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*J Immunol* 2008; 181:5660-5672; doi: 10.4049/jimmunol.181.8.5660

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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Although a considerable number of reports indicate an involvement of the Hox-A10 gene in the molecular control of hemopoiesis, the conclusions of such studies are quite controversial given that they support, in some cases, a role in the stimulation of stem cell self-renewal and myeloid progenitor expansion, whereas in others they implicate this transcription factor in the induction of monocyte-macrophage differentiation. To clarify this issue, we analyzed the biological effects and the transcriptome changes determined in human primary CD34⁺ hemopoietic progenitors by retroviral transduction of a full-length Hox-A10 cDNA. The results obtained clearly indicated that this homeogene is an inducer of monocyte differentiation, at least partly acting through the up-regulation of the MafB gene, recently identified as the master regulator of such a maturation pathway. By using a combined approach based on computational analysis, EMSA experiments, and luciferase assays, we were able to demonstrate the presence of a Hox-A10-binding site in the promoter region of the MafB gene, which suggested the likely molecular mechanism underlying the observed effect. Stimulation of the same cells with the vitamin D₃ monocyte differentiation inducer resulted in a clear increase of Hox-A10 and MafB transcripts, indicating the existence of a precise transactivation cascade involving vitamin D₃ receptor, Hox-A10, and MafB transcription factors. Altogether, these data allow one to conclude that the vitamin D₃/Hox-A10 pathway supports MafB function during the induction of monocyte differentiation. The Journal of Immunology, 2008, 181: 5660–5672.
both self-renewal and commitment-differentiation processes (13, 14). In this regard, a number of reports indicate an involvement of the Hox-A10 gene in the regulation of monocyte commitment that is fundamentally based on three experimental observations. First, Hox-A10 expression is restricted to the CD34+ progenitor and myeloid precursor phase of hemopoiesis (15); secondly, this gene is a primary response gene of the VD monocyte differentiation inducer (16, 17); and finally, retroviral vector-mediated expression of its cdNA induces the monocyte differentiation of U937 (16) and CD34+ hemopoietic progenitors (18) inhibiting, at the same time, the commitment to lymphoid, erythroid, and granulocyte lineages (19). Despite this, a microarray study performed on CD34+ cells retrovirally transduced with Hox-A10 disclosed the up-regulation of genes belonging to the Wnt pathway, suggesting a role in the commitment to lymphoid and erythroid lineages (19). Similarly, in a distinct report, transplantation of Hox-A10-transduced CD34+ cells in NOD/SCID mice resulted in a proliferative expansion of the myeloid progenitor compartment (19). Both reports confirmed the previously reported inhibition of erythroid differentiation, whereas the former also claimed a down-regulation of monocyte-related genes thus contradicting the previously mentioned data. This controversy is further complicated by the observation that transplantation of Hox-A10-transduced murine stem cells in recipient mice gives rise to an expansion of megakaryocyte progenitors, observed only in vitro, and the appearance of acute myeloid leukemias in vivo (21). A recent report, based on a transgenic mouse model in which transcription of the transgene is controlled through an inducible system, indicated Hox-A10 expression levels as the crucial parameter able to determine different biological responses (22). In this study, a low/intermediate expression of the transgene was associated with increased self-renewal activity of HSCs, whereas a higher expression resulted in a remarkable inhibition of erythroid and megakaryocyte differentiation. Both the conditions led to increased numbers of macrophages and neutrophils. Although some of these findings could be simply explained in terms of species specificity of the biological actions promoted by the considered gene, the role exerted by Hox-A10 in the molecular regulation of hemopoiesis is, according to the previously cited literature data, quite controversial. To better characterize this role we constructed a bicistronic retroviral vector expressing a full-length Hox-A10 cdNA and a truncated version of low-affinity nerve growth factor receptor (ΔLNGFR) used as marker gene. The biological effects exerted by Hox-A10 on hemopoiesis were assessed, infecting the U937 monocytic cell line initially and primary CD34+ hemopoietic cells subsequently. Modulation of gene transcription determined in these last cells by Hox-A10 overexpression was also evaluated by means of the microarray methodology. The results obtained clearly suggested that the analyzed transcription factor is an inducer of monocyte-macrophage differentiation at least partly acting through the up-regulation of the MafB gene, recently indicated as master regulator of such maturation pathway. Additional investigation based on computational analysis, EMSA experiments, and luciferase assays allowed the identification of a Hox-A10-binding site in the promoter region of MafB, providing a plausible molecular mechanism explaining the observed differentiation effect.

Materials and Methods

Hemopoietic cell lines

K562, KG1a, KG1, KASUMI1, HL60, NB4, THP1, and U937 cell lines were obtained from American Type Culture Collection and cultured in RPMI 1640 (Euroclone), supplemented with 10% heat-inactivated FBS (BioWhittaker) and 1 mM l-glutamine (Euroclone). Stimulation of U937 cells with VD was achieved by treatment with a 5 × 10−8 M concentration of this nuclear hormone (Hoffman-Laroche).

Primary hemopoietic cells

Human CD34+ hemopoietic stem-progenitor cells were purified from umbilical cord blood samples as described (10, 23) and maintained in liquid culture for 2 wk. During the initial 5 days of culture, necessary for retroviral transduction, these cells were seeded at a 5–10 × 10^5/ml density in IMDM (Euroclone) containing 10% human serum (BioWhittaker) and early-acting human hemopoietic cytokines: 50 ng/ml stem cell factor and Flt3 ligand, 20 ng/ml thrombopoietin, 10 ng/ml IL-6 and IL-3 (R&D Systems). The subsequent phase of culture was accomplished under similar conditions without thrombopoietin and in the presence of 20% FBS. These experimental conditions normally promote a mixed granulomonocyte differentiation of CD34+ cells that is generally achieved within 14 days from plating (10, 24, 25). Treatment with VD was conducted with the same modalities of U937 cells. Myeloblasts, monoblasts, erythroblasts, and megakaryoblasts were generated by in vitro culture of cord blood CD34+ hemopoietic progenitors performed as already described (23, 25, 26). Normal human monocytes, neutrophils, and eosinophils were selected from Ficoll-separated PBMCs of adult samples and collected by immunomagnetic systems (8). Purity of isolated primary cell populations, determined by flow cytometry and morphological analysis (May-Grünwald-Giemsa staining), always exceeded 95%.

