

Differences in Gene Expression between Wild Type and Hoxa1 Knockout Embryonic Stem Cells after Retinoic Acid Treatment or Leukemia Inhibitory Factor (LIF) Removal*

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Homeobox (Hox) genes encode a family of transcription factors that regulate embryonic patterning and organogenesis. In embryos, alterations of the normal pattern of Hox gene expression result in homeotic transformations and malformations. Disruption of the Hoxa1 gene, the most 3' member of the Hoxa cluster and a retinoic acid (RA) direct target gene, results in abnormal ossification of the skull, hindbrain, and inner ear deficiencies, and neonatal death. We have generated Hoxa1^{-/-} embryonic stem (ES) cells (named Hoxa1-15) from Hoxa1^{-/-} mutant blastocysts to study the Hoxa1 signaling pathway. We have characterized in detail these Hoxa1^{-/-} ES cells by performing microarray analyses, and by this technique we have identified a number of putative Hoxa-1 target genes, including genes involved in bone development (e.g. *Col1a1*, *Postn/Osf2*, and the bone sialoprotein gene or *BSP*), genes that are expressed in the developing brain (e.g. *Nnat*, *Wnt3a*, *BDNF*, *RhoB*, and *Gbx2*), and genes involved in various cellular processes (e.g. *M-RAS*, *Sox17*, *Cdkn2b*, *LamA1*, *Col4a1*, *Foxa2*, *Foxq1*, *Klf5*, and *Igf2*). Cell proliferation assays and Northern blot analyses of a number of ES cell markers (e.g. *Rex1*, *Oct3/4*, *Fgf4*, and *Bmp4*) suggest that the Hoxa1 protein plays a role in the inhibition of cell proliferation by RA in ES cells. Additionally, Hoxa1^{-/-} ES cells express high levels of various endodermal markers, including *Gata4* and *Dab2*, and express much less *Fgf5* after leukemia inhibitory factor (LIF) withdrawal. Finally, we propose a model in which the Hoxa1 protein mediates repression of endodermal differentiation while promoting expression of ectodermal and mesodermal characteristics.

Transcription factors are proteins that can activate or repress target genes by binding directly to specific DNA sequences in the target gene promoters or enhancers. One group of such transcription factors is the homeobox (Hox)¹ family of

proteins that regulates embryonic patterning and organogenesis (reviewed in Refs. 1–4). In mammals, 39 of the Hox genes are located in four separate chromosome clusters, *Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*. The expression of Hox genes during development depends upon their position in the chromosomal cluster: genes positioned at the 3'-end are expressed earlier and more anterior, whereas 5'-end genes are expressed at later times and more posterior (reviewed in Refs. 1 and 5). Colinear gene expression from these Hox gene clusters may involve an initial change in chromatin structure (histone modification and chromatin decondensation) of the entire loci, which would then facilitate the programmed expression of genes along the clusters by a progressive 3' to 5' change in higher order chromatin structure (6). Treatment of teratocarcinoma cells or embryonic stem cells with retinoic acid (RA), which acts via the retinoic acid receptors (RAR α , - β , and - γ and their isoforms (reviewed in Refs. 7 and 8), results in the sequential activation of several Hox genes in a manner that resembles their positions in the clusters, e.g. 3' genes are activated before 5' genes (7–9). Moreover, our laboratory discovered the presence of a retinoic acid response element (RARE) in the 3' enhancer of the *Hoxa1* gene (10–13). This RARE is also functional in transgenic mice (14–16). We also demonstrated that unlike F9 Wt cells, F9 RAR γ ^{-/-} cells fail to express the *Hoxa1* gene in response to RA (17).

Alterations in the normal pattern of Hox gene expression in embryos result in homeotic transformations and malformations, and frequently, in perinatal lethality (3, 18). For instance, the targeted inactivation in mice of both alleles of the most 3' member of the Hoxa cluster, the *Hoxa1* gene, leads to numerous developmental defects, including hindbrain deficiencies and abnormal skull ossification, and ultimately, to neonatal death (19–22). Numerous studies have demonstrated that the hindbrain defects in Hoxa1^{-/-} mice result in part from the failure of *Hoxb1* to reach its anterior limit of expression at the presumptive rhombomere 3/rhombomere 4 (r3/4) boundary, which initiates a cascade of gene misexpression that results in the misspecification of the hindbrain compartments from r2 to r5 (23–26). Furthermore, the ectopic expression of *Hoxa1* in transgenic mice leads to the ectopic activation of *Hoxb1* in r2, produces anterior abnormalities, including the reorganization of the developing hindbrain, and ultimately results in embryonic death (27). These observations highlight the importance of the *Hoxa1* gene and suggest that the regulatory effects of retinoids on cell growth and on embryonic patterning may be

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¹ The abbreviations used are: Hox, homeobox; RAL, retinaldehyde; RAR, retinoic acid receptor; RA, all-trans-retinoic acid; BSP, bone sialoprotein; Col1a1, α -1 type I procollagen; Col4a1, α -1 type IV procollagen; Cdkn2b, cyclin-dependent kinase inhibitor 2B; ES cells, embryonic stem cells; Gbx2, gastrulation brain homeobox 2; LamA1, laminin A1

chain; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; Nnat, neuronatin; Postn/Osf2, periostin/osteoblast-specific transcription factor 2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Wnt3a, wingless-related MMTV integration site 3A; Wt, wild type; RT, reverse transcriptase; BDNF, brain-derived neurotrophic factor.

exerted, at least in part, through the activation of specific levels of *Hoxa1* expression in cells and tissues.

Earlier work by this laboratory (28) initiated the isolation of *Hoxa1* target genes by overexpression of *Hoxa1* in F9 teratocarcinoma cells and subsequent comparison of gene expression with that of wild-type F9 cells by subtractive hybridization. These studies resulted in the isolation and identification of novel *Hoxa1* target genes, including *HAIR-62* (29). Other researchers have identified *Hoxa1* binding sites in the promoter regions of the neural cell adhesion molecule *L1* gene (30), the *Epha2* gene (31), and the *Hoxb1* gene (24, 32). The identification of additional *Hoxa1* target genes is required to understand fully the mechanism of action of the *Hoxa1* protein in cells and tissues. In this research we have generated embryonic stem (ES) cells from wild-type, *Hoxa1*^{+/−}, and *Hoxa1*^{−/−} blastocyst-stage mouse embryos to use as a model system in which to characterize in detail the putative genes involved in the *Hoxa1* signaling pathway and to identify *Hoxa1* target genes by microarray analyses.

MATERIALS AND METHODS

Isolation of *Hoxa1* Homozygous ES Cell Lines—C57Bl/6 *Hoxa1*^{+/−} mice, generated by replacing the 5′-end of the *Hoxa1* coding region with a neomycin^R cassette by homologous recombination (19), were kindly provided by Dr. Pierre Chambon (Strasbourg-Cédex, France). *Hoxa1*^{+/−} mice were mated in order to obtain mouse embryos at the blastocyst stage. Female mice were examined every morning for the presence of plugs. The day on which a plug was found was considered to be embryonic day 0.5 (E0.5). Blastocysts were harvested on day E3.5 and individually cultured in ES cell medium (containing 20% fetal calf serum and 2× leukemia inhibitory factor (LIF)) on a feeder layer of mitomycin C-treated embryonic fibroblasts, as previously described (33). Cells from the inner cell mass were trypsinized and replated 8 days after hatching from the zona pellucida. After several rounds of propagation, the murine ES cells were genotyped by PCR, and the cell lines were frozen.

Four different Wt cell lines were obtained (EMC-ES-*Hoxa1*-1, -11, -12, and -17), one heterozygous *Hoxa1*^{+/−} (EMC-ES-*Hoxa1*-3), and one homozygous *Hoxa1*^{−/−} (EMC-ES-*Hoxa1*-15). Cell lines 1, 3, and 15 were subsequently single-cell cloned and selected for further analyses.

PCR Genotyping—The genotypes of the newly generated EMC-ES-*Hoxa1* ES cell lines were initially determined by PCR analyses on genomic DNA. Isolation of genomic DNA was performed as described by Lufkin *et al.* (19). The sequences of the primers used were provided by Dr. Pierre Chambon. Primers 1 and 3 (P1 and P3) were used to detect the wild-type allele (expected size of the PCR fragment is 567 bp). Primers 2 and 3 (P2 and P3) were employed for the detection of the disrupted *Hoxa1* allele (expected size of PCR fragment is 700 bp). The positions of these PCR primers for the detection of each allele are shown in Fig. 1A. The sequences of the primers used for PCR genotyping are as follows: P1, 5′-GCCATTGGCTGGTAGAGTCACGTGT-3′; P2, 5′-GATGGAAGCCGGCTTGTGTCAG-3′; P3, 5′-CATGGAGTCGAGAGGTTTCCAGAG-3′. For PCR reactions, samples were subjected to 45 amplification cycles at 92 °C for 1 min; 62 °C for 2 min, and 72 °C for 1 min; followed by an extension cycle at 72 °C for 10 min. PCR products were separated on a 1% agarose gel and detected by ethidium bromide staining.

Karyotyping of EMC-ES-*Hoxa1* Cell Lines—Karyotyping of the six EMC-ES-*Hoxa1* cell lines was performed by Dr. Margaret Laversha from the Molecular Cytogenetics Core Facility of the Memorial Sloan Kettering Cancer Center (New York, NY). Metaphases were prepared from subconfluent cultures by standard cytogenetic procedures. Briefly, after a 1-h incubation with colcemid (final concentration 0.02 μg/ml), trypsinized cells were pelleted (250 × g, 5 min) from 25-cm² flasks and washed briefly in 4 ml of warm 0.075 M KCl, then incubated in 8 ml of fresh hypotonic solution for 5 min at 37 °C before mixing in 2 ml of fresh 3:1 methanol/glacial acetic acid fixative. The cells were washed in three changes of 5 ml of fixative before being resuspended in ~2 ml of fixative for slide preparation. Fixed cell suspensions were stored at −20 °C. Analysis was performed on 20–25 GTG-banded metaphases from each clone. Images were digitally captured using a Nikon E800 microscope equipped with a SenSys KAF1400-G2 CCD camera (Photometrics, Tucson, AZ), supported by QUIPS imaging and analysis software (Applied Imaging Corp., San Jose, CA). The analysis indicated that most EMC-ES-*Hoxa1*-1 cells had a trisomy 11 and an unbalanced translocation

between chromosomes 8 and 14. Approximately 50% of EMC-ES-*Hoxa1*-3 cells were of normal karyotype, and the remainder had sporadic structural changes. Approximately 70% of EMC-ES-*Hoxa1*-15 cells had a chromosome 14 with additional chromosomal material of unknown origin, and 20% of the cells had trisomy 11. About 10% of the cells had a trisomy 1 as a consequence of a Robertsonian fusion between chromosomes 1 and 6.

Cell Culture and Drug Treatments—Cells were cultured in ES medium (Dulbecco's modified Eagle's medium supplemented with 10% ES cell qualified fetal calf serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 100 μM MEM nonessential amino acids, and 0.1 mM β-mercaptoethanol. 1 × 10³ units/ml leukemia inhibitory factor (LIF, Esrgo, Invitrogen) was added prior to use. Cells were treated with 1 μM RA (Sigma) 24 h after plating on gelatin-coated 100-mm tissue culture plates. Control cells were treated with 0.1% ethanol (vehicle). After various times of drug treatment, cells were harvested for total RNA extraction.

For cell proliferation studies, ES cells were plated on 12-well plates at a density of 1 × 10⁴ cells/well. The day after plating, cells were treated in triplicate wells with various doses of RA (1 × 10^{−9}, 1 × 10^{−8}, 1 × 10^{−7}, 5 × 10^{−7}, and 1 × 10^{−6} M) and incubated for 96 h. After various times in culture, cells were counted using an electronic particle counter (model: Coulter Z1; Beckman Coulter, Inc., Fullerton, CA). Control cells were treated with 0.1% ethanol (vehicle).

