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The LIM homeobox transcription factor Lhx2 is required to specify the retina field and synergistically cooperates with Pax6 for Six6 trans-activation

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ABSTRACT

In mammals, a limited set of homeobox-containing transcription factors are expressed in the presumptive eye field and required to initiate eye development. How these factors interact together at the genetic and molecular level to coordinate this developmental process is poorly understood. We found that the Lhx2 and Pax6 transcription factors operate in a concerted manner during retinal development to promote transcriptional activation of the *Six6* homeobox-gene in primitive and mature retinal progenitors. *Lhx2* demarcates the presumptive retina field at the neural plate stage and *Lhx2* inactivation delays initiation of *Rx*, *Six3* and *Pax6* expression in this domain. The later expressed *Six6* is properly activated in the pituitary/hypothalamic axis of *Lhx2*$^{-/-}$ embryos, but expression fails to be initiated in the optic vesicle. Lhx2 and Pax6 associate with the chromatin at several regions of *Six6 in vivo* and cooperate for trans-activation of *Six6* regulatory elements *in vitro*. In retinal progenitor/stem cells, both *Lhx2* and *Pax6* are genetically required for proper *Six6* expression and forced co-expression of Lhx2 and Pax6 can synergistically trans-activate the *Six6* locus. Our work reveals how two master regulators of eye development coordinate their action to sequentially promote tissue-specific transcriptional initiation and full activation of a retinal determinant gene.

Introduction

The optic vesicle of vertebrates originates from the neural tube and is first detected between embryonic stages (e) 8.25 and e8.5 as a bilateral evagination of the anterior neural plate, named the optic sulcus (Marquardt, 2003; Oliver and Gruss, 1997). Around e9.5, the intimate contact between the optic vesicle and the eye surface ectoderm induces the transformation of the ectoderm into a lens placode, which is revealed by the proliferation and thickening of the ectoderm (Oliver and Gruss, 1997). In turn, the lens placode induces the evagination of the optic vesicle into an optic cup around e10.0. The optic cup ultimately gives rise to the fully mature neural retina. How retinal specification is established at the neural plate stage and how definitive retinal identity is promoted and maintained in retinal progenitors remain important issues in developmental biology. The homeobox-containing transcription factors Pax6, Rx, Six3 and Lhx2 are genetically required for eye formation and over-expression of Pax6, Rx or Six3/Six6 can induce ectopic retinal tissues in frog and fish embryos (Bernier et al., 2000; Carl et al., 2002; Chow et al., 1999; Hill et al., 1991; Loosli et al., 1999; Mathers et al., 1997; Porter et al., 1997; Zuber et al., 1999). Although the genetic function of these factors has been highly studied, the molecular mechanisms by which they coordinate retinal development and establish definitive retinal identity remain poorly understood.

Pax6 is a member of the paired-box and homeobox-containing gene family (PAX) of transcription factors and has been used as a prototype to study eye development in several model organisms (Gehring and Kazuho, 1999; Gehring, 2002). In mice, *Pax6* is expressed starting at e8.0 in the eye surface ectoderm, and in the eye neural ectoderm, which gives rise to the optic vesicle (Walther and Gruss, 1991). Despite being anophthalmic at later stage of development, *Pax6*-null embryos form an optic vesicle that arrests in development prior to the optic cup stage (Grindley et al., 1995; Hogan et al., 1986). In the optic vesicle of *Pax6* mutants, neuroepithelial (NE) progenitors over-proliferate and display an abnormal cell cycle kinetic, possibly owing to downregulation of cyclin-dependent kinase inhibitors (Duparc et al., 2007). Conditional mutagenesis of *Pax6* in the e11.0 distal retina revealed that *Pax6* is required at the time of retinogenesis to maintain progenitor cells proliferation and generate retinal cell types diversity, in part through transcriptional activation of pro-neural genes (Marquardt et al., 2001). Although *Pax6* is being considered to operate at the apex of the genetic cascade governing eye and retinal development, expression of *Lhx2*, *Rx*, *Otx2*, *Six3* and *Six6* in the optic vesicle of *Pax6* mutants is unaffected, revealing that early retinal specification does occur in the absence of *Pax6* (Bernier et al., 2001; Jean et al., 1999).

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Lhx2 is a transcription factor that plays an essential role in mammalian's eye development and that is conserved in lower vertebrate species (Porter et al., 1997; Zuber et al., 2003). *Lhx2* encodes a member of the LIM homeobox-containing transcription factors family (*LHX*). LHX proteins can activate or repress gene transcription by direct DNA binding and association with co-activators or co-repressors through their LIM domain (Agulnick et al., 1996). In mice, *Lhx2* is required for the development of numerous organs, including the eye, the telencephalon and blood system (Porter et al., 1997). While little is known about how *Lhx2* operates, experiments have showed that red blood cell defects in *Lhx2*-null mice are non-cell autonomous and mediated by abnormalities that lie within the liver (Porter et al., 1997). In contrast, most abnormalities present in the CNS appear to be cell autonomous, as revealed by chimera aggregation studies (Porter et al., 1997). *Lhx2* is also expressed by immature B and T lymphocytes, but not by hematopoietic stem cells (Wu et al., 1996). Notably, some chromosomal translocations involved in human leukemia's appear to include the *LHX2* locus, and Lhx2 over-expression can immortalized human hematopoietic stem cells (Wu et al., 1996). Recent genetic studies have also revealed that *Lhx2* is required for the self-renewal of epithelial stem cells, but the underlying molecular mechanism remains elusive (Rhee et al., 2006).

