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TGIF, a homeodomain transcription factor, regulates retinal progenitor cell differentiation

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ABSTRACT

TG-interacting factor (TGIF) is a TALE homeodomain protein expressed predominantly in the central nervous system and functions as a transcriptional repressor. Several mutations in *TGIF* have been identified in patients with holoprosencephaly, the most common congenital malformation of the developing human forebrain. However, the precise role of TGIF in neural development is not well understood. We found that TGIF was expressed strongly in the mouse retina during early stages of development, and that its expression gradually decreased as retinal development progressed. *In vitro* explant cultures of mouse retina mimic the *in vivo* development of retinal subtypes. Forced expression of TGIF using a retrovirus in explant culture induced the differentiation of amacrine cells from retinal progenitor cells. A TGIF paralog, TGIF2, showed a similar transition in expression during retinal development, and TGIF2 also promoted amacrine cell differentiation in a retinal explant culture system. However, no apparent difference between wild-type and TGIF-knockout mouse retina was observed, suggesting that TGIF and TGIF2 function redundantly in that tissue. Forced expression of TGIF homeodomain (HD)-EnR (repressing) rather than TGIF HD-VP16 (activating) resulted in a phenotype similar to that induced by wild-type TGIF, suggesting that TGIFs may act as transcriptional repressors to induce amacrine genesis.

1. Introduction

The vertebrate neural retina consists of six types of neurons and Müller glial cells, which are organized into a laminar structure. During retinogenesis, these cell types are derived from a common population of multipotent retinal progenitor cells in a relatively fixed chronological sequence (Marquardt and Gruss, 2002). Intrinsic cues and extrinsic signals play critical roles in defining the types of cells generated from the retinal progenitors (Harris, 1997; Cepko, 1999). During development, retinal progenitor cells are believed to change their intrinsic properties at some point in their environmental transition, so that they can respond to extrinsic signals and generate the appropriate types of retinal cells (Cepko et al., 1996). This retinal competence model suggests that retinal progenitors are not a temporally homogeneous population of cells. Such heterogeneous intrinsic properties had been studied at the molecular level using high-throughput screening technologies such as SAGE and microarray (Mu et al., 2001; Blackshaw et al., 2004; Livesey et al., 2004; Zhang et al., 2006; Trimarchi et al., 2008).

Homeodomain proteins play important roles in regulating many developmental processes, including retinogenesis, acting as either transcriptional activators or repressors (Marquardt, 2003). TG-interacting factor (TGIF) is a three-amino-acid loop extension (TALE) homeodomain protein that functions as a transcriptional repressor (Bertolino et al., 1995; Wotton et al., 1999a, b). Pbx1, Prep1, and the Meis proteins are also members of the TALE family, and all of them have been identified in diverse species (Burglin, 1997). TGIF was initially identified by its ability to bind to a retinoid X receptor response element in the cellular retinol-binding protein II promoter, and the preferred DNA sequence to which TGIF binds was identified *in vitro* (Bertolino et al., 1995). It has recently been demonstrated that TGIF interacts with RXRα and subsequently recruits a corepressor, CtBP, to RXRα, resulting in the inhibition of retinoid X receptor-dependent transcription (Bartholin et al., 2006). The interaction between TGIF and RXRα can be inhibited by the addition of retinoic acid (RA), which is consistent with the observation that TGIF represses RXRα-dependent transcriptional activation. In addition, TGIF represses TGFβ-activated transcription (Wotton et al., 1999a, b). TGIF binds to Smad2, a key substrate of the receptors for TGFβ signalling, and competes for Smad2 binding with factors that promote TGFβ-mediated transcriptional activation, such as p300/CBP. As a result, corepressor complexes replace the coactivator complexes. Interestingly, both RA and TGFβ signals

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are involved in retinal development (Kastner et al., 1997; Ikeda et al., 1998; Close et al., 2005). However, it is not known whether TGIF is also involved in this process.

Holoprosencephaly (HPE) is the most common birth defect in forebrain and craniofacial development (Cohen, 2006). HPE is aetiologically heterogeneous, with both environmental and genetic causes. Mutations in several genes have been identified in human HPE, and more than 10 mutations or truncations in the *TGIF* gene have been reported in HPE patients to date (Gripp et al., 2000; Cohen, 2006). This suggests that TGIF plays important roles in central nervous system development. However, TGIF-null mice had no apparent abnormalities in any major organ system, including the forebrain (Shen and Walsh, 2005; Bartholin et al., 2006; Jin et al., 2006; Mar and Hoodless, 2006).

We found that TGIF was expressed in developing neural retina and examined its role in retinogenesis by gain-of-function analysis using a retinal explant culture system. TGIF was found to bias the fate of retinal progenitor cells in favour of amacrine cells. In addition, a TGIF paralog, TGIF2, also showed a similar effect on retinogenesis in a retinal explant culture system when it was overexpressed. However, detailed examination of TGIF-knockout mice showed no apparent phenotype in retinal structure or in the cell types present. Taken together, these results suggest that TGIF and TGIF2 function redundantly in the retina.

