Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development

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During eye development in D. melanogaster, the TALE-homeodomain protein Homothorax (Hth) is expressed by progenitor cells ahead of the neurogenic wave front, promotes rapid proliferation of these cells and is downregulated before cells exit the cell cycle and differentiate. Here, we present evidence that hth function is partially conserved in vertebrates. Retinal progenitor cells (RPCs) in chicks and mice express two Hth-related proteins, Meis1 and Meis2 (Mrg1), in species-specific temporal sequences. Meis1 marks RPCs throughout the period of neurogenesis in the retina, whereas Meis2 is specific for RPCs prior to the onset of retinal differentiation. Transfection of Meis-inactivating constructs impaired RPC proliferation and led to microphthalmia. RNA-interference-mediated knock-down of expression indicated that progenitor cells expressing Meis1 together with Meis2 proliferate more rapidly than cells expressing Meis1 alone. Transfection of Meis-inactivating constructs reduced the expression of cyclin D1 (Ccnd1) in the eye primordium and co-transfection of cyclin D1 partially rescued RPC proliferation. Collectively, these results suggest that (1) Meis1 and Meis2, similar to hth, maintain retinal progenitor cells in a rapidly proliferating state; (2) they control the expression of some ocular-determination genes and components of the cell cycle machinery; and (3) together with the species-specific differences in Meis1/Meis2 expression, combinatorial expression of Meis family proteins might be a candidate mechanism for the differential regulation of eye growth among vertebrate species.

KEY WORDS: Proliferation, Retina, Meis, Cyclin D1, Pax6, Mouse, Chick

INTRODUCTION

Despite the different structure and developmental origin of the vertebrate lens-eye and the invertebrate compound eye, some components of the regulatory network that directs eye development are conserved across the animal kingdom. The transcription factor eyeless/Pax6, for instance, is essential for eye development in vertebrates and invertebrates, and atonal and its vertebrate homolog Ath5 (also known as Atoh7) are important regulators of retinal neurogenesis in both systems (Koznik, 2005; Vetter and Brown, 2001). However, examples of non-conservation are also known, which include dachshund (dac) and its vertebrate homologs Dach1 or Dach2, or apterous and the closely related Lhsx2 (Davis et al., 2001; Davis et al., 2006; Porter et al., 1997). The molecular network that controls ocular differentiation is, thus, only partially conserved.

During development of the vertebrate neural retina, six classes of neurons and one class of glia are generated from multipotent progenitor cells over a long developmental period and in overlapping neurogenic waves (Livesey and Cepko, 2001). Retinal ganglion cells (RGCs) are always born first (beginning by E10.5 in the mouse or late E2 in the chick), followed by the other retinal cell classes (Prada et al., 1991; Young, 1985). Postmitotic neurons in the compound eye of Drosophila melanogaster, by contrast, are generated in the wake of a single neurogenic wave, the morphogenetic furrow (MF), which sweeps across the eye imaginal disc during the third larval instar (Heberlein and Treisman, 2000).

Proliferating progenitor cells ahead of the MF express the TALE-homeodomain protein Homothorax (Hth), which together with the Pax6 homolog Eyeless (Ey) and the zinc-finger protein Teashirt (Tsh) promotes rapid, asynchronous proliferation of retinal progenitor cells and prevents their premature differentiation (Bessa et al., 2002). The targets of hth in the eye imaginal disc and its mechanism of action are not yet known.

Vertebrate homologs of Hth are the Meis family proteins Meis1, Meis2 (Mrg1) and Meis3 (Mrg2). Meis proteins were first identified as co-factors of other homeodomain-containing proteins and play multiple roles in development and disease (Berkes et al., 2004; Burglin, 1997; Mercader et al., 1999; Moens and Selleri, 2006; Nakamura et al., 1996). Meis1 and Meis2 are expressed in the eye, but only Meis1-deficient embryos have been generated to date, and these display defects in angiogenesis and eye development (Azcoitia et al., 2005; Hisa et al., 2004). The precise function of Meis1 and Meis2 in the retina is still largely unknown. Here, we provide evidence based on gain-of-function and knock-down experiments, and on the expression of function-blocking constructs in chick embryos that both proteins play an evolutionary conserved role in maintaining the rapidly proliferating state of early retinal progenitor cells.

