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COUP-TFI and -TFII nuclear receptors are expressed in amacrine cells and play roles in regulating the differentiation of retinal progenitor cells

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ABSTRACT

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are members of the steroid/thyroid hormone receptor superfamily. We have shown that two homologous COUP-TF genes, *COUP-TFI* and *COUP-TFII*, are expressed in developing mouse retina with a unique gradient along the dorsal–ventral axis. In this work, we aimed to characterize the detailed expression patterns of COUP-TFs in mature retina. Their functions in retinal progenitor cell differentiation into subtypes of mature retinal cells were also examined. Immunostaining of frozen mouse retinal sections with antibodies against COUP-TFs and markers for retinal subtypes revealed that COUP-TFI and -TFII are expressed in amacrine cells, especially in a glycinergic subtype in mature mouse retina. Forced expression of COUP-TFI and -TFII in mouse retinal explant culture by retrovirus-mediated gene transfer promoted amacrine and cone photoreceptor cell differentiation, whereas that of rod photoreceptors decreased. Cell proliferation and apoptosis were not affected by the perturbation of COUP-TFI and -TFII expression levels. Using the Y79 retinoblastoma cell line, we observed that COUP-TFI and -TFII suppressed the transcriptional activation of the *Nrl* gene. We then analyzed one another member of COUP-TF transcription factors, COUP-TFγ, whose structure is relatively distant from those of COUP-TFI and -TFII. It is expressed mainly in horizontal cells and has weak activity in inducing amacrine cells when COUP-TFγ was ectopically expressed in retinal explants. In summary, we found that COUP-TFI and -TFII play roles in amacrine cell differentiation, and COUP-TFγ has distinct expression pattern and roles during retinal development.

1. Introduction

Nuclear receptors (NRs), a large family of ligand-dependent transcription factors, are involved in gene networks during embryonic development, as well as in adult physiology. Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are highly conserved members of the steroid/thyroid hormone receptor superfamily (Tsai and Tsai, 1997). Mice contain two COUP-TF homologs, COUP-TFI and -TFII, which possess an exceptionally high degree of homology at the amino acid level, with 99% identity in the DNA-binding domain and 96% identity in the putative ligand-binding domain (Qiu et al., 1997; Tsai and Tsai, 1997). COUP-TFs are expressed in several organs during embryonic development, and COUP-TFI-null mice die perinatally from starvation and dehydration and exhibit defects in neuronal development and axon guidance (Qiu et al., 1997). In contrast, COUP-TFII-null mutants exhibit defects in angiogenesis and heart development and die before embryonic day (E) 10.5 (Pereira et al., 1999). The importance of COUP-TFs in retinogenesis was demonstrated initially in *Drosophila*. The *Drosophila* homolog of COUP-TF, seven-up (svp), is involved in the determination of photoreceptor cell fate (Mlodzik et al., 1990). In addition, the zebrafish COUP-TF homologs svp[40] and svp[44] are expressed in the retina (Fjose et al., 1993). However, the role of COUP-TFs in vertebrate retinal development has not been demonstrated. There is one other member of COUP-TF family, COUP-TFγ (also known as Nr2f6 or Ear2), which has only limited homology with other members (Avram et al., 1999).

We have found that COUP-TFI and -TFII are expressed in the inner nuclear layer (INL) of mouse retina, especially in amacrine cells. Amacrine cells are interneurons present in the INL and ganglion cell layer (GCL); they create synapses on bipolar cell terminals and ganglion cell dendrites and modulate the synaptic connection between bipolar and ganglion cells (Kolb, 1997). Multiple morphologically and functionally distinct subtypes of amacrine cells exist. In the mouse retina, at least 26 morphological types of amacrine cells have been identified (MacNeil and Masland, 1998). The majority contain either glycine- or GABA-inhibitory neurotransmitters, and are categorized into two major groups,

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glycinergic and GABAergic amacrine cells (Vaney et al., 1998). Glycinergic amacrine cells account for ~35% of the amacrine cell population in mouse (Marquardt et al., 2001) and are usually small-field amacrine cells with diffuse dendritic trees (Menger et al., 1998). GABAergic amacrine cells generally have wider dendritic fields than those of glycinergic cells and account for ~40% of amacrine cells in the mouse retina (Marquardt et al., 2001). Additionally, other minor amacrine subtypes are present, including cholinergic and dopaminergic cells, which express choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH), respectively.

In this study, we found that COUP-TFI, -TFII, and -TFγ are expressed in most INL cells in mature retina, and the overexpression of one of them perturbed retinal progenitor cell differentiation. COUP-TFI and -TFII promote a subset of amacrine cells and suppress rod photoreceptor differentiation. These results suggest that the COUP-TFs play a critical role in retinal development.

2. Material and methods

2.1. Mice and reagents

ICR mice were obtained from Japan SLC Co. All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo. Nine-cis-retinoic acid (RA) was obtained from Sigma (St. Louis, MO) and stock solution was prepared in dimethylsulfoxide.

