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*J Immunol* 2007; 178:3038-3047; doi: 10.4049/jimmunol.178.5.3038

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Early Growth Response Transcriptional Regulators Are Dispensable for Macrophage Differentiation

John H. Carter* and Warren G. Tourtellotte2*†‡

Early growth response (Egr) proteins comprise a family of transcriptional regulators (Egr1-4) that modulate gene expression involved in the growth and differentiation of many cell types. In particular, Egr1 is widely believed to have an essential role in regulating monocyte/macrophage differentiation. However, Egr1-deficient mice have normal numbers of functional macrophages, an observation that has led to the hypothesis that other Egr proteins may compensate for Egr1 function in vivo. We examined whether other Egr transcription factors have a functionally redundant role in monocyte/macrophage differentiation. Egr1 and Egr3 expression was found to be induced in myeloid cells when they were differentiated into macrophages by treatment with M-CSF, whereas Egr2 was minimally induced and Egr4 was not detected. In either Egr1/Egr3 or Egr1/Egr2 double homozygous mutant mice, macrophage differentiation and function remained unimpaired. Additionally, the expression of molecules that broadly inhibit Egr function failed to block commitment to the monocytic lineage or inhibit the maturation of monocyte precursors. Finally, several hemopoietic growth factors were found to induce Egr gene expression, indicating that Egr gene expression is not cell lineage specific. Taken together, these results demonstrate that Egr transcription factors are neither essential for nor specific to monocyte/macrophage differentiation. The Journal of Immunology, 2007, 178: 3038–3047.

Mammalian hemopoietic development requires the coordinated regulation of cellular lineage commitment, growth arrest, and differentiation. Lineage-specific transcriptional regulators direct cellular differentiation by modulating gene expression that is at least partially influenced by extracellular signaling molecules such as cytokines (1). For example, GM-CSF and IL-3 cytokines regulate the expansion and maturation of early myeloid precursors, while M-CSF and G-CSF promote terminal differentiation of monocyte and granulocytic cells, respectively (2). Myeloid cell lines that differentiate in response to cytokines or their analogues have made it possible to identify some of the gene regulatory networks involved in myeloid differentiation. A greater understanding of the molecular mechanisms mediating myeloid differentiation is of considerable significance for understanding myeloid leukemogenesis, the hallmark of which involves the abnormal proliferation and differentiation of myeloid progenitor cells.

Egr1, the archetypal member of the early growth response (Egr) transcriptional regulators, is thought to be essential for monocyte/macrophage differentiation (3–6). Egr transcriptional regulators are rapidly induced by a variety of extracellular signaling molecules, consistent with their role in regulating the gene expression required for cellular differentiation and/or homeostasis. There are four Egr proteins, Egr1 (7, 8), Egr2 (9), Egr3 (10), and Egr4 (11), which contain highly homologous DNA binding domains that bind GC-rich Egr response elements (ERE) in the promoter regions of target genes (12). Functional EREs have been identified in gene promoters involved in both diverse processes as cell growth, differentiation, apoptosis, inflammation, synaptic transmission, and endocrine function (reviewed in Ref. 13).

Numerous studies have demonstrated Egr mRNA induction in myeloid cells in response to cytokine stimulation. In leukemia cell lines, Egr1 is up-regulated by IL-3 and GM-CSF (14), G-CSF (15), and M-CSF (3), although only M-CSF is known to induce Egr1 in normal bone marrow progenitors (3). Furthermore, both Egr1 and Egr2 are up-regulated during the PMA-stimulated monoctytic differentiation of HL60 cells and primary human monocytes (16). Although the precise function of Egr proteins as effectors of cytokine signaling in myeloid cells has not yet been fully elucidated, previous studies demonstrated an essential role for Egr1 during macrophage differentiation. For example, Egr1 was shown to be rapidly induced in normal mouse bone marrow myeloid precursors in response to M-CSF, but not G-CSF, and in leukemic cell lines undergoing macrophage differentiation. Moreover, Egr1 antisense oligonucleotides blocked macrophage but not granulocyte differentiation of both leukemia cell lines and normal bone marrow precursors (3). In further support of a role for Egr1 in macrophage differentiation, later studies reported that overexpression of Egr1 in cell lines and primary bone marrow promoted macrophage differentiation at the expense of other myeloid lineages (4–6). Taken together, Egr1 appeared to have an essential role in the lineage commitment of bipotential granulocyte/macrophage precursors and in the maturation of committed monocytic precursor cells.

Surprisingly, Egr1−/− mice showed unimpaired macrophage differentiation both in vivo and in bone marrow cells cultured in vitro with M-CSF (17). Thus, it has since been widely presumed that other Egr proteins compensate for the loss of Egr1 function...

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Received for publication November 9, 2006. Accepted for publication December 21, 2006.

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1 This study was supported by National Institutes of Health Grants (NS046468 and NS040748) and a Howard Hughes Faculty Scholar Award (to W.G.T.). J.H.C. was supported by a predoctoral fellowship from the National Institutes of Health (CA009560) and the National Institutes of Health Medical Scientist Training Program (GM008152).

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3 Abbreviations used in this paper: Egr, early growth response; dnEgr, dominant negative; Egr, EGFp, enhanced GFP; ERE, Egr response element. IRES, internal ribosome entry site; MSCV, main stem cell virus; P/S, penicillin/streptomycin; WT, wild type.