Retroviral vector construction and packaging

Construction of the LXIΔN and LmAfBNΔN retroviral vectors have been already described (8). To obtain the LmAfBΔNΔN retroviral vector, a full-length Hox-A10 cdNA was generated by RT-PCR performed on total RNA extracted from U937 cells using Hox-A10 primers (Table I). PCR amplification was conducted using a proofreading thermostable DNA polymerase (Fast Start High Fidelity PCR System; Roche Diagnostics) and an annealing temperature of 60°C. The amplified fragment was then inserted in the pcR2.1 TOPO T/A cloning vector (Invitrogen) generating the ΔLNGFR cDNA. The LmAfBΔNΔN retroviral vector was constructed by transduction of U937 cells and CD34+ hemopoietic progenitors with VD, as described (8, 9).

Hemopoietic cell transduction and LNGFR purification

U937 hemopoietic cell line was transduced by two to three cycles of infection (4 h each) with viral supernatant in the presence of polybrene (8).
μg/ml). CD34+ hematopoietic progenitors, preactivated for 48 h in liquid culture, were transduced by two cycles of infection (12 h each) with viral supernatant on retinacon-coated plates (10 μg/cm²). NGFR purification of transduced cells was performed after a 48-h post-transduction incubation using a purified mouse anti-human p75NGFR mAb and tiny FACS-compatible magnetic nanoparticles in a column-free magnetic system (EasySep Do-It-Your Self Selection Kit; Stem Cells Technologies) following the manufacturer’s guidelines.

Flow cytometric, cytochemical, and morphological analysis of differentiated hematopoietic cells
The extent of myeloid differentiation in transduced U937 and CD34+ cells was monitored by flow cytometric, cytochemical, and morphological analysis. Flow cytometry analysis of CD11b, CD14, and CD163 Ags was conducted as already described (8) using a Coulter Epics XL flow cytometer. Cytoplasts from normal or transduced cell samples were fixed using the naphthol AS-D chloroacetate (specific esterase) kit (Sigma-Aldrich). Morphological analysis was accomplished on cytocentrifuged cell samples upon May-Grünwald-Giemsa staining.

RNA purification, semiquantitative and quantitative RT-PCR
Total RNA was extracted from the various analyzed cell populations by using total RNA purification kits as recommended by the manufactures (Qiagen). RNA integrity and concentration was then verified by the BioAnalyzer technique (Applied Biosystems). Semiquantitative RT-PCR analysis was performed as previously described (28), using oligonucleotide primers reported in Table I. Normalization of the amplified samples was obtained by the GAPDH housekeeping gene (10). Results of a representative experiment of three performed were presented in each corresponding figure. Quantitative real-time RT-PCR (QRT-PCR) was performed with an ABI PRISM 7900 sequence detection system (Applied Biosystems) as already described (8), amplifying GAPDH mRNA as endogenous control. Statistical analysis of the QRT-PCR results was obtained using the (2^-ΔΔCt) method (29) which calculates relative changes in gene expression of the target gene normalized to the endogenous control and relative to a calibrator sample. QRT-PCR reactions were performed on three independent experiments and always presented as ± SEM.

Nuclear extract (NE) preparation and Western blot analysis
NEs were conducted as previously described with minor modifications (10) and used either for gel shift experiments (see EMSA) or Western blot analysis. The latter was performed as follows. Briefly, 20 μg of NEs of the analyzed cell populations were loaded onto 10% SDS-PAGE and blotted as described (10). Membrane was preblocked in blocking solution, supplemented with 5% nonfat powdered milk (Roche Diagnostics) for 1 h at room temperature, and then incubated with a 1/200 dilution of goat polyclonal anti-Hox-A10 (Santa Cruz Biotechnology), followed by a 1-h incubation at room temperature with a secondary Ab anti-goat conjugated to HRP (Amersham) for 30 min, resolved using a nondenaturing 4% polyacrylamide gel and used either for gel shift experiments (see EMSA) or Western blot analysis. Membrane was preblocked in blocking solution, supplemented with 5% nonfat powdered milk (Roche Diagnostics) for 1 h at room temperature, and then incubated with a 1/200 dilution of goat polyclonal anti-Hox-A10 (Santa Cruz Biotechnology), followed by a 1-h incubation at room temperature with a secondary Ab anti-goat conjugated to HRP (Amersham). Detection was conducted by using the BM chemiluminescence blotting substrate (Roche Diagnostics). Results of a representative experiment of three performed were presented in each corresponding figure.