MATERIALS AND METHODS

In situ hybridization and immunohistochemical detection

The following probes were used to analyse gene expression in mice and chicks: chick Meis1 [nucleotides (NT) 1-711], chick Meis2 (NT 1-818), mouse Meis1 (NT 247-1122), mouse Meis2 (NT 558-1056) and mouse Pax6 (Grindley et al., 1995). All other probes were gifts of C. Cepko (Harvard Medical School, Boston, MA), C. Tabin (Harvard Medical School, Boston, MA), F. P. Pittel (University P. Sabatier, Toulouse, France) and J.-M. Matter (University of Geneva, Geneva, Switzerland) or were cloned with gene-specific primers (primer sequences are available upon request). The dilutions of primary antibodies were: polyclonal anti-Meis2 1:2000 (A. Buchberg); purified monoclonal anti-Pax6 Fab-fragments 1:5000 (Developmental Studies Hybridoma Bank), polyclonal anti-RCAS (p27) 1:10000 (Charles River Laboratories, CT), polyclonal anti-GFP 1:1000 (Molecular Probes, OR), polyclonal anti-phosphorylated histone H3 (pH3) 1:1000 (Upstate
Biotechnology, NY), polyclonal anti-Myc tag 1:300 (Upstate Biotechnology). In situ hybridization and immunohistochemical analyses were performed as described previously (Engelkamp et al., 1999; Schulte et al., 1999; Bumsted-O’Brien et al., 2007).

Construction of gene-delivery vectors
The coding sequence of Meis2a was cloned by RT-PCR from total RNA extracted from HH10-14 chick embryonic heads using the primers 5’-CCATGGCCGAAAGGGTC and 5’-AGTTGATCTTGGCTGTG. Meis2HA is full-length chick Meis2a fused to a triple HA-tag. For Meis2EnR, Meis2a was fused to the repressor domain of D. melanogaster engrailed (EnR), which contained a single Myc-tag at its C-terminus for immunohistochemical detection. Meis1EnR is a fusion of the chick Meis1a partial coding sequence (NCBI AF202933) extended by the first four amino acids of mouse Meis1 (NCBI NM_010789). Controls included SOHo1, GH6 or Nkx5.1 (Hmx3) fused to EnR. The expression vector was either pMIWIII or pMES, which contains an IRES-GFP cassette (Suemori et al., 1990; Swartz et al., 2001). Unless stated otherwise, 1 μg/μl of the expression constructs were electroporated into the right optic vesicle as described (Schulte et al., 1999). In experiments performed with pMIWIII, 0.7 μg/μl pMIWIII-GFP was co-electroporated for visualization. RIS(B) viral particles were produced in cell culture by supplying the avian replication-incompetent derivative of RCASBP(B), in which the pMIWIII-GFP was co-electroporated for visualization. RIS(B) is a replication-incompetent derivative of RCASBP(B), in which the pMIWIII-GFP was co-electroporated for visualization.

Analysis of cell proliferation and cell death
pMES or pMES-MeisEnR were electroporated into the right optic vesicle at HH11. Forty-eight hours later, at HH22, BrdU labeling was performed with the BrdU Labeling and Detection Kit 1 (Roche, Mannheim, Germany); labeling duration was 2 hours. Apoptotic cells were determined with the In Situ Cell Death Detection Kit (Roche). As a control for the labeling procedure, sections were treated with 200 U/ml DNase I for 10 minutes at room temperature.

RESULTS AND DISCUSSION
Spatial-temporal expression of Meis family members in mouse and chicken
Meis2 is expressed in the anterior neural plate of Hamburger-Hamilton stage 7 (HH7) chick embryos (Fig. 1A). Later, Meis2 was strongly expressed in the optic vesicle and optic cup, where it co-localized with Pax6 (Fig. 1B-F). Strikingly, expression of Meis2 was downregulated in the retina after late E2 in a central-to-peripheral wave, which paralleled the appearance of RGC-competent, Ath5-expressing cells (Fig. 1C,D). By HH25, Meis2 was absent from RPCs with the exception of the nasal retinal periphery, which reflects the slight delay in maturation of the nasal compared with the temporal retina in chicken (Fig. 1E, arrowhead) (Prada et al., 1991). Meis2 expression in the chick is, therefore, specific for progenitor cells ahead of the RGC differentiation wave. This expression pattern is unique amongst known RPC markers (e.g. Pax6, Six3 and Optx2), which continue to be expressed when postmitotic neurons and glia are generated. The related protein Meis1 was also expressed by RPCs, but onset of expression lagged behind that of Meis2 and expression continued throughout the period of proliferation (Fig. 1G-J). Meis2-specific transcripts were also detected in the mouse eye anlage, but expression was lost prior to optic vesicle invagination (Fig. 1K,L). Meis1 was strongly expressed in the mouse optic vesicle and expression continued into postnatal development (Fig. 1M,N and data not shown). Meis3 was not detected in the eyes of either species at the embryonic stages analyzed (data not shown). RPCs in chicks and mice, thus, express different combinations of Meis1 and Meis2 over time: self-renewing RPCs in the early eye anlage of both species express Meis1 together with Meis2. Meis2 expression is terminated before the onset of retinal differentiation, at the late optic vesicle stage in mice, but just ahead of the RGC production front in chicken.