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were made between WT and Egr gene-deficient mice. All experimental WT mice were used as controls for all experiments in which comparisons were used in Ref. 22 and had the identical genetic background. Littermate strain. The Egr1 and Flt3L (PeproTech) each for 3 days, at which time nonadherent cells were collected for further differentiation.

In this study, we show that primary mouse myeloid precursors treated with M-CSF induce Egr1 and Egr3, and to a lesser extent, Egr2. However, bone marrow-derived myeloid precursors from germ-line Egr1−/−/Egr3−/− double homozygous (Egr1/Egr3 DKO) mice exhibited macrophage differentiation that was qualitatively and quantitatively identical with that of wild-type (WT) littermates. Similarly, fetal liver-derived myeloid precursors from Egr1−/−/Egr2−/− double homozygous mice (Egr1/Egr2 DKO) mice showed no abnormalities in M-CSF induced macrophage differentiation. Furthermore, broad inhibition of Egr transcriptional activity through overexpression of a dominant negative Egr (dnEgr) molecule or the endogenous Egr repressor Nab2 likewise did not affect the ability of myeloid cells to differentiate along the macrophage lineage. Finally, multiple Egr genes were found to be induced by a variety of hemopoietic growth factors including G-CSF. These results indicate that Egr transcription factors are neither specific to nor essential for monocyte/macrophage differentiation.

Materials and Methods

**Animals**

Egr1-deficient (Egr1−/−), Egr2-deficient (Egr2−/−), and Egr3-deficient (Egr3−/−) mice were generated and genotyped as previously described (17, 23–26). Egr1−/− and Egr2−/− mice were backcrossed 10 generations and Egr3−/− mice were backcrossed four generations to the C57BL/6J inbred background. Littermate WT mice were used as controls for all experiments in which comparisons were made between WT and Egr gene-deficient mice. All experimental procedures complied with protocols approved by the Northwestern University Institutional Animal Care and Use Committee (Chicago, IL).

**Cell culture and primary cell isolation**

U937, HL60, and M1 cell lines (all from American Type Culture Collection) were maintained in RPMI 1640 medium with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (P/S). FDCP-mix cells were maintained in RPMI 1640 medium with 20% horse serum, P/S, 1% MEM non-essential amino acids, and 10% WEHI-3 conditioned medium as a source of IL-3. Phoenix-Eco and Phoenix-Ampho cells (27) (obtained from American Type Culture Collection) were maintained in DMEM medium with 10% FCS and P/S. Primary bone marrow cells were isolated from the tibias and femora of 6- to 8 wk-old mice that had been injected 3 days prior with 10% sodium caseinate to enrich for myeloid progenitors. Cells were then spin-infected in the presence of Wright-Giemsa-stained cytospin preparations, or FACS staining with anti-F4/80 (clone CI:A3-1) and stained with PE-conjugated anti-F4/80 (eBioscience) and stained with PE-conjugated anti-F4/80 (clone CI:A3-1) and stained with PE-conjugated anti-F4/80 (clone CI:A3-1) and stained with PE-conjugated anti-F4/80 (clone CI:A3-1) and stained with PE-conjugated anti-F4/80 (clone CI:A3-1) and stained with PE-conjugated anti-F4/80 (clone CI:A3-1).

**Assays in vitro differentiation**

Primary bone marrow cells were cultured at a density of 5 × 10^5 cells/ml in RPMI 1640 medium with 10% FCS, P/S, 30 μM 2-ME, 1% nonessential amino acids, and 10 ng/ml M-CSF, G-CSF, GM-CSF, and IL-3 (PeproTech) each for 3 days, at which time nonadherent cells were collected for further differentiation.

**Growth factor treatment**

Myeloid progenitor-enriched bone marrow cells and FDCP-mix cells were rested for 8 h in RPMI 1640 medium with 10% FCS and P/S to minimize Egr gene expression caused by serum-containing medium. In some cases, bone marrow was first stained with biotin-labeled Abs that cross-reacted with the lineage markers CD11b, Gr-1, B220, TERR1.9, and CD45 followed by allophycocyanin-streptavidin, and the lineage-negative population was isolated using FACS before growth factor stimulation. Cells were then stimulated with 10 ng/ml M-CSF, G-CSF, IL-3, or GM-CSF (PepreTech) for various lengths of time, after which total RNA was isolated from the cells. The medium for FDCP-mix cells also contained 0.1 ng/ml IL-3. The effects of these growth factors on growth and differentiation of both bone marrow cells and FDCP-mix cells were confirmed by cell counts, cell cycle analysis, and analysis of the cellular morphology of Wright-Giemsa-stained cytospin preparations.

**Quantitative real-time PCR**

RNA isolation, reverse transcription, and quantitative real-time PCR analysis were performed as described (28). Quantification was performed by generating standard curves using control cDNA samples, and all samples were normalized relative to the expression of GAPDH. Primer sequences are available upon request.

**Plasmid construction**

The full-length Nab2 cDNA and the dominant negative form of the Egr3 DNA-binding domain (29, 30) were cloned into the replication defective murine stem cell virus (MSCV) retroviral vector (31). The vector provides a bicistronic message using the encephalomyelitis virus internal ribosome entry site (IRES) followed by the enhanced GFP (EGFP). A vector containing EGFP alone was used as a control.

