CYP26A1 knockout embryonic stem cells exhibit reduced differentiation and growth arrest in response to retinoic acid

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Abstract

CYP26A1, a cytochrome P450 enzyme, metabolizes all-trans-retinoic acid (RA) into polar metabolites, e.g. 4-oxo-RA and 4-OH-RA. To determine if altering RA metabolism affects embryonic stem (ES) cell differentiation, we disrupted both alleles of Cyp26a1 by homologous recombination. CYP26a1−/− ES cells had a 11.0±3.2-fold higher intracellular RA concentration than Wt ES cells after RA treatment for 48 h. RA-treated CYP26A1−/− ES cells exhibited 2–3 fold higher mRNA levels of Hoxa1, a primary RA target gene, than Wt ES cells. Despite increased intracellular RA levels, CYP26a1−/− ES cells were more resistant than Wt ES cells to RA-induced proliferation arrest. Transcripts for parietal endodermal differentiation markers, including laminin, J6 (Hsp 47), and J31 (SPARC, osteonectin) were expressed at lower levels in RA-treated CYP26a1−/− ES cells, indicating that the lack of CYP26A1 activity inhibits RA-associated differentiation. Microarray analyses revealed that RA-treated CYP26A1−/− ES cells exhibited lower mRNA levels than Wt ES cells for genes involved in differentiation, particularly in neural (Epha4, Pmp22, Nrp1, Gap43, Ndn) and smooth muscle differentiation (Madh3, Nrp1, Tagln Calponin, Caldesmon1). In contrast, genes involved in the stress response (e.g. Tlr2, Stk2, Fcgr2b, Bnip3, Pdk1) were expressed at higher levels in CYP26A1−/− than in Wt ES cells without RA. Collectively, our results show that CYP26A1 activity regulates intracellular RA levels, cell proliferation, transcriptional regulation of primary RA target genes, and ES cell differentiation to parietal endoderm.

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Introduction

Retinoids, a group of natural and synthetic analogs of retinol (vitamin A), play essential roles during embryogenesis and have profound effects on biological processes, such as cell differentiation, cell proliferation, and cell death (Blomhoff and Blomhoff, 2006; Mark et al., 2006). The biological effects of retinoid signaling are primarily mediated through retinoic acid receptors (RARs) and retinoid X receptors (RXRs), members of the nuclear receptor superfamily that act as ligand-inducible transcription factors (Ahuja et al., 2003; Bastien and Rochette-Egly, 2004; Mark et al., 2006; Mongan and Gudas, 2007). All-trans-retinoic acid (RA), the most potent retinoid, binds and activates RARs, and 9-cis-RA binds and activates both RARs and RXRs (Bastien and Rochette-Egly, 2004). RXR–RAR heterodimers bind to the RA response elements (RAREs) located in the promoter or enhancer regions of target genes, and upon ligand binding, the receptors activate transcription of the target genes (Bastien and Rochette-Egly, 2004; Gillespie and Gudas, 2007a,b; Su and Gudas, in press).

The metabolism of retinol (ROL) into its bioactive derivatives, including RA, is mediated by different enzymes (Blomhoff and Blomhoff, 2006). Cytochrome P450 RA hydroxylase (CYP26), a family of cytochrome P450 enzymes which has three known members in mammals (CYP26A1, B1, and C1), is responsible for the oxidation of ROL and RA into more polar metabolites (Abu-Abed et al., 2002; Lane et al., 1999, 2008; MacLean et al., 2001; Reijntjes et al., 2003; Tahayato et al., 2003; Taimi et al., 2004; White et al., 1997). CYP26A1 was the first identified CYP26 member and was cloned in zebrafish (White et al., 1996), mouse (Genbank No. NM_007811), and human (Genbank No. NM_000783) (Fujii et al., 1997; Lee et al., 2007; Ray et al., 1997; White et al., 1997).
The Cyp26a1 gene is RA-inducible and has two functional RAREs in its promoter region (Loudig et al., 2005). The transcriptional activation of Cyp26a1 by RA acts to regulate RA levels (Abu-Abed et al., 1998; Liu and Gudas, 2005; White et al., 1997). Polar RA metabolites generated by CYP26A1 include 4-oxo-RA, 4-OH-RA, 18-OH-RA and other more polar products (Chithalen et al., 2002; White et al., 1997).

All three CYP26s are expressed during murine embryonic development in various tissues, with some spatial and temporal differences. Cyp26a1 is expressed at the anterior end of the embryo, and also in the tail bud and hindgut at the posterior end of the embryo (Abu-Abed et al., 2002; de Roos et al., 1999; MacLean et al., 2001; Reijntjes et al., 2004; Swindell et al., 1999). Differential expression is seen in the branchial arches, with Cyp26a1 mainly expressed in neural crest-derived mesenchyme, Cyp26b1 in specific ectodermal and endodermal areas (MacLean et al., 2007), and Cyp26c1 in the rostral portion (Abu-Abed et al., 2002; MacLean et al., 2001; Reijntjes et al., 2003; Tahayato et al., 2003). All three Cyp26 transcripts have been detected in the hindbrain (MacLean et al., 2001; Tahayato et al., 2003). Cyp26c1 is also expressed in the inner ear and tooth buds of the mouse (Tahayato et al., 2003). Since the expression patterns of the three Cyp26 genes are generally non-overlapping, each enzyme most likely has an individual role in RA metabolism during embryogenesis.

During embryonic development, precise levels of retinoids must be generated in different developing structures at different times. Inadequate levels of retinoids during embryonic development result in a phenotype termed vitamin A deficiency syndrome, which includes defects in the heart, brain, urogenital and respiratory systems, and in the development of the skeleton and limbs (Clagett-Dame and DeLuca, 2002; Means and Gudas, 1995; Zile, 2001). However, excess RA during murine embryogenesis, as a result of the disruption of the Cyp26a1 gene, also causes many developmental defects, including caudal truncation, abnormal patterning of the hindbrain, posterior transformation of the cervical vertebrae, and defects in the urogenital system (Abu-Abed et al., 2001; Sakai et al., 2001). The major morphogenetic phenotypes resulting from the disruption of CYP26A1 point to the importance of precisely regulating retinoid levels during embryonic development for appropriate retinoid signaling.

P19 embryonal carcinoma (EC) cells that stably overexpress exogenously transfected Cyp26a1 were generated, and these studies provided some insights into how CYP26A1 activity affects cell differentiation (Sonneveld et al., 1999a). The P19 EC cells which overexpress Cyp26a1 differentiated into neurons with low RA (1 × 10⁻⁸ M) treatment, unlike Wt P19 EC cells, and inhibition of CYP26A1 by the cytochrome P450 inhibitor, liarozole, was sufficient to inhibit this differentiation. These data suggested that the increased expression of Cyp26a1 and the subsequent generation of a higher concentration of polar RA metabolites caused neuronal differentiation of P19 EC cells (Sonneveld et al., 1999a).

These data prompted us to examine the role of Cyp26a1 in the differentiation of murine embryonic stem (ES) cells. ES cells are capable of self-renewal and have the ability to differentiate into all cell types of the three germ layers. This unique characteristic makes ES cells an ideal cell culture model for investigating the mechanisms that control retinoid metabolism, retinoid signaling pathways, and cell differentiation in early embryonic development (Burdon et al., 2002; Gudas, 1994; Wobus and Boheler, 2005). Furthermore, the directed differentiation of ES cells in culture is extensively being studied and optimized for the use of ES cells as a cell transplant source (Wobus and Boheler, 2005). Many directed differentiation protocols use RA as a differentiating agent, so dissecting the molecular pathways activated during RA-induced cell differentiation is critical for understanding the molecular decisions that result in differentiation. Our hypothesis is that disruption of CYP26A1 activity would result in increased intracellular RA levels, increased sensitivity to RA-induced proliferation arrest, and increased transcription of RA-inducible genes. We generated ES cell lines with both alleles of the Cyp26a1 gene disrupted by homologous recombination to use as a model system in which to characterize in detail the effects of RA metabolism on ES cell proliferation, gene expression, and differentiation.

Materials and methods

Cell culture and chemicals

AB1 murine ES cells, cultured in monolayer on gelatin-coated culture dishes, were maintained as described in (Chen and Gudas, 1996), 1 × 10⁸ units/ml leukemia inhibitory factor (LIF) (Millipore-Chemicon LIF2010, Temecula, CA) was added prior to use. All-trans-retinoic acid (Sigma Chemicals Co., St. Louis, MO) and 4-oxo-RA (Hoffman-LaRoche, Nutley, NJ) were dissolved in 100% ethanol and diluted in ES medium to obtain final concentrations of 1 × 10⁻⁹ to 5 × 10⁻⁶ M. [³H]RA and [α-³²P]dCTP were obtained from DuPont NEN (Boston, MA). All experiments involving retinoids were performed in dim light.

