β-galactosides on the cell surface, and sucrose was used as a negative control. The results showed that lac-
Galectin-1 mediates HIF-1α or C/EBPα-induced U937T cell differentiation through its CRD activity. A and B, U937THIF-1α and U937TC/EBPα cells were grown in tetracycline-plus (Tet+Tet) or tetracycline-free (Tet) medium for 4 days and 8 days, respectively. The galectin-1 protein secreted into the culture medium was quantified (A), and the percentage of galectin-1-positive cells was detected (B). *p values are shown for comparison between cells in Tet+ and Tet− medium. C–E, U937THIF-1α and U937TC/EBPα cells were grown for 4 days and 8 days in the indicated treatments. Then, the percentage of galectin-1-positive cells (C); the N/C ratio (D); the percentage of CD11b+/CD11c+ cells (E, upper panel); and the mRNA levels of M-CSFR, G-CSFR, and GM-CSFR (E, bottom panel) were measured. All values represent mean ± S.D. of three independent experiments. *, p < 0.05 for comparison between the lactose and sucrose treatments.
Tose, but not sucrose, dramatically reduced the percentage of galectin-1-binding U937T cells (Fig. 6C), suggesting the effectiveness of lactose for the inhibition on galectin-1 binding. As expected, lactose, but not sucrose, also partially inhibited the differentiation of U937T cells induced by HIF-1α/H9251 or C/EBPα/H9251 after tetracycline removal (Figs. 4E and 6, D and E).

Recombinant Galectin-1 Restores HIF-1α-induced Differentiation of U937T Cells under the Suppression of Endogenous Galectin-1 by shRNA—The recombinant galectin-1 protein was expressed and purified in a prokaryotic system (supplemental Fig. S3A). The recombinant protein was active because it was capable of inducing apoptosis of Jurkat T cells (supplemental Fig. S3, B and C) (5, 33, 34). Using the solvent of galectin-1 protein as a control buffer, the recombinant galectin-1 was added into the culture medium of U937T HIF-1α-sh-G8 cells after HIF-1α induction by tetracycline removal. Compared with the solvent treatment, recombinant galectin-1 protein could remarkably rescue sh-G8-inhibited differentiation under HIF-1α induction in U937T HIF-1α cells (Figs. 4F and 7).

Inducible Expression of Galectin-1 or Recombinant Galectin-1 Treatment Is Not Sufficient for Myeloid Differentiation—To further determine whether galectin-1 was sufficient for myeloid differentiation, we established a U937T Gal-1 cell line. As depicted in supplemental Fig. S4A, galectin-1 protein was significantly induced after tetracycline removal in this cell line. According to the N/C ratio (supplemental Fig. S4B), the percentage of morphologically differentiated cells (supplemental Fig. S4C) and the percentage of CD11b/c-positive cells (supplemental Fig. S4D), the inducible expression of galectin-1 only induced U937T cells to undergo a lower degree of differentiation. In addition, recombinant galectin-1 treatment only triggered U937 cells to present...
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incomplete differentiation-related morphological features (supplemental Fig. S4, E and F) but did not change the percentage of CD11b/c-positive cells (supplemental Fig. S4G).

DISCUSSION

Here we provide the first demonstration that C/EBPα binds to the 48-TGCAAT-42 region of the promoter of the galectin-1 gene and induces its expression. On the basis of the fact that C/EBPα interacts with HIF-1α and its transcriptional activity is enhanced (14) and that galectin-1 is also a direct target of these two transcriptional factors, it would be rational to ask whether C/EBPα and HIF-1α could comodulate galectin-1 expression. Our results revealed that C/EBPα and HIF-1α synergistically regulated galectin-1 expression because the silencing of C/EBPα expression by its specific shRNA could remarkably suppress HIF-1α- or hypoxia-induced expression of galectin-1, whereas ectopic expression of HIF-1α remarkably enhanced C/EBPα-increased activity of the galectin-1 promoter. Such a synergistic effect of C/EBPα and HIF-1α on the endogenous expression of galectin-1 could also be found in the gastric cancer cell line SGC7901. More intriguingly, the ChIP-reChIP assay showed that C/EBPα and HIF-1α could both bind to the promoter of the galectin-1 gene, in which both HREs and C/EBPα-binding sites were present. Furthermore, the silencing of C/EBPα and HIF-1α expressions by their cognate shRNAs partially suppressed the binding activity of HIF-1α to HREs and C/EBPα to its consensus sequence in the galectin-1 promoter, respectively.

Recently, Takubo et al. (35) reported that normal hematopoietic stem cells (HSCs) maintain intracellular hypoxia and stabilize HIF-1α protein and that HSCs maintain cell cycle quiescence through the precise regulation of the HIF-1α level. It was also shown that HIF-1α plays a role in myeloid leukemic cell differentiation induced by hypoxia (14, 28, 36–38) and that a clinically useful differentiation-inducing drug, all-trans retinoic acid, stabilizes HIF-1α protein (30). On the other hand, C/EBPα contributes to the granulocytic differentiation of hematopoietic cells, and its abnormality is related to the pathogenesis of many kinds of AML (39, 40). Thus, we addressed whether increased galectin-1 expression contributed to HIF-1α- and/or C/EBPα-mediated AML cell differentiation. Our results showed that the silencing of galectin-1 expression by shRNA blocked HIF-1α or C/EBPα-induced differentiation of U937T cells as well as U937 and NB4 cells treated with CoCl₂ or hypoxia. These results collectively suggested a role of galectin-1 in HIF-1α- or C/EBPα-induced leukemic cell differentiation. Furthermore, galectin-1, which was modulated by HIF-1α or C/EBPα after tetracycline removal in U937T(HIF-1α or U937T(C/EBPα, cells, also externalized and, subsequently, bound to the cell surface in an autocrine-like manner. Moreover, inhibition of galectin-1 CRD activity by lactose reduced the HIF-1α- or C/EBPα-induced galectin-1-mediated differentiation, suggesting that CRD activity of galectin-1 was crucial in mediating U937T cell differentiation. Notably, although all of the expression of the differentiation-associated genes, M-CSFR, G-CSFR, and GM-CSFR, could be induced after tetracycline removal in U937T(HIF-1α or U937T(C/EBPα cells, only the expression of M-CSFR and GM-CSFR could be inhibited, whereas the expression of G-CSFR could be activated by lactose treatment. One probable explanation was that the galectin family proteins such as galectin-4 and galectin-8 play opposite roles to galectin-1 (41–43) so that in this case, the expression of G-CSFR might be more potently regulated by the other members of the galectin family via a different pathway. Even though lactose treatment might inhibit galectin-1-induced G-CSFR expression, as a common inhibitor of the galectin family, lactose might recover the G-CSFR expression inhibited by the other galectins, and the final result might be the activation of G-CSFR. Interestingly, the contradictory effect of lactose on G-CSFR expression did not affect the inhibitory effect of lactose on HIF-1α- or C/EBPα-induced differentiation of U937T(HIF-1α or U937T(C/EBPα cells. Moreover, an active recombinant galectin-1 could restore HIF-1α-induced differentiation of U937T(HIF-1α cells under the suppression of galectin-1 by its shRNA. However, the inducible expression of galectin-1 or recombinant galectin-1 treatment alone was not sufficient for the myeloid differentiation. Taken together, galectin-1 is synergistically induced by C/EBPα and HIF-1α and contributes to HIF-1α-induced and C/EBPα-dependent differentiation, although these findings remain to be further investigated in the primary cells and in vivo.

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