Fig. 1. Spatial and temporal expression of Meis1 and Meis2. (A–C,E) Expression of Meis2 in chick at (A) HH7 (arrow, anterior neural plate), (B) HH15 (E2; arrow, eye cup), (C) HH19 (early E3) and (D) HH25 (E4.5). Meis2 expression is lost in RPCs after late E2. Arrows in C indicate the central domain, where Meis2 is downregulated. (D) Expression of Ath5 in chick at HH19. Arrows indicate where Ath5* cells appear at HH19. (F–P) Double immunostaining for Meis2 and Pax6 at HH11. (G–J) Expression of Meis1 in chick at (G) HH11, (H) HH15, (I) HH19 and (J) HH25. Arrow in J indicates Meis1 downregulation in the RGC layer. (K) Expression of Meis2 in mouse eye anlage at E9.5. (L) Meis2 is not expressed in the E12.5 mouse retina. (M,N) Meis1 expression in the mouse optic vesicle (M) and neural retina (N). Expression is downregulated in the RGC layer (arrow). Boxed areas in E and J are shown at high magnification in the insets. Le, lens; Ip, lens placode; mes, mesencephalon; nR, neural retina; ov, optic vesicle; PE, pigmented epithelium; SE, surface ectoderm. Scale bars: 200 μm in C,D,I, 500 μm in B,E,H,J, 50 μm in K,M, 100 μm in L,N.
Meis1 and 2 are required for rapid progenitor cell proliferation

The presence of Meis1 and Meis2 in proliferating and undifferentiated cells of the early retina suggests that both proteins promote self-renewal of these cells. Moreover, rapid asynchronous cell proliferation in the D. melanogaster eye imaginal disc is restricted to cells expressing the Meis-related protein Hth, and loss-of-function and gain-of-function studies have demonstrated that hth is both necessary and sufficient for progenitor cell proliferation (Bessa et al., 2002; Dominguez and Casares, 2005; Heberlein and Treisman, 2000). We therefore hypothesized that the normal proliferative capacity of early vertebrate RPCs might, at least in part, depend on the presence of functional Meis proteins in these cells.

To test this idea, we first blocked Meis function by ectopically expressing the coding region of Meis1a or Meis2a fused to the engrailed repressor domain (Meis1EnR or Meis2EnR, collectively termed MeisEnR) from D. melanogaster. These fusion proteins compete with the endogenous Meis proteins for DNA binding and can act as true antimorphs (Dibner et al., 2001; Inbal et al., 2001). Twenty-four hours following introduction of MeisEnR and/or GFP into the right optic vesicle (HH15-16), the right transfected and left control eyes were similar in size (Fig. 4 and data not shown). From HH21-22 onwards, however, the transfected eyes were severely microphthalmic (80%, n=12/15) compared with non-electroporated, GFP- or Meis2HA-transfected eyes (wild type, n=13 embryos; GFP, n=12; Meis2HA, n=9; Fig. 2A-B’ and data not shown) and often exhibited an apparent partial transformation of the pigmented epithelium to retina (Fig. 2C). MeisEnR-induced microphthalmia was associated with a reduction in the total number of cells per retina. Upon dissociation of transfected HH25 retinæ, we counted an average of 9.06×10^5 cells/GFP-transfected retina (s.e.m.=0.3001; n=3) compared with 4.41×10^5 cells/MeisEnR-transfected retina (s.e.m.=0.6899; n=3). Retinæ transfected with RNAi-targeting constructs against Meis1 and Meis2 contained an average of 5.15×10^5 (s.e.m.=0.856; n=3) cells per retina. These figures correlate well with those determined by others (Dutting et al., 1983) and demonstrate that transfection of Meis-inactivating constructs reduced retinal cell counts. To determine whether this resulted from reduced progenitor cell proliferation or enhanced cell death, we labeled retinae 24 hours after transfection, a time before morphological changes of the eye became apparent, with the thymidine analog BrdU. Two hours after BrdU injection, 61.7% (±1.07 s.e.m.; n=13 embryos) of the GFP-expressing cells, but only 27.8% (±2.63 s.e.m.; n=17) of the MeisEnR/GFP-expressing cells had incorporated the label (Fig. 2D-F). To control for possible non-specific effects, several other homeodomain-EnR fusion proteins were misexpressed in the...
eye and were found to not affect progenitor cell proliferation (data not shown). TUNEL labeling indicated that MeisEnR transfection does not compromise cell survival (Fig. 2G-I).