For proliferation studies, ES cells were plated on 12-well plates at a density of 1 × 10³/well for the cell doubling experiments and 1 × 10⁴/well for the RA-dose response experiments. For the cell doubling experiments, cells were counted 96 h after plating using an electron particle counter (model: Coulter Z, Beckman Coulter, Inc., Fullerton, CA). For the RA-dose response study, various doses of RA (1 × 10⁻⁷ to 5 × 10⁻⁶ M) were added to the medium 24 h after plating. Cells were cultured in the presence of RA for either 6 h and replenished with ES medium without retinoids or for 72 h with a medium change after 48 h. Control cells were treated with 0.1% ethanol (vehicle). Cells were counted using an electron particle counter. Each experiment was performed in triplicate and the entire experiment was repeated three times.

Generation of CYP26A1−/− ES cell lines

The murine Cyp26a1 targeting construct was a generous gift from Dr. Martin Petkovich (Abu-Abed et al., 2001). The targeting construct is flanked by 2.8 kb of 5’ homology and 4.3 kb of 3’ homology, and contains a Cyp26a1 genomic sequence with a loxP site in intron 1 that is preceded by a novel BgII restriction site, and a PGK-neomycin cassette in intron 6 that is flanked by loxP sites (Fig. 1A). The EcoRI-linearized targeting construct was introduced into AB1 ES cells by electroporation using the BioRad GenePulser Xcell (BioRad Laboratories, Hercules, CA) at a voltage of 240 mV and capacitance of 500 μF. The murine Cyp26a1 targeting construct was a generous gift from Dr. Martin Petkovich (Abu-Abed et al., 2001). The targeting construct is flanked by 2.8 kb of 5’ homology and 4.3 kb of 3’ homology, and contains a Cyp26a1 genomic sequence with a loxP site in intron 1 that is preceded by a novel BgII restriction site, and a PGK-neomycin cassette in intron 6 that is flanked by loxP sites (Fig. 1A). The EcoRI-linearized targeting construct was introduced into AB1 ES cells by electroporation using the BioRad GenePulser Xcell (BioRad Laboratories, Hercules, CA) at a voltage of 240 mV and capacitance of 500 μF. Positive colonies were isolated with G418 selection (300 μg/ml active G418) and expanded. A total of 122 cell colonies were screened, and 2 positive cell lines were obtained. The cell lines that had undergone homologous recombination were identified by Southern blot analysis with Probe 1 (Fig. 1B). Genomic DNA was digested with BgII for Southern blot analysis. The two cell lines were expanded in the presence of 6 mg/ml active G418 to select for cell
DNA probes for Southern blot and Northern blot analyses

To confirm homologous recombination of the targeting construct, a 1 kb DNA fragment (Probe 1 in Figs. 1A) that is outside of the construct (4.1 kb upstream of the Cyp26a1 start site) was amplified from genomic DNA from AB1 Wt ES cells with the forward primer, 5′-ACA CCG CAG TGG AGA AGA-3′, and the reverse primer, 5′-GAA GGC AGA TAG TGG TTG CT-3′. A 310 bp DNA fragment that is part of exon 7 of the Cyp26a1 gene (Probe 2) was amplified from genomic DNA from AB1 Wt ES cells using the forward primer, 5′-AGG GAT ACC AGA TCC CCA AG-3′ and the reverse primer, 5′-GGG AGA TTT TCC ACA GGG TA-3′. The 609 bp cDNA fragment containing Cyp26a1 exons 2–4 (Probe 3) was prepared by digesting a plasmid containing the full-length Cyp26a1 cDNA in plasmid vector with PstI (New England Biolabs).

The following cDNA probes were used in the Northern blot assays: Hoxa1 (Genbank No. NM_010449) inserted into the EcoRI site of the pUC-9 vector (LaRosa and Gudas, 1988). LamA1 (Genbank No. NM_008480) was obtained from Dr. Y. Yamada (Sasaki et al., 1988). LamB1 (Genbank No. NM_008482), LamC1 (Genbank No. NM_010683), J31 (Genbank No. NM_009242; also known as SPARC and osteonectin), and J6 (Genbank No. NM_009825; also known as Hsp47 and Serpintii) cDNAs were originally cloned from a F9 EC cell cDNA library in our laboratory (Wang and Gudas, 1983; Wang et al., 1985).

Retinoid extraction and high performance liquid chromatography (HPLC) analysis

Cells were seeded in 60 mm tissue culture dishes at a density of 5 × 10⁵ cells/dish in ES medium with or without LIF. The next day, medium containing 1 μM non-radiolabeled RA was added to the cells for 24 h. Then, the medium was replaced with medium containing 50 nM [3H]RA for 8 h. In a different experiment, 24 h after seeding cells, medium containing 100 nM [3H]RA was added to the cells for 8 h. The following steps were the same for both experiments. A separate control consisting of radiolabeled RA in medium

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without cells was included concurrently during the incubation period. Medium was collected and cells were scraped into PBS for extraction. Radiolabeled retinoids were extracted from both cells and media under dim light at room temperature. Non-radiolabeled retinoid standards were added to the samples before extraction so that their elution profiles could be followed by absorbance. Retinoids were extracted immediately as previously described by Guo and Gudas (1998). The HPLC analysis was performed using a Waters Millennium system (Waters Corp., Milford, MA) equipped with a photodiode array detector to separate the various retinoids as previously described (Suh et al., 2006). Non-radiolabeled retinoid standards in each sample were run concurrently and were monitored at a wavelength of 340 nm while a Packard A-500 radiochromatography detector (Packard Instruments, Downers Grove, IL) was employed to monitor the radiolabeled retinoids. The criteria used to identify all-trans-RA and 4-oxo-RA were coelution with known standards and UV spectral analysis of peaks in this region of the chromatogram. The amounts of retinoids were calculated on the basis of their peak areas, volume of the sample, concentration of the starting [3H]all-trans-RA, and the total radioactive counts of the starting [3H]all-trans-RA. The lower limit of detection, calculated from standard controls, is approximately 0.4 pmol.

**Drug treatments for microarray analysis experiments**

For the microarray analysis, three different conditions were tested in Wt AB1 ES cells and CYP26A1−/− ES cells: Control (+LIF), 8 h; RA (+LIF), 8 h; and RA (+LIF), 72 h. Cells were treated with 100 nM all-trans RA or 0.1% ethanol (control vehicle) in the presence of LIF. Total RNA was harvested from the plates at 8 h and 72 h. Two conditions, Control (+LIF), 8 h and RA (+LIF), 8 h, were repeated independently, starting with freshly thawed cells, three times in the microarray chips. One condition, RA (+LIF), 72 h, was repeated independently two times.

**Microarray analysis**

Biotinylated cRNA targets were prepared using the One-Cycle cDNA Synthesis Kit (Affymetrix P/N 900431, Santa Clara, CA) by following the manufacturer’s instructions. Briefly, double-stranded cDNA was synthesized from 5 μg total RNA with Superscript II™ reverse transcriptase. After purification, the cDNA was used as a template in the subsequent in vitro transcription reaction for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were fragmented and hybridized to the GeneChip® Mouse Genome 430 2.0 arrays, which have 45,101 probe sets (each with a unique Affymetrix Identifier) representing over 34,000 mouse genes (Affymetrix 900496). The hybridization and subsequent scanning of the chips were performed by the microarray core facility at Weill Cornell Medical College.

Data from the chips were recorded as .cel files, which were directly imported into the software program GeneSpring GX 7.3.1 (Redwood, CA) for computational analysis. The data preprocessing method used was GC-RMA. The normalization involved two sequential steps: (1) Data transformation of all data less than 0.01 was reset to 0.01. (2) Each chip was normalized to 50th percentile of the total intensity. After normalization, the data were filtered by expression. Probe sets with expression values over 100 arbitrary units were filtered by fold-change. Samples were compared, and the log ratio expression of probe sets that had changed by 2-fold or more was statistically tested for significance. Probe sets that passed these filters were studied further by RT-PCR and bioinformatics analyses. Consultation on the analyses, including statistical analysis, was done with Piali Mukherjee, a microarray analysis expert from the Computational Genomics Core Facility at Weill Cornell Medical College.

All microarray data from this study have been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and the Series accession number will be released to the public, pending publication of this manuscript.

**Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)**

For the semi-quantitative RT-PCR analysis of differentially expressed genes, Wt and CYP26A1−/− ES cells were plated on gelatin-coated, 10-cm tissue culture plates, and treated with various concentrations of all-trans RA as indicated. After various times of drug treatment, cells were harvested for total RNA extraction. As a control, cells were treated with 0.1% ethanol (control vehicle). Total RNA (5 μg) was reverse-transcribed with Superscript II (Invitrogen) and primed with 1 μg of random hexanucleotides. Samples were
amplified in the linear range by PCR using the following primers. Samples were amplified in the linear range by PCR using the following primers: 36B4, 448 bp (upper primer, 5'-AGA ACC CAG CTC TGG AGA AA-3'; lower primer, 5'-TGG ATT CCA GAT CTG TTC AGG GAG-3'); lower primer, 5'-ATT TCA ATG TAC ACC TCC GTC GCC GGC-3'); Ldyh3, 281 bp (upper primer, 5'-AAC ATG ACG CTC ATC GGA GAG AAG-3'; lower primer, 5'-TGG TTT GTA CTA GCC ACA GAC TCT-3'); Tagl, 441 bp (upper primer, 5'-GGT GGA GGA GCC ACT AGT GG-3'; lower primer, 5'-GCT CCT GCA GTT GGC TG-3'); PtkWhk3, 995 bp (upper primer, 5'-GTG GAG TAG TAA GCT GGA-3'; lower primer, 5'-ATG GCA GGC TCC ACT TC-3'); Pmp22, 398 bp (upper primer, 5'-ATA GCT GGT GGC TTC TGA ACA CTT-3'; lower primer, 5'-ACA GGG TCC GCG CAG ACA GTC CTT-3'); Aass, 256 bp (upper primer, 5'-AGC TTC CAA TCG AGG CTA CCG AAT-3'; lower primer, 5'-ACA TAG CGA GAC CCA AGG ACC AAC-3'); Lamb1, 563 bp (upper primer, 5'-GAT AAC TGT CAG CAC AAC ACC-3'; lower primer, 5'-GGT GAG TAG TAA CCG GAC TTC-3'). The primers were designed to span two exons, and checked for specificity to the gene and lack of hybridization to pseudogenes using the University of California at Santa Cruz In-Silico PCR online program (http://genome.ucsc.edu).

Gene ontology annotation and enrichment analysis in DAVID

Affymetrix Identifiers for genes that passed the filters in GeneSpring GX 7.3.1 (Redwood, CA) were uploaded into the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software program (http://david.abcc.ncifcrf.gov/) for functional annotation study. The functional annotation clustering tool was used to sort genes according to their Gene Ontology (GO) terms and to identify functional categories that were over-represented in a gene list relative to the representation within the genome of a given species. The species Mus musculus was selected for this study. Affymetrix Mouse 430 2.0 was chosen as the background. Genes with positive fold-changes and negative fold-changes from each drug condition were analyzed separately in DAVID to understand how up-regulated genes in Wt ES cells related to biological function in each of the two cell lines.

Statistical methods

The means±standard errors of the mean (S.E.M.) were calculated using GraphPad Prism 4.0 software (San Diego, CA) and Microsoft Excel (Redmond, WA). The Student’s t-test was used to determine differences between two groups, and the one-way analysis of variance was used to determine differences among multiple experimental groups. Differences with a p-value of <0.05 were considered to be statistically significant.

Results

Generation of multiple, independent Cyp26a1−/− ES cell lines

To address the biological functions of Cyp26a1 in ES cells, both alleles of the Cyp26a1 gene were disrupted in AB1 Wt ES cells by two rounds of homologous recombination. AB1 Wt ES cells were transfected with a targeting construct containing the Cyp26a1 genomic DNA with a loxP site in intron 1 that is preceded by a novel BgII restriction site and a PGK-neomycin resistance cassette in intron 6 that is flanked by loxP sites (Fig. 1A). Of 122 colonies screened by Southern blot analysis using Probe 1, two colonies were found to have integrated the targeting construct by homologous recombination (Fig. 1B). These colonies were grown in 6 mg/ml G418 to select for colonies that had two copies of the targeting construct. A total of 48 surviving colonies were picked and screened by Southern blot analysis using Probe 1. One colony out of 48 was found to have both Cyp26a1 alleles replaced by the targeting construct (Fig. 1C). This colony (KO/KO or Cyp26Δ) was expanded and then transiently transfected with a Cre expression vector by electroporation. Cre recombinase expression that resulted in the deletion of Cyp26a1 exons 2–6 as a result of the removal of the neo cassette in the ES cells was determined by Southern blot analysis using Probe 2 and Probe 3 (Figs. 1D and E). A total of 216 colonies were picked, and 6 colonies were found to lack Cyp26a1 exons 2–6. These AB1 ES cell-derived cell lines were named CYP26A1−/− #22, 59, 142, 165, 169, and 184.

The CYP26A1−/− #59, 142, and 165 ES cell lines were tested for Cyp26a1 mRNA expression by Northern blot analysis to confirm the absence of the Cyp26a1 message. The CYP26A1−/− ES cells were treated with 1 μM RA for 24 h. The Northern blot analysis results show that CYP26A1−/− ES cells do not express the 1.7 kb, full-length Cyp26a1 mRNA seen in Wt ES cells, but they do express a truncated transcript of 0.7 kb, presumably containing exons 1 and 7, after RA treatment (Fig. 1F). To confirm that the truncated transcript did not contain any enzymatic activity, we assayed CYP26A1 enzyme activity by reverse-phase HPLC.

All-trans-RA metabolism in CYP26A1−/− ES cells cultured in the presence of LIF

Two independent CYP26A1−/− ES cell lines, #59 and #142, were tested by HPLC to examine RA metabolism. Wt, CYP26A1−/− #59, and CYP26A1−/− #142 ES cells were cultured in the presence of 1 μM non-radiolabeled RA for 24 h and then treated with 50 nM radiolabeled RA for 8 h. The 24-h treatment with the unlabeled RA was used to induce expression of metabolic enzymes, i.e. CYP26A1, while the radiolabeled RA was used to measure RA metabolism by reverse-phase HPLC. The Wt ES cells exhibited high levels of polar RA metabolites, while both CYP26A1−/− #59 and #142 ES cell lines did not show any radioactive counts above background where RA metabolites elute, indicating that both CYP26A1−/− #59 and #142 ES cell lines did not generate any polar RA metabolites from RA (data not shown, but see below for more detailed HPLC analyses). Thus, the removal of exons 2–6 resulted in an inactive CYP26A1 enzyme and the inability of the CYP26A1−/− #59 and #142 cell lines to metabolize RA. These experiments show that any protein translated from the truncated transcript is not functional in the CYP26A1−/− ES cells.

We then assessed RA metabolism in AB1 Wt versus CYP26A1−/− ES cells. Wt and CYP26A1−/− #59 ES cells were cultured with 100 nM [3H]RA for 8, 24, and 48 h, either in the presence or absence of LIF. LIF is a critical growth factor that is added to the ES medium to maintain pluripotency of murine ES cells; its absence promotes ES cell differentiation spontaneously in cell culture (Boeuf et al., 1997).

RA treatment of Wt ES cells induced a rapid and efficient metabolism of RA into polar RA metabolites after 8 h of RA treatment, and by 48 h, all of the RA had been metabolized into...
polar RA metabolites (Figs. 2A–H). Metabolism of RA into polar RA metabolites was not seen in the CYP26A1−/− ES cell line after 8 h of RA treatment (Fig. 2B). In CYP26A1−/− ES cells cultured with LIF (+LIF), polar RA metabolites were not detected until after 48 h of RA treatment (Fig. 2D). In addition, the levels of intracellular RA, normalized to cell number, were higher in the CYP26A1−/− cells than in the Wt cells at all times tested (Fig. 2E). CYP26A1−/− ES cells (+LIF) had 2.0±0.5-fold higher intracellular RA levels than Wt ES cells (+LIF) after 8 h RA treatment (p<0.05), 3.7±0.4-fold higher intracellular RA levels after 24 h of RA treatment (p<0.05), and 11.0±3.2-fold higher intracellular RA levels after 48 h of RA treatment (p<0.05, Fig. 2E). These data prove that CYP26A1 is the major and only enzyme responsible for metabolizing RA into polar RA metabolites in murine ES cells during the first 48 h of RA treatment.

Higher levels of polar RA metabolites were found in the Wt ES cells than in the CYP26A1−/− ES cells at all times tested (Figs. 2G and H). Wt ES cells (+LIF) exhibited an average intracellular RA polar metabolite (sum of all metabolites) concentration of 670±251 nM after 8 h, 671±166 nM after 24 h, and 500±68 nM after 48 h of RA treatment. The concentration of all polar RA metabolites does not increase over time because these polar RA metabolites are excreted from the cells and/or further metabolized by CYP26A1 to compounds not extracted by acetonitrile/butanol (Chen and Gudas, 1996; Chithalen et al., 2002). After 8 and 24 h of RA treatment, CYP26A1−/− ES cells (+LIF) exhibited no radioactive counts above background where polar RA metabolites normally elute; therefore, polar RA metabolites were not detected in CYP26A1−/− ES cells after 8 and 24 h of RA treatment. After 48 h of RA treatment, CYP26A1−/− ES cells (+LIF) exhibited an average intracellular RA polar metabolite concentration of 119.8±10 nM, which is approximately 20% of the level in Wt ES cells at the same time point (Fig. 2G). We do not know what enzyme is producing the polar metabolites in the CYP26A1−/−
ES cells at the later (>48 h) time points. Cyp26b1 mRNA was expressed at similar levels in both Wt and CYP26A1−/− ES cells after RA treatment for 72 h, so it is possible that CYP26B1 activity is responsible for the metabolism of RA at the later time points (data not shown). Thus, we conclude that: a) during the first 48 h in the presence of 100 nM RA +LIF the CYP26A1−/− ES cells do not metabolize RA, and b) the intracellular RA concentration is several fold higher in RA treated CYP26A1−/− ES cells than in Wt ES cells.

