Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 301 (2007) 374–387
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Pax6 controls the proliferation rate of neuroepithelial progenitors from the mouse optic vesicle

Robert-Hugues Duparc [a], Mohamed Abdouh [a], Jocelyn David [a], Mireille Lépine [a], Nicolas Tétreault [a], Gilbert Bernier [a,b,*]

[a] *Developmental Biology Laboratory, Maisonneuve-Rosemont Hospital, 5415 Boul. l'Assomption, Montreal, Canada H1T 2M4*
[b] *Department of Ophthalmology, University of Montreal, Montreal, Canada*

Received for publication 11 May 2006; revised 7 September 2006; accepted 4 November 2006
Available online 10 November 2006

Abstract

In vertebrates, a limited number of homeobox-containing transcription factors are expressed in the optic vesicle primordium and are required and sufficient for eye formation. At present, little is known about the distinct functions of these factors in optic vesicle growth and on the nature of the main neuroepithelial (NE) progenitor population present in this organ. We have characterized a multipotent cell population present in the mouse optic vesicle that shows extensive proliferation potential and which expresses NE progenitor and retinal markers *in vitro*. In *Pax6* mutant embryos, which form an optic vesicle, we found that the number of resident NE progenitors was greater than normal. *In vitro*, *Pax6*-null NE progenitors overproliferate and display reduced $p16^{Ink4a}$, $p19^{Arf}$, $p27^{kip1}$, $p57^{kip2}$, and $p21^{cip1}$ expression. Pax6 overexpression repressed cellular proliferation and secondary colonies formation, supporting the hypothesis that Pax6 acts cell-autonomously on NE progenitors cell cycle. Notably, these *in vitro* data correlated with aberrant numbers of mitosis observed in the optic vesicle of early stage *Pax6* mutants, with Pax6 association with the chromatin upstream of $p27^{kip1}$ promoter region, and with reduced expression levels of $p27^{kip1}$, $p57^{kip2}$, and $p21^{cip1}$ in the primitive forebrain of *Pax6* mutants. Taken together, our results suggest that, prior to retinal progenitor cell identity and neurogenesis, *Pax6* is required to regulate the proliferation rate of NE progenitors present in the mouse optic vesicle.
© 2006 Elsevier Inc. All rights reserved.

Keywords: Pax6; Optic vesicle; Retina; Homeobox transcription factor; Cell cycle; Neuroepithelial progenitors; Neurosphere assay; Lentivirus

Introduction

Neuroepithelial (NE) progenitors represent the most primitive neural progenitors from which radial glial cells and adult neural stem cells of the central nervous system (CNS) are thought to derive (Gotz and Huttner, 2005; Huttner and Kosodo, 2005). NE progenitors form a stratified epithelium that lies within the ventricular wall of the developing neural tube (Huttner and Kosodo, 2005). The optic vesicle originates from the neural tube and is first detected around embryonic stage (e) 8.25 as a bilateral evagination of the anterior neural plate, named the optic sulcus (Oliver and Gruss, 1997; Marquardt, 2003). 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 neural retina and optic nerve fibers, while the surrounding neuroepithelium gives rise to the retinal pigment epithelium, optic stalk, and optic nerve myelinating sheet. These developmental processes are controlled by several morphogens and transcription factors which expression is finely regulated in time and space. One of these factors, 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, 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). Mice,

* Corresponding author. Maisonneuve-Rosemont Hospital, 5415 Boul. l'Assomption, Montreal, Canada H1T 2M4. Fax: +1 514 252 3569.
E-mail address: gbernier.hmr@ssss.gouv.qc.ca (G. Bernier).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.11.006

humans, and flies carrying homozygous mutations in *Pax6* are eyeless (Hogan et al., 1986; Glaser et al., 1994; Quiring et al., 1994; Grindley et al., 1995). Conversely, overexpression of *Pax6* in flies or frog embryos results in the formation of ectopic eyes, supporting the hypothesis that *Pax6* operates as a "master regulator of eye development in multi-cellular organisms" (Halder et al., 1995; Chow et al., 1999; Gehring, 2002; Gehring and Kazuho, 1999). 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 (Hogan et al., 1986; Grindley et al., 1995). Tissue recombination and chimera aggregation experiments have revealed that *Pax6* is required cell autonomously for the specification of the lens ectoderm and formation of the lens placode (Fujiwara et al., 1994; Collinson et al., 2000). *Pax6* was also showed to control the proximo-distal patterning and homophilic and heterophilic cellular adhesion properties of the optic vesicle (Collinson et al., 2000). Notably, expression of *Rx*, *Lhx2*, *Otx2*, and *Six3*, all encoding transcription factors involved in early eye development, is apparently unaffected by the *Pax6* mutation, revealing that in mice, eye specification does occur in the absence of *Pax6* (Bernier et al., 2001). Conditional mutagenesis of *Pax6* in the retina around e10.5 also revealed that *Pax6* is required at the time of retinogenesis (Marquardt et al., 2001). In $Pax6^{\alpha\text{-}Cre}$ mutants, retinal progenitors underproliferate and give rise only to amacrine neurons, in part due to a failure to activate a specific set of pro-neural genes.