**Retroviral production and infection**

Replication-deficient retroviruses were generated by the transient transfection of Phoenix-Eco cells Phoenix-Eco cells with the MSCV plasmid using Lipofectamine 2000 (Invitrogen Life Technologies). Viral supernatants were collected 2 days after transfection, filtered through a 45-μm filter, and either used immediately or stored at −80°C.

For the infection of primary bone marrow cells, myeloid progenitor-enriched cells were first stimulated in medium supplemented with 20% FBS and 10 ng/ml IL-3, IL-6, SCF, and Flt3L (PeproTech) each for 2 days. Cells were then spin-infected in the presence of a viral supernatant supplemented with Polybrene (4 μg/ml) for 1 h at 1800 rpm, after which the cells were incubated at 32°C for 4 h. Viral supernatants were removed, and cells were grown for an additional 2 days in the presence of IL-3, IL-6, SCF, and Flt3L before assessing for macrophage differentiation. Infection efficiency ranged from 30 to 40%.

Exponentially growing FDCP-mix, M1, U937, and HL60 cells were resuspended in ecotropic (FDCP-mix and M1 cells) or amphotropic (U937 and HL60 cells) retroviral supernatant with 8 μg/ml Polybrene and similarly spin-infected, after which viral supernatants were removed and cells were cultured in the appropriate complete medium for 2 days before assessing for macrophage differentiation. Infection efficiency was 10–20% (M1), 20–30% (FDCP-mix), 40–50% (U937), and 10–20% (HL60).

**Western blotting**

Cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris (pH 7.7), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 10 nM NaF and Complete protease inhibitors (Roche). Eighty micrograms of total cellular protein was resolved by SDS-PAGE on a 10% acrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with anti-Egr3 (Santa Cruz Biotechnology; catalog no. sc-191), anti-ERK (Santa Cruz Biotechnology; catalog no. sc-94), or anti-Nab2 (M1510) (US Biological) followed by a HRP-conjugated goat anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce). The Egr3 Ab, raised against a C-terminal fragment of Egr3, detects the recombinant dnEgr protein as a 25-kDa band.

**Analysis of the cellular morphology**

Cells for FACS analysis were performed as described (28). Quantification was performed by flow cytometry on a FACS Calibur cytometer (BD Biosciences).
IgG1 (Caltag Laboratories). M1 cells were stained with anti-F4/80 or isotype control as described above. FDCP-mix cells were pelleted and resuspended at a density of 5 x 10^5 in RPMI 1640 supplemented with 10% FBS, 0.1 ng/ml IL-3, and 10 ng/ml M-CSF, G-CSF, GM-CSF, or IL-3. Analysis of retrovirus-infected cells was performed by first gating on infected EGFP-positive cells.

**Phagocytosis assay**

Murine bone marrow-derived macrophages were gently scraped, plated at 10^5/well in a 48 well plate, and allowed to reattach for 1 h at 37°C. Green fluorescent latex beads (1 x 10^6; Sigma-Aldrich) were then added to each well and incubated for an additional 1.5 h. Parallel control incubations were performed at 4°C to prevent phagocytosis. After five washes with PBS, the cells were examined under a Nikon TE200 fluorescent microscope.

**Immunohistochemistry**

For immunohistochemical analysis, anesthetized mice were perfused through the heart, first with PBS and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The thymi, livers, and spleens were removed, cryoprotected in 30% sucrose-PBS overnight, frozen in OCT embedding medium (VWR Scientific Products), and stored at −80°C. Frozen blocks were cut on a freezing microtome at 16-μm thickness. The sections were blocked (3% normal serum and 0.3% Triton X-100 in PBS) for 1 h at room temperature, incubated with purified F4/80 Ab (Caltag Laboratories), diluted in blocking buffer overnight at room temperature, and subsequently incubated with a biotinylated, anti-mouse secondary Ab (Jackson ImmunoResearch Laboratories), diluted in blocking buffer overnight at room temperature, incubated with a biotinylated, anti-mouse secondary Ab (Vector Laboratories) according to the manufacturer’s specifications (Vector Laboratories).

**Transient transfection and luciferase assays**

The luciferase reporter vector containing four tandem Egr consensus binding sites (ERE-luc) was used to evaluate Egr-mediated transcriptional activity (32). HL60 cells were nucleofected according to manufacturer’s protocol (Amaxa) with 1.5 μg of MSCV-IREs-EGFP, MSCV-duEgr-IREs-EGFP, or MSCV-Nab2-IREs-EGFP, 0.5 μg of ERE-luc, and 0.05 μg of pRL-CMV for transfection control (Promega). NIH3T3 cells were similarly transduced using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen Life Technologies). Egr transcriptional activity was then induced using 10 μM PMA (HL60) or 10% serum (NIH3T3). Twenty-four hours after stimulation, lysates were processed for luciferase activity using the Dual luciferase assay (Promega) according to the manufacturer’s specifications.

** Colony forming assays**

Cells were resuspended in RPMI 1640 medium with 10% FCS and P/S at 20,000 cells per 0.3 ml. The cell mixture was added to 2.7 ml of a methylcellulose solution (MethoCult M3234; Stem Cell Technologies) containing 0.9% methylcellulose in IMDM supplemented with 20% FBS, 1% bovine albumin, 100 μM 2-ME, 2 mM t-glutamine, and 10 ng/ml the appropriate cytokine. Cultures were maintained at 37°C in a humidified incubator for 12 days, at which time morphologic analysis was performed. Between 100 and 350 colonies were scored for each group, and 10–20 colonies from each group were picked, dissociated, cytospun, and stained with Wright-Giemsa to confirm that the colony morphology corresponded to precise cell types.