EMSA
The following single-strand oligomers and their complement, corresponding to putative Hox-A10-binding Hox response elements (HRE) 1 and 2, were used as probes in gel shift assay: HRE1 probe: 5'-GCACTTATGCCTGG-3' (spanning from -943 bp to -929 bp from transcriptional start site); HRE2 probe: 5'-TCTGTTAAGCCTA-3' (spanning from -896 bp to -881 from transcriptional start site). Complementary oligonucleotides were annealed and 5' end labeled using [γ-32P]ATP (6000 Ci/mmol; GE Healthcare Europe) and T4 polynucleotide kinase (New England Bio-labs) and purified with Microspin G-25 columns (GE Healthcare). The mobility shift reactions were conducted in gel shift buffer (10 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 20% glycerol, 1 mM DTT) supplemented with 1 μg of polydeoxyinosin-polycytoxytidylic acid, 1.0 × 10^−6 M of the indicated labeled probe, and 10 μg of NE, in a total volume of 20 μl. Supershift was obtained by adding, to the gel shift mix, an anti-Flag Ab (Sigma-Aldrich). Binding reactions were incubated at room temperature for 30 min, resolved using a nondenaturing 4% polyacrylamide gel in 0.5× Tris-buffered EDTA, and prerun for 1 h at room temperature. Finally, the gel was fixed, vacuum dried, and exposed to x-ray films.

Plasmid expression vectors and cell transfection
An overhang double-strand oligomer HRE1 probe, obtained by annealing single-strand oligonucleotides modified at the 5' end with the BamHI and SalI restriction sites, was inserted into the BamHI/SalI-digested pT811Luc reporter vector generating the pT811LucHRE1 plasmid. Nucleotide sequence analysis of this plasmid demonstrated that HRE1 site had been inserted as single-copy oligomer. Hox-A10 cDNA was excised with EcoRI from pCR2.1HoxA10 construct and then inserted in the EcoRI site of pcDNA3 vector (Invitrogen), obtaining the pcDNA3HoxA10 construct. The pCMVFlagHox-A10 plasmid, coding for a N-terminal Flag-tagged Hox-A10 protein under the control of the CMV enhancer/promoter, was kindly provided by Prof. Fulvio Mavilio’s laboratory. HEK293T cells used in gel shift experiments were transfected with 20 μg of the pCMVFlagHox-A10 vector using the calcium phosphate procedure. For luciferase assays, transient transfection of HEK293T cells was conducted in a 24-well plate with Lipofectamine 2000 (Invitrogen), using 200 ng of pT811LucHRE1 reporter plasmid, 50 ng of pcDNA3HoxA10 expression vector, 200 ng of pCMVβ-galactosidase (Clontech) to normalize for transfection efficiency, and a CMV-luciferase vector, as described previously (23). Images obtained by scanning chips of untransduced and XIβ-transduced 800 ng. Mock-transfected HEK293T cells were used as control.

Reporter gene assay
Forty-eight hours after transfection, cells were washed twice in cold 1 × PBS, resuspended in lysis buffer (1% Triton X-100, 25 mM glycylicin (pH 7.8), 15 mM MgSO4, 4 mM EDTA (pH 8), 1 mM DTT), and assayed for β-galactosidase and luciferase expression. Luciferase measurements were performed using a Lumat LB 9501 (Berthold) luminometer. Each transfection was done in duplicate in the same experiment and luciferase activities were represented as the mean ± SEM values of three independent experiments.

Biotin-labeled transcription, GeneChip hybridization, and microarray data analysis
RNA pools (100 ng) of LXIαN- and LHoxA10LαN-transduced CD34+ cells, obtained from three independent experiments, were converted in labeled cRNA according to the two-cycle protocol advised by Affymetrix. cRNA has been used to hybridize Affymetrix HG-U133A GeneChip arrays (already described (23)). Images obtained by scanning chips of untransduced and LXIαN- and LHoxA10LαN-transduced CD34+ cells were processed using GeneChip operating software. This software allows assignment of a detection (absent or present), to quantify mRNA expression levels in terms of a signal value and to attribute a change, increased, decreased, or not changed to each transcript when comparing two analyzed cell populations. Changes in the expression levels of the analyzed RNAs were observed comparing the considered sample (LHoxA10I transduced) with the baseline sample (LXIαN-transduced cells) to the baseline (LXIαN-transduced cells), were then expressed as signal log ratio (SLR; fold change, 2^SLR). The transcripts showing change equal to increased, SLR ≥1, signal ≥100, and detection present were selected as increased; on the contrary, the transcripts showing change equal to decreased, SLR ≤-1, and, in the baseline sample, signal ≥100 and detection present were selected as decreased.

To identify similarities between gene expression profiles of Hox-A10-transduced CD34+ progenitors and the transcriptome of other myeloid cells, we analyzed a wider dataset including data previously obtained in our laboratory (30) and concerning hematopoietic CD34+ stem/progenitors, in vitro-differentiated precursors, HoxA-10- or MegF12-transduced CD34+ cells. Gene expression data were scaled to target intensity 150 using GeneSpring operating software (Agilent Technologies). Gene expression data were scaled to target intensity 150 using GeneSpring operating software (Agilent Technologies) and used as input for unsupervised hierarchical clustering analysis, using Pearson correlation as a similarity measure and average linkage. Supervised analysis was subsequently conducted to select genes differentially expressed among CD34+ cells and myeloid precursors. With the use of GeneSpring GX software, the selected gene list was then analyzed performing a one-way ANOVA.
Results

Endogenous expression of Hox-A10 gene in hemopoietic cells

To define the appropriate cell context in which the effect exerted by Hox-A10 in the commitment/differentiation process could be investigated, we analyzed the endogenous expression of this Hox gene in primary cells and cell lines of the hemopoietic system.

Microarray data obtained in our laboratory using primary hemopoietic cells evidenced that Hox-A10 mRNA is expressed in CD34+ stem/progenitor cells, in myeloblasts, in monoblasts, and to a lesser extent in megakaryoblasts, whereas it is completely absent in erythroblasts and in terminally differentiated cells, such as granulocytes and monocytes (Fig. 1a). Accordingly, semiquantitative RT-PCR analysis, performed in hemopoietic cell lines, revealed that Hox-A10 is selectively expressed in early myeloblastic (KG1a, KG1) and monocytic cell lines (Kasumi1, THP1, U937; Fig. 1b). These data globally suggest that Hox-A10 expression is restricted to the myeloid progenitor/precursor compartment of hemopoiesis.