All-trans-RA metabolism in CYP26A1−/− ES cells cultured in the absence of LIF

As compared to culture in the presence of LIF, Wt ES cells cultured in the absence of LIF exhibited 43% and 10% increases in RA metabolism at 8 and 24 h after RA treatment, respectively, as calculated by the total amounts of polar RA metabolites detected in the cell extracts and medium (Figs. 2G and H). By 48 h, nearly all of the RA was metabolized in Wt ES cells cultured in either the presence or absence of LIF (Figs. 2E and F). Low levels of polar metabolites of RA were detected in the cell extracts (206 nM ± 43.5) and in the media (4.2 ± 0.1 nM) of CYP26A1−/− cells cultured without LIF, but not in the cell extracts or in the media of these cells cultured with LIF at the 24-h time point (Figs. 2G and H). After 48 h of RA treatment, low levels of polar RA metabolites were detected in cell extracts (119.8 ± 10 nM, +LIF; 151.3 ± 4.2 nM, −LIF) and in the media (7.4 ± 1.5 nM, +LIF; 27.6 ± 9.8 nM, −LIF) of CYP26A1−/− ES cells cultured with or without LIF (Figs. 2G and H). We conclude that the absence of LIF increases RA metabolism in both Wt and CYP26A1−/− ES cells in a CYP26A1-independent manner.

Cell proliferation of Wt and CYP26A1−/− ES cells

To determine whether disrupting Cyp26a1 affects ES cell proliferation, we measured the cell proliferation rates of two CYP26A1−/− ES cell lines, #59 and #142, compared to Wt ES cells. Wt ES cells doubled in 14.67 ± 2.85 h (mean ± range). CYP26A1−/− #59 and #142 ES cells doubled in 13.76 ± 2.2 h and 12.27 ± 1.15 h, respectively. The population doubling rates among all three ES cell lines were not statistically different, showing that the disruption of both alleles of Cyp26a1 did not affect ES cell proliferation in normal ES medium containing LIF.

RA inhibits proliferation of various cell types, including murine ES cells (Martinez-Ceballos et al., 2005). Since the CYP26A1−/− ES cells had higher intracellular levels of RA than the Wt ES cells after RA treatment (Fig. 2), we wanted to determine how RA treatment would affect proliferation of these cells. Wt and CYP26A1−/− #59 ES cells were treated with varying concentrations (1 × 10−9 to 5 × 10−6 M) of RA for 72 h and counted. Like Wt ES cells, the CYP26A1−/− ES cells were growth inhibited by RA treatment in a dose-dependent manner (Fig. 3). However, the CYP26A1−/− ES cells were growth inhibited to a lesser degree than AB1 Wt ES cells at the same doses of RA, indicating that the CYP26A1−/− ES cell lines were more resistant to growth inhibition by RA than Wt ES cells (Fig. 3). To investigate whether short treatments of RA were sufficient for growth arrest we cultured ES cells with RA for 6 h and then replenished with fresh medium without RA for the remainder of the experiment. We observed that in Wt cells, 72 h of RA treatment was more effective in inhibiting proliferation than 6 h of RA treatment (Fig. 3). However, in the CYP26A1−/− ES cells the 6- and 72-h RA treatments resulted in similar degrees of growth inhibition (Fig. 3), suggesting that the metabolism of RA by CYP26A1 in Wt ES cells over time contributed to the proliferation arrest.

Hoxa1 mRNA expression

Northern blot analysis was performed to determine if the disruption of the Cyp26a1 gene would affect transcription of the direct RA target gene, Hoxa1. Hoxa1 has a DR5 RARE in the 3′ enhancer, which is required for transcriptional activation by RA (Boylan et al., 1993; Langston and Gudas, 1992; Langston et al., 1997; LaRosa and Gudas, 1988). Since the CYP26A1−/− ES cells have higher intracellular RA levels (Fig. 2), we expected to observe higher expression of Hoxa1 mRNA in the CYP26A1−/− ES cells. At both the 24- and 72-h time points, the Hoxa1 gene was transcriptionally activated in an RA dose-dependent manner in both Wt and CYP26A1−/− ES cell lines, but was expressed at 3.65 ± 1.39 and 1.97 ± 0.31-fold higher levels in CYP26A1−/− ES cells than in Wt ES cells treated with 1 and 100 nM RA, respectively, for 72 h (Fig. 4).

We also included the KO/KO cell line in our analyses (Fig. 1D). This cell line has both of the “floxed” Cyp26a1 alleles in it,
but the cell line was not transfected with cre so it still contains the neomycin resistance gene and exons 2 through 6. This KO/KO cell line behaved like the J1 Wt cells, as expected, with the neomycin resistance gene and exons 2 through 6. This KO/KO cell line was not transfected with cre so it still contains

Differentiation specific gene expression

Our laboratory isolated cDNA clones for several RA regulated, differentiation-specific “late-response” genes, LamB1, J6, and J31, from RA-treated F9 WT EC cells, and characterized their expression profiles (Chen and Gudas, 1996; Wang and Gudas, 1988; Wang et al., 1985). These genes exhibit steady state mRNA levels that can be detected approximately 24 h after RA treatment and that reach maximal levels around 48 to 72 h after RA treatment (Wang et al., 1985). Both RNA synthesis and protein synthesis were shown to be required for the induction of these genes by RA, indicating that RA regulation of these genes is indirect (Wang and Gudas, 1988; Wang et al., 1985). LamB1 is a gene that encodes the laminin β1 chain, a glycoprotein that is a major constituent of the extracellular matrix (Ekblom et al., 2003). Our laboratory had previously described cis-acting DNA regulatory elements, including an RARE, that regulate tissue-specific LamB1 expression (Li and Gudas, 1997; Sharif et al., 2001; Vasiòs et al., 1991, 1989). LamB1 mRNA expression was 5.4±1.4-fold, 4.8±0.3-fold, and 1.8±0.2-fold higher in RA-treated Wt ES cells than in CYP26A1−/− ES cells at 24, 48, and 72 h, respectively (Fig. 5B). The basal level of LamB1 mRNA was also reduced in CYP26A1−/− ES cells (Fig. 5C). Culturing Wt and CYP26A1−/− ES cells with increasing concentrations of RA activated transcription of LamB1 in a dose-responsive manner, but the LamB1 mRNA levels were higher in Wt than in CYP26A1−/− ES cells at all RA concentrations (Fig. 5C). These results show that the lack of CYP26A1 activity is associated with a reduction in LamB1 mRNA levels. Culture in the presence of the polar RA metabolite, 4-oxo-RA, increased LamB1 mRNA levels in both Wt and CYP26A1−/− ES cells. However, 4-oxo-RA was less potent than RA at a similar dose in both Wt and CYP26A1−/− ES cells (Fig. 5D), indicating that the reduced LamB1 mRNA levels seen in RA-treated CYP26A1−/− ES cells did not result from the absence of the polar RA metabolite, 4-oxo-RA. Next, we cultured CYP26A1−/− ES cells in the presence of both RA and 4-oxo-RA to determine if treating with both retinoids would restore LamB1 mRNA levels in CYP26A1−/− ES cells to those of Wt ES cells, but cotreatment did not increase LamB1 mRNA levels in CYP26A1−/− ES cells to those of Wt ES cells (data not shown).

We also examined LamA1 and LamC1, other members of the laminin family, which encode the laminin α1 chain and the laminin γ1 chain, respectively (Aberdam et al., 2000; Ekblom et al., 2003). LamA1 mRNA levels were 1.4±0.03-fold higher in 100 nM RA-treated Wt ES cells than in 100 nM RA-treated CYP26A1−/− ES cells (Fig. 5E). Culture in the presence of 4-oxo-RA increased LamA1 mRNA levels in both Wt and CYP26A1−/− ES cells, but 4-oxo-RA was less potent than RA (data not shown). LamC1 mRNA levels were 1.1±0.01-fold higher in 100 nM RA-treated Wt ES cells than in 100 nM RA-treated CYP26A1−/− ES cells (data not shown). These data show that CYP26A1 is important for the appropriate increases in LamA1, LamB1, and LamC1 mRNA levels.