Herein we report on the characterization of *Lhx2* function during the earliest steps of retinal development. We found that *Lhx2* represents the first retinal determinant gene with Rx within the presumptive retina field, and that *Lhx2* mutation results in delayed induction of *Six3*, *Rx* and *Pax6* expression in this domain. Later on, *Lhx2* is also required for *Six6* expression initiation in the optic vesicle. In retinal tissue, Lhx2 and Pax6 proteins are bound to the chromatin at the *Six6* locus, and can cooperate to trans-activate Six6 regulatory elements *in vitro*. In retinal progenitor/stem cells, Lhx2 and Pax6 are genetically required for *Six6* expression and can cooperate to synergistically trans-activate the *Six6* gene. Our work reveals that *Lhx2* is required to establish primitive retinal identity at the neural plate stage by allowing initiation of retinal-determinant genes expression, and how later *Lhx2* cooperates with *Pax6* to establish definitive retinal identity and promote cellular proliferation.

Materials and methods

Animals

Adult mice from the albinos CD1 or 129sv strains were purchased from Charles River (St-Constant, Qc., Canada). *Pax6* mutant mice are a gift from Peter Gruss (Max-Planck Institute, Goettingen) and *Lhx2* mutant mice from Heiner Westphal (National Institute of Health, Bethesda). Embryos stage was determined according to the time of vaginal plug.

In situ hybridization

Embryos were dissected in PBS, fixed overnight in 4% paraformaldehyde at 4 °C and embedded in Paraplast (Monoject Scientific). Sections (10 μm) were cut and dried onto super-frost glass slides (Fisher Scientific). ^{35}S-labelled RNA probes using SP6, T3 or T7 RNA polymerase were done with Boehringer enzyme according to the directive of the company. Exposure time for the radioactive RNA *in situ* hybridization was 15 days. For *in situ* hybridizations on whole embryos, preparations were hybridized with digoxigenin-labelled RNA probes and visualized with alkaline phosphatase-coupled anti-digoxigenin antibody (1/2000) (Boehringer) and NBT/BCIP substrate (Boehringer) at pH 9.5. For cryosection, embryos were cryoprotected in 30% sucrose/PBS overnight at 4 °C, embedded in cryomatrix solution and snap-frozen in liquid nitrogen. Specimens were cut using a cryostat (Leica) at 10 μm and used for non-radioactive *in situ* hybridization.

RT-PCR and quantitative real-time PCR

All primers were designed to flank individual exons and tested by PCR in RT+ and RT− control extracts. Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed using 1 μg of total RNA and the MML-V reverse transcriptase (Invitrogen). PCR amplification was performed using the HotStar TAQ polymerase (Invitrogen). PCR was run as follow; 94 °C for 10 min, followed by 30 cycles of denaturing at 94 °C, annealing at 57 °C and extension at 72 °C in an Applied Biosystems thermal cycler. Real-time PCR was performed using the Platinum SYBRGreen SuperMix (Invitrogen) and a Real-Time PCR apparatus (BioRad).

Chromatin immunoprecipitation

ChIP was performed using the ChIP Assay kit (Upstate) according to the manufacturer's instructions. Briefly, 0.5×10^6 cells were sonicated to shear the chromatin. Chromatin-associated proteins were precipitated using goat anti-Lhx2 (Santa Cruz), rabbit anti-Pax6 (US Biological) or rabbit anti-IgG (Upstate) antibodies. Samples were heated to reverse the protein–DNA crosslinks and the DNA recovered by phenol/chloroform/isoamyl alcohol extraction. Genomic DNA was used as template for PCR amplification using primers to the *Six6* loci.

Luciferase assay

Four distinct genomic DNA fragments from the *Six6* locus (−3964 to −2465, −2480 to −1263, −1284 to −75 and +638 to +1703) were cloned into the pGL3 Luciferase Reporter Vector (Promega). *Pax6*, *Lhx2*, *Six3* and *Otx2* cDNAs were cloned into the expression vector pCS2+. Reporter vectors were transfected with either one expression vector or a combination using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, cells were lysed using Passive Lysis Buffer (Promega). Cells lysates were analyzed using the Dual Glow Luciferase Assay System (Promega).

Cell culture

Optic vesicles of e9.5 embryos were dissected-out with tungsten needles in HBSS, as described (Duparc et al., 2007). Optic vesicles were directly triturated in HBSS using needles (20G-10x; 22G-5x), in order to obtain a suspension of single cells. After centrifugation, cells were placed in neural stem cell (NSC) media: DMEM/F12 (Invitrogen) containing 0.25% glucose, B27 supplement, Heparin (2 μg/ml; SIGMA), Gentamycin (25 μg/ml; Invitrogen) and human recombinant FGF2 (10 ng/ml; Preprotech). Cells were cultured in 6 well plates (Sarstedt) for 3 to 10 days at 37 °C in 5% CO_2 atmosphere. When applicable, BrdU (SIGMA) was added to the culture media at 10 μg/ml. For passage, single retinal spheres were dissociated with an enzyme-free solution (CHEMICON). After trituration, the single cell suspension was harvested at 300 g for 5 min and washed twice with HBSS. Cells were plated at 2000 cells/ml in neural stem cell media. Cell viability was evaluated using a hemacytometer and trypan blue exclusion staining.

Immunofluorescence and immunohistochemistry

For BrdU-labeling experiments, retinal spheres were directly frozen in liquid nitrogen and post-fixed after sectioning using 100% ETOH for 30 min and 4% PFA/PBS for 10 min. Sections or cells were treated with DNase I/0.05% HCl for 30 min in order to reveal BrdU epitopes. Samples were blocked in 1% BSA (Vector laboratories)/0.1% Tween 20/PBS solution and incubated with the primary antibodies overnight at 4 °C. After washes with PBS, samples were incubated with appropriate secondary antibodies for 1 h at RT. Antibodies