For the LIF withdrawal studies, EMC-ES-*Hoxa1*-1 and EMC-ES-*Hoxa1*-15 were cultured in ES cell medium containing 10% fetal calf serum plus LIF. Cells were then plated on gelatinized 100-mm plates at a density of 1 × 10⁶ cells/plate in the presence or absence of LIF for 24 h, and then they were treated with 1 μM RA or ethanol vehicle for various periods of time and harvested for subsequent Northern blot analyses.

Northern Blot Assays—Total RNA samples were isolated from cells and then subjected to Northern blot analysis as described previously (34). RNA was fractionated by size on 1% agarose, 2.2 M formaldehyde gels, transferred to nylon filters, and cross-linked using a UV Stratalinker 1800. Filters were hybridized to radiolabeled cDNA probes in standard hybridization buffer containing 10% dextran (17). After washing under high stringency conditions (0.2× SSC, 0.1% SDS for 20 min at 60 °C), hybridization filters were exposed to Kodak BioMax films. Results from Northern blot assays were normalized relative to *GAPDH* mRNA levels; *GAPDH* is constitutively expressed in these cells. Quantitation of Northern blots was performed by PhosphorImager analysis (Amersham Biosciences, Piscataway, NJ), with the aid of the computer software NIH Image 1.62 or Image Quant 1.2. Statistical analyses of Northern blot quantitations were performed by a 2-way analysis of variance using the software GraphPad Prism 4.0. For the statistical analyses of Northern blot assays, normalized quantitations from *Hoxa1*-3^{+/−} or *Hoxa1*-15^{−/−} cells were compared with the normalized quantitation from *Hoxa1*-1 (Wt) cells at the respective time points and drug treatments. Significance indicates a *p* value of < 0.05.

Probes Used for Northern Blot Analyses—The following cDNA probes were used in the Northern blot assays: *Hoxa1* (1-kb fragment, initially isolated as ERA-1-993) inserted into the EcoRI site of the pUC-9 vector (10); the full-length *Hoxa2* cDNA, generated in this laboratory, (~1.6 kb) inserted into the EcoRI site of pBluescript; a cDNA fragment of *Hoxa3* inserted into the HindIII site of pGem1 (obtained from Dr. Frank Ruddle, Yale University, New Haven, CT). For *Hoxa5*, a 637-bp fragment was excised with EcoRI-BglII from pUC18-*Hoxa5* (formerly known as pUC18-Hox1.3, a gift from Dr. W. F. Odenwald). *Hoxb1* was excised as a 435-bp EcoRI-HindIII fragment from pGEM72f-*Hoxb1* (35). A 620-bp fragment of *Fgf4*, cloned into the SmaI site of pBluescript, and an 800-bp fragment of *Fgf5*, cloned into the SmaI site of pBluescript, were obtained from Dr. Gail Martin (UCSF, San Francisco, CA). The plasmid pRex-1 2-5R contains a 1.7-kb fragment of *Rex-1* (Zfp-42) inserted into the EcoRI site of the pUC9 vector (36). An Oct3/4 cDNA fragment (800 bp) was excised with PstI-EcoRI from the pBSII-Oct3/4 plasmid (a gift from Dr. Yehudit Bergman). *Bmp4* was obtained from Dr. John Wozney (37). *Gata-4* was obtained from Dr. Stuart H. Orkin (38). A *Pbx1* cDNA fragment (750 bp) was obtained by restriction digestion of the plasmid pBS-*Pbx1* with EcoRI-NcoI (obtained from Drs. Dwaine Wright and Mark P. Kamps, UC San Diego, San Diego, CA). Similarly, a 1.6-kb cDNA fragment of *Meis1* was excised from the EcoRI site of the pMSCV vector (a gift from Drs. Scott Steelman and Arthur M. Buchberg). *LamA1* was obtained from Dr. Y. Yamada (39). A 2.4-kb cDNA fragment of *Col4a1* was excised from the EcoRI site of the pUC9 vector (40). *Foxa2* (HNF3β) was obtained from Dr. K. Anderson (Sloan-Kettering Institute, New York, NY). *Dab2* and *Foxq1* cDNAs were obtained from a F9 cDNA library and cloned into the pT-Adv vector (Clontech, Palo Alto, CA) by TA cloning (41). The following clones were

obtained from ATCC: Bmi1 (BE854719), Cdx1 (AA509618), Klf5 (1331265), and Nnat (BG962074).

The following cDNAs were amplified by PCR from RA-treated EMC-ES-Hoxa1-1 cells (for genes up-regulated in Wt) or EMC-ES-Hoxa1-15 cells (for genes up-regulated in Hoxa1^{-/-} cells) and subsequently cloned into the Topo TA vector (Invitrogen): Postn/Osf2, 758 bp (upper primer, 5'-GACGAAGATCCAAATTGAAGGTGA-3'; lower primer, 5'-CTGAGGCGGAAGGTTTTTACTGT-3'); Col1a1, 424 bp (upper primer, 5'-CTGACCTTCCTGCGCTAATGT-3'; lower primer, 5'-TTGAGTTTGGGTTGTTCGTCTGTT-3'); Wisp1, 685 bp (upper primer, 5'-CCAGAGGAGGCCACGAACCTT-3'; lower primer, 5'-GTCCCCAATCCCATCCATCTGCTCT-3'); Hoxc9, 561 bp (upper primer, 5'-GGCAGCAAGCACAAGAGGAGAA-3'; lower primer, 5'-TTGCGCTGGGGAAGAGAACG-3'); RAE3, 479 bp (upper primer, 5'-CGGCGCTGGTCTGAACATCCT-3'; lower primer, 5'-TCAGCCCTGCAGCCCCATCTA-3'). The identities of the amplified cDNAs were confirmed by DNA sequencing in the sequencing facility of Cornell University in Ithaca, NY.

RT-PCR Analysis—Total RNA was extracted from both wild-type and Hoxa1^{-/-} ES cells treated with RA for 0, 24, and 48 h. RNA was treated with RNase-free DNase I (Invitrogen) to eliminate any residual genomic DNA, and then reverse-transcribed with Superscript II (Invitrogen) and primed with 1 μ g of random hexanucleotides. Samples were amplified in the linear range by PCR using the following primers: Wnt3a, 507 bp (upper primer, 5'-CGGGCGCGGCATAACG-3'; lower primer, 5'-CCATCCGCACGAACCCACCTA-3'); RhoB, 442 bp (upper primer, 5'-CAGGCAGGCCAGGAGGACTAC-3'; lower primer, 5'-GC-AAGGGCGTGAGGCAGGAC-3'); Cdkn2b, 647 bp (upper primer, 5'-CGTGACATTGCGAGGTATC-3'; lower primer, 5'-CAGAATCCAGGCATCAAGGCAACT-3'); Gbx2, 767 bp (upper primer, 5'-ATCGCCACGCCCTCAAACTC-3'; lower primer, 5'-TGCTTCCGACATGGCTCAGATAGG-3'); M-Ras, 529 bp (upper primer, 5'-ACCAGGGACCAAGGAAAAAATG-3'; lower primer, 5'-CTGGTGCCTTGTATGTGGGTAAGC-3'); Sox17, 477 bp (upper primer, 5'-GCCGGCACCTACACTTACG-3'; lower primer, 5'-GTCGGACACCGAGGAGAAATG-3'); Gapdh, 432 bp (upper primer, 5'-ACCACAGTCCATGCCATCAC-3'; lower primer, 5'-TCCACCACCCTGTTGCTGTA-3').

Microarray Analysis—Wild-type and Hoxa1^{-/-} ES cells were treated with 1 μ M RA for 24 and 48 h in the presence of LIF, and total RNA was extracted using RNeasy-60 (Tel-Test, Inc.; Friendswood, TX) as indicated by the manufacturers. The differential expression of putative Hoxa1 target genes in EMC-ES-Hoxa1-1 versus EMC-ES-Hoxa1-15 ES cells was examined according to the Affymetrix microchip array protocol (Affymetrix, Santa Clara, CA). Briefly, single strand cDNA was prepared from 20 μ g of total RNA by reverse transcription. After second strand synthesis, cRNA was prepared by *in vitro* transcription of double strand cDNA with biotinylated ribonucleotides (Enzo Diagnostics, Farmingdale, NY). cRNA was then fragmented by heating at 95 °C for 35 min and then mixed with control oligonucleotide, control cRNA (composed of biotin B, biotin C, biotin D, and Cre), and herring sperm DNA. This mixture was hybridized to MG-U74Av2 microarray chips for 16 h at 45 °C. The MG-U74Av2 microarray chips contain all sequences (~6000) in the mouse UniGene data base that have been functionally characterized, plus ~6000 ESTs (Affymetrix, Santa Clara, CA). The hybridized microarray chips were washed and stained in a Fluidics station in the Microarray Core facility of the Weill Medical College of Cornell University. The microarray analyses were performed twice on two different starting preparations of ES Wt and Hoxa1^{-/-} ES RNA. Microarray results were analyzed using the computer software Microarray Suite 5.0 from Affymetrix. Scatter plots were performed with Microarray Suite 5.0 software. Data analysis was performed using the computer program GeneSpring (Redwood, CA).

RESULTS

Establishment of the Wt, Hoxa1^{+/-}, and Hoxa1^{-/-} ES Cell Lines—The C57BL/6 Hoxa1^{+/-} mice used in this research were provided by Dr. Pierre Chambon. Heterozygous Hoxa1 ES cells were generated by transfection of a construct containing 8 kb of homologous Hoxa1 genomic DNA into wild-type D3 ES cells, followed by homologous recombination (see Fig. 1A). The heterozygous ES line was employed to generate the Hoxa1 knockout mice (19). To generate Hoxa1 ES cell lines, heterozygous C57BL/6 Hoxa1 mice were mated, and day 3.5 blastocyst-stage embryos were extracted from pregnant females by uterine flushing as previously described (33). Six different ES cell lines were initiated, each from a different blastocyst, and these were genotyped by PCR. Of these, four were wild type (EMC-ES-

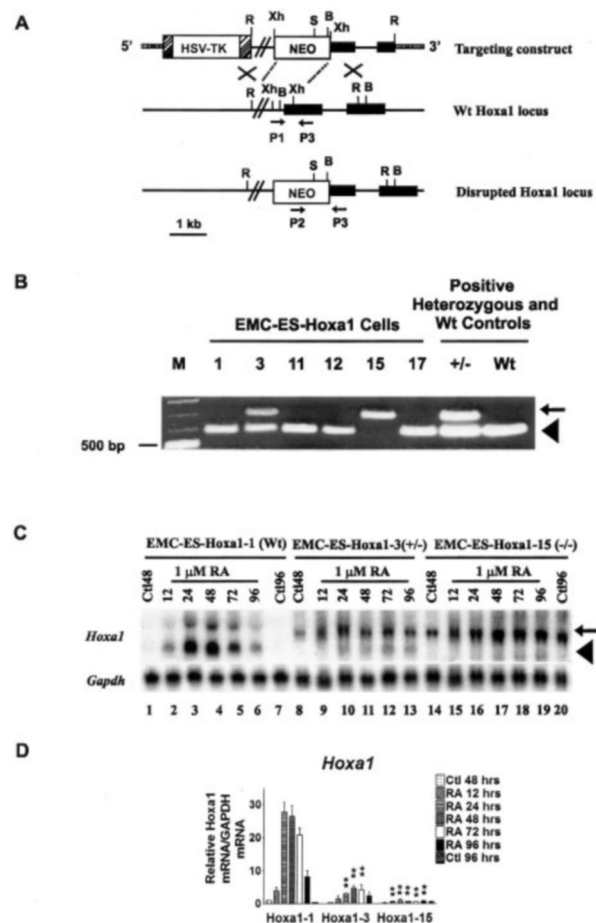


FIG. 1. PCR genotyping of EMC-ES-Hoxa1 cell lines and Northern blot analysis. A, diagram showing the position in Wt and the disrupted locus of the primers used for genotyping (adapted from Lufkin *et al.*, Ref. 19). P1 and P3 recognize the Wt allele. P2 binds to the NeoR cassette (open box), and together with the P3 primer, can be used to identify the disrupted Hoxa1 alleles. Hatched lines in the targeting construct represent vector DNA; black boxes, Hoxa1 exons; thin lines, genomic DNA. B, four different Wt cell lines were obtained (EMC-ES-Hoxa1-1, -11, -12, and -17), one heterozygous (Hoxa1-3), and one homozygous (Hoxa1-15). Cell lines 1, 3, and 15 were selected for further analyses. Positive controls are from Hoxa1 heterozygous and wild-type mouse tails. M, 1-kb ladder molecular DNA markers. Arrow, disrupted allele (~4 kb). Arrowhead, wild-type allele (2.2 kb). C, Northern blot analysis using a radiolabeled cDNA probe for Hoxa1 confirmed the genotype of EMC-ES-Hoxa1-1 (Wt), -3^{+/-}, and -15^{-/-}. Cells were cultured plus or minus RA and plus LIF. Arrow, expression of the gene containing the NeoR locus. Arrowhead, expression of the wild-type Hoxa1 mRNA. D, quantitation of Northern blots shown in C after normalization to Gapdh mRNA levels was performed with the software program GraphPad Prism Version 4.0. **, very significant ($p < 0.001$). B, BamHI; R, EcoRI; S, SphI; Xh, XhoI.