J31 and J6 are two other RA-inducible genes that were isolated from RA-treated F9 EC cells and they show expression...
kinetics similar to those of LamB1 (Wang et al., 1985). The J31 sequence was found to be identical to the gene, secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin) (Mason et al., 1986). RA treatment of both Wt and CYP26A1−/− ES cells increased J31 mRNA levels in a dose-dependent manner, but the mRNA levels were 1.54±0.04-fold higher in 100 nM RA-treated Wt as compared to 100 nM RA-treated CYP26A1−/− ES cells (Fig. 6A). J6 encodes a gene for a heat shock protein, HSP47, that is involved in collagen biosynthesis (Bielinska and Wilson, 1995; Clarke et al., 1991; Henle et al., 1994). The carboxy-terminal portion has sequence similarity to members of the serpin (serine protease inhibitor) family, but the molecule does not possess protease inhibitor activity (Wang and Gudas, 1990). J6 mRNA was increased by 100 nM RA in both Wt and CYP26A1−/− ES cells, but the J6 mRNA levels were 1.49±0.04-fold higher in Wt ES cells than in CYP26A1−/− ES cells (Fig. 6B).

In addition, while the gene expression profiles for RA-inducible genes were very similar in Wt and CYP26A1−/− ES cells, other molecular markers of parietal endoderm, including tissue plasminogen activator, GATA-4, and parathyroid hormone receptor, were expressed at 3–5 fold higher levels in
monolayer cultures of Wt than in CYP26A1−/− ES cells at 72 h after RA addition in the presence of LIF (data not shown). Collectively, our data show that the genes expressed in response to RA after later times (e.g. 72 h) in the differentiated parietal endoderm cells, such as the laminin genes, are induced to a lesser extent in the CYP26A1−/− ES cells. These lower mRNA levels in the CYP26A1−/− ES cells indicate that differentiation of ES stem cells to parietal endoderm is reduced by the lack of CYP26A1 activity.

Identification of genes differentially expressed in response to RA in Wt ES cells versus CYP26A1−/− ES cells by microarray analyses

Next, we performed microarray analyses using the Affymetrix GeneChip Mouse 430 2.0 arrays, which are genome-wide arrays with 45,101 unique probe sets representing transcripts and variants for over 34,000 mouse genes. Three conditions were used to examine AB1 Wt and CYP26A1−/− #59 gene expression patterns: (A) Control vehicle for 8 h, (B) 100 nM RA for 8 h, and (C) 100 nM RA for 72 h (Fig. 7A), as described in Materials and methods. The data were imported into the software program GeneSpring GX 7.3.1 (Redwood, CA) for analyses.

To identify genes that are either up-regulated or down-regulated in the Wt versus CYP26A1−/− ES cells, three independent analysis steps were taken for each condition (Fig. 7B). Each of the 45,101 unique probe sets on the microarray chips were analyzed individually, even though some genes had multiple probe sets. First, probe sets with low expression (less than 100 arbitrary units of raw data) in samples from both Wt and CYP26A1−/− ES cells were filtered out because genes with background expression levels are not reliable for calculating fold-change due to exaggerated ratios. In Condition A, Control for 8 h, 14,703 probe sets passed the expression filter. In Condition B, RA for 8 h, 14,708 probe sets passed the expression filter. In Condition C, RA for 72 h, 15,021 probe sets passed the expression filter.

Probe sets that passed the first filter were subjected to a second filter that was based on a fold-change of at least 2 between Wt and CYP26A1−/− ES cells. In Condition A, Control

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<td>B</td>
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<td>C</td>
<td>100 nM RA +LIF (72h)</td>
<td>100 nM RA +LIF (72h)</td>
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<table>
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<tr>
<th>A. Control 8 hr</th>
<th>B. RA 8 hr</th>
<th>C. RA 72 hr</th>
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<tr>
<td>45101 probes</td>
<td>45101 probes</td>
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Fig. 6. Northern blot analyses of J31 and J6 genes. (A) J31 mRNA levels after 72 h treatment with varying doses of RA (0–1000 nM). The y-axis shows J31/GAPDH mRNA levels for each sample compared to the J31/GAPDH mRNA levels for the WT ES cells control sample. The differences in the mRNA levels between WT and CYP26A1−/− ES cell after RA treatment at 0, 100, and 1000 nM were statistically significant (p<0.05). In most cases, symbol points were larger than the S.E.M. (B) J6 expression after 72 h treatment with varying doses of RA (0–1000 nM). The differences in the mRNA levels between WT and CYP26A1−/− ES cell after RA treatment at 1, 100, and 1000 nM were statistically significant (p<0.05). In most cases, symbol points were larger than the S.E.M. Statistical significance is indicated by * (p<0.05).

Fig. 7. Design of microarray experiment and analysis of differentially expressed genes. (A) Three drug conditions were used to examine gene expression in WT and CYP26A1−/− ES cells. A "#" indicates the number of times the microarray analysis was performed, starting with fresh cells. (B) Genes had to pass three filters to be considered differentially expressed between WT and CYP26A1−/− ES cells. The first step filtered out probes with raw expression less than 100 arbitrary units of raw data. The second step filtered out probes with expression levels not reliable for calculating fold-change due to exaggerated ratios. The third step filtered out statistically insignificant means based on the Student’s t-test (p>0.05). The remaining probes were considered to be genes with a 2-fold or greater difference in expression between WT and CYP26A1−/− ES cells. The top 20 and bottom 20 transcripts are listed in Tables 1–3.
for 8 h, 258 probe sets passed the fold-change filter. In Condition B, RA for 8 h, 259 probe sets passed the fold-change filter. In Condition C, RA for 72 h, 645 probe sets passed the filter. Finally, these probe sets were subjected to statistical analysis, and probe sets that had significantly different means (log ratio) based on the Student’s t-test ($p<0.05$) passed this last filter and designated as differentially expressed transcripts.

A total of 165 transcripts were either up- or down-regulated in WT versus CYP26A1−/− ES cells by 2-fold or greater in condition A, Control for 8 h (Table 1 lists the top 20 and bottom 20 transcripts. For the complete list, see Supplemental Table 1). Of these 165 differentially expressed transcripts, 108 transcripts were down-regulated in CYP26A1−/− ES cells compared to WT. Some of the genes differentially expressed in the absence of RA include Bnip3, Epha4, Fcgr2b, Gja1, Igfbp3, LamB1, Leftb, Pdk1, Prkwnk3, Stk2, and Trl2. It is of interest that we did not observe any differences in Nanog, Oct3/4, or Sox2 mRNA levels between the AB1 WT and the Cyp26A1−/− ES cells cultured without RA (control) and plus LIF, either in these microarray experiments or in Northern analyses we performed (data not shown).

A total of 183 transcripts were either up- or down-regulated in WT versus CYP26A1−/− ES cells by 2-fold or greater in Condition B, RA for 8 h (Table 2 lists the top 20 and bottom 20 transcripts. For the complete list, see Supplemental Table 2). Of these 183 differentially expressed transcripts, 136 transcripts were down-regulated in CYP26A1−/− ES cells relative to WT. Among the differentially expressed genes in Condition B are

### Table 1

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<td>Protein kinase, lysine deficient 3</td>
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<td>–</td>
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<td>Palladin</td>
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<td>Eph receptor A4</td>
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### Table 2

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</tbody>
</table>

a Average from three experiments.

b Some genes have multiple distinct probes on the GeneChip® resulting in different fold-changes from the different probes.

c Prkwnk3 mRNA is expressed at a 97.16±32.75-fold higher level in control WT ES cells versus CYP26A1−/− ES cells.

d Fold change means ratio of Wt to CYP26A1−/− when CYP26A1−/− is set at 1 (e.g. 0.40 in Table 1—2.5 fold change).
A total of 187 transcripts were either up- or down-regulated in Wt versus CYP26A1−/− ES cells by 2-fold or greater in Condition C, RA for 72 h (Table 3 lists the top 20 and bottom 20 transcripts. For the complete list, see Supplemental Table 3). Of these 187 differentially expressed transcripts, 119 transcripts were down-regulated in CYP26A1−/− ES cells relative to Wt. Some of the differentially expressed genes in the presence of RA for 72 h are Calponin, Cadillacon, Eph4, Hist1h1c, Hist1h2hp, Hoxb4, Nrp1, Pmp22, Tagln, and Thbs1.

To validate the microarray data, new cell samples were used to confirm the results. In addition to CYP26A1−/− #59, which was the cell line used for the microarray analyses, other independently derived CYP26A1−/− ES cell lines, #142 and #165, were tested during the validation studies. Several differentially expressed genes were selected for examination by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). As a loading control, RT-PCR of 36B4, a housekeeping gene that encodes the acidic ribosomal protein P0, was set at 1 (e.g. 0.40 in Table 1—2.5 fold change).

Aire, Bnip3, Cyp26a1, Eph4, FcgR2b, Ltbp3, PrkWnk3, Sox3, and Trl2.

Table 2

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a Average from three experiments.
b Some genes have multiple distinct probes on the GeneChip® resulting in different fold-changes from the different probes.
c PrkWnk3 mRNA is expressed at a 79.57±27.66-fold higher level in RA-treated WT ES cells relative to Wt.
d Fold change means ratio of Wt to CYP26A1−− when CYP26A1−− is set at 1 (e.g. 0.40 in Table 1—2.5 fold change).
Epha4, PrkWnk3, Aass, LamB1, and Lthp3 (Fig. 8A). These genes were selected for validation because they had relatively high fold-changes and/or were found to have interesting biological functions.