**Statistical analysis**

Statistical analysis of replicate experiments was performed using Student’s t test. Significance was defined as p < 0.05.

**Results**

**Egr gene expression in M-CSF-treated, bone marrow-derived myeloid and FDCP-mix cells**

In the absence of Egr1, other Egr genes may regulate the target gene expression necessary to sustain macrophage differentiation.

![FIGURE 1](http://www.jimmunol.org/)  
**Egr transcription factor gene expression induced by M-CSF treatment of myeloid cells. A**, Quantitative real-time PCR analysis of Egr1, Egr2, Egr3, and Egr4 gene expression after M-CSF treatment of bone marrow-derived cells. Egr1 was maximally induced 6.7-fold relative to medium without M-CSF, whereas Egr2 and Egr3 were maximally induced 1.7- and 4.3-fold, respectively. Egr4 expression was not detected. **B**, Quantitative real-time PCR analysis of Egr1, Egr2, Egr3, and Egr4 gene expression FDCP-mix cells after M-CSF treatment. Egr1 and Egr3 were induced 5.2- and 2.3-fold, respectively, relative to untreated cells. Egr2 expression was unchanged after M-CSF treatment and Egr4 was not detected (results are representative of two experimental replicates).
However, it has not been clear which Egr genes are up-regulated after M-CSF treatment of myeloid precursors. Both primary bone marrow cells and the FDCP-mix progenitor cell line were used to address this question. FDCP-mix is a progenitor cell line that is able to differentiate into all myeloid lineages in response to cytokine treatment, making it a useful tool for studying lineage commitment and the maturation of myeloid cells. M-CSF treatment of either bone marrow cells or FDCP-mix cells was confirmed to induce macrophage differentiation (Table I). By contrast, cells cultured in the absence of cytokines resulted in the accumulation of high numbers of apoptotic cells (data not shown). Both Egr1 and Egr3 mRNAs were rapidly and transiently up-regulated 6.7- and 2.3-fold, respectively, after M-CSF treatment of primary bone marrow-derived cells (Fig. 1A). Egr2 was detected at lower overall levels and was up-regulated 1.7-fold after M-CSF treatment. This finding corresponds to Northern blot analysis that demonstrated robust Egr1 expression after an overnight exposure, whereas weak Egr2 induction could only be detected after 5 days of exposure to film (data not shown). Likewise, Egr1 was highly expressed in FDCP-mix cells and induced 5.2-fold by M-CSF treatment (Fig. 1B). Egr3 was also induced 2.3-fold; however, the absolute levels were ~50 times lower than those of Egr1. Egr2 expression was detectable in FDCP-mix cells but unchanged by M-CSF treatment. Egr4 expression was not detected in either cell type after M-CSF treatment. Thus, Egr1 and Egr3 appear to be the major M-CSF-responsive Egr genes in both primary bone marrow-derived and FDCP-mix myeloid progenitor cells.

Unimpaired macrophage differentiation in Egr1/Egr3 DKO mice

In previous studies, Egr1−/− mice did not demonstrate any defects in macrophage differentiation in vitro or in vivo, raising the possibility that another Egr protein may compensate for the loss of Egr1 function (33). Because Egr1 and Egr3 are the most abundantly induced Egr genes in primary myeloid cells (Fig. 1A), we first examined germline Egr1/Egr3 DKO mice to determine whether simultaneous loss of both Egr1 and Egr3 had any effect on macrophage differentiation. Newborn WT and Egr1/Egr3 DKO bone marrow cells treated with M-CSF showed no morphological differences in their capacity to differentiate into macrophages in vitro (Fig. 2, A and B). Before M-CSF treatment only blast-like cells were identified (data not shown), whereas after treatment typical macrophages containing abundant vacuolated cytoplasm and round to oval nuclei were identified in cells derived from both WT and Egr1/Egr3 DKO mice. Quantitative flow cytometric analysis for the macrophage-associated marker F4/80 revealed no statistically significant differences between WT and Egr1/Egr3 DKO bone marrow-derived cells after differentiation with M-CSF (Fig. 2C). To assess whether Egr1/Egr3 DKO bone marrow-derived cells contained fewer monocyctic progenitors due to impaired monocyctic lineage commitment, bone marrow-derived cells from WT and Egr1/Egr3 DKO littermates were cultured in methylcellulose supplemented with M-CSF. Both WT and Egr1/Egr3 DKO bone marrow cultures contained similar numbers of M-CSF-responsive colony forming progenitors, indicating their intact commitment to the monocytic lineage (Fig. 2D).

LPS was used to compare the capacity of M-CSF-differentiated WT and Egr1/Egr3 DKO bone marrow-derived macrophages to respond to the cytokine inducing stimuli. LPS-mediated induction of cytokine expression (IL-1β, IL-6, IL-12, MCP-1 (CCL2), RANTES (CCL5), TNF, and tissue factor) was similar between WT and Egr1/Egr3 DKO macrophages after 2 h (Fig. 2E) and 4 h (data not shown).
of treatment. Additionally, both WT and Egr1/Egr3 DKO macrophages showed comparable fluorescent latex bead phagocytosis (Fig. 2F). These data indicate that Egr1/Egr3 DKO myeloid progenitors show normal morphologic differentiation, cell surface protein expression, cytokine induction, and phagocytosis after M-CSF mediated differentiation.