To substantiate these results, we performed clonal analysis with a replication-incompetent avian retrovirus [RIS(B)]. We infected chick embryos at the optic vesicle stage with low-titer RIS(B) viruses that contained either GFP, Meis2HA or the dominant-negative MeisEnR, and analyzed the number of progeny that infected cells had given rise to 48 hours later (HH20, Fig. 2J-L). Following infection with RIS(B)-GFP, the majority of clones contained 5-7 cells/section with a second peak of 13 cells/clone/section, which most likely reflects a cohort of cells that had undergone an additional round of cell division (n=74 clones; Fig. 2J). Clone sizes did not change upon retroviral misexpression of Meis2, suggesting that RPCs do not overproliferate in the presence of elevated Meis2 levels (n=80 clones; Fig. 2K and data not shown). This contrasts with published data on homothorax, as clones overexpressing hth in the D. melanogaster eye imaginal disc can grow very large (Bessa et al., 2002). Upon retroviral misexpression of MeisEnR, the vast majority of clones contained only 2-3 cells/section with a second peak of 5 cells/clones/section (n=85 clones; Fig. 2L). Although retinal progenitor cells can divide in the presence of MeisEnR, Meis function nevertheless appears to be required for the rapid proliferation characteristic of early RPCs (Li et al., 2000).

Fig. 3. Expression of Meis-inactivating constructs reduces RPC proliferation and cyclin D1 expression. (A-E) HH15 chick eyes transfected with the listed expression constructs together with GFP (green) and stained for phosphorylated histone H3 (pH3, red). (A) Control siRNA construct containing a randomized targeting sequence. pH3+/GFP+ indicates percentage of transfected cells in mitosis. (F-J) HH15 chick eyes stained for cyclin D1 or cyclin D2 following transfection with the constructs indicated. (F,H) cyclin D1, (G,I) cyclin D2. (K-M) Retinae transfected with MeisEnR+GFP (K), GFP (L) or MeisEnR+GFP together with cyclin D1 (M), stained for GFP (green) and pH3 (red); cell nuclei are counterstained with DAPI (blue). The position of pH3-labeled cell nuclei is indicated in the right-hand and middle panels of K. (N) Percentage of cells in mitosis (y-axis) for each transfected construct (x-axis). (O-R) Expression of Ath5 (O,Q) following transfection with the constructs indicated. The boxed areas in O,Q are shown at high magnification in O’,Q’, respectively. FG, H-J, O,P and Q,R show neighboring sections. (I,P,R) Transfection controls.
chick development when cells downregulate Meis2 expression (but retain Meis1) after late E2. Indeed, the BrdU index of RPCs was seen to decline between HH18 and HH25 following a central-to-peripheral gradient (see Fig. S2 in the supplementary material).

To pinpoint the molecular basis of this effect, we transfected retinal progenitor cells at HH10-11 with MeisEnR (to simultaneously block Meis1 and Meis2 function) and assessed the expression of cell cycle components 16-20 hours later. D-type cyclins are important regulators of the progression through the G1 phase of the cell cycle and the retinae of cyclin D1-deficient animals are hypoplastic, with each retinal layer containing significantly fewer cells than those of wild-type littersmates (Fantl et al., 1995; Sicinski et al., 1995). Following MeisEnR expression, cyclin D1 was markedly reduced (in 86% of the embryos, n=6/7; Fig. 3F-H,I), whereas expression of the related cyclin D2 was normal (Fig. 3G,J).