Epha4 belongs to the ephrin receptor subfamily of the protein–tyrosine kinase family and has been shown to play roles in axonal guidance signaling and segmentation of the hindbrain during development (Holmberg and Frisen, 2002; Nittenberg et al., 1997). Epha4 mRNA levels were 5.1±1.8-fold and 8.7±3.1-fold lower in CYP26A1−/− ES cells than in Wt ES cells in both Conditions A and B, respectively, and semi-quantitative RT-PCR confirmed that Epha4 expression was higher in Wt than in CYP26A1−/− ES cells (Fig. 8A). Epha4 mRNA levels were also 3.6±0.6-fold higher in Wt ES cells than in CYP26A1−/− ES cells in Condition C, and this was confirmed by semi-quantitative RT-PCR (Figs. 8C, D).

PrkWnk3 (also known as WNK3) is a member of the “with no lysine” (WNK) serine–threonine protein kinase family that activates Na+–K+–Cl transporters by increasing their phosphorylation (Kahle et al., 2005). PrkWnk3 was shown to interact with procaspase 3 and heat shock protein 70 to increase cell...
survival in HeLa cells (Verissimo et al., 2006). PrkWnk3 mRNA levels were higher in Wt than in CYP26A1−/− ES cells by 97.2±32.8-fold in Condition A and by 79.6±27.6-fold in Condition B, and semi-quantitative RT-PCR confirmed that PrkWnk3 mRNA levels are higher in Wt ES cells than in CYP26A1−/− ES cells (Figs. 8C, D).

Aminoadipate-semialdehyde synthase (Aass) encodes a bifunctional enzyme that catalyzes the conversion of lysine to alpha-aminoadipic semialdehyde in the mammalian lysine degradation pathway (Markovitz and Chuang, 1987; Sacksteder et al., 2000). Aass mRNA was higher in Wt than in CYP26A1−/− ES cells by 12.8±6.9-fold in the Condition B, and semi-quantitative RT-PCR validated this (Fig. 8A).

Lamb1 mRNA expression has been shown to be transcriptionally regulated by RA by our laboratory (Li and Gudas, 1996, 1997; Sharif et al., 2001; Vasiou et al., 1991). Lamb1 mRNA levels in Wt were 2.7±0.6-fold higher than in CYP26A1−/− ES cells in Condition B and 3.5±1.3-fold higher in Wt ES cells in Condition B, consistent with our prior data (Figs. 5 and 8A).

Latent transforming growth factor beta binding protein 3 (Ltbp3) has been shown to bind transforming growth factor β (TGFβ) and to play a role in cell growth and skeletal development (Dabovic et al., 2005). Ltbp3 mRNA levels were 2.4±0.3-fold lower in Wt ES cells as compared to CYP26A1−/− ES cells in Condition B, and semi-quantitative RT-PCR confirmed that Ltbp3 mRNA levels are higher in CYP26A1−/− ES cells than in Wt ES cells (Fig. 8A).

For validation of genes differentially expressed in Condition C (100 nM RA for 72 h), Wt and CYP26A1−/− #59 and #142 ES cells were treated with varying concentrations of RA for 72 h. For validation of the 72-h time point, semi-quantitative RT-PCR analyses of Epha4, Tagln, and Pmp22 were performed (Fig. 8B).

Transgelin (Tagln), also known as Smooth muscle α22 is expressed in smooth muscle cells and fibroblasts (Lawson et al., 1997). Tagln mRNA levels were 56.9±17.7-fold higher in Wt ES cells than in CYP26A1−/− ES cells in Condition C, and this was validated by semi-quantitative RT-PCR (Figs. 8C, D).

The Peripheral myelin protein 22 (Pmp22) gene encodes a glycoprotein that is a major component of myelin in the peripheral nervous system (Amici et al., 2006), and Pmp22 complexes with extracellular matrix molecules to mediate interactions of glial cells with the extracellular environment (Amici et al., 2006). Pmp22 mRNA levels were 9.9±1.9-fold higher in Wt ES cells than in CYP26A1−/− ES cells in Condition C, and semi-quantitative RT-PCR confirmed that Pmp22 mRNA levels are lower in CYP26A1−/− ES cells than in Wt ES cells (Figs. 8C, D).

Functional analyses of differentially expressed genes by Gene Ontology classification

Assigning biological functions to the differentially regulated genes is a first step in understanding how Wt ES cells and CYP26A1−/− ES cells are functionally different from each other. The Gene Ontology (GO) Consortium developed three structured, controlled vocabularies (ontologies) that describe the gene products in terms of their associated biological processes, molecular functions, and cellular components in a species-independent manner (Ashburner et al., 2000). Biological processes include broad biological goals that are based on multi-step molecular functions, such as metabolism, cell differentiation, and transcription. Molecular functions describe the activities performed by the individual gene product and include such functions as kinase activity and ion binding. Cellular components are the physical locations of the gene product, such as nucleus and cytoskeleton. For the purpose of understanding how the loss of Cyp26a1 affects the biology of ES cells, we chose to identify the biological processes associated with genes that show reduced or increased expression independently.

DAVID, which stands for Database for Annotation, Visualization, and Integrated Discovery, is a web-based tool that analyzes and annotates genome-scale data sets based on GO terms and identifies categories that are over-represented in the gene list relative to the representation within the genome of a given species. First, the functional annotation clustering tool displays similar annotations together in clusters. The grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. The degree of common genes between two annotations is measured by Kappa statistics, and the Kappa values are then used to classify groups of similar annotations. Next, DAVID calculates the chances of over-representation of the clusters using the Fisher Exact test. The main benefit of over-representation analysis is to order categories associated with a gene list in order to focus on those processes most likely associated with the biological phenomenon under study; in this case, the biological phenomenon is the lack of CYP26A1 enzyme activity in ES cells.

Based on the genes that were expressed at higher levels in the CYP26A1−/− ES cells relative to Wt ES cells, the biological processes termed DNA metabolism and chromosome organization were the top GO terms to be enriched in all conditions (Table 4 and Fig. 9). Five histone genes were expressed at higher mRNA levels in CYP26A1−/− ES cells than in Wt ES cells, including Hist1h2bf, Hist1h2bc, Hist1h2bp, Hist1h1c, and Hist1h3c1.

In Conditions A and B, Wt ES cells were highly enriched for genes involved in cell proliferation and cellular morphogenesis, such as Igfhp3, Gja1, and Leftb, suggesting that CYP26A1 activity is important for normal ES cell proliferation and morphogenesis (Table 4). Igfhp3 binds IGF (Insulin-like growth factor) to inhibit IGF activity, which stimulates cell proliferation and differentiation (Ranke and Elmlinger, 1997). Igfhp3 has also been shown to bind RXR, resulting in reduced RAR signaling (Liu et al., 2000). Leftb (also known as Lefty) is a direct transcriptional target of Oct-4, and is expressed in pluripotent ES cells (Babaie et al., 2007; Tabibzadeh and Hemmatti-Brivanlou, 2006). Gja1 encodes a gap junction protein that is a member of the TGFβ family and plays a role in maintaining pluripotency in ES cells (Tabibzadeh and Hemmatti-Brivanlou, 2006). Gja1 encodes a gap junction protein that is a member of the TGFβ family and plays a role in maintaining pluripotency in ES cells (Tabibzadeh and Hemmatti-Brivanlou, 2006).
membrane channel protein alpha 1, a stem cell marker in human ES cells, and is involved in signal transduction (Bhattacharya et al., 2004; Player et al., 2006).

In Conditions A and B, CYP26A1−/− ES cells relative to Wt ES cells were highly enriched for genes involved in stress response and defense response, suggesting that CYP26A1
activity plays a role in regulating and reducing the cellular response to stress in ES cells (Fig. 9). The stress response-related genes that displayed higher mRNA levels in CYP26A1−/− ES cells than in Wt ES cells included Tlr2, Stk2, Fcgr2b, Bnip3, and Pdk1. Tlr2 was found to have the largest positive fold-change in CYP26A1−/− ES cells versus Wt ES cells in both the absence and presence of RA for 8 h (Conditions A and B). Serine/threonine kinase 2 (Stk2), also known as ste20-like kinase (slk), is involved in caspase-dependent apoptosis induced by various stress stimuli (Sabourin et al., 2000). Fcgr2b is the only known immunoglobulin G receptor capable of inducing apoptosis, which was shown in B-cells to involve pro-apoptotic Bcl-2 members Bid and Bad (Carter and Harnett, 2004). Bnip3 expression is regulated by hypoxia-inducible factor-1α (HIF-
1α) and encodes a mitochondrial pro-apoptotic protein that has a role in hypoxia-induced cell death (Guo et al., 2001; Lee and Paik, 2006). Pyruvate dehydrogenase kinase 1 (Pdk1) is also induced by HIF-1α as a cellular response to hypoxia, resulting in reduced mitochondrial function and oxygen consumption (Kim et al., 2006; Papandreou et al., 2006). The increased expression of these genes in CYP26A1−/− ES cells indicates that the lack of CYP26A1 function results in increased cellular stress, even in the absence of RA. A previous study showed that retinol levels above the physiological limit resulted in oxidative stress and apoptosis in human dermal fibroblasts (Gimeno et al., 2004). It is possible that, in the absence of RA, basal levels of CYP26A1 protein in Wt ES cells metabolize retinol to maintain precise levels that do not elicit the stress response, while the lack of CYP26A1 basal activity in CYP26A1−/− ES cells results in high retinol levels that do elicit the stress response.