Hoxa1-1, -11, -12, and -17), one was Hoxa1 heterozygous (EMC-ES-Hoxa1-3), and one Hoxa1 homozygous knockout (EMC-ES-Hoxa1-15; Fig. 1B). Of the wild-type ES cell lines, EMC-ES-Hoxa1-1 was selected for subsequent experiments, in addition to the EMC-ES-Hoxa1-3 and EMC-ES-Hoxa1-15 ES cells. The genotypes of these three ES cell lines were further confirmed by Northern blot analysis (Fig. 1C). RA induced the transcription of the wild-type Hoxa1 gene (transcript size about 2.2 kb, arrowhead in Fig. 1C) in both Hoxa1-1 (Wt) and Hoxa1-3^{+/-} ES cells, although RA-inducible Hoxa1 mRNA levels were about 4–7-fold lower in heterozygous cells than in wild-type cells, as demonstrated by quantitative analyses of the Northern blot assays (Fig. 1D). The upper band in Fig. 1C (about 4 kb; arrow) results from the transcription of the disrupted allele.

Induction of Hoxa Cluster Gene Expression by RA in the Absence of Hoxa1—As a first step toward the characterization of our Hoxa1^{-/-} ES cells, we asked whether the expression of other Hox genes is altered in these Hoxa1-null cells lines. Knockout inactivation of the *Hoxa1* gene has demonstrated that Hoxa1 is not required for the correct expression of *Hoxa* cluster genes (e.g. *Hoxa2*, *Hoxa3*, and *Hoxa4*) in the Hoxa1 mutant embryos (42). However, these experiments do not exclude the possibility that Hoxa1 may be required for the RA-induced colinear expression of *Hoxa* cluster genes upstream of *Hoxa1*. To test whether a functional Hoxa1 protein is required for the induction of the more 5' *Hoxa* cluster genes by RA in ES cells, Wt, Hoxa1^{+/-}, and Hoxa1^{-/-} ES cell lines were treated with 1 μ M RA or vehicle only for different periods of time and the expression of various *Hoxa* cluster genes, along with the expression of *Hoxb1*, was assayed by Northern blot analysis. Expression of *Hoxa2*, *Hoxa3*, *Hoxa5*, and *Hoxb1* mRNA was detected in all three ES cell lines by Northern blot assays (Fig. 2A). Quantitation of Northern blots, after normalization against the loading control, demonstrated that there were no significant differences in *Hoxa2*, *Hoxa3*, or *Hoxa5* mRNA levels (by two-way analysis of variance) after treatment of the three cell lines with RA (Fig. 2B). Taken together, these results indicate that Hoxa1 expression is not required for the colinear induction of these *Hoxa* cluster genes by RA.

Hoxb1 mRNA expression Is Reduced in RA-treated Hoxa1^{+/-} and Hoxa1^{-/-} ES Cells—It has been demonstrated that Hoxa1 is required for the correct expression levels of *Hoxb1* both *in vitro* and *in vivo* (23, 26, 32, 43). To determine whether this is the case in our model system, we examined the expression of *Hoxb1* in the three ES cell lines with or without RA treatment (Fig. 2A). RA-induced *Hoxb1* mRNA expression was about 2–4-fold lower in the Hoxa1-3^{+/-} and the Hoxa1-15^{-/-} cells than in Hoxa1-1 (Wt) cells (Fig. 2A, lanes 4–7 versus lanes 11–14 and 18–21, also, see quantitation in Fig. 2B). These results demonstrated that Hoxa1 is required for the transcriptional activation of *Hoxb1* by RA in these ES cell lines. Our results using these ES cells are in agreement with previous observations reported in the literature for other model systems (23, 24, 26, 32, 43).

Expression of Regulators of Hox Activity and Hox Gene Transcription in the ES Cell Lines: Pbx1 and Meis1—Hox proteins exert their activity by forming protein complexes with Meis1 or Pbx1 that bind to specific DNA sequences in the promoters of their target genes (44–48). To test whether Hoxa1 can regulate the expression of *Meis1* and/or *Pbx1*, we next examined the expression of these two genes in the three ES cell lines treated with RA. As has been previously reported (49), we observed that *Meis1* and *Pbx1* mRNAs were induced by RA. No differences in the levels of *Meis1* mRNA induction were observed among the three ES cell lines (Fig. 3A). However, quantitative analyses demonstrated that *Pbx1* mRNA levels were significantly lower in the Hoxa1-15^{-/-} cells at 96 h after RA addition than in either Hoxa1-1^{+/-} or Hoxa1-3^{+/-} cells at 96 h plus RA (Fig. 3A, sample 21 versus samples 7 and 14).

Expression of Polycomb Group Genes in the EMC-ES-Hoxa1-15 Cell Line—Another type of regulator of Hox gene expression is the polycomb group (PcG) of transcriptional repressors (50). To determine whether Hoxa1 regulates PcG gene expression, we examined the mRNA levels of *Cdx1* and *Bmi1* in the ES cell lines by Northern blot analysis. No differences in either *Cdx1* or *Bmi1* mRNA expression were observed among the different ES cell lines tested (Fig. 3B). Our results indicate that Hoxa1 does not play a transcriptional role in the expression of either *Cdx1* or *Bmi1*.

Identification of Putative Hoxa1 Target Genes in Hoxa1 ES Cells by Microarray Analysis—The targeted inactivation of

both copies of the *Hoxa1* gene results in a variety of defects and ultimately, in death (19–22). Likewise, overexpression of *Hoxa1* driven by the actin promoter leads to embryonic lethality (27). These results clearly demonstrate the importance of the *Hoxa1* gene for the proper development and survival of the vertebrate embryo. Despite this evidence, the actions of the Hoxa1 protein are not yet well understood. Thus, in order to identify putative Hoxa1 target genes and to characterize in more detail our Hoxa1-null cells, we compared the gene expression of Hoxa1-1^{+/-} versus Hoxa1-15^{-/-} ES cells by gene microarray analysis. Total RNA was obtained from Hoxa1-1 versus Hoxa1-15 ES cells after RA treatments for 24 and 48 h; all cells were cultured in the presence of LIF. RNA was converted to biotin-labeled cRNA as described under “Materials and Methods.” After hybridization to microarray chips, washing, and staining, the signals were corrected for intensity via internal controls in a Fluidics Station. The comparative microarray analyses were performed in duplicate for each time point assayed, starting with different RNA preparations, with similar results. The expression profile from one experiment at 48 h of RA treatment is shown in Fig. 4 as a scatter plot. For clarity, only genes (each dot represents one gene) that were differentially expressed in Hoxa1-1 versus Hoxa1-15 cells by greater than 2-fold are shown.

A total of 71 genes were down-regulated in the Hoxa1-15^{-/-} versus Hoxa1-1^{+/-} cells by 2-fold or greater. Of these, 27 were unknown genes. Of 74 genes up-regulated in Hoxa1-15^{-/-} versus Hoxa1-1^{+/-} cells by 2-fold or greater, 26 were unknown genes.

Some of the genes that exhibited decreased expression in Hoxa1-15^{-/-} cells as compared with Hoxa1-1^{+/-} cells and thus are putative target genes activated by Hoxa1 are shown in Table I. Genes that exhibited increased expression in Hoxa1-15^{-/-} cells are shown in Table II. These are putative target genes repressed by the Hoxa1 protein. Some of the genes (17 genes) shown in Tables I and II were examined by either Northern blot assays or RT-PCR for the purpose of characterizing the Hoxa1^{-/-} ES cells in more detail and validating the results obtained from the microarray analyses. The results from these analyses are described below.

Putative Hoxa1 Target Genes Involved in the Development of the Nervous System and/or Brain Development—One gene that exhibits reduced expression in Hoxa1-15^{-/-} cells is neuronatin (*Nnat*), whose expression in the hindbrain of the developing embryo is restricted to rhombomeres 3 and 5 (51). In the neonate, *Nnat* expression is restricted to the brain (52). We examined the effect of RA treatment on the expression of *Nnat* mRNA in the three Hoxa1 ES cell lines to validate the microarray results. For comparison purposes, the effect of RA was also examined in the well established AB1 murine embryonic stem cell line (34) (Fig. 5A, lanes 1–7). One representative Northern blot is shown (Fig. 5A), and the quantitation of *Nnat* mRNA levels from three separate experiments is also shown (Fig. 5B). High levels of *Nnat* mRNA were observed in both untreated Hoxa1-1^{+/-} and Hoxa1-3^{+/-} cell lines (Fig. 5A, lanes 8, 9 and lanes 15, 16 and B, samples 1, 2 and samples 8, 9). A decrease in *Nnat* mRNA levels was observed after 12 h of RA treatment in these cell lines, but by 48 h of RA treatment *Nnat* mRNA levels were similar to those of untreated controls. *Nnat* mRNA levels in untreated Hoxa1-15^{-/-} ES cells were about 10-fold lower than those in untreated Hoxa1-1^{+/-} cells (Fig. 5B, samples 15–21 versus samples 1–7). RA treatment for 12 and 24 h also decreased *Nnat* mRNA levels in the Hoxa1-null ES cells (Fig. 5A, lanes 24, 25).