To address the ability of Egr1/Egr3 DKO mice to generate mature macrophages in vivo, the spleen, liver, and thymus from WT and Egr1/Egr3 DKO mice were examined for the presence of F4/80/H11001 macrophages. Comparable F4/80 immunoreactivity was observed in the splenic red pulp and hepatic sinusoids and scattered throughout the thymic parenchyma in both WT and Egr1/Egr3 DKO mice (Fig. 3). Taken together, the results indicate that simultaneous loss of Egr1 and Egr3 function is dispensable for macrophage differentiation in vitro and in vivo.

Unimpaired macrophage differentiation in Egr1/Egr2 DKO mice

Although our results showed that Egr2 was only minimally induced by M-CSF in both primary bone marrow-derived and FDCP-mix cells (Fig. 1), it has recently been reported that Egr1 and Egr2 have an essential and overlapping role in macrophage differentiation (22). Egr2-deficient mice die postnatally due to defects in hindbrain formation (23), and Egr1/Egr2 DKO mice die shortly after birth, precluding an ex vivo analysis of bone marrow-derived myeloid cells. However, it was possible to analyze liver-derived myeloid precursors from newborn Egr1/Egr2 DKO mice. Liver-derived myeloid cells were first expanded in vitro by treatment with SCF, IL-3, IL-6 and Flt3-L and then differentiated with M-CSF (see Materials and Methods). There were no qualitative differences in the phenotype of liver-derived myeloid cells from WT and Egr1/Egr2 DKO mice (Fig. 4). These data indicate that Egr2 is not essential for macrophage differentiation in vivo, and that simultaneous loss of Egr1 and Egr3 function is dispensable for macrophage differentiation in vitro and in vivo.

FIGURE 3. Egr1 and Egr3 are dispensable for macrophage differentiation in vivo. Immunohistochemical staining for the macrophage marker F4/80 demonstrates equivalent staining patterns in splenic red pulp (A and B), liver sinusoids (C and D), and thymus (E and F) of WT (A, C, and E) and Egr1/Egr3 DKO (1/3 DKO) (B, D, and F) mice. A and B. In the spleen, F4/80 immunoreactivity was localized to the red pulp (RP) but not the white pulp (WP). C and D. Kupffer cells in the liver sinusoids stain equivalently in WT and Egr1/Egr3 DKO mice. Nonspecific staining is localized to the hepatic vein (arrowhead) and arteries. E and F. No differences are noted in the distribution of scattered F4/80+ cells in the WT and Egr1/Egr3 DKO thymus (scale, 50 μm).

FIGURE 4. Egr1 and Egr2 are dispensable for macrophage differentiation in vitro. WT (A) and Egr1/Egr2 DKO (1/2 DKO) (B) liver-derived myeloid cells differentiated with M-CSF showing characteristic and qualitatively similar macrophage morphology (scale bar, 50 μm). Similar numbers of F4/80+ cells were obtained from WT (C) and Egr1/Egr2 DKO (D) liver-derived hemopoietic precursors after differentiation with M-CSF.

FIGURE 5. Global inhibition of Egr transcription factor activity. A, NIH3T3 cells were transfected with a synthetic reporter construct consisting of four tandem EREs driving a firefly luciferase gene along with plasmids that express dnEgr, the endogenous Egr corepressor Nab2, or an empty expression vector. The cells were starved in 0.1% serum for 24 h and restimulated by the addition of serum to a final concentration of 10% for 8 h. Renilla luciferase driven by the CMV promoter was used to normalize for transfection efficiency. Expression of either dnEgr or Nab2 was capable of inhibiting ERE-mediated transactivation by Egr1, Egr2, and Egr3, which are all up-regulated in NIH3T3 cells by serum stimulation. B, Similarly in HL60 cells treated with PMA (final concentration 10 μM) for 8 h, ERE-mediated transactivation by Egr1 and Egr2 induced by PMA was abrogated by the coexpression of either dnEgr or Nab2 (values represent the mean fold luciferase induction of stimulated cells relative to unstimulated cells ± SEM for three independent experiments).
FIGURE 6. Inhibition of Egr transcriptional activity does not impair macrophage differentiation of normal bone marrow-derived progenitor cells. A, Cells infected with recombinant retroviruses engineered to express either dnEgr plus EGFP or Nab2 plus EGFP expressed high levels of the recombinant proteins after infection (Erk p42/p44 levels were used to normalize protein loading). B, Bone marrow-derived myeloid progenitor cells were infected with retroviruses expressing EGFP, dnEgr plus EGFP, or Nab2 plus EGFP. Forty-eight hours after infection, cells were either treated (differentiated) with M-CSF or untreated for an additional 4 days. The relative frequency of infected (EGFP+) F4/80+ macrophages was determined by flow cytometry (values represent the mean percentage of F4/80− ± SEM for four independent experiments; p > 0.5 for each comparison). C, Myeloid progenitor cells were infected with retrovirus and seeded into a methylcellulose-containing dish supplemented with GM-CSF. After 12 days, individual colonies were scored as either monocyte/macrophage (CFU-M), mixed granulocytic/monocytic (CFU-GM), or granulocytic (CFU-G). Left panel, Mean percentage of each colony type ± SEM from three to six replicate experiments. There were no statistically significant differences between any colony type in any infected population (Student’s t test, p > 0.5 for all comparisons). Right panel, Representative photomicrographs of dissociated/cytospin prepared cells from CFU-M colonies derived from vector-only, dnEgr, or Nab2-infected myeloid cells (scale bar, 25 μm).

differences in macrophage differentiation between WT and Egr1/Egr2 DKO liver-derived myeloid progenitor cells (Fig. 4, A and B). Similarly, there was no quantitative difference in the relative efficiency of myeloid progenitor cells to differentiate and up-regulate the macrophage-related F4/80 cell surface marker (Fig. 4, C and D). These results demonstrate that neither Egr1 nor Egr2 are necessary for M-CSF-mediated macrophage differentiation in vitro.