In Condition C, genes up-regulated in Wt ES cells relative to CYP26A1−/− ES cells were found to be enriched in the biological processes, cell differentiation, nervous system development, vasculature development, and angiogenesis categories, which indicates that Wt ES cells are induced by RA to differentiate into certain cell lineages, and that these developmental programs require CYP26A1 activity to proceed normally (Table 4 and Fig. 9). Some of the differentiation-related genes expressed at higher levels in Wt versus CYP26A1−/− ES cells were Tagln, Calponin, Eph4a, Pmp22, and Nrp1. The functional annotation analysis results indicate that with the addition of RA for 72 h, Wt ES cells adopt specific characteristics of differentiating cells. However, differentiation appears to be more limited in CYP26A1−/− ES cells relative to Wt ES cells.

**Discussion**

**Lack of RA metabolism and reduced differentiation in the CYP26A1−/− ES cell lines**

Here we report the generation and characterization of cultured murine ES cells with a targeted disruption of both alleles of Cyp26a1. This method was chosen for several reasons. First, the targeting construct had been used to generate Cyp26a1-null murine ES cells with a targeted disruption of both alleles of Cyp26a1. This method was chosen for several reasons. First, the targeting construct had been used to generate Cyp26a1-null
mice (Abu-Abed et al., 2001). Second, the homologous recombination process ensures that no expression of Cyp26a1 will occur. Other methods to reduce gene expression, such as RNA-interference and antisense technology, often result in some expression of the target gene, as well as induction of non-specific pathways (off target effects) (Houdebine, 2002; Marques and Williams, 2005; Sledz and Williams, 2005). The phenotype of the CYP26A1\(^{-/-}\) mouse indicates that the areas in which CYP26A1 is normally expressed develop morphogenetic defects (Abu-Abed et al., 2001; Sakai et al., 2001). For example, CYP26A1\(^{-/-}\) mouse embryos show abnormal patterning of the hindbrain and posterior transformation of the cervical vertebrae (Abu-Abed et al., 2001; Sakai et al., 2001). These areas most likely have increased RA levels resulting from the lack of CYP26A1 activity. Deletion of one copy of retinaldehyde dehydrogenase-2 (RALDH2) in CYP26A1\(^{-/-}\) mice rescued many of the defects attributable to the lack of CYP26A1, and the authors speculated that this resulted from the reduction of RA levels to a range that is compatible with proper embryonic development (Niederreither et al., 2002). In the present study we show that disruption of both copies of Cyp26a1 in ES cells resulted in increased intracellular RA levels (Fig. 2), but decreased differentiation, as assessed by parietal endoderm-related gene expression (Fig. 5), by neuronal differentiation gene expression (Figs. 8 and 9, and Table 4), and by smooth muscle cell differentiation gene expression (Figs. 8 and 9, and Table 4).

The role of CYP26A1 in metabolizing RA is not limited to the embryo since CYP26A1 is also expressed in various adult tissues, including brain, spleen, lung, liver, skeletal muscle, and testis (Choudhary et al., 2003; White et al., 1997). CYP26A1 is involved in retinoid signaling in the endometrium, as it is transcriptionally activated by progesterone in mice and by estrogen in humans (Fritzsche et al., 2007). CYP26A1 is also a gene target for cancer treatment because of its role in metabolizing RA and decreasing the sensitivity of various cancer cells to RA-induced differentiation or apoptosis (Armstrong et al., 2005; Klaassen et al., 2001; Ozpolat et al., 2005). Recently, Shelton et al. (2006) discovered that the CYP26A1 protein is up-regulated by a Wnt-dependent mechanism in tissues deficient in the colon tumor suppressor gene, adenomatous polyposis coli (APC), in zebrafish, mice, and humans. Their findings suggest that APC promotes intestinal cell differentiation in part by suppressing the catabolism of RA by CYP26A1 (Shelton et al., 2006). In acute promyelocytic leukemia (APL) cells, Cyp26a1 gene transcription was enhanced by Hoxa10v2, a homeobox transcription factor, after all-trans-RA treatment (Quere et al., 2007). Highly RA-sensitive APL blasts expressed higher levels of Cyp26a1 compared to low RA-sensitive APL blasts (Quere et al., 2007). Collectively, these data show that RA metabolism and signaling play important roles in differentiation and cancer progression.

The use of liarozole or ketoconazole, both broad-spectrum cytochrome P450 inhibitors, to inhibit RA metabolism in various normal and cancer cell lines also resulted in increased intracellular RA levels (Sonneveld et al., 1999b). Recently, newer, more specific inhibitors of CYP26s, including the highly CYP26-specific R116010 compound, have been described (Armstrong et al., 2005; Njar et al., 2006; Van Heusden et al., 2002). One major limitation of general cytochrome P450 inhibitors is that they inhibit many or all CYPs, whereas our study targeted only Cyp26a1 and is therefore more specific to RA metabolism. Our results demonstrate that the disruption of this single gene results in increased intracellular RA, but in decreased sensitivity to growth inhibition by RA (Fig. 3).

Previously, our lab showed that the removal of LIF resulted in the increased differentiation and metabolism of retinol to 4-oxo-retinol in murine CCE ES cells without the addition of exogenous RA (Lane et al., 1999, 2008). Induction of Cyp26a1 mRNA was correlated with the conversion of retinol to 4-oxo-retinol, suggesting that Cyp26a1 was responsible for the increased metabolism (Lane et al., 1999). We show here that the absence of LIF also increased retinoid metabolism in murine AB1 ES cells and that this effect was observed in both Wt and CYP26A1\(^{-/-}\) ES cells (Fig. 2). More polar RA metabolites were detected in the samples without LIF than with LIF at the 8- and 24-h time points for Wt ES cells, and at the 24- and 48-h time points for CYP26A1\(^{-/-}\) ES cells. These data suggest that LIF removal results in increased RA metabolism at later times (>48 h) that is independent of CYP26A1 function in AB1 ES cells. The removal of LIF could be associated with increased retinoid uptake, increased metabolic enzyme activity, increased CYP26B1 or CYP26C1 activity, or decreased efflux of RA. Recently, the identification of Stra6 as the receptor for the serum retinol binding protein (RBP4) indicates that an active transport system for retinol exists, at least in some cell types (Kawaguchi et al., 2007).

CYP26A1\(^{-/-}\) ES cells are more resistant to RA-induced growth arrest

The molecular mechanisms involved in the anti-proliferative properties of RA have been shown to include both cell cycle arrest and apoptosis in tumor cell lines (Mongan and Gudas, 2007), but the mechanism by which RA inhibits growth in cells of embryonic origin remains unclear (Faria et al., 1999; Huynh et al., 2006; Osanai and Petkovich, 2005; Walkley et al., 2004). We have shown previously that in F9 EC cells, signaling through RAR\(\beta\)2 is required, since RAR\(\beta\)2 knockout cells exhibited no growth arrest in response to RA (Faria et al., 1999). RAR\(\beta\)2 mRNA levels, examined by semi-quantitative RT-PCR, were similar in both Wt and CYP26A1\(^{-/-}\) ES cells (data not shown). In addition, RA-induced growth arrest of F9 EC cells is associated with up-regulation of p27 protein and down-regulation of cyclin D1, D3, and E proteins (Li et al., 2004b).

Despite higher intracellular RA levels, CYP26A1\(^{-/-}\) ES cell were more resistant to RA-induced growth inhibition than Wt ES cells (Fig. 3). Polar RA and ROL metabolites have been shown to inhibit the proliferation of various cell types (Faria et al., 1998; Idres et al., 2001; van der Leede et al., 1997), so we speculate that their presence enhances ES cell growth arrest by RA. 4-oxo-RA has been shown to bind and transactivate RAR\(\alpha\) more efficiently than all-trans-RA (Idres et al., 2002), so the presence of 4-oxo-RA in Wt ES cells may heighten the growth arrest response of RA by acting through the RAR\(\alpha\) isoform.
Both RARα and RARγ mRNA levels, examined by microarray analyses, were similar between Wt and CYP26A1−/− ES cells (data not shown).