WISP1, an extracellular matrix-associated signaling molecule that can be induced by Wnt-1 (53, 54), also exhibited a reduced expression in Hoxa1^{-/-} ES cells. Wnt-1 encodes a

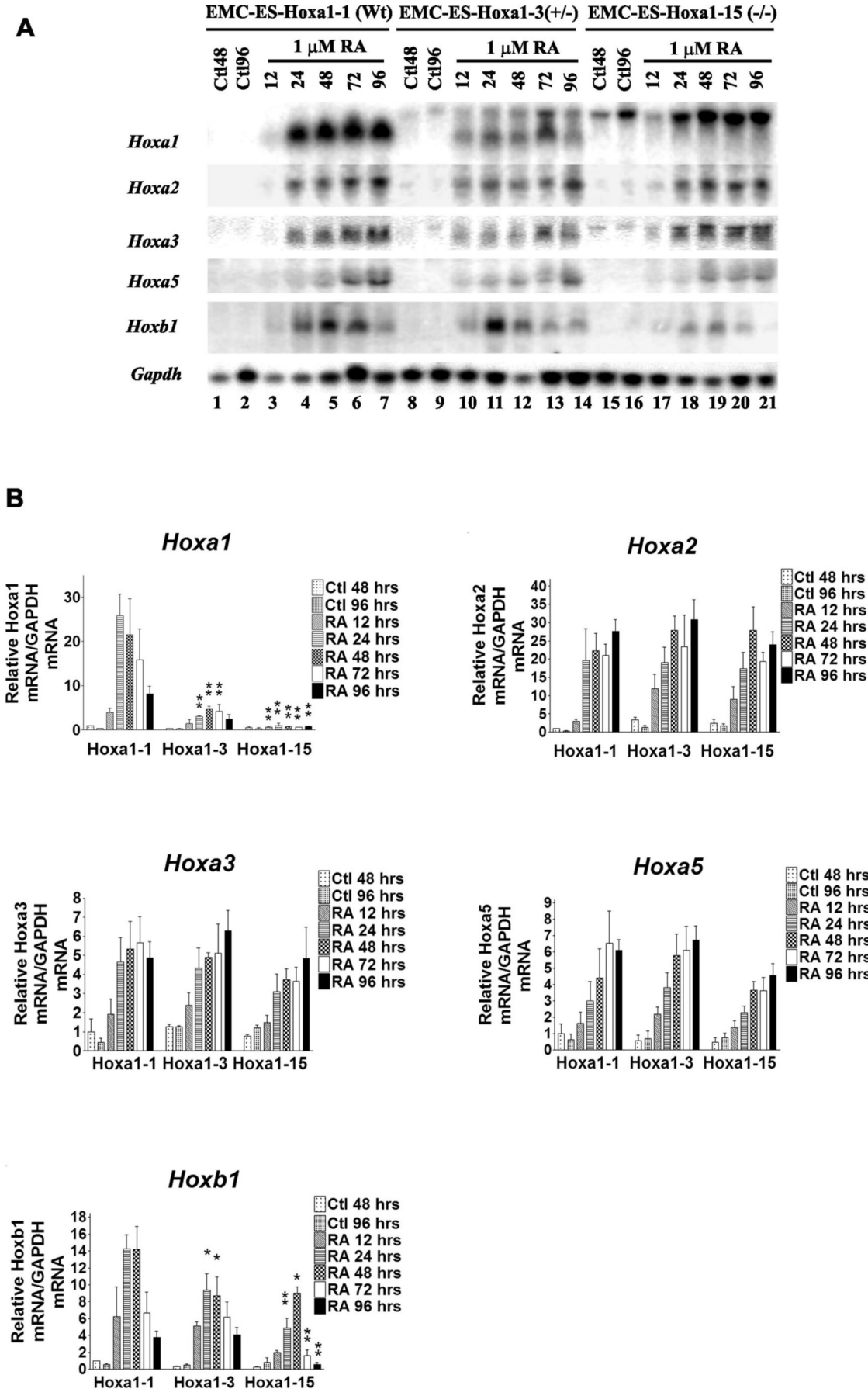
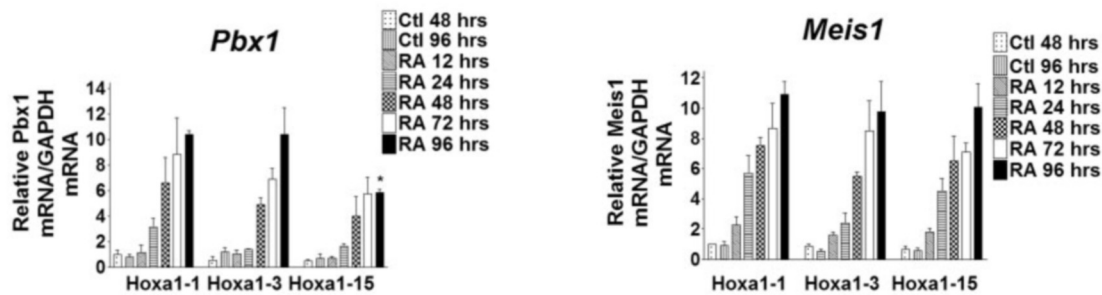


FIG. 2. Induction by RA of *Hoxb1* and *Hoxa* cluster gene expression in ES cell lines cultured plus or minus RA and plus LIF. A, Northern blot analysis of *Hoxb1* and *Hoxa* cluster genes in RA-treated (1 μ M) ES cells; B, quantitative analysis of *Hox* gene expression in the cell lines. Hox mRNA levels were normalized to *Gapdh* mRNA levels, and the data are shown with the 48-h control (Ctl 48 h) as the unit. Approximate

A



B

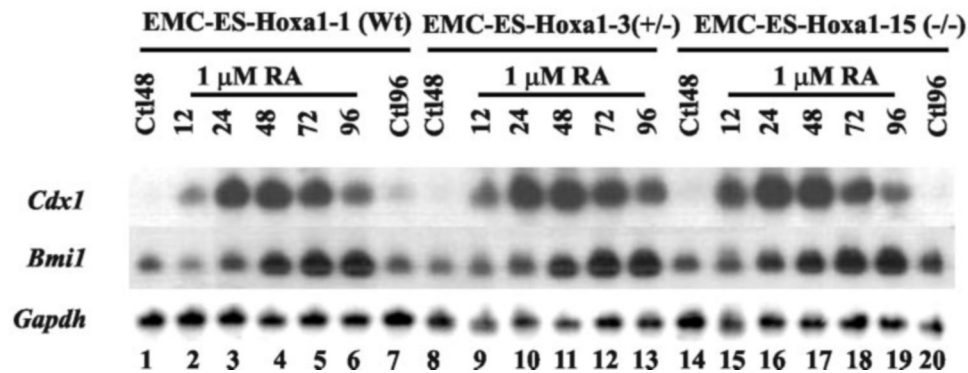


FIG. 3. **Induction of expression of Hox cofactor mRNA by RA.** Cells were cultured plus or minus RA and plus LIF. **A**, quantitative analysis of *Meis1* and *Pbx1* mRNA levels after Northern blot analyses. Northern blots were performed three times with different RNA preparations, and these Northern blots were quantitated by phosphorimager to generate the data in **A**. **B**, expression of polycomb group genes *Cdx1* (~1.7 kb) and *Bmi1* (~3.6 kb) in various ES cell lines. Only one Northern blot is shown in **B**, but Northern blots were performed three times with different RNA preparations with similar results. S.D. was calculated using GraphPad Prism 4.0. *, significant ($p < 0.05$).

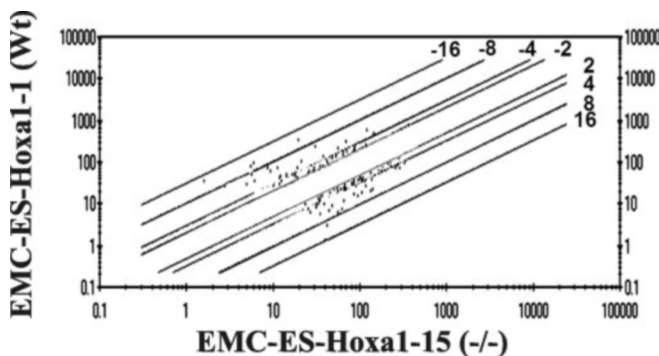


FIG. 4. **Logarithmic scatter plot of microchip array results showing the expression intensities of individual genes in EMC-ES-Hoxa1-1 versus EMC-ES-Hoxa1-15 cell lines.** Dots represent genes that are differentially expressed in EMC-ES-Hoxa1-1^{+/+} versus EMC-ES-Hoxa1-15^{-/-} ES cell lines. For clarity, only dots representing genes with a fold change of 2 or greater are shown. Intensity levels of genes in EMC-ES-Hoxa1-1 cells are shown on the ordinate, and intensity levels of genes in EMC-ES-Hoxa1-15 cells are shown on the abscissa. *Diagonals* represent fold difference (negative numbers, higher levels in EMC-ES-Hoxa1-1; positive numbers, higher expression levels in EMC-ES-Hoxa1-15).

secreted protein implicated in the regulation of neural development in vertebrates (55–59). Compared with untreated controls, *WISP1* mRNA levels were increased by 11-fold after 72 h

and by 17-fold after 96 h of RA in Hoxa1-1^{+/+} and Hoxa1-3^{+/+} cells (Fig. 5A, lanes 13, 14 and 20, 21, and B, samples 6, 7 and 13, 14). In Hoxa1-15^{-/-} ES cells, *WISP1* mRNA levels increased only 2- and 4-fold at 72 and 96 h after RA addition, respectively (quantitation in Fig. 5B).

From our microarray analyses, we identified additional genes that are involved in the development of the nervous system. One of these genes is *Wnt3a*, which is highly expressed in the developing nervous system (60, 61) and whose gene product has been shown to be essential for neural tube patterning (62), for the promotion of proliferation of dopaminergic neuronal precursor cells (59), and for induction of neuronal differentiation of ES cells *in vitro* (63). The differential expression of *Wnt3a* in wild-type versus Hoxa1 knockout cells was demonstrated by RT-PCR (Fig. 5D).

An additional gene that exhibited reduced expression in Hoxa1^{-/-} ES cells is *RhoB*, a member of the rho family of GTP-binding proteins. *RhoB* is expressed in migrating neural crest cells during the early stages of hindbrain development (64, 65). RT-PCR demonstrated that wild-type ES cells express higher *RhoB* mRNA levels than Hoxa1 knockout ES cells after RA treatment for 24 and 48 h (Fig. 5D).

RT-PCR also demonstrated the differential expression of the cyclin-dependent kinase inhibitor 2B (*Cdkn2b*), which is frequently mutated in tumors of the central nervous system (for a

transcript sizes are: *Hoxa1*, 2.2 kb; *Hoxa2*, 1.7 kb; *Hoxa3*, 1.9 kb; *Hoxa5*, 1.9 kb; *Hoxb1*, 1.8 kb. Only one of the Northern blots is shown in **A** but the data in **B** are from three repetitions with different RNA preparations. S.D. was calculated using the software program GraphPad Prism 4.0 as described under “Materials and Methods.” Hoxa1^{-/-} and Hoxa1^{-/-} ES cells were compared with Wt at each time point for statistical significance. *, significant ($p < 0.05$). **, very significant ($p < 0.001$).

TABLE I

Microarray analysis of genes decreased in EMC-ES-Hoxa1-15^{-/-} cells relative to Wt after RA treatment for 48 h

Gene name	Fold change ^a
Periostin (<i>Postn</i> , previously named <i>Osf2</i>)	12.68
α -1 Type 8 procollagen (<i>Col8a1</i>)	10.80
Insulin-like growth factor-binding protein 3 (<i>IGFBP3</i>)	8.90
Small inducible cytokine A2	5.63
α -1 Type I procollagen (<i>Col1a1</i>)	4.85
Aortic carboxypeptidase-like protein (<i>ACLP</i>)	4.29
Short-chain dehydrogenase 2 (<i>CRAD2</i>)	3.90
Mouse plasminogen activator inhibitor (<i>PAI-1</i>)	3.69
Homeobox protein PSX	3.41
Connective tissue growth factor-related protein (<i>Wisp1</i>)	3.16
Neuronatin (<i>Nnat</i>)	3.06
Vascular smooth muscle α -actin	2.93
Wingless-related MMTV integration site 3A (<i>Wnt3a</i>)	2.82
Bone sialoprotein gene (<i>BSP</i>)	2.60
<i>RhoB</i>	2.57
Cell death activator CIDE-A (<i>Cide-a</i>)	2.45
Serum amyloid A3	2.40
CCAAT/enhancer-binding protein, β (<i>C/EBP</i>)	2.30
Brain-derived neurotrophic factor (<i>BDNF</i>)	2.30
Cyclin-dependent kinase inhibitor 2B (<i>Cdkn2b</i>)	2.27
Adenylate kinase 3 (<i>Ak3</i>)	2.22
Insulin-like growth factor 2 (<i>IGF2</i>)	2.09

^a Average of two experiments.

review see Ref. 66), in wild type *versus* Hoxa1-null ES cells (Fig. 5D).