Biological effects promoted by retroviral Hox-A10 transduction in U937 cells

On the basis of data described thus far, we initially overexpressed Hox-A10 cDNA in the U937 hemopoietic cell line, due to its monoblastic phenotype and responsivity to compounds inducing monocyte-macrophage differentiation. For this purpose, we constructed the LHoxA10LN retroviral vector expressing a Hox-A10 full-length cDNA and a truncated version of ΔNGFR, used as marker gene, in the context of a bicistronic transcript driven by the viral long terminal repeat (LTR). Biological effects promoted by Hox-A10 overexpression were always assessed by comparison with the LXIΔN retroviral vector (empty vector), containing only the marker gene, and all assays necessary to monitor such effects were conducted on cells transduced with the mentioned vectors and purified for NGFR expression.

RT-PCR and Western blot analysis performed on transduced U937 cells evidenced that the retroviral transcript was regularly produced (Fig. 2b), and expression of the transgene at the protein level was comparable with that obtained upon VD stimulation, as demonstrated by densitometric analysis showing respectively a 5.1- and 3.3-fold increase over the corresponding controls, i.e., “empty” vector for Hox-A10-transduced cells and untreated cells for VD stimulation, respectively (Fig. 2, c, lanes 1 and 2, and d, lanes 1 and 3).

A preliminary assessment of Hox-A10 differentiation capacity was performed by flow cytometry analysis evidencing, in cells transduced with this transcription factor, a gradual increase of...
MafB IS A TARGET GENE OF VD/Hox-A10 SIGNALING PATHWAY

To confirm these data in a primary cell model, the retroviral vectors described thus far were used to transduce cord blood CD34+ hemopoietic stem/progenitor cells expanded in liquid culture (see Materials and Methods for details). The experimental plan adopted for these cells included a 48-h pre-activation period, a 24-h transduction conducted by two cycles of infection (12 h each), and a further 48-h post-transduction incubation that was immediately followed by NGFR purification. Myeloid differentiation was then monitored on transduced/NGFR-purified cells by a combination of immunophenotypic, cytotoxic, and morphological analysis that were performed at day 14 of liquid culture.

Transgene dosage analysis, performed in these cells by QRT-PCR, indicated that Hox-A10 mRNA was $25 \pm 4$ (mean $\pm$ SEM) times more expressed in Hox-A10-transduced CD34+ cells as compared with VD-stimulated CD34+ cells, i.e., the biological sample used in our experimental system as control for endogenous Hox-A10 expression. Although this result is in apparent contrast with that observed in U937 cells, it is substantially the consequence of a different basal mRNA endogenous levels of Hox-A10, which is remarkably higher in hemopoietic cell lines (including U937) as compared with primary hemopoietic cells (Ref. 15 and data not shown). In addition, by using a sophisticated transgenic mouse model allowing an inducible expression of Hox-A10 in vivo, Magnusson et al. (22) have recently demonstrated that the monocytopoietic effect driven by this transcription factor is observed in a wide range of transgene expression levels up to ~500-fold higher than endogenous controls.

Flow cytometry analysis of Hox-A10-transduced cells revealed an evident up-regulation of several monocyte specific surface markers such as the CD11b and CD163 Ags. In a representative experiment, reported in Fig. 4a, CD14 Ag reached a $44.4\%$ positivity in Hox-A10-transduced cells vs $23.5\%$ of control cells infected with the empty vector. Similarly, these values appeared to be, respectively, $52.9$ and $26.6\%$ for the CD163 Ag (Fig. 4a). The up-regulated expression of these surface Ags was also observed on mean fluorescence intensities varying from $20.7$ to $44.6$ for CD14 and from $4.9$ to $10.7$ for CD163 (Fig. 4a).

CD11b expression that reached $33.8 \pm 0.5\%$ positivity (mean $\pm$ SEM) at day 7 post-infection (Fig. 3a), and by morphological analysis indicating that these cells assumed a pro-monocytic phenotype (Fig. 3b).

To provide additional evidence of such capacity, we also investigated, by means of semiquantitative RT-PCR, the induction at the mRNA level of a number of myeloid differentiation markers selected on the basis of expression profiling data previously obtained in our laboratory (8, 23). Beside the already mentioned CD11b Ag, these reactions also disclosed the up-regulation of other monocyte-related genes, such as the CD163 Ag, scavenger receptor for hemoglobin-aptoglobin complex, the CD115 Ag, receptor for the M-CSF, the IL-1$\beta$ and IL-7R, previously detected at high levels in monocyte precursors (23), the MafB transcription factors (8) and the p21$^{waf-1}$ growth arrest gene (Fig. 3c).

Among the others, the induced expression of MafB appeared of particular interest due to the crucial role that this transcription factor plays in the molecular control of monocyte differentiation. In this regard it is worth underlining that QRT-PCR, performed on the same cell samples, confirmed this finding evidencing a $3.9 \pm 0.2$ (mean $\pm$ SEM) increase of MafB mRNA levels.

Cell counts demonstrated that the induction of monocyte differentiation observed in Hox-A10-transduced cells was accompanied by a reduced proliferation activity given that the number of expansions in these cells averaged 6.9 vs 13.8 of control cells transduced with the empty vector (Fig. 3d). This observation was in agreement with the up-regulated expression of the p21$^{waf-1}$ gene detected in Hox-A10-transduced cells (Fig. 3c).