2.2. DNA construction and retrovirus production

The mouse *COUP-TFI*-, *COUP-TFII*-, and *COUP-TFγ*-open reading frames (ORFs) were cloned by RT-PCR using mouse retinal RNA, and the products were inserted into pGEM-T-Easy vector (Promega, Madison, WI). The ORF fragments were subcloned into pMX-IRES-EGFP (Tabata et al., 2004) to obtain the retrovirus expression vector designated as pMX-COUP-TFI-IRES-EGFP, pMX-COUP-TFII-IRES-EGFP, or pMX-COUP-TFγ-IRES-EGFP. To obtain mouse *Nrl* reporter plasmid (pGL4-Nrl), 1.2 kb *Nrl* gene upstream promoter region (Khanna et al., 2006) was amplified by PCR using mouse genomic DNA and subcloned into pGL4.10 (Promega). Retrovirus production was described previously (Tabata et al., 2004) using the retrovirus packaging cell line PLAT-E (Morita et al., 2000).

2.3. Cell lines and retinal explant culture

Y79 cells (Reid et al., 1974) were obtained from the Riken Cell Bank (no. RCB1645) and cultured in RPMI 1640 medium (Nakarai, Tokyo, Japan) supplemented with 10% fetal calf serum. Retinal explant cultures and retrovirus infection were prepared as described previously (Tabata et al., 2004).

2.4. Immunostaining

Immunostaining of frozen-sectioned eyes or retinal explants was done as described previously (Tabata et al., 2004) using the following primary antibodies: mouse monoclonal antibodies against COUP-TFI (PPMX, Tokyo, Japan), COUP-TFII (PPMX), COUP-TFγ (PPMX), RXRγ (PPMX), rhodopsin (Rho4D2, kind gift from Dr. RS Molday, The University of British Columbia), glutamine synthetase (GS, Chemicon, Temecula, CA), HuC/D (Invitrogen, San Diego, CA), and PKC (Oncogene, Boston, MA), goat polyclonal antibodies against glycine transporter 1(Chemicon) and choline acetyltransferase (Chemicon), rabbit polyclonal antibodies against S-opsin (1:2000, Chemicon), M-opsin (1:2000, Chemicon), γ-aminobutyric acid (Chemicon), Calbindin D28k (Chemicon), parvalbumin (Sigma), and GFP (Takara, Tokyo, Japan), and rat monoclonal antibody against GFP (Nakarai). Binding of primary antibodies was visualized using appropriate secondary antibodies conjugated to Alexa-488 or Alexa-594 (Invitrogen). In the case of double-immunostaining using two mouse antibodies of the same isotype, we used Zeon Tricolor Mouse IgG2a labeling kit (Invitrogen) as manufacturer's recommendation. DAPI was used for nuclear staining. The samples were mounted in 50% glycerol and analyzed using a Zeiss Axioplan.

2.5. Semi-quantitative and quantitative RT-PCR

Y79 cells were transfected with pMX-IRES-EGFP, pMX-COUP-TFI-IRES-EGFP or pMX-COUP-TFII-IRES-EGFP plasmid by GeneJuice Transfection Reagent (Merck). After 1 day of transfection, cells were treated with or without 5 μM RA, and cultured for 1 day. The EGFP-positive cells were isolated using FACSAria (BD Biosciences, San Diego, CA). Total RNAs were purified from sorted EGFP-positive cells using the TRIZOL reagent (Invitrogen), and cDNAs were synthesized by Superscript II (Invitrogen). Semi-quantitative PCR was performed using rTaq (Takara). We performed three independent experiments and obtained essentially the same results. To quantify the levels of G3PDH and Nrl mRNA expression, SYBR Green-based quantitative PCR (qPCR) was performed using the Roche LightCycler 1.5 apparatus and was analyzed by the Second Derivative Maximum Method for quantification (Roche).

2.6. Luciferase assay

For *Nrl* promoter assay, Y79 cells transfected with pMX-IRES-EGFP, pMX-COUP-TFI-IRES-EGFP or pMX-COUP-TFII-IRES-EGFP plasmid together with pGL4-Nrl plasmid were cultured in the presence or absence of RA for 1 day, and then harvested with passive lysis buffer (Promega). Luciferase activity was examined by a luminometer. Values were expressed as relative luciferase activities to protein concentration. Experiments were performed in triplicate, and all assays were repeated at least two times.

2.7. Fluorescence activated cell sorting (FACS) analysis

Retinal explants were dispersed to single cells using trypsin, and then stained with antibodies, as described previously (Koso et al., 2006). The following antibodies were used: anti-Ki67 (BD Biosciences); anti-CD73 (BD Biosciences); anti-rhodopsin (Rho4D2). Non-labeled antibodies were visualized with the appropriate secondary antibody conjugated with phycoerythrin (BD Biosciences). Cells were analyzed in FACSCalibur (BD Biosciences).

3. Results

3.1. COUP-TFs are expressed in most cell subtypes in the INL of the mature retina

We first examined the detailed expression patterns of COUP-TFI and -TFII in mature mouse retina using immunostaining. Almost all of the cells in the INL expressed COUP-TFI at variable levels (Fig. 1A). COUP-TFII was detected broadly on the dorsal side of the INL, and only in small subsets of cells on the ventral side (Fig. 1A). Very weak expression of COUP-TFII was detected in the ONL on the dorsal side. We then examined which subtypes of retinal cells expressed COUP-TFs in the INL, using double immunostaining with antibodies against COUP-TFs and marker proteins for retinal cell subtypes along with frozen sectioned retina prepared from mouse at P15. The markers examined in the INL included glutamine synthetase (GS), HuC/D, PKC, and Islet1, which are markers for Müller glia, amacrine, bipolar, and bipolar/amacrine cells, respectively. All of these markers