The gene product of another putative Hoxa1 target (Table I), brain-derived neurotrophic factor (*BDNF*), has a role during embryogenesis in the survival of the neurons of vertebrate sympathetic and sensory ganglia (67). More recently, *BDNF* has been shown to act as a neuromodulator of the central rhythmic respiratory circuits (68, 69).

These results suggest that Hoxa1 may be involved in the RA-induced neuronal differentiation of ES cells *in vitro*. Our data also provide information about important components of this pathway that act downstream of Hoxa1.

Putative Hoxa1 Targets Involved in Bone Formation—Among the genes identified by microarray analyses that exhibited reduced expression in EMC-ES-Hoxa1-15^{-/-} cells relative to Wt ES cells were genes that are involved in the differentiation of connective tissue or in bone formation. These genes are periostin (*Postn*, previously named osteoblast-specific factor 2 or *Osf2*) (70–72), the procollagen A1 α 1 gene (*Col1a1*) (73–76), and the bone sialoprotein gene (*BSP*) (77, 78). The mRNA levels of *Col1a1* and *Postn* were examined by Northern blot analyses in the three Hoxa1 ES cell lines and in AB1 ES cells in order to validate our microarray results. RA increased the *Col1a1* mRNA levels in Hoxa1-1 ES cells by about 16-fold at 72 h and 23-fold at 96 h, with respect to untreated Wt cells. In Hoxa1^{-/-} cells only 3- and 4-fold increases in *Col1a1* mRNA levels were observed after RA treatment for 72 and 96 h, respectively (Fig. 5A, lanes 27, 28, quantitation in Fig. 5B). Thus, the differences in *Col1a1* mRNA levels between Hoxa1-1^{+/+} and Hoxa1-15^{-/-} ES cells were about 5-fold at 72 h and about 6-fold at 96 h after RA addition, which correlates with the results obtained from the microarray assays (see Table I).

Northern blot analyses also demonstrated that the expression of *Postn*, whose gene product is a secreted protein involved in cell adhesion and differentiation of osteoblasts (70–72), was increased in Wt and in Hoxa1 heterozygous ES cells 10- and 16-fold by RA at 72 and 96 h, respectively (Fig. 5A, lanes 13, 14 and lanes 20, 21, quantitation in Fig. 5B). In contrast, only 4–6-fold increases in *Postn* mRNA levels were observed after RA treatment in Hoxa1^{-/-} cells (Fig. 5A, lanes 27, 28, quantitation in Fig. 5B). These results implicate the activity of Hoxa1

TABLE II

Microarray analysis of genes increased in EMC-ES-Hoxa1-15^{-/-} cells relative to Wt after RA treatment for 48 h

Gene name	Fold change ^a
SRY-box containing gene 17 (<i>Sox17</i>)	25.63
Laminin, α 1 (<i>LamA1</i>)	8.02
Fork head-related protein a2 (<i>Foxa2/HNF-3b</i>)	6.30
Proteoglycan core protein	5.70
Disabled 2 (<i>Dab2</i>)	5.50
Mus musculus LERK-4 (<i>Epl4</i>)	4.63
Histone H2A	4.40
Retinoic acid-inducible E3 (<i>RAE3</i>)	4.34
Kruppel-like factor 5 (<i>Klf5</i>)	3.53
Fork head-related protein q1 (<i>Foxq1</i>)	3.36
Homeobox C9 (<i>Hoxc9</i>)	3.31
Platelet-derived growth factor receptor, α (<i>PDGFRα</i>)	2.93
<i>M-Ras</i>	2.79
Procollagen, type IV, α 1 (<i>Col4a1</i>)	2.67
Laminin γ 1	2.60
SRY-box-containing gene 7 (<i>Sox7</i>)	2.36
Gastrulation brain homeobox 2 (<i>Gbx2</i>)	2.36
Ephrin A2 (<i>EphA2</i>)	2.33
Growth factor receptor-bound protein 10 (<i>Grb10</i>)	2.32
Mouse carbonic anhydrase II (<i>CAII</i>)	2.22
Laminin β 1 C terminus (<i>LamB1</i>)	2.12
Cathepsin C	2.10
Lactate dehydrogenase A-4 (<i>LDH-A</i>)	2.05

^a Average of two experiments.

during the initial steps in the RA-induced differentiation of ES cells toward a chondrogenic and/or osteogenic pathway.

Putative Target Genes Inhibited by Hoxa1—Microarray analyses identified a number of genes that exhibited increased expression in Hoxa1-15^{-/-} ES cells as compared with Hoxa1-1^{+/+} cells, which suggests that these genes are normally repressed by the Hoxa1 protein. From this group of genes (see Table II), *Hoxc9* (79–81) and *RAE3* (82) were selected for further examination by Northern blot analyses. *Hoxc9* was expressed at low levels in both Hoxa1-1^{+/+} and Hoxa1-3^{+/+} ES cell lines (Fig. 5A, lanes 8–14 and 15–21), and after treatment with RA for 72 or 96 h, the mRNA levels increased by about 2–3-fold (Fig. 5A, lanes 13, 14 and lanes 20, 21, quantitation in Fig. 5B). A significant increase ($p < 0.05$) in *Hoxc9* mRNA levels was observed in Hoxa1-15^{-/-} ES cells treated with RA for 48 to 96 h (about 4–5-fold with respect to Hoxa1-1-untreated controls) (Fig. 5A, compare lanes 26–28 *versus* lanes 12–14; also see quantitation in Fig. 5B).

The *RAE3* gene was found to be directly activated by RA in cells of the hematopoietic lineage (82). Our microarray assays showed that this gene was up-regulated in Hoxa1-15^{-/-} ES cells compared with Hoxa1-1^{+/+} cells. *RAE3* was expressed at very low levels in AB1, Hoxa1-1^{+/+}, and Hoxa1-3^{+/+} ES cells (Fig. 5A, lanes 1–21). No increase in *RAE3* mRNA levels by retinoic acid treatment was observed in these cell lines. In contrast, *RAE3* mRNA was highly expressed in Hoxa1-15^{-/-} cells even before RA treatment (about 6-fold higher than in the Hoxa1-1-untreated control cells) (Fig. 5A, lanes 22, 23 *versus* lanes 8, 9). At 48 h after the addition of RA, a significant increase of *RAE3* mRNA levels was observed in Hoxa1-15^{-/-} ES cells (more than 10-fold with respect to Hoxa1-1^{+/+} untreated controls) (quantitation in Fig. 5B). In summary, no effect of RA on *RAE3* mRNA expression was observed in Hoxa1-1^{+/+} or Hoxa1-3^{+/+} ES cell lines, but RA increased *RAE3* mRNA levels in Hoxa1-15^{-/-} cells.

The mRNA levels of additional genes that exhibited increased expression in Hoxa1^{-/-} ES cells were examined by Northern blot analyses (e.g. *Col4a1*, *Foxa2/Hnf3 β* , *Foxq1*, *LamA1*, and *Klf5*; Fig. 5C) or by RT-PCR (*Gbx2*, *M-Ras*, and *Sox17*; Fig. 5D). Interestingly, *Sox17*, *Col4a1*, *Foxa2*, and *LamA1* are known markers of endodermal differentiation,

which suggests that in the absence of Hoxa1, ES cells commit to an endodermal lineage after treatment with RA.

Hoxa1 Plays a Role in the Antiproliferative Effect of RA on ES Cells—To continue with the characterization of the Hoxa1 knockout ES cells, we compared their proliferative capacity in the presence of RA treatment to that of Hoxa1 wild-type or Hoxa1 heterozygous ES cells. Although it has been reported that Hoxa1 is required for correct neuronal differentiation in mice (83) and that the overexpression of *Hoxa1* in zebrafish results in alterations similar to those observed after RA treatment of fertilized eggs (84), whether Hoxa1 plays a role during the RA-induced ES cell differentiation and/or inhibition of cell growth has not been determined. For the proliferation analyses, EMC-ES-Hoxa1-1, -3, and -15 cells were treated for 96 h with various concentrations of RA (1×10^{-9} , 1×10^{-8} , 1×10^{-7} , 5×10^{-7} , and 1×10^{-6} M) in triplicate wells and counted as described under "Materials and Methods." These assays demonstrated that EMC-ES-Hoxa1-15 (Hoxa1^{-/-}) cells were more resistant to the growth inhibitory effects of RA than EMC-ES-Hoxa1-1 (Hoxa1^{+/+}) and EMC-ES-Hoxa1-3 cells (Hoxa1^{+/+}) (Fig. 6). These results suggest that the Hoxa1 protein plays a role in the RA-induced inhibition of proliferation in ES cells.

Oct 3/4 and Bmp4 mRNA Levels Are Higher in RA-treated Hoxa1^{-/-} ES Cells—Because Hoxa1-15^{-/-} ES cells showed a differential proliferative response to RA with respect to both wild-type and heterozygous ES cells, we next sought to investigate this differential response at the molecular level by assessing the effects of RA on the expression of the ES stem cell markers *Fgf4* (85–87) and *Oct3/4* (88, 89) by Northern blot analysis. We also examined the expression levels of *Bmp4* because its expression has been shown to decrease during the transition from ES cells to epiblast (90), and its gene product has been implicated in the self-renewal of ES cells (91). RA inhibited the expression of *Fgf4* by more than 2-fold in all three cell lines in a time-dependent manner (Fig. 7A), but no differences in *Fgf4* mRNA levels were observed by quantitative analysis among the different ES cell lines, with the exception of a decrease in *Fgf4* mRNA levels (<2-fold) at 96 h plus RA in both Hoxa1-3^{+/+} and Hoxa1-15^{-/-} cells, as compared with wild-type ES cells (Fig. 7D, samples 12, 18 versus sample 6). However, 3–4-fold higher *Oct3/4* mRNA levels were observed in the ES-Hoxa1-15^{-/-} cells compared with Hoxa1-1^{+/+} or Hoxa1-3^{+/+} cell lines after RA treatment for 48 to 96 h (Fig. 7B, lanes 5–7 versus 19–21). The differential down-regulation of *Oct3/4* mRNA expression in response to RA in Hoxa1-15 cells versus Hoxa1-1 or Hoxa1-3 cell lines was demonstrated by quantitative analysis from three different RNA preparations (Fig. 7D, samples 5–7 and samples 12–14 versus samples 19–21).

Higher levels of *Bmp4* mRNA expression were also detected in Hoxa1-15^{-/-} cells than in Hoxa1-1^{+/+} ES cells (Fig. 7C). The levels of *Bmp4* mRNA were 6-fold higher in Hoxa1-null ES cells than in Wt ES cells (Fig. 7D, samples 1–3 versus samples 7–9). Moreover, in both cell lines, RA induced a time-dependent down-regulation of *Bmp4* mRNA. Taken together, these results suggest that the Hoxa1 protein may play a role in the RA-induced differentiation of ES cells toward specific cell lineages.

Expression of the ES Stem Cell Markers *Fgf4*, *Oct3/4*, and *Rex1* in the ES Cell Lines Cultured with or without LIF—It has been reported by Niwa *et al.* (92) that a specific level of *Oct3/4* is required to maintain pluripotency in ES cells. A decrease in *Oct3/4* expression leads to dedifferentiation to trophectoderm, whereas a less than 2-fold increase promotes differentiation into primitive endoderm and mesoderm. Another factor which modulates ES cell renewal and differentiation is the LIF (93).