Validation of dominant negative Egr constructs

Although neither simultaneous loss of Egr1/Egr2 nor Egr1/Egr3 had any apparent effect on macrophage differentiation, it was still possible that in each mutant mouse line the remaining expressed Egr protein could functionally compensate for the others during macrophage differentiation. It was not possible to analyze Egr1+/−/Egr2−/−/Egr3−/− triple knockout (Egr1/Egr2/3 TKO) mice, because they could not be identified from the >30 litters received from compound heterozygous matings. Therefore, we expressed two inhibitory molecules in myeloid progenitor cells to simultaneously inhibit Egr1, Egr2, and Egr3 function in vitro: 1) truncated Egr3, which contains the DNA binding domain but lacks the transcriptional activation and Nab protein-binding domains (dnEgr; see Ref. 30); and 2) the endogenous Egr corepressor Nab2 (34). Both dnEgr and Nab2 have been previously shown to inhibit the transcriptional activity of multiple Egr family members in a wide variety of cell types (30, 35–39). Nab2 binds Egr1, Egr2, and Egr3 and recruits repressor molecules to target gene promoters bound by Egr proteins (40). The dnEgr molecule is a competitive inhibitor of both Egr-dependent transcriptional activation and Egr/Nab-dependent transcriptional repression by competing for target gene DNA binding (12, 22).

To express these molecules in hematopoietic cells, retroviruses were generated that simultaneously express the appropriate inhibitory molecule and EGFP using the MSCV promoter. The ability of these constructs to inhibit endogenous Egr transcriptional activity was confirmed in cells where Egr1, Egr2, and Egr3 are known to be coexpressed. NIH3T3 cells were transfected with MSCV-dnEgr/EGFP, MSCV-Nab2/EGFP, or MSCV-EGFP vectors along with a luciferase expression plasmid containing four tandem consensus EREs driving a prolactin minimal promoter. After transfection, the cells were serum starved for 24 h and subsequently restimulated with 10% serum, a stimulation paradigm confirmed in these experiments and known to up-regulate Egr1, Egr2, and Egr3 in NIH3T3 cells (data not shown and see Refs. 8–10). Egr-dependent transcriptional activity was induced by serum in the vector-transfected cells, and this induction was completely abrogated by the expression of either dnEgr or Nab2, indicating that these constructs were able to inhibit the activity of multiple endogenous Egr transcription factors (Fig. 5A). To confirm the activity of the inhibitory molecules in myeloid cells, HL60 cells were similarly transfected and stimulated with the macrophage differentiation-inducing phorbol ester PMA. As with NIH3T3 cells, the dnEgr and Nab2 constructs completely abrogated transactivation of the ERE luciferase reporter in HL60 cells (Fig. 5B).

Egr inhibition does not affect monocytic lineage commitment or maturation

To determine whether broad inhibition of Egr transcriptional activity could affect macrophage differentiation from normal myeloid progenitors, murine bone marrow-derived cells were stimulated with a cytokine mixture containing SCF, IL-3, IL-6, and Flt3-L. Proliferating cells were infected with MSCV-EGFP, MSCV-dnEgr/EGFP, or MSCV-Nab2/EGFP retroviruses. Robust expression of the transgene in infected cells was confirmed by Western blot analysis (Fig. 6A). Two days after infection, the nonadherent cells were cultured in the presence of M-CSF for an additional 4 days to facilitate macrophage differentiation. After gating on
EGFP cells to limit the analysis to infected cells, the proportion of F4/80-expressing cells was determined by flow cytometry. Compared with cells that received only the empty EGFP-expressing retrovirus, the presence of dnEgr or Nab2 had no effect on macrophage differentiation (Fig. 6B). To examine whether blocking Egr transcriptional activity could influence the lineage commitment of granulocyte/monocyte precursors, infected progenitors were sorted for EGFP expression and seeded into methylcellulose supplemented with GM-CSF. If Egr transcriptional activity is important for granulocyte vs macrophage lineage commitment, then inhibiting Egr function should lead to increased numbers of granulocyte colonies at the expense of macrophage and mixed granulocyte/macrophage colonies. The frequency of macrophage, granulocyte, and granulocyte/macrophage colonies was determined for cells infected with control (MSCV-EGFP)-, dnEgr-, or Nab2-expressing retroviruses. The overall colony forming ability did not significantly vary between experimental groups (1.26, 1.43, and 1.24% for MSCV-EGFP, MSCV-dnEgr, and MSCV-Nab2, respectively; \( p > 0.05 \) for all comparisons). In addition, analysis of the recovered colonies revealed no statistically significant differences between control, dnEgr, or Nab2-infected cells (\( p > 0.05 \) for all comparisons) (Fig. 6C). Moreover, the morphology of individual cells from macrophage colonies was normal in all experimental groups.