Herein we report on the characterization of multipotent and long-term proliferating NE progenitors located in the mouse optic vesicle. This cell population expresses NE and radial glia markers as well as early retinal patterning genes, including *Pax6*. We found that NE progenitors located in the optic vesicle of *Pax6* mutants are more abundant than normal and over-proliferate. Clonal cell dissociation assays revealed that Pax6 is expressed in colony-forming units. *In vitro*, NE progenitors lacking *Pax6* generate more symmetric cell divisions and have a reduced tendency to exit the cell cycle. *Pax6* overexpression repressed NE progenitors proliferation and secondary colonies formation in clonal assays, suggesting that *Pax6* act cell autonomously on NE progenitors proliferation. *Pax6* mutation results in reduced expression levels of several negative regulators of the cell cycle *in vitro* and *in vivo*, which may explain the observed overproliferation phenotype.

Materials and methods

Animals

Adult mice from the albino CD1 strain were purchased from Charles River (St-Constant, Qc., Canada). *Pax6* mutant mice are a gift from Peter Gruss (Max-Planck Institute, Goettingen). *Pax6* mutant mice and embryos were genotyped accordingly to St-Onge (St-Onge et al., 1997) and using LacZ staining in complementation. Embryo's stage was determined according to the time of vaginal plug.

Cell cultures

Optic vesicles of e9.5 embryos were dissected out with tungsten needles in HBSS. Optic vesicles were directly triturated in HBSS using needles (20G-10×; 22G-5×) in order to obtain a suspension of single cells. After centrifugation, cells were placed in neural stem cell 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; SIGMA). 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 0.25% trypsin (SIGMA)/culture media at 37°C for a period of time that was empirically established and that is proportional to spheres' size (1 min/13.5 μm of diameter). Trypsin was inhibited with 0.05% of trypsin inhibitor (SIGMA) in culture media. 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.

Cell differentiation assays

Retinal spheres were allowed to differentiate in chamber slides (Lab-tek) coated with poly-L-lysine (0.5 mg/ml; SIGMA) and laminin (10 ng/ml; SIGMA) in DMEM/F12 with 0.25% glucose, B27 supplement, Gentamycin (25 μg/ml) and 1% Fetal Bovine Serum (Invitrogen) at 37°C in 5% CO_2 atmosphere. After 10 days, cells were exposed to serum-free differentiation media containing BDNF (10 ng/ml) and NGF (50 ng/ml) for 5 days (Invitrogen).

Cell cycle analyses

Propidium iodide staining was performed following the procedure described by Krishan (1975). 10^6 cells were fixed overnight in 75% ethanol and incubated for 1 h on ice with Krishan solution containing PI (50 μg/ml; Molecular Probes). Acquisition of nuclei preparation was performed employing a FACScan flow cytometer (Becton Dickinson) and Lysis II acquisition software with the electronic doublet-discrimination module (DDM) activated. 20,000 events were recovered for each sample. The percentage of cells in the cell cycle phases was evaluated using the ModFit LT 3.0 software.

Immunofluorescence (IF)

Samples were fixed in 4% PFA/PBS for 1 h, washed in PBS, and equilibrated overnight at 4°C in 30% sucrose/PBS. Samples were then immersed in cryomatrix solution (Shandon, Pittsburgh) and snap frozen in liquid nitrogen. Cryosections were performed at 7 μm, air dried on slides for 10 min, and washed 3× with PBS prior to blocking. For cell culture experiments, cells were fixed in 4% PFA/PBS for 10 min and washed with PBS. For BrdU labeling experiments, retinal spheres were directly frozen in liquid nitrogen and post-fixed after sectioning using 100% ETOH for 10 min and 1% PFA/PBS for 1 h. 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: Six3 (G. Oliver, St. Jude Children's Research Hospital); Nestin (BD Transduction Laboratory); BrdU and syntaxin, clone HPC-1 (SIGMA); Blbp (N. Heintz, The Rockefeller University); 4D2 (R. Molday, UBC); β-galactosidase (CORTEX BIOCHEM); P-H3 (Upstate); Thy1.2 (Cedarlane); and Bmi1 (US Biological). All others were from CHEMICON. Specificity of all antibodies was tested on frozen sections from whole embryos and adult mouse eyes. Secondary antibodies were used as negative controls in all experiments. TUNEL assay was performed accordingly to manufacturer instructions (Roche).

Viruses

Pax6 cDNA (P. Gruss, Max-Planck Institute, Goettingen) was cloned into EF.V.CMV.GFP (L. Cheng, Johns Hopkins University) and transfected in 293T cells with helper vectors (F. Boudreau, Sherbrooke University) using Lipofectamine 2000 (Invitrogen). Viral supernatants were ultra-centrifuged and exposed to single cell suspensions O/N. Aggregates were dissociated to single cells and plated at 2000 cells/ml in NSC media for 1 week. GFP

Download English Version:

https://daneshyari.com/en/article/2175297

Download Persian Version:

https://daneshyari.com/article/2175297

Daneshyari.com