These results substantially confirm previous studies indicating that retroviral transduction of Hox-A10 induces the monocyte differentiation of U937 cells. At the same time, they allowed us to sensibly enrich the list of monocyte-related genes up-regulated in this cell context by transduction with the analyzed transcription factor.

**Differentiation effect observed in human CD34+ hemopoietic progenitors upon retroviral Hox-A10 transduction**

To confirm these data in a primary cell model, the retroviral vectors described thus far were used to transduce cord blood CD34+ hemopoietic stem/progenitor cells expanded in liquid culture (see Materials and Methods for details). The experimental plan adopted for these cells included a 48-h pre-activation period, a 24-h transduction conducted by two cycles of infection (12 h each), and a further 48-h post-transduction incubation that was immediately followed by NGFR purification. Myeloid differentiation was then monitored on transduced/NGFR-purified cells by a combination of immunophenotypic, cytotoxic, and morphological analysis that were performed at day 14 of liquid culture.

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Morphological analysis, performed on cytological specimens upon May-Grünwald-Giemsa staining, confirmed the results of previous assays evidencing an enrichment of neutrophils and their immediate precursors in control cells and a clear increase of monocytes and macrophages among Hox-A10-transduced cells (Fig. 4c).

Our phenotypic analysis consequently indicates that the induction of monocyte differentiation observed in Hox-A10-transduced CD34+ cells is accompanied by a parallel and remarkable inhibition of granulocyte differentiation.

**Gene expression profiling of Hox-A10-transduced CD34+ hemopoietic progenitors**

To characterize the genetic program underlying the observed differentiation capacity, we analyzed transcriptome changes induced by retroviral transduction of Hox-A10 in CD34+ hemopoietic progenitors using the Affymetrix microarray methodology. The expression profile determined in this cell background by Hox-A10 overexpression was, again, assessed by comparison with control cells infected with the empty vector. All of the data have been deposited in the Gene Expression Omnibus MIAME-compliant public database at http://www.ncbi.nlm.nih.gov/geo and are accessible through GEO Series accession number GSE12396.

Microarray analysis was conducted at the end of the transduction/NGFR purification procedure (day 5 of liquid culture), therefore under experimental conditions in which the stem/progenitor phenotype of analyzed cells was substantially maintained, as evidenced by the 70–80% positivity of CD34Ag (data not shown).

This experimental design allowed performance of mRNA expression profiling in the differentiation window in which, standing on our data, endogenous Hox-A10 is preferentially expressed. The results of this analysis disclosed the up-regulation of 167 genes and the down-regulation of 64 genes (SLR ≥+1 and ≤−1, respectively). Detailed analysis of the obtained expression profiles indicated that the majority of up-regulated genes could be classified into the following functional categories: CD Ags; degradation enzymes; secretory proteins; surface receptors; cell cycle/apoptosis regulators; and transcription factors.

In agreement with flow cytometry data, Hox-A10 transduction led to an increased mRNA expression of the CD14 and CD11b monocyte-related markers. More in general, virtually all up-regulated CD Ags have been previously detected on the monocyte/macrophage surface where they mediate a variety of biological functions related to innate and specific immunity and particularly: cell adhesion and migration (CD9, CD11b, CD24, CD39, CD44, CD54, CD87); recognition and scavenging of inflammatory molecules (CD14 and CD163); and Ag presentation and activation of T cell-mediated immune responses (CD1a–e, CD83, CD85D) (Fig. 5). For a detailed description regarding expression pattern and biological functions of the above-mentioned CD Ags, see www.ncbi.nlm.nih.gov/prow.

Consistent with these findings, Hox-A10-transduced CD34+ cells also showed an increased expression of genes coding for proteins mediating inflammatory processes related to monocyte-macrophage activation and inflammation response. Among them we detected: 1) granule proteins, such as cathepsins (CTSB, CTSG, CTSL1; Refs. 31–33), CPVL (34), and RNase 1 (33) all involved in intracellular Ag degradation; 2) STX 12, a protein that, together with STX 7, is responsible for fusion of endosomes and lysosomes with the phagosome, i.e., the intracellular compartment where phagocytic cells kill and degrade internalized foreign particles (35); 3) MMP9 and MMP12, responsible for the remodeling of extracellular matrix that is necessary for cell migration (36); 4) other enzymes such as PTGS2, catalyzing the first rate-limiting step in the conversion of arachidonic acid to prostaglandins (37),

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**FIGURE 4.** Analysis of myeloid differentiation in Hox-A10-transduced CD34+ cells. Representative experiment showing the results of flow cytometry, cytochemical and morphological analysis performed at day 14 of liquid culture in CD34+ cells transduced with the empty vector (LXIΔN, left) and with the Hox-A10 transcription factor (LHox-A10ΔN, right). a. Flow cytometry analysis of CD14 (top) and CD163 (bottom) Ag expression. Entities of surface Ag expression are shown inside the histograms in terms of positivity percentage and mean fluorescence intensity (MFI). b. Cytochemical analysis of naphthol AS-D chloroacetate-specific esterase; c, morphological analysis, performed on May-Grünwald-Giemsa staining, on cytocentrifuged specimens.

The effect that Hox-A10 overexpression was able to exert on granulocyte differentiation was assessed by cytochemical analysis of the chloroacetate-specific esterase. By using this assay, activity of the studied enzyme, indicated as mean ± SEM, was detected in 52 ± 5% of controls cells and only 8 ± 2% of Hox-A10-transduced cells (Fig. 4b). Again, the decrease of positivity percentage was coupled with a reduced staining score on single-reacting cells (Fig. 4b).
PLA2G7, encoding for platelet activating factor acetylhydrolase (38), and HMOX1, involved in the cytoprotection against inflammation induced oxidative stress (39) (Fig. 5).