Newer CYP inhibitors have been generated and shown to inhibit RA metabolism specifically, as well as to exert retinoidal effects (Njar et al., 2006; Patel et al., 2004). Recently, studies with a second generation RAMBA, R116010, showed that a combination of R116010 and RA increased intracellular levels of RA in neuroblastoma cell lines by at least 2-fold, but the growth inhibitory effects of RA were enhanced by only a modest 11% when combined with R116010 (Armstrong et al., 2005). These results demonstrate that specific inhibition of CYP26A1, either by removal of the gene in our case or by inhibiting activity with R116010 (Armstrong et al., 2005), leads to increased intracellular RA levels, but that this increase does not result in a concomitant increase in growth inhibition. Thus, the effect of RA on growth inhibition is not as simple as previously believed, and the assumption that a higher internal concentration of RA equals more growth inhibition is not true in the CYP26A1−/− versus Wt ES cells.

The early-response, direct RA target gene, Hoxa1, is induced to a higher level in CYP26A1−/− ES cells

Hoxa1 gene is a direct, transcriptional RA target gene with a classical DR5 RARE in its regulatory region (Langston and Gudas, 1992; Langston et al., 1997). The high affinity RARE is very sensitive to retinoids and acts as a “direct readout” of intracellular RA concentration in ES cells (Dupe et al., 1997; Langston and Gudas, 1992). Ectopic Hoxa1 expression driven by a β-actin promoter in mice results in major developmental defects, including embryonic lethality (Zhang et al., 1994). Interestingly, as in CYP26A1−/− mice, rhombomeres of the developing hindbrain are transformed in mouse embryos with ectopic Hoxa1 expression (Abu-Abed et al., 2001; Sakai et al., 2001; Zhang et al., 1994). Specifically, rhombomeres 2 and 3 are transformed into a rhombomere-4-like identity, and this defect is seen in both CYP26A1−/− mice and mice with ectopic Hoxa1 expression. Previous studies from our laboratory showed that overexpression of ectopic Hoxa1 affected a wide range of genes, including genes encoding enzymes, growth factors, chromatin and cell cycle regulatory proteins, and other homeodomain-containing proteins (Shen et al., 2000). In this study, CYP26A1−/− ES cells exhibited higher levels of Hoxa1 mRNA than Wt ES cells after RA treatment (Fig. 4), indicating that the loss of CYP26A1 activity leads to an increase in retinoid signaling, including increased Hoxa1 transcriptional activation. These data indicate that the appropriate level of Hoxa1 is critical for normal embryonic development and that CYP26A1 can function to regulate Hoxa1 transcription by controlling the levels of intracellular RA.

Parietal endoderm differentiation is reduced in CYP26A1−/− ES cells

LamB1, J31 (also known as SPARC), and J6 encode proteins involved in organizing the extracellular matrix and assembling the basement membrane, which occurs during parietal endodermal differentiation (Breken and Sage, 2001; Ekbloom et al., 2003; Leivo and Wartiovaara, 1989; Li et al., 2004a). These RA-inducible, late-response genes are induced by RA with maximal expression around 48 to 72 h (Wang and Gudas, 1988; Wang et al., 1985). How RA metabolism plays a role in the transcriptional regulation of these genes remains unclear. To explain why these genes require a longer time to reach maximal induction by RA, we hypothesized that these late-response genes may require RA metabolism into polar RA metabolites for transcriptional activation (Fig. 9). While we observe reduced levels of expression of Lamb1, J31, and J6 mRNA in CYP26A1−/− ES cells compared to Wt ES cells, treatment with the polar RA metabolite, 4-oxo-RA, did not restore the expression of these genes to that found in Wt ES cells (Figs. 5 and 6). However, a caveat is that exogenously added 4-oxo-RA and RA may differ from each other in terms of cellular uptake. In addition, we cannot rule out the possibility that other polar RA metabolites, such as 4-OH-RA, are involved in the regulation of transcription of these RA-inducible late-response genes. The possibility that CYP26A1 metabolizes a non-retinoid that affects the expression of these “late” genes exists, even though there is no evidence for this. The reduced mRNA levels of these and other parietal endoderm-related genes in CYP26A1−/− ES cells indicate that CYP26A1 activity is required for proper endodermal differentiation. Lastly, some of the differences noted may reflect differences between the Wt parental ES cells and the Cyp26a1 knockout/knockout cells, and thus may not be due to inactivation of the Cyp26a1 gene. We did not examine the mRNA levels of all of the genes in the Cyp26a1 knockout/knockout cells, but rather compared the Wt ES cells with the Cyp26a1−/− lines in our analyses.

CYP26A1−/− ES cells express lower levels of genes involved in smooth muscle cell differentiation and migration

Major progress has been made to differentiate embryonic and adult stem cells into smooth muscle cells, but the exact factors involved are still unclear (Owens et al., 2004). RA has been shown to induce smooth muscle cell differentiation in P19 EC cells (Blank et al., 1995), murine ES cells (Drab et al., 1997), and human ES cells (Huang et al., 2006). The mRNA levels of Madh3, Nrp1, Tagln, calponin2, and caldesmon1 were lower in CYP26A1−/− ES cells than in Wt ES cells treated with RA for 72 h (Figs. 8 and 9, Table 3). Madh3, also known as Smad3, is an effector molecule of TGFβ signaling that acts as a transcription factor to mediate biological effects of TGFβ, which include differentiation of smooth muscle cells from ES cells (Kretschmer et al., 2003; Sinha et al., 2004). Neuropilin 1 (Nrp1) is a vascular endothelial growth factor (VEGF) receptor that is expressed in smooth muscle cells (Ishida et al., 2001). Calponin2 is important for migration of endothelial cells and for proper vascular development (Tang et al., 2006). Taken together, the down-regulation of these genes indicates that the lack of CYP26A1 may have a negative influence on smooth muscle cell differentiation and/or endothelial cell migration.
CYP26A1 function is important for proper differentiation of neuronal cells

Epha4, Nrp, Pmp22, Gap43, and Ndn are other genes involved in nervous system development that we found to be down-regulated in CYP26A1−/− ES cells relative to Wt ES cells treated with RA for 72 h (Figs. 8 and 9, and Table 3). Epha4 expression has been shown to be regulated by Krox-20, which is a transcription factor crucial for proper hindbrain patterning (Theil et al., 1998). Besides acting as a VEGF receptor to induce smooth muscle cell growth, Nrp also plays an important role in axon guidance by interacting with class 3 semaphorins (Van Vactor and Lorenz, 1999). Another gene that plays a role in nervous system development, but which we found to be up-regulated in CYP26A1−/− ES cells, was Hoxb4. We found increased expression of Hoxb4 in CYP26A1−/− ES cells treated with RA for 72 h, which was in agreement with a previous finding that Hoxb4 expression in the Cyp26a1-null mouse embryo was increased and had expanded beyond its normal boundaries in the somites (Sakai et al., 2001). Taken together, the aberrant expression of these neural-associated genes in CYP26A1−/− ES cells suggests that RA metabolism by CYP26A1 is necessary for proper differentiation of ES cells into neurons.

CYP26A1−/− ES cells express higher levels of genes involved in DNA metabolism and chromosome organization

The expression of histones is tightly coupled to DNA replication and mainly occurs during the S-phase of the cell cycle (Marzluff and Duronio, 2002). The correct stoichiometric amounts of core histones are necessary for proper nucleosome formation and chromosome function (Marzluff and Duronio, 2002). Inappropriate ratios of histone proteins can result in increased frequency of chromosome loss (Meeks-Wagner and Hartwell, 1986). Deletion of the Cyp26a1 gene resulted in greater than 2-fold increased expression of various histone genes, including Hist1h2bf, Hist1h2bc, Hist1h2bp, Hist1h1e, and Hist1h3c1, in all three conditions, A, B, and C, suggesting that CYP26A1 activity may be involved in replication-dependent gene transcription or regulation of the cell cycle (Fig. 9, Tables 1–4). Fen1 and Nxf1 were also expressed 2.0 and 2.1-fold higher, respectively, in CYP26A1−/− ES cells relative to Wt ES cells. Fen1 is responsible for removing Okazaki DNA–RNA flaps and for repairing DNA mismatches during DNA synthesis (Kao and Bambara, 2003; Kim et al., 1998; Stewart et al., 2006). Nxf1 encodes Nuclear RNA export factor 1, which mediates the general cellular export of mRNA from the nucleus for translation of the mRNA into protein (Erkmann and Kutay, 2004). Further analysis of CYP26A1−/− ES cells, cultured in the absence of RA, is warranted to determine the relationship between CYP26A1 activity and DNA synthesis. Our current hypothesis is that retinol in the serum may play a role in these differences in gene expression in the Cyp26a1−/− cells cultured without added RA, but this has not been tested.

Biological implications of the inhibition of RA metabolism in murine ES cells by the disruption of CYP26A1

The ability of ES cells to self-renew and differentiate into cells of all three germ layers makes ES cells a tool of great potential in the study of developmental biology as well as in the study of cell differentiation. Our microarray data suggest that CYP26A1 activity is involved in at least three differentiation programs in ES cells: parietal endoderm, smooth muscle, and neural differentiation (Fig. 9).

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Appendix A. Supplementary data


References


