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ScienceDirect
Developmental Biology 315 (2008) 331–354
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

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

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Received for publication 6 August 2007; revised 13 December 2007; accepted 17 December 2007
Available online 27 December 2007

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.

Keywords: Retinoic acid; Metabolism; Stem cells; Endoderm differentiation; Vitamin A; CYP26A1; Microarray; Knockout; Cytochrome P450; Review

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 oxidization 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).

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doi:10.1016/j.ydbio.2007.12.021

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). 1×10^3 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}–5×10^{-6} M. [^{3}H]RA and [α-^{32}P]dCTP were obtained from DuPont NEN (Boston, MA). All experiments involving retinoids were performed in dim light.

For cell proliferation studies, ES cells were plated on 12-well plates at a density of 1×10^3/well for the cell doubling experiments and 1×10^4/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 *Bgl*II restriction site, and a PGK-neomycin cassette in intron 6 that is flanked by *loxP* sites (Fig. 1A). The *Eco*RI-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 microfarads. 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 *Bgl*II for Southern blot analysis. The two cell lines were expanded in the presence of 6 mg/ml active G418 to select for cell