Primary bone marrow progenitors consist of a heterogeneous group of lineage-committed and multipotent precursor cells. To further clarify a possible role for Egr transcription factors in lineage commitment vs maturation, the differentiation experiments were repeated in clonal cell lines. These included a cell line with broad myeloid differentiation potential (FDCP-mix), a cell line with granulocytic and monocytic differentiation potential (HL60), and two cell lines representing committed monocytic precursors (U937 and M1). HL60 and U937 cells undergo a well-defined macrophage differentiation program characterized by morphologic changes and up-regulation of the integrin CD11b after treatment with PMA (41). Similarly, M1 and FDCP-mix cells differentiate to monocytes/macrophages in response to IL-6 and M-CSF treatment, respectively (41–43). As with bone marrow-derived precursors, dnEgr and Nab2 were unable to inhibit macrophage differentiation as determined by flow cytometry for either CD11b or F4/80 cell surface marker expression in any of these four cell lines (Fig. 7). Further analysis of EGFP-sorted U937 cells infected with dnEgr, Nab2, or control retroviruses revealed no differences in the kinetics of morphologic macrophage differentiation, cell cycle arrest, CD11b up-regulation, or the acquisition of robust phagocytic activity over a 4-day treatment with PMA (data not shown). Taken together, these data do not support the long-standing model in which Egr transcriptional activity is required for monocytic lineage commitment and/or macrophage differentiation of either normal murine myeloid precursors or murine and human differentiation-competent cell lines (3).

**Egr mRNAs are up-regulated by multiple myeloid growth factors**

It has been reported that Egr1 is specifically induced by M-CSF and not G-CSF in normal bone marrow (6), consistent with previously reported data indicating that Egr1 induction is specific for monocyte but not granulocyte differentiation. However, Egr1 appears to be induced by additional hematopoietic growth factors in...
different cell contexts (6, 14, 15, 44, 45), raising questions about its specificity for monocyte lineage commitment or differentiation. To examine whether any Egr genes are induced specifically by cytokines known to direct cell lineage-specific differentiation, Egr mRNA induction was examined in response to GM-CSF, IL-3, or G-CSF treatment of WT normal bone marrow and multipotent FDCP-mix cells. Consistent with previously reported data, Egr1, Egr2, and Egr3 were induced by GM-CSF and IL-3 in bone marrow cells, with typical immediate-early kinetics (Fig. 8A and data not shown). Surprisingly, Egr1 was also up-regulated 5-fold by G-CSF 30 min after treatment, an induction of similar magnitude to that seen with M-CSF. Egr2 and Egr3 were also induced by G-CSF, albeit only ~2-fold and to a much lower overall level. In FDCP-mix cells, Egr1, Egr2, and Egr3 were also induced by all three cytokines tested (Fig. 8B). Notably, a high concentration of IL-3, which promotes proliferation and inhibits terminal differentiation of FDCP-mix cells (Table I) (42), was the most potent inducer of all Egr transcription factors. Egr4 was not detected in either primary bone marrow or FDCP-mix cells with any of the three cytokines tested.

Although both M-CSF and G-CSF could rapidly induce Egr gene expression, it remained possible that these factors differentially regulate long term Egr expression during differentiation. To test this hypothesis, lineage negative bone marrow precursors form WT mice were cultured for 2 or 4 days in G-CSF or M-CSF. Egr gene expression was first analyzed by RT-PCR. Egr1, Egr2, and Egr3 transcripts were detectable in all conditions (Fig. 9, left panel). Egr4 was expressed at very low levels in cells treated with G-CSF but not M-CSF. Quantitative real-time PCR analysis revealed moderate expression of Egr1 with lower levels of Egr2 and Egr3, consistent with the results obtained from common myeloid progenitors and differentiation-competent cell lines (Fig. 9, right panel). Thus, these data demonstrate that neither M-CSF nor G-CSF treatment differentially regulates transient or sustained Egr gene expression.