In fact, when cultured in the absence of LIF, murine ES cells differentiate and give rise to a variety of cell types, including cells of the hematopoietic, cardiac, and neuronal cell lineages (94, 95). In addition, our laboratory has demonstrated that RA can override the self-renewing effects of LIF on ES cells, partially by down-regulating the expression of the LIF receptor (LIFR) (34, 96). Because higher levels of *Oct3/4* mRNA expression after RA treatment were observed in Hoxa1^{-/-} ES cells than in the other ES cell lines, we hypothesized that Hoxa1 may be involved in the RA-induced differentiation of ES cells grown in monolayer. In order to test this hypothesis, EMC-ES-Hoxa1-1^{+/+} and EMC-ES-Hoxa1-15^{-/-} cells were cultured in the presence or absence of LIF, and plus or minus RA for different periods of time. After harvesting and total RNA extraction, the expression of different ES markers was examined by Northern blot analysis.

We first examined the expression of the ES cell marker *Rex1* (Fig. 8A) (97), which we previously showed was transcriptionally inhibited by RA (98). Quantitative analysis demonstrated that in the presence of LIF and after 12 and 24 h with RA, Hoxa1-15^{-/-} ES cells expressed between 1.5- and 2-fold higher levels of *Rex1* mRNA than Hoxa1-1^{+/+} ES cells (Fig. 8B, samples 4, 5 versus samples 16, 17). No significant differences among the two cell lines after RA treatment were observed in the absence of LIF (Fig. 8B, samples 10–12 versus samples 22–24).

We next examined *Fgf4*, and we observed that LIF removal resulted in the down-regulation of *Fgf4* mRNA expression in both ES cell lines at 72 h (Fig. 8A, lane 3 versus lane 9, and lane 15 versus lane 21). Similarly, treatment with RA in the presence of LIF resulted in the down-regulation of *Fgf4* mRNA levels in both cell lines (Fig. 8A, lanes 1–3 versus lanes 4–6 and lanes 13–15 versus lanes 16–18). Quantitative analyses demonstrated that no further decrease in *Fgf4* mRNA was achieved when either wild-type or Hoxa1^{-/-} ES cells were cultured in the presence of RA and in the absence of LIF at early times (Fig. 8B, samples 4–6 versus samples 10–12). However, a 5-fold decrease in *Fgf4* mRNA levels was observed in Hoxa1-15^{-/-} ES cells at 72 h, as compared with RA treatment plus LIF (Fig. 8B, sample 18 versus sample 24).

LIF removal resulted in the down-regulation of *Oct3/4* mRNA levels in both Hoxa1 wild type and Hoxa1-null cells either with or without RA treatment (Fig. 8A, lanes 1–6 versus lanes 7–12 and lanes 13–18 versus lanes 19–24). In addition, higher levels of *Oct3/4* mRNA were observed in Hoxa1-15^{-/-} cells than in Hoxa1-1 (Wt) cells after RA treatment (Fig. 8A, lanes 4–6 versus lanes 16–18), LIF withdrawal only (Fig. 8A, lanes 7–9 versus 19–21), or RA treatment plus LIF removal (Fig. 8A, lanes 10–12 versus lanes 21–24). Thus, the Hoxa1-15^{-/-} ES cells cultured without LIF express higher levels of *Oct3/4* mRNA than Hoxa1-1 (Wt) cells that have been cultured in the absence of the cytokine.

Expression of *Fgf5*, *Gata4*, and *Dab2* in EMC-ES-Hoxa1 Cell Lines with or without LIF—The characterization of the Hoxa1^{-/-} ES cells by microarray analyses suggested that these cells preferentially differentiate along the endodermal pathway following treatment with RA. As the next step in the characterization of our Hoxa1^{-/-} ES cells, we sought to investigate whether the differentiation of Hoxa1-1^{+/+} versus Hoxa1-15^{-/-} ES cell lines gave rise to different cell lineages after RA treatment and/or LIF removal. First, the expression of a differentiation marker expressed in the primitive ectoderm of the early embryo but not in ES cells, *Fgf5* (99–102), was examined in Hoxa1-1^{+/+} versus Hoxa1-15^{-/-} ES cells cultured in the presence or absence of LIF, and with or without RA treatment (Fig. 8A). A 4-fold induction of *Fgf5* expression (Fig. 8B) was ob-

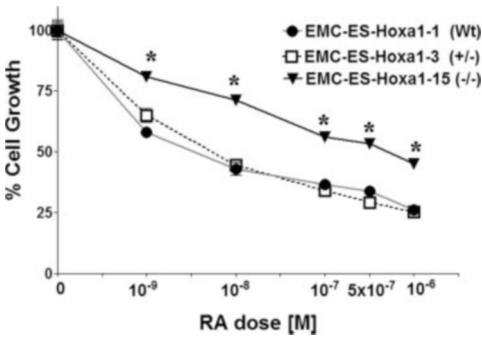
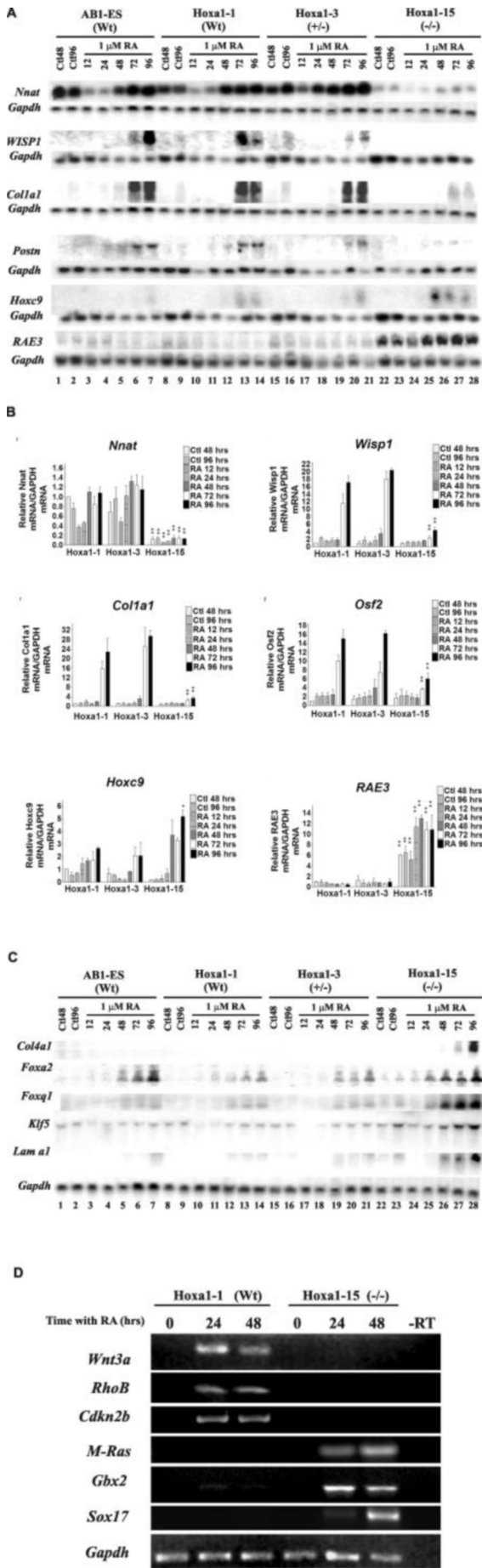
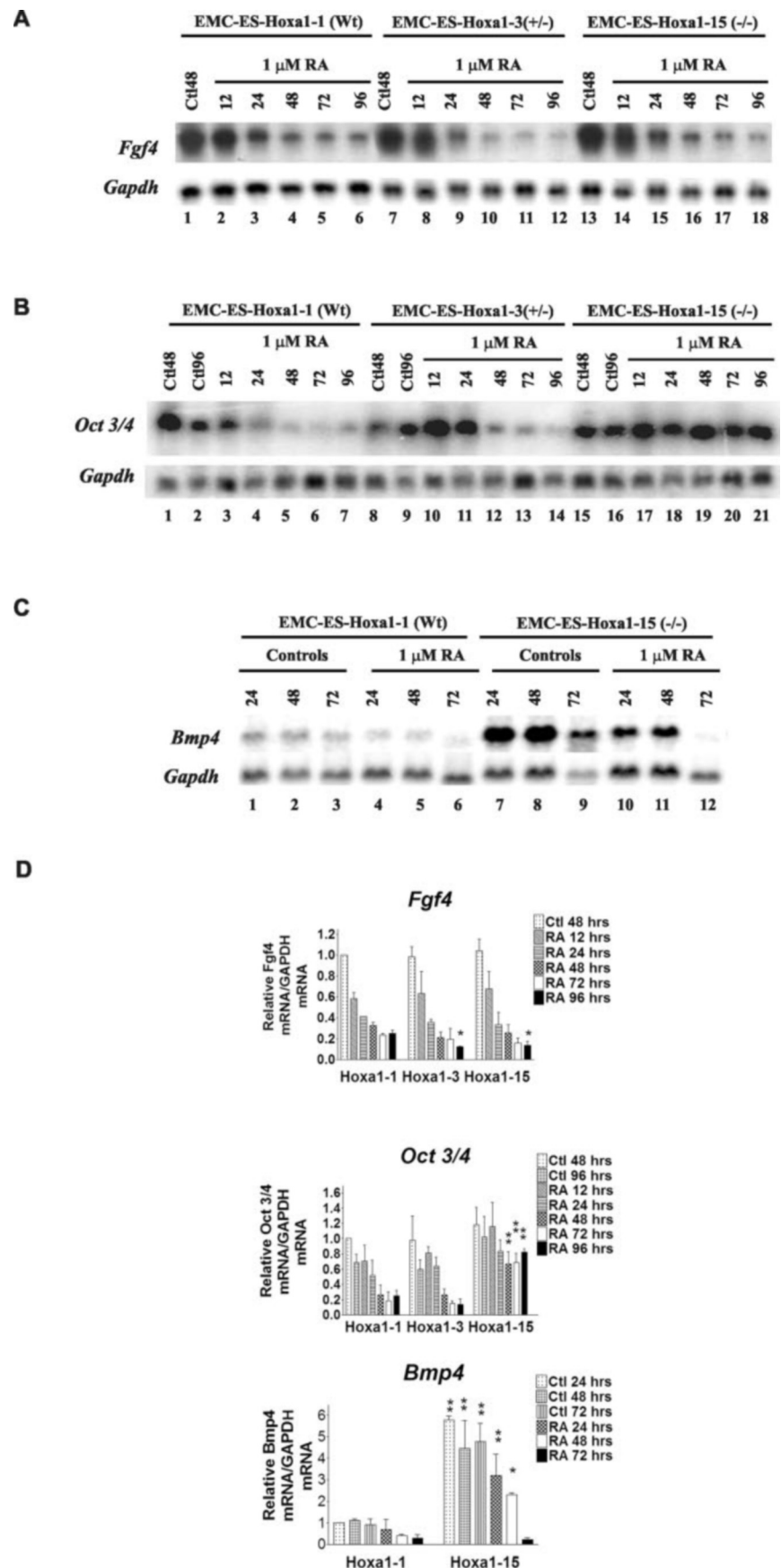


FIG. 6. Proliferation responses of ES cell lines to different concentrations of RA. Cells were plated in the presence of LIF on 12-well plates at a density of 1×10^4 cells/well. The day after plating, cells were treated with various doses of RA (1×10^{-9} , 1×10^{-8} , 1×10^{-7} , 5×10^{-7} , and 1×10^{-6} M) or with ethanol (vehicle) for 96 h. The results are plotted as the percentage of cell growth versus the dose of RA (M, molar). The statistical analysis was performed using the software program GraphPad Prism Version 4.0. Data points, means of triplicate samples; bars, S.D. In most cases, symbol points are larger than the S.D. Significance ($p < 0.05$) is shown by an asterisk (*).

served in Wt ES cells at 24 h after LIF removal and without RA treatment, whereas a 6-fold increase in *Fgf5* mRNA expression in Wt cells occurred at 72 h. In contrast, only a slight (< 2 -fold) induction of *Fgf5* mRNA was observed in Hoxa1-15^{-/-} cells at 24 and 72 h after LIF removal (Fig. 8A, lanes 8, 9 versus lanes 20, 21). In both cell lines, *Fgf5* mRNA expression was inhibited by RA. Thus, because the neuroectoderm arises from the primitive ectoderm (103) and in ES cells the sustained expression of *Fgf5* is an indicator of neuroectodermal differentiation (104), these results suggest that Hoxa1-null ES cells possess a reduced capacity to differentiate into neuroectoderm when cultured in the absence of LIF.