2. Materials and methods

2.1. Mice

ICR and C57BL/6J mice were obtained from Japan SLC Co. TGIF-knockout mice were kindly provided by Dr C.A. Walsh (Beth Israel Deaconess Medical Center) (Shen and Walsh, 2005). The day that the vaginal plug was found was designated as embryonic day 0 (E0) and the day of birth as postnatal day 0 (P0).

2.2. Retinal explant culture, re-aggregation culture, and retroviral infection

Retinal explant cultures were prepared as described previously (Tabata et al., 2004). Re-aggregation cultures were prepared as described previously (Koso et al., 2006), with minor modifications. One day after retrovirus infection of retinal explant cultures, cells were dissociated and re-aggregated for clonal analysis.

2.3. FACS analysis

FACS analysis was used to monitor the proliferation and expression of rhodopsin in retinal cells from explant cultures using monoclonal antibodies against Ki67 (1:140, BD Clontech) and rhodopsin (Rho4D2, 1:100, kindly donated by Dr R.S. Molday, The University of British Columbia), respectively, with a FACSCalibur (Becton-Dickinson) and Cell Quest™Pro ver5.1.1 software (BD Bioscience) (Koso et al., 2006).

2.4. DNA construction

The mouse TGIF and TGIF2 open reading frames (ORFs) were cloned by RT-PCR using mouse retinal cDNA, and the products were cloned into pGEM-T-Easy vector (Promega). The primers used for amplification were as follows: TGIF-F, 5′-ATG AAA AGC AAG AAG GGT CTT G-3′; TGIF-R, 5′-TTA AGC TGT GAG TTT GGC CT-3′; TGIF2-F, 5′-ATG TCG GAC AGC GAT CTA G-3′; and TGIF2-R 5′-CTA CTT GGC GTT TTC TGA GA-3′. Retroviral vectors containing TGIF (pMX/TGIF-IE) and TGIF2 (pMX/TGIF2-IE) were made by inserting each ORF fragment into the NotI site of pMX-IRES-EGFP.

Construction of TGIF homeodomain (HD)-VP16, which consisted of the VP16 transcriptional activation domain (amino acids 414–490) fused to the C-terminus of the HD (amino acids 31–112) of TGIF, was made by inserting the coding regions of the TGIF HD into pCS2-VP16. The TGIF HD-VP16 fragment was then subcloned into pMX-IRES-EGFP to produce pMX/HD-VP16. TGIF HD-EnR was constructed by fusing the engrailed transcriptional repressor domain (amino acids 1–298) from pCS2-EnR to the C-terminus of the TGIF HD. TGIF HD-EnR was subcloned into pMX-IRES-EGFP to produce pMX/HD-EnR. pCS2-VP16 and pCS2-EnR were kindly provided by Drs R. Davis and M. Watanabe.

A luciferase reporter plasmid containing three tandem repeats of the TGIF-HD binding consensus sequence (Bertolino et al., 1995) was constructed by inserting the double-stranded oligonucleotide (5′-CTA GCT GTC ACA GCT GTC AGG ATC CTG TCA C-3′) into the NheI and XhoI sites of pGL3/S-opsin pro (Lin et al., in press).

2.5. Luciferase reporter assay

Y79 retinoblastoma cells were grown in RPMI (Nakarai) with 10% FCS and seeded in a 12-well plate. The cells were transfected with 100 ng of reporter and 900 ng of plasmid pMX-IRES-EGFP, pMX/HD-VP16, pMX/HD-EnR, pMX/TGIF-IE or pMX/TGIF2-IE by GeneJuice (Merck). After 48 h of culture, cells were harvested and luciferase activity was examined. Luciferase activity was normalized to lysate protein concentration. Determinations were performed in triplicate, and each experiment was repeated more than twice.

2.6. Immunostaining

Immunostaining of frozen sectioned retina or retinal explants was done as described previously (Tabata et al., 2004) using the following primary antibodies: mouse monoclonal antibodies against HuC/HuD (1:500, Molecular Probes), PNR (1:500, PPMX), PKC (1:100, Oncogene), cyclin D3 (1:500, Santa Cruz Biotechnology), glutamine synthetase (1:500, Chemicon), rhodopsin (Rho4D2, 1:500), and parvalbumin (parv-19, 1:100, Sigma), and rabbit polyclonal antibodies against GFP (1:5000, Clontech), Pax6 (1:500, Covance), Calbindin D28k (1:500, Chemicon), GABA (1:200, Chemicon), tyrosine hydroxylase (1:500, Chemicon), and active caspase3 (1:250, Promega). The primary antibodies were visualized using appropriate Alexa-488- or Alexa-546-conjugated secondary antibodies (Molecular Probes). The samples were mounted in a VectaShield (Vector Laboratories) and analyzed using a Zeiss Axioplan.

2.7. Semi-quantitative RT-PCR

Total RNA was purified from retinal cells at various time points using TRIZOL reagent (Invitrogen), and cDNA was synthesized using Superscript II (Invitrogen). All primer sets were tested using different numbers of cycles (20–30 cycles) with rTaq (Takara), and the semi-quantitative cycle number was determined for each primer set. The products were visualized with ethidium bromide.

2.8. In situ *hybridization*

In situ hybridization was performed using a DIG (digoxigenin)-labelled RNA probe as described previously (Zhao et al., 2007).