Discussion

In a previous report, treatment of bone marrow progenitors and several leukemic cell lines with Egr1 antisense oligonucleotides suppressed their macrophage differentiation potential (3). However, Egr1-deficient mice were subsequently shown to generate normal numbers of functional macrophages in vitro and in vivo (33). The disparity between these two results has been widely assumed to relate to functional compensation by other Egr proteins. Although such an explanation never fully accounted for why other Egr proteins could not compensate when Egr1 was knocked down by ostensibly specific antisense oligonucleotides, it has remained a
formal possibility because multiple Egr genes are often coordinately expressed, both within and outside the hematopoietic system. In a recent report, Laslo and colleagues (22) attempted to address the question of Egr functional redundancy and found that Egr2 was induced by the restoration of PU.1 in PU.1-deficient myeloid cells. Moreover, they found that both Egr1 and Egr2, but not Egr3, were expressed in fetal liver myeloid progenitor cells during macrophage differentiation and that Egr2 was important for mediating differentiation. In the present study however, we found that Egr3 was coincided with Egr1 by M-CSF and at substantially higher levels than Egr2 in both normal bone marrow progenitor cells and FDCP-mix cells. Indeed, although we found that Egr2 was induced in agreement with the results of Laslo et al. (22), it was induced to a much lower level than either Egr1 or Egr3, and Egr2 was not induced at all by M-CSF treatment of FDCP-mix cells during macrophage differentiation. From these results, we reasoned that if there is functional redundancy between Egr proteins to mediate macrophage differentiation, then Egr1 and Egr3 would be the most likely candidates. However, bone marrow-derived myeloid cells from mice that lack both Egr1 and Egr3 were capable of generating functional macrophages both in vitro and in vivo. Reasoning that perhaps even low levels of Egr2 expression could compensate for Egr1 function, we examined Egr1/Egr2 DKO mice. By contrast, Laslo et al. (22) did not directly examine Egr1/Egr2 DKO mice but instead examined Egr1−/−; Egr2−/− haploinsufficient mice. We found that liver-derived myeloid progenitor cells isolated from Egr1/Egr2 DKO mice showed no impairments in M-CSF mediated macrophage differentiation, clearly indicating that neither Egr1, Egr2 nor the combination of the two transcriptional regulators is absolutely required for macrophage differentiation. Given that neither the simultaneous loss of Egr1 and Egr3 nor Egr1 and Egr2 impaired macrophage differentiation, it seemed plausible that any one of the Egr proteins (Egr1, Egr2, or Egr3) could be sufficient to mediate macrophage differentiation. Because we were unable to generate mice that simultaneously lacked Egr1, Egr2, and Egr3, two different dominant negative constructs were used to broadly inhibit Egr transcriptional activity within differentiating myeloid precursors. However, despite confirmation that both dnEgr and Nab2 proteins inhibit Egr-dependent transcription, they had no qualitative or quantitative effect on macrophage differentiation in any differentiation-competent cell type tested. A lack of effect by Nab2 may be explainable if Egr-mediated repression, not activation, is required for macrophage differentiation (46). However, our dnEgr construct lacks both transcription activation domains and repression (Nab2-binding) domains. Interestingly, a very similar construct inhibited both the repression of Gfi-1 and the induction of macrophage differentiation (22). In the present study however, the dnEgr molecule was unable to interfere with the commitment of monocyctic precursors or the differentiation of normal bone marrow progenitor and differentiation-competent cell lines.

Considering the results presented in this study, it is surprising that Egr2 has been recently implicated as an important regulator of macrophage differentiation (22). We were not able to identify any alteration in macrophage differentiation in Egr1/Egr2 DKO liver-derived hematopoietic cells despite the fact that the genetically modified mice were the same and it appears that the genetic backgrounds were also similar in both studies. It is possible that there may have been some inherent differences in the myeloid precursors from newborn P0 Egr1/Egr2 DKO liver (this study) and those obtained from adult Egr1−/− Egr2−/− haploinsufficient bone marrow (22). It is also possible that the expansion of liver-derived myeloid precursors with growth factors before differentiation by M-CSF may have up-regulated the M-CSF receptor sufficiently to no longer require Egr1 or Egr2 activation during the subsequent phases of macrophage differentiation. However, as we also observed Egr3 expression through all stages of macrophage differentiation, defective differentiation in Egr1−/− Egr2−/− haploinsufficient progenitors would indicate that Egr3 was present but unable to compensate for a reduction of Egr1 and Egr2 protein levels. Although this is a formal possibility, it seems unlikely that Egr3 would have no complementing function in this context given the high degree of structural homology between Egr2 and Egr3.

In addition to examining whether Egr transcription factors have a role in macrophage differentiation, we examined whether Egr gene expression was specifically coupled to M-CSF signaling in normal bone marrow and in FDCP-mix cells. Despite the fact that M-CSF, G-CSF, GM-CSF, and IL-3 exert very different effects on proliferation, lineage commitment, and maturation, we found that all four cytokines were able to induce Egr gene expression. Although the expression of Egr transcription factors does not appear to correlate with a specific myeloid lineage, these results may still be consistent with previously published evidence showing that Egr1 overexpression in various myeloid cell lines or normal progenitors promotes monocytic differentiation (4–6). It is likely that cellular context is important, as Egr family members presumably act in concert with other lineage-specific transcription factors. Therefore, it is reasonable to assume that the high levels of Egr1 induced by IL-3 may not lead to the same cellular response as would high levels of Egr1 expression enforced by a retroviral promoter. The kinetics of Egr induction may also play an important role in determining the cellular response. For example, transient up-regulation of Egr1 in vascular endothelial cells in response to basic fibroblast growth factor signaling leads to the production of proangiogenic growth factors (reviewed in Ref. 47), whereas sustained adenoaviral overexpression of Egr1 leads to the induction of inhibitory feedback mechanisms and an antiangiogenic state (48).

In vivo biology is often significantly more complex than models based on in vitro studies would predict. Our conclusion that Egr transcription factors are not essential for monocytic lineage commitment or differentiation is consistent with the report that Egr1−/− Egr2−/− haploinsufficient mice appear to have a normal hemopoietic system (22). Further studies will be required to more precisely define the role of Egr proteins in myeloid differentiation and to determine what role, if any, they have in macrophage differentiation and function in vivo.

Acknowledgments
We thank members of the Tourtellotte Laboratory and N. Yaseen for comments and review of the manuscript. The FDCP-mix cell line was provided by D. Hockenbery (Fred Hutchinson Cancer Center, Seattle, WA). We thank T. Gridley for providing the Egr2-heterozygous mice and J. Milbrandt for providing the Egr1 heterozygous mice.

Disclosures
The authors have no financial conflict of interest.

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