Analyzing the secretory proteins and surface receptors categories, we could detect a number of up-regulated genes that are known to play a role in other aspects of innate immunity. In particular, inside the former we observed inflammatory cytokines (Ref. 40; IL-1β, IL-8, TGFβ1) and molecules exerting a chemotactic activity on monocytes/macrophages (chemokines, described in Ref. 41; galectin-3, described in Ref. 42), whereas the latter disclosed the presence of receptors involved in chemokine/cytokine signaling (CCRI5, described in Refs. 43 and 44; IL-1R1 and IL-1R2, described in Ref. 45), recognition and scavenging of bacterial products (TLR2, described in Ref. 46; MRC1, described in Ref. 47), and activation of monocytes (IFNGR, TNFRSF21, TREM1, IL1RN, all described in Ref. 33) (Fig. 5).

The majority of transcription factors exhibiting an up-regulated expression in Hox-A10-transduced CD34+ cells have been already implicated in monocyte-macrophage differentiation. In fact: 1) the Hox-A9 gene, localized upstream of the Hox-A10 gene on chromosome 7 (13), has been involved in the molecular pathogenesis of a subset of acute myeloid leukemias (48–50); 2) endogenous expression of MafB and MafF is remarkably induced during monocyte-macrophage differentiation of CD34+ hemopoietic progenitors (23) and viral transduction of these cells with a MafB cDNA induces a massive commitment toward the macrophage lineage (8); 3) c-jun determines a partial monocyte differentiation when transfected into hemopoietic cell lines (51) and is a recognized dimerization partner of Maf family proteins (52, 53); 4) expression of KLF4 (54), STAT4 (55), and NR4A2 (56) is normally induced in macrophages in response to inflammation stimuli.

Analysis of cell cycle regulators revealed an increased mRNA expression of the GADD45B (57), CFLAR (57), BTG1 (58), and DUSP2 (59) growth arrest/proapoptotic gene and a clear down-regulation of universally recognized proliferation markers as the c-myc proto-oncogene (60) and cyclin A1 (CCNA1; Ref. 61) (Fig. 5).

Down-regulated genes included a remarkable number of markers belonging to nonmonocytic differentiation lineages. Among them, we observed transcription factors (KLF1, GATA2, and NFE2), globin chains (α, β, γ), and surface Ags (glycophorin C), associated with erythroid differentiation (62, 63) and granule proteins related to neutrophil (ELA2B, RNase 2, CLC, PRG2, MPO; Ref. 64), eosinophil (EPX; Ref. 65) and basophil maturation (HDC; Refs. 66 and 67) (Fig. 5).

These results are consistent with the biological effects observed in CD34+ cells upon retroviral transduction with Hox-A10 (see previous paragraph). More importantly, they confirm in a primary hemopoietic cell context our observation, previously obtained in U937 cells, indicating a capacity of the investigated transcription factor to up-regulate MafB expression. Similarly to the U937 cells, this finding was validated by QRT-PCR showing a 2.7 ± 0.3 induction of MafB mRNA levels following Hox-A10 transduction of CD34+ cells. Altogether these data raise the possibility that MafB might be a direct target gene of Hox-A10.
Clustering analysis on expression profiles of human primary hemopoietic cells under different experimental conditions

As already underlined in Introduction, it is to date still controversial whether Hox-A10 is implicated in hemopoietic stem and progenitor expansion or in the differentiation of specific hemopoietic lineages. To clarify this issue and to better understand the relationship existing between genetic programs activated by Hox-A10 and MafB upon transduction of CD34+ cells, we used GeneSpring software to perform a supervised hierarchical clustering analysis in which the expression profiles of such cell populations were compared with those of fresh CD34+ cells and CD34+-derived normal monoblasts, myeloblasts, erythroblasts, and megakaryocytes. These samples were analyzed using a probe list containing all genes exhibiting a differential expression in the comparison among the CD34+ cells and the already-mentioned hemopoietic precursor populations. Results of this analysis, reported in Fig. 6 as Eisen map, allowed to distinguish several classes of samples corresponding to the various analyzed cell types. Examination of the condition tree and gene tree indicated that, as expected, MafB- and Hox-A10-transduced CD34+ cells clustered with the monoblast cell populations (Fig. 6). Unsupervised hierarchical clustering analysis, performed on all expressed genes, confirmed this observation (data not shown). These data provided additional evidence supporting the involvement of Hox-A10 in the molecular control of monocyte-macrophage differentiation.

Identification of a Hox-A10-binding site in the promoter region of MafB gene

Molecular analysis of U937 and CD34+ cells transduced with Hox-A10 clearly indicated MafB as a possible direct target gene of the analyzed transcription factor. To verify this hypothesis, we assessed the extent of interspecies conservation in the 5'-flanking region of MafB gene using the UCSC Genome Browser (68).

This preliminary analysis was based on the principle that sequence conservation frequently discloses the presence of relevant transcription regulatory elements. The results obtained revealed that the highest degree of conservation is observed in the region spanning from the −1000 to the +1 nt, whereas sequence similarity exhibits a gradual decrease up to the −2000 nt and a substantial drop further upstream (Fig. 7a). For this reason, we focused our attention on the 1-kb region placed immediately upstream the transcription start site of the MafB gene. The presence of Hox-binding sites within this region was then investigated by a computational search of the TAAT and TTAT core motifs. This approach resulted in the identification of two putative binding sites for Hox-A10, conventionally named HRE1 and -2, placed, respectively, at position −934 and −886 nt inside highly conserved genomic regions (Fig. 7b).