Because the ES-Hoxa1^{-/-} cells showed a higher level of Oct3/4 than wild-type cells after RA treatment and showed a decreased capacity to express the ectodermal marker *Fgf5*, it is possible that the Hoxa1-15^{-/-} ES cells preferentially differentiate along the endodermal or mesodermal pathways upon LIF removal. To test the first possibility, the expression of the early (extraembryonic) and late (embryonic) endodermal marker *Gata4* (38, 105, 106) was examined in Wt versus Hoxa1^{-/-} ES cells treated with RA or vehicle alone and in the presence or absence of LIF. Low levels of *Gata4* mRNA were detected in

Fig. 5. Expression of putative Hoxa1 target genes in EMC-ES-Hoxa1-1 versus EMC-ES-Hoxa1-15 ES cell lines. A, total RNA prepared from AB1 or EMC-ES-Hoxa1 cell lines treated with RA or ethanol vehicle in the presence of LIF were subjected to Northern blot analysis and probed with various potential Hoxa1 target genes shown in Tables I and II. AB1, a well characterized mouse ES cell line, was included for comparison to our C57Bl/6 ES cell lines. AB1, Hoxa1-1^{+/+}, and Hoxa1-3^{+/+} ES cell lines showed similar patterns of gene expression. *Col1a1*, ~4.7 kb; *Osf2*, ~3.1 kb; *Nnat*, ~1.2 kb; *Wisp1*, connective tissue growth factor-related protein (~5 kb); *RAE3*, retinoic acid-inducible E3 (~2.4 kb); *Hoxc9*, Homeobox C9 (~1.7 kb). One representative Northern blot is shown, although the experiments were performed three times with different RNA preparations with similar results. B, quantitation of the mRNA levels of putative Hoxa1 target by phosphorimager analysis from three independent experiments. Northern blots were normalized to *GAPDH* mRNA levels, and the data are shown as fold increase with the relative mRNA levels for the 48 h controls expressed as the unit. Quantitation results for the AB1 ES cell line are not shown. *, significant ($p < 0.05$). **, very significant ($p < 0.001$). C, additional Northern blot analyses of genes that exhibited an increased expression in Hoxa1-15^{-/-} ES cells by microarray analyses. *Col4a1*, procollagen type IV $\alpha 1$ (~7.6 kb); *Foxa2*, forkhead-related protein a2 (previously *HNF3B*; ~2kb); *Foxq1*, forkhead-related protein q1 (~3.4 kb); *Klf5*, Kruppel-like factor 5 (~1.7 kb); *LamA1*, ~9.5 kb. D, additional validation of the microarray results by RT-PCR in Hoxa1-1 (Wt) versus Hoxa1-15^{-/-} ES cells treated with RA for 0, 24, or 48 h. These assays were repeated with similar results obtained.



Hoxa1-1^{+/+} ES cells treated with vehicle only in the presence of LIF (Fig. 8A, lane 1 and B, sample 1). In contrast, *Gata4* expression levels were about 3-fold higher in Hoxa1-15^{-/-} ES

cells under the same conditions (Fig. 8A, lane 13, and B, sample 13). Treatment of Hoxa1-1^{+/+} ES cells with RA for 72 h increased the expression of *Gata4* mRNA by 2-fold in the pres-

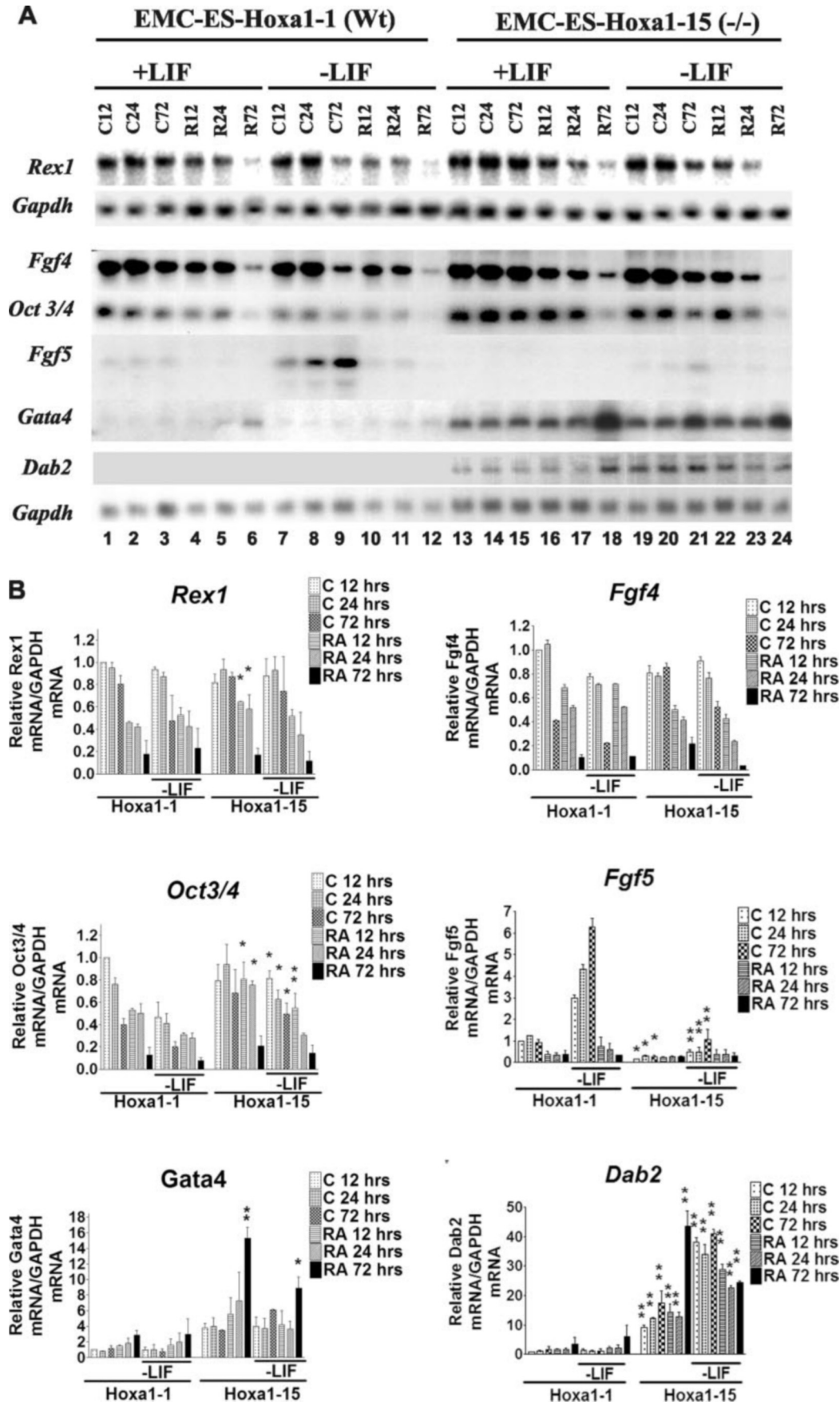


FIG. 8. Expression of ES differentiation markers in EMC-ES-Hoxa1 cell lines cultured \pm LIF and \pm RA. A, Northern blot analysis of lineage differentiation markers in ES cell lines cultured in the presence or absence of LIF, and plus or minus RA. B, quantitation of Northern blots for the markers shown in A after normalization to *Gapdh* mRNA levels. *Fgf4*, *Oct3/4*, and *Rex1* (~1.7 kb): ES cell markers. *Fgf5* (~2.5 kb): ectodermal marker. *Gata4* (~3.4 kb) and *Dab2* (~3.3 kb): endodermal markers. Northern blots were performed using three different RNA preparations with similar results. Quantitations were performed with GraphPad Prism 4.0. *, significant ($p < 0.05$). **, very significant ($p < 0.001$).

ence of LIF (Fig. 8A, lane 6). Remarkably, a 14-fold increase in *Gata4* mRNA expression was observed in Hoxa1-15^{-/-} ES cells after treatment with RA for 72 h as compared with RA-treated Hoxa1-1^{+/+} ES cells (Fig. 8A, lane 18 versus lane 6; Fig. 8B, sample 18 versus sample 6). Induction by RA of *Gata4* mRNA expression was observed in the absence of LIF in both ES cell lines but in general, these levels were lower than in the presence of LIF (Fig. 8B, sample 6 versus sample 12, and sample 18 versus sample 24).

The gene encoding the signal transduction adapter protein Disabled-2 (*Dab2*) is another marker for early and late endodermal differentiation and its expression has been shown to be regulated by *Gata4* and *Gata6* (107). Because *Gata4* expression was up-regulated in Hoxa1-15^{-/-} versus Hoxa1-1^{+/+} ES cell lines, we examined the expression of *Dab2* in these ES cell lines cultured with or without LIF and plus or minus RA. As seen in Fig. 8A (lanes 1–12) little to no *Dab2* mRNA expression was detected in Wt ES cells. However, about 10–20-fold higher *Dab2* mRNA levels were detected in Hoxa1-15^{-/-} ES cells in the absence of RA-treatment plus LIF (Fig. 8A, lanes 1–3 versus lanes 13–15; 8B, samples 1–3 versus samples 13–15). The greater expression of *Dab2* in Hoxa1-15^{-/-} cells versus Hoxa1-1 (Wt) cells was further enhanced (up to 50-fold) by the absence of LIF (Fig. 8A, lanes 7–9 versus lanes 19–21). LIF removal from Hoxa1-null cells resulted in an increase in *Dab2* mRNA levels (Fig. 8B, samples 13–15 versus samples 19–21).

Taken together, these results suggest that EMC-ES-Hoxa1-1 (Wt) ES cells are able to initiate differentiation into neuroectoderm (increased *Fgf5*) when cultured in monolayer without LIF, whereas EMC-ES-Hoxa1-15 (Hoxa1^{-/-}) ES cells may preferentially initiate differentiation along the endodermal pathway upon LIF removal and/or RA treatment, as suggested by their high expression of *Gata4* and *Dab2*, as well as *Col4a1*, *Foxa2*, *LamA1*, and *Sox17* (see Fig. 5, C and D).

The generation and in-depth characterization of the Hoxa1 knockout ES cells described in this work may be of assistance to researchers in the elucidation of the Hoxa1 signaling pathway. These data may also lead to a greater understanding of the molecular origins of the defects observed in the Hoxa1 knockout animals reported in the literature.

DISCUSSION

Low levels of Hoxa1 expression in Hoxa1^{+/-} Cells Are Sufficient for a Normal Activation of the Hoxa1 Pathway—Although the levels of Hoxa1 mRNA expression after RA treatment in Hoxa1 heterozygous (+/-) ES cells were between 3- and 4-fold lower than in Hoxa1-1 (Wt) ES cells under the same conditions, in general, these two cell lines displayed similar gene expression profiles as demonstrated by Northern blot assays. A likely explanation is that the low levels of Hoxa1 in Hoxa1-3^{+/-} ES cells that are induced by RA (see Fig. 1D, Hoxa1-1^{+/+} versus Hoxa1-3^{+/-}; also see Fig. 2B) are sufficient to activate a normal Hoxa1 genetic pathway. This hypothesis is in agreement with the normal phenotype observed in the Hoxa1 heterozygous mice (19, 20).