Transfection of cyclin D1 together with MeisEnR partially rescued the RPC proliferation defects we had observed after transfection of MeisEnR alone (Fig. 3K-N; n=5/5). Together, these results place MeisEnR-transfected cells also failed to express the proneural genes NeuroM and Ath5 (Fig. 3O-R and data not shown; n=5/5).

In addition, few MeisEnR-expressing cells could be detected in the retina past HH24, and those that were present were clumped together at the vitreal surface and did not show features of differentiated neurons (see Fig. S3 in the supplementary material). This was surprising, as retinal differentiation is normal in cyclin D1 mutant mice despite the insufficient cell number in their retinae. We therefore investigated whether the expression of other genes that are essential for eye development also depends on Meis function.

**Meis proteins are components of the regulatory network that controls eye development in vertebrates**

The paired-type transcription factor Pax6 is crucial for vertebrate eye development and Meis proteins have been shown to directly regulate expression of Pax6 in the developing lens and pancreas (Zhang et al., 2002; Zhang et al., 2006). Pax6-specific transcripts were decreased in eyes transfected with MeisEnR (Fig. 4A-D and see Fig. S3 in the supplementary material). This suggests that Meis1 and Meis2 contribute to the regulation of retinal Pax6 expression. This view is supported by a series of co-transfection experiments in which transfection of Meis1 or Meis2 together with Pax6 stimulated the activity of an α-enhancer-driven β-galactosidase reporter construct about 1.6-fold over the previously reported autostimulation by Pax6 alone, whereas co-transfection of Pax6 with unrelated homeodomain proteins did not elevate enhancer activity (see Fig. S3 in the supplementary material) (Kammandel et al., 1999). Transfection of MeisEnR effectively repressed expression of Six3 and Chx10 (Vsx2), but not that of other ocular-determination genes (Fig. 4E-I). RNAi-mediated knock-down of Meis1 together with Meis2 also reduced retinal Pax6 transcript levels, albeit to a lesser degree than did MeisEnR transfection (Fig. 4J,K).
Meis1/Meis2 upstream of Pax6 in retinal development and indicate that Meis proteins are part of the genetic cascade that drives neuroepithelial cells towards ocular differentiation.

Progenitor cell proliferation in the developing retina and cortex is sensitive to changes in the level of Pax6 expression (Berger et al., 2007; Grindley et al., 1995; Manuel et al., 2007; Schedl et al., 1996). This raises the possibility that the observed reduction in cyclin D1 expression by Meis1/EnR might be a secondary effect of the concomitant decrease in Pax6 expression in the eye. We therefore tested whether cyclin D1 expression could be rescued by cotransfection with Pax6. This was, however, not observed (Fig. 4L.M). Overexpression of Pax6 also failed to elevate cyclin D1 or cyclin D2 expression in the retina, and cyclin D1 and cyclin D2 expression levels were normal following expression of a Pax6-EnR fusion protein, which had previously been shown to effectively block Pax6 function in the developing central nervous system (Fig. 4N-P) (Yamasaki et al., 2001). Therefore, Meis1/Meis2 control over cyclin D1 expression appears to be independent of their ability to regulate Pax6 expression.

Collectively, our data integrate Meis proteins into the genetic network that regulates eye development in vertebrates, place them upstream of cyclin D1 and Pax6 in RPCs and suggest that they, similar to their invertebrate homolog Hth, help to maintain retinal progenitor cells in a rapidly self-renewing state. Homeodomain transcription factors have been implicated in the tissue-specific regulation of progenitor cell proliferation in the nervous system and several of the homeodomain proteins that are expressed in the vertebrate eye anlage, such as Chx10 and Optx2, are directly or indirectly required for retinal progenitor proliferation or enhance eye growth when overexpressed (Burmeister et al., 1996; Green et al., 2003; Zuber et al., 1999). Unlike these proteins, the duration of Meis expression is highly species-specific and correlates well with the different eye sizes in birds and mammals. For instance, chicken, which express Meis2 together with Meis1 over a longer developmental period than mice, contain approximately 50-fold more RGCs than do mice (Rager and Rager, 1976; Strom and Williams, 1998). Although other genes clearly contribute to eye growth too, the species-specific regulation of Meis family proteins together with their influence on retinal progenitor cell proliferation present a likely mechanism as to how species-specific differences in retinal size could have evolved.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/805/DC1

References