The capacity of Hox-A10 protein to bind these elements was assessed by means of EMSA experiments for which we used NEs of U937 cells under different treatment conditions and 14-mer double-strand oligonucleotide probes encompassing the two putative sites. Results of this assay, presented in Fig. 8a, showed that incubation of NE obtained from VD-treated or Hox-A10-transduced U937 cells with HRE1 oligonucleotide probe generated an evident shift complex (Fig. 8, lanes 2 and 4) that was not observed using NE of untreated or empty vector-transduced U937 cells (Fig. 8, lanes 1 and 3). This complex was on the contrary undetectable when EMSA was performed with HRE2 oligonucleotide probe (not shown). To further characterize the specificity of the shift complex observed with HRE1 oligonucleotide probe, we optimized a supershift assay performed using NE of HEK293T cells transiently transfected with the pCMVFlagHox-A10 plasmid expression vector, coding for a Flag-tagged version of Hox-A10 protein. Under these experimental conditions, we were able to reproduce the shift complex formed with HRE1 oligonucleotide probe (Fig. 8a, lane 3, bottom arrow). Incubation of the same sample with an anti-Flag Ab resulted in a mixed pattern of competition and supershift (Fig. 8a, lane 4, top arrow), indicating that the observed shift complex actually contains Hox-A10 protein.

To verify the capacity of the identified Hox-A10-binding site to activate gene transcription, the HRE1 oligonucleotide probe was...
cloned in the pT81Luc plasmid, where it was placed upstream a minimal promoter and the luciferase reporter gene, obtaining the pT81LucHRE1 construct. This vector was then transiently transfected in HEK293T cells together with the pcDNA3Hox-A10 plasmid, coding for wild-type Hox-A10 protein, to perform a set of transactivation assays based on the luciferase reporter system. The results of these experiments clearly demonstrated that the analyzed element was able to determine a 3.6-fold activation of the reporter gene in the presence of the required Hox-A10 transcription factor (Fig. 8c).

Based on our bioinformatic analysis, EMSA experiments and transactivation assays, it is therefore possible to state that the MafB gene is a direct target of Hox-A10 transcription activity.

**Activation of Hox-A10 and MafB expression in response to VD stimulation of CD34+ hemopoietic stem progenitor cells**

The observation that Hox-A10 is a VD target gene has been, to date, exclusively obtained in cell lines and never verified on primary normal cell populations, especially those belonging to the hemopoietic system.

On the basis of this consideration and data presented thus far, we performed a set of time course experiments in which CD34+ hemopoietic progenitors were stimulated with VD for 72 h and then the mRNA levels of the Hox-A10 and MafB genes were estimated by QRT-PCR at various treatment times. As reported in Fig. 9a (top), exposure of CD34+ hemopoietic progenitors to the mentioned nuclear hormone gave rise to a rapid and transitory increase of Hox-A10 mRNA peaking at 24 h of treatment. The entity of the observed up-regulation, reported as mean ± SEM value, was 3.7 ± 0.5. Consistent with this result, MafB mRNA underwent a 2.6 ± 0.4 (mean ± SEM) increase that was detected at a later time (72 h; Fig. 9a, middle). CD14 mRNA levels, analyzed as control, exhibited the expected increase over time that reached a 21 ± 2.4 (mean ± SEM) value at 72 h of treatment (Fig. 9a, bottom).

Data obtained by this quantitative analysis of endogenous Hox-A10 and MafB expression in response to VD treatment is therefore consistent with the existence of a transactivation cascade involving sequentially VDR, Hox-A10, and MafB transcription factors during the induction of monocyte-macrophage commitment.
Direct comparison of the monocyte-macrophage differentiation effect determined by VD stimulation and Hox-A10 or MafB retroviral transduction of CD34⁺ hemopoietic progenitors

Although the biological effects exerted on CD34⁺ hemopoietic progenitors by VD stimulation and Hox-A10 or MafB transduction have been already studied in previous reports, we decided to perform a number of similar experiments to obtain a direct comparison among all these monocyte differentiation inducers and to provide a more accurate estimation of their relative efficiency. As control for these experiments we used empty vector-transduced/VD-untreated cells.

Flow cytometry analysis, performed on the various samples at day 14 of liquid culture, evidenced a 4-fold increase of CD14 positivity in Hox-A10- and MafB-transduced CD34⁺ cells and a 3-fold induction of the same Ag in VD-stimulated CD34⁺ cells (Fig. 9b). The CD163 Ag exhibited a similar trend even if with less pronounced variations of expression (Fig. 9b). These data substantially indicate that treatment of CD34⁺ cells with the analyzed inducers results in comparable differentiation effects. In this regard, the weaker effect promoted by VD is not surprising in light of the different nature of the compared stimuli, i.e., hormone stimulation in one case and retroviral transduction in the other two.

Cell counts showed that, although all samples underwent a certain degree of proliferative expansions, the three analyzed stimuli determined an about double reduction of cellularity in comparison with control cells at day 14 of liquid culture (day 9 post-transduction-NGFR purification; Fig. 9c). This result confirms the proliferation inhibitory activity of Hox-A10 already observed in U937 cells.