Hoxa1^{-/-} ES Cells Express Lower Levels of Genes Involved in the Chondrocyte and/or Osteoblast Differentiation of ES Cells—Microarray and Northern blot analyses suggest a role for Hoxa1 in the expression of various genes involved in the osteogenic differentiation of ES cells upon RA treatment. These genes include *Col1a1*, *Postn/Osf2*, and *BSP*. Our analyses show that Hoxa1 may be required for the RA-induced expression of *Postn*, a specific marker for preosteoblasts that may also play an important role in the early stages of fracture healing (71, 108–111). This effect of Hoxa1 on *Postn* expression seems to be contrary to that of Hoxa2, which has been shown to repress the expression of another osteogenic marker, *Runx2* (also called

Cbfa1) in the second branchial arch of mouse embryos (112). Although the expression of *Postn* has not been examined in Hoxa1^{-/-} mice, the smaller size of second branchial arch-derived structures in these mutant embryos (19, 20) suggests that in the domain of Hoxa1 action, the Hoxa1 protein may affect the progress from condensation to initiation of chondrogenesis, which is the size determining step in the initiation of skeletogenesis (113). Because chondrocytes and osteoblasts arise from mesodermal precursors (95), our results suggest that Hoxa1 is involved in some aspects of the mesodermal differentiation of ES cells *in vitro*.

Hoxa1^{-/-} ES Cells Express Lower Levels of Genes Involved in the Development of the Nervous System—Our microarray analyses show that Wt and Hoxa1-15^{-/-} cells differentially express a number of genes whose pattern of expression or known function implies a role in brain development (*Nnat*, *Wnt3a*, *BDNF*, *RhoB*, *Gbx2*, *LamA1*, and perhaps *WISP1*). Hoxa1 homozygous mutant embryos have multiple developmental defects but they die at birth because of their failure to initiate respiration (19, 20). Although it has been reported that the physiological origin of this defect is the presence of an ectopic neuronal circuit affecting the respiration-controlling region in the brain of the mutant embryos (114), little is known about the molecular causes that lead to the alteration of normal neurogenesis in this region. The identification by microarray analyses of the putative Hoxa1 target gene, *BDNF*, whose gene product acts as a neuromodulator of the central rhythmic respiratory circuits (69), may provide an additional clue about the molecular events involved in the perinatal lethality of the Hoxa1 homozygous mice. Analysis of the expression of the additional brain-related Hoxa1 putative targets in this region of the brain in mutant embryos may help to elucidate the cascade of genetic events that induces the formation of novel neuronal circuits.

Hoxa1^{-/-} ES Cells Are Less Able to Differentiate than Wt ES Cells upon RA Treatment or LIF Withdrawal—ES cells that have been induced to differentiate by either LIF removal or RA treatment give rise to cell populations with diverse lineages (95). Our results suggest that Wt and Hoxa1^{-/-} ES cells grown in adherent monolayer culture respond differentially to RA treatment or LIF removal. Our proliferation analyses, together with our molecular studies involving RA treatment and/or LIF removal, suggest that more Hoxa1^{-/-} ES cells remain undifferentiated than Wt cells under these conditions. However, an important difference between these two ES cell lines exists: whereas Wt cells tend to differentiate toward the neuroectodermal pathway in the absence of LIF, Hoxa1^{-/-} ES cells show a decreased ability to differentiate along this lineage since they express very low levels of *Fgf5* after LIF removal. Furthermore, our microarray assays, together with Northern blot and RT-PCR analyses, indicate that Hoxa1^{-/-} ES cells express higher levels of a number of known endodermal markers (*Sox17*, *Gata4*, *Dab2*, and *LamA1* among others) than Wt cells, which suggests that Hoxa1-15^{-/-} ES cells, cultured under differentiating conditions, most likely follow an endodermal pathway. Interestingly, some of the genes that exhibited increased expression in Hoxa1-15^{-/-} ES cells, as shown by Northern blot assays and microarray analyses, have also been identified as genes positively regulated by RAR β in F9 teratocarcinoma stem cells by this laboratory (e.g. *Dab2*, *PDGFR α* , and *Gata* family members) (41). Because we have genetic evidence that an increase in *Hoxa1* gene expression by RA occurs through the action of the RAR γ nuclear receptor (17, 115), these observations suggest opposing roles for RAR β and RAR γ during the differentiation of stem cells into endodermal precursors. It would be of interest to examine this further by using specific

RAR β agonists to explore the RAR β signaling pathway in Hoxa1-15^{-/-} ES cells.

Hoxa1^{-/-} Cells Express Higher Bmp4 mRNA Levels than Wt ES Cells—The observation that *Bmp4* is a target of Gata4 and Gata6 (116) may explain the high levels of *Bmp4* mRNA observed in the Hoxa1^{-/-} ES cells. In addition, the high *Bmp4* mRNA levels observed in Hoxa1^{-/-} ES cells may result from the greater number of ES cells that remain undifferentiated after RA treatment in Hoxa1^{-/-} versus Wt cells. The observation that *Bmp4* mRNA levels are higher in cells that do not express the Hoxa1 protein is in accord with previous results by this laboratory, which showed that overexpression of Hoxa1 in mouse F9 cells resulted in the specific down-regulation of a group of genes, including *Bmp4* (28), although to date is not known whether the *Bmp4* gene is directly regulated by Hoxa1. Although Hoxa1^{-/-} ES cells express higher levels of *Bmp4* mRNA than Wt cells, our microarray assays did not identify any of the BMP4 mesodermal targets (e.g. *Msx-1*, *Msx-2*, and *Id* genes) (117) as differentially expressed among Hoxa1-1^{+/+} and Hoxa1-15^{-/-} ES cells. We speculate that the reason for this is that endodermal markers expressed at high levels repress the Bmp4 signaling pathway involved in mesodermal differentiation.

Neuroectodermal versus Endodermal Differentiation in Hoxa1^{-/-} ES Cells—Taken together, our data demonstrate the generation and detailed characterization of Hoxa1-null ES cells that may be of further utility in the study of the Hoxa1 signaling pathway *in vitro*. Our data also suggest that Hoxa1 may regulate the neuroectodermal and mesodermal differentiation of ES cells and that these actions of the Hoxa1 protein may be exerted, at least in part, by repression of the expression of important regulators of endodermal differentiation (see model in Fig. 9). We speculate that in the absence of Hoxa1, the increased expression of endodermal genes by RA (e.g. *Gata4*) (38) would lead to the repression by their gene products of neuroectodermal and mesodermal markers. The model in Fig. 9 may also explain why *Fgf5* fails to be up-regulated in vehicle-treated Hoxa1-15^{-/-} cells after LIF withdrawal: low retinoid levels from the serum in the culture medium may be responsible for the activation of endodermal-specific gene expression (e.g. *Gata4*, *Dab2*, *Sox17*). The activation of endodermal gene expression, which in wild-type cells would normally be repressed by the low Hoxa1 levels induced by serum retinoids, would result in a decrease of *Fgf5* mRNA levels and repression of neuroectodermal differentiation in Hoxa1-null cells.

This hypothesis concerning the repression of neuroectodermal differentiation in ES cells by endodermal proteins is supported by the observation that the forced expression of the endodermal gene *Sox17* in ectodermal animal caps of *Xenopus* embryos resulted in the endodermal differentiation of this structure (118). In addition, two endodermal factors, *Dab2* and *Sox17*, can directly inhibit the Wnt signaling pathway, which has been shown to play important roles during the development of the vertebrate nervous system (59, 61, 62, 119–121) and during the neural differentiation of ES cells *in vitro* (63). Because our microarray analyses (see Table I and Fig. 5) indicate that two members of the Wnt signaling pathway, *Wnt3a* and *WISP1*, exhibit decreased mRNA expression levels in the Hoxa1^{-/-} ES cells, high expression of endodermal markers may interfere with the activation of Wnt signaling and perhaps with the ability of these cells to produce neuroectodermal progenitors upon RA treatment, which is in accord with our proposed model for the action of Hoxa1 during ES differentiation.

Although the expression of *Hoxa1* comprises a broad ectodermal and mesodermal domain in the early embryo (15, 122, 123), the targeted inactivation of both *Hoxa1* alleles affects only

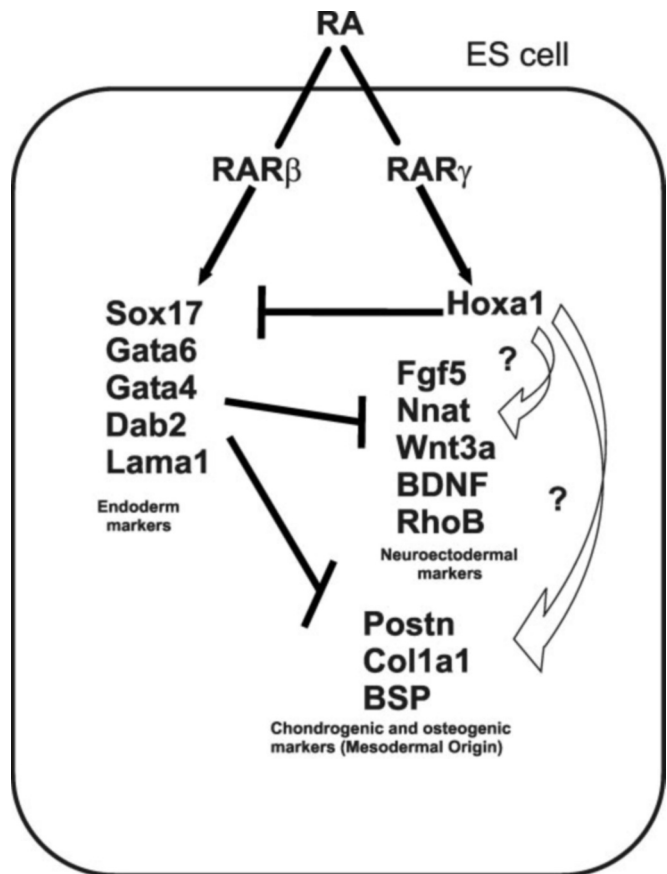


FIG. 9. Proposed model for the regulation of ectodermal and mesodermal versus endodermal markers by different signaling pathways in ES cells. In this model, activation of Hoxa1 by RA, acting through RAR γ , would repress the RAR β -induced endodermal differentiation of ES cells and promote or maintain the RA-induced differentiation of ES cells toward a neuroectodermal and/or mesodermal lineage. Hoxa1 would act as a repressor of RA-responsive endodermal genes (*Sox17*, *Gata4*, *Dab2*, and *Lama1*, among others). In the absence of Hoxa1, the protein products of these RA-induced endodermal genes would act as repressors of neuroectodermal and mesodermal ES differentiation. The question marks in the open arrows indicate that it is not yet known whether the genes identified by the microarray analyses are direct targets of Hoxa1. Thus, this model suggests a role for Hoxa1 as a positive regulator of neuroectodermal and/or mesodermal differentiation of ES cells and a repressor of RA-induced endodermal differentiation of ES cells *in vitro*.

anterior structures. An explanation for this limited effect relies upon the concept of posterior dominance, which postulates that the main site of individual *Hox* gene action is in the most anterior subdomain of their expression (5, 124, 125). Consistent with this, the proposed action of Hoxa1 as a repressor of embryonic endoderm formation and a stimulator of neuroectodermal and mesodermal differentiation would take place only at its most anterior subdomain of expression: the prospective rhombomere 4. An alteration in the balance between neuroectodermal/mesodermal and endodermal commitment in this part of the embryo would then result in the rhombomere mis-specifications observed in the Hoxa1 mutant mice (19–22).

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