Discussion

A significant number of reports have demonstrated the role played by Hox genes in the molecular control of self-renewal and differentiation of hemopoietic stem/progenitor cells. These observations are substantially based on endogenous expression studies and viral vector mediated overexpression experiments (14). Since the middle of the 1990s, the former approach has led to the evidence that, among Hox genes, Hox-A10 exhibited the highest specificity of expression pattern. In fact, in normal cell populations, its transcript was detected in CD34⁺ progenitors but not in terminally differentiated neutrophils and monocytes; and in neoplastic tissues (fresh leukemic populations and cell lines) Hox-A10 expression appeared to be restricted to the myeloblast-monoblast cell contexts (15). Although these last findings had not been verified on normal
hemopoietic precursors, data reported thus far globally indicated a myeloid-restricted and differentiation stage-related expression of Hox-A10, suggesting a role in the regulation of myeloid commitment. Overexpression experiments, performed in subsequent years, gave rise to contradictory results since they evidenced, in some cases, a role in stem cell maintenance (20) and myeloid progenitor expansion (19) whereas, in others, a remarkable differentiation activity (16, 18). In addition, the precise maturation lineage affected by the latter effect varied significantly among the different studies, allowing one to conclude that the exact function of Hox-A10 inside the hemopoietic system is still unclear. The considered species (murine or human) also appeared as a crucial factor, able to deeply influence the nature and entity of observed effects. The aim of the experimental work performed in our laboratory was to clarify the controversy focusing the attention on human models of hemopoiesis. For this purpose, we analyzed endogenous Hox-A10 mRNA expression in a number of hemopoietic cells of human origin and transduced the U937 monoblastic cell line and cord blood CD34+ hemopoietic stem/progenitor cells with a retroviral vector expressing full-length Hox-A10 cDNA and the ΔLNGFR marker gene.

Semiquantitative RT-PCR analysis showed that, as expected, Hox-A10 mRNA expression is restricted to cell lines having a myeloblastic or monoblastic phenotype. Consistently, microarray analysis performed on eight normal primary hemopoietic cell populations at different maturation levels (progenitors, precursors, terminally differentiated cells) evidenced that Hox-A10 is expressed in CD34+ cells, in myeloblasts and in monoblasts, whereas it is low or not expressed in other precursor contexts or in more mature cells. These data, for the first time including a statistical conditions, paired statistical analysis performed on eight normal primary hemopoietic stem/progenitor cells with a retroviral vector expressing full-length Hox-A10 cDNA and the ΔLNGFR marker gene.

Under the experimental conditions used in our laboratory, retroviral vector-mediated expression of Hox-A10 led to a massive induction of monocyte-macrophage differentiation, observed in both the analyzed cell types (U937 and CD34+ cells) and accompanied by an evident inhibition of proliferation activity. Although these results represent a confirmation of the data published by the Freedman (16) and Leclercq (18) groups, respectively, the differentiation effect was corroborated, in our study, by the up-regulation of a broader panel of monocyte-macrophage-related genes assessed, in U937 cells, by RT-PCR analysis and, in CD34+ cells, by microarray analysis conducted using Affymetrix methodology. Among them, we were able to identify the MafB transcription factor, previously described as the master regulator of monocyte-macrophage commitment (8); this observation was also confirmed by means of the QRT-PCR reaction. Computational analysis, subsequently validated by gel shift experiments and by transactivation assays, demonstrated that MafB is a direct target of the Hox-A10 transcription factor. This finding suggests a plausible molecular mechanism explaining the biological effects promoted by Hox-A10 transduction of human hemopoietic cells.

Microarray analysis of Hox-A10-transduced CD34+ cells provided a substantial contribute for a better comprehension of the biological effects driven by this transcription factor in human primary hemopoietic stem/progenitor cells. Results of this analysis confirmed the stimulatory effect exerted by Hox-A10 on monocytopoiesis, disclosing an up-regulated expression of transcription factors and differentiation markers (CD Ags, granule proteins, cytokines/chemokines) that are typically associated with this maturation lineage. They also evidenced a decreased expression of genes related to erythroid and granulocyte differentiation programs. This last effect was also confirmed by cytochemical and morphological evaluation of Hox-A10-transduced CD34+ cells. Modulation of cell cycle-related genes was consistent with the inhibitory effect exerted by Hox-A10 on proliferation activity.

The microarray data reported in this paper are significantly divergent in comparison with those recently published by the H. J. Lawrence (20) group using a similar approach in terms of target cells, culture conditions, and viral transduction, but different microarray platform, probe preparation, and analysis procedure. A number of comparative studies have clearly shown that these are crucial parameters resulting in remarkable differences in the final output of microarray experiments and, in this regard, oligonucleotide platforms based on the Affymetrix technology are the most reliable for accuracy and precision (69–71). An additional aspect contributing to these controversial results might be represented by the different transgene levels obtained in the two reports. Regardless of the possible factors accounting for data discrepancy, the main claims of the cited paper (induction of HSC self-renewal and inhibition of myeloid differentiation) are based on limited gene subsets and are not supported by the proper functional demonstration. Nevertheless, the compared studies are in complete agreement with regard to the down-regulation of erythroid-related genes.

Clustering analysis of expression profiles, performed on a considerable number of hemopoietic cell contexts under rigorous statistical conditions, paired Hox-A10- and MafB transduced CD34+ cells with mononuclear cells rather than with cell populations representative of stem/progenitor or non-monocyte lineage phenotypes (fresh CD34+ cells and non-monocyte precursors, respectively). This observation indicates that, at least in our experimental conditions, the Hox-A10 genetic program is univocally associated with monocyte-macrophage differentiation.

Taken together, our data demonstrate the existence of a precise transactivation cascade in which stimulation of the VDR-dependent pathway leads to the induction of Hox-A10 gene in turn up-regulating MafB expression, allowing us to conclude that the vitamin D3/Hox-A10 pathway cooperates MafB function in the regulation of monocyte commitment. This conclusion is also supported by the observation that all these inducers (VD, Hox-A10, MafB) comparably stimulated the monocyte-macrophage differentiation of CD34+ cells and that treatment of the same cells with VD resulted in the sequential induction of Hox-A10 and MafB expression.

Acknowledgments
This paper is dedicated to the memory of Stefano Ferrari, Professor of Biochemistry at the University of Modena and Reggio Emilia (Italy).

Disclosures
The authors have no financial conflict of interest.

References


