Histol Histopathol (2006) 21: 1331-1337

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Fetal ocular movements and retinal cell differentiation: analysis employing DNA microarrays

M. Baguma-Nibasheka, T. Reddy, A. Abbas-Butt and B. Kablar

Dalhousie University, Faculty of Medicine, Department of Anatomy and Neurobiology, Halifax, NS, Canada

Summary. As developmental biologists we study the role of fetal movements in providing continuity between prenatal and postnatal life. There are two major categories of fetal motility. The first category consists of movements that have an obvious effect on the survival or development of the fetus (e.g., changes of position, sucking and swallowing). The second category consists of fetal movements that anticipate postnatal functions. For example, fetal ocular movements (FOMs) predict postnatal eye function (e.g., motion vision) of the newborn and therefore represent an important indicator of fetal health. However, while the clinical significance of fetal motility is obvious, its biological significance is elusive. We propose to use retina of genetically modified mouse embryos to study the biological role of FOMs in the genesis of cell diversity and organ functional maturation. Our results have already demonstrated the importance of fetal eye motility in the differentiation of cholinergic amacrine cells (CACs) in the retina (Kablar, 2003). Apparently, these cells are sensitive to motion and also responsible for motion vision. In the current report, we suggest employing the unique opportunity provided by the mouse Myf5$^{-/-}$:MyoD$^{-/-}$ knock-outs that lack skeletal musculature and FOMs, microarray analysis and the follow-up experiments to identify a group of candidate genes that are essential for the molecular regulation of CAC differentiation and in turn for the functional maturation of the visual system towards its ability to perform motion vision. Finally, the molecules identified via this approach may be important in the mechanochemical signal transduction pathways employed during the process of conversion of a mechanical stimulus into an instruction understandable by the developing retinal neurons and glia cells.

Key words: Mouse embryo, Myf5, MyoD, Retinal cell differentiation, Mechanical stimuli, Microarray analysis

Offprint rquests to: Dr. Boris Kablar, Dalhousie University, Faculty of Medicine, Department of Anatomy and Neurobiology, 5850 College Street, Halifax, NS, Canada B3H 1X5. e-mail: bkablar@dal.ca

Introduction

A paradigm in developmental biology is that both intrinsic and extrinsic factors can influence cell fate. The gene expression of a cell will often impose some limitations on available fates (i.e., cell competence), whereas final determination of a specific cell type may depend on environmental influence. For example, two different progenitors in the rat retina are restricted to producing 3 types of neurons or 2 types of neurons and a glial cell (Turner and Cepko, 1987). In culture these cells are also subject to regulation by their cellular environment. Placing embryonic day (E) 16 retinal progenitor cells in an *in vitro* post-partum (P) 0 cellular environment reduces the normal production of amacrine cells and increases production of cone photoreceptors (Belliveau and Cepko, 1999). In contrast, a P0 amacrine cell-depleted culture environment allows the E16 cells to produce more amacrine cells than would normally be produced *in vivo* (Belliveau and Cepko, 1999). Thus, we may also conclude that feedback inhibition on cell fate determination is a likely mechanism to regulate cell type ratios in the vertebrate neural retina. Treatment of E16 cells with size-excluded cell lysates has the same effect suggesting that diffusible ligands are likely involved in retinal cell fate determination.

Although the cellular microenvironment can clearly influence cell fate determination, it is likely that the large-scale physical environment is also involved. Indeed, we have used *Myf5$^{-/-}$:MyoD$^{-/-}$* mouse embryos (amyogenic, double-mutant or DM), which completely lack striated muscle, to study the developmental dependence of the neural retina on mechanical stimulation of the eye (Kablar, 2003). Since neither of the removed myogenic regulatory factors have been detected in the mouse retina at various stages of development nor do the single mutants have distinct retinal phenotypes (Kablar, 2004 and the references therein), the amyogenic embryos are ideal for studying the role of FOMs in retinal differentiation. While the basic laminar organization of the retina, cell cycle kinetics (i.e., cell proliferation and cell death), and the total number of cells present in each of the layers were not altered in amyogenic fetuses, there was a notable

absence of CACs (Kablar, 2003). We also observed that many amacrine cell subtypes were present in larger numbers, that amacrine precursors were significantly depleted, and that ganglion cells did not completely differentiate. Thus, by loss of function, we ascertained that FOMs are important for maintaining the proper ratios of differentiated cells as well as required for the differentiation of at least one amacrine cell subtype.

This review first encompasses studies of FOMs, most of which involve real-time ultrasound observation of human fetuses. Second, the intrinsic (genetic) control of cellular competence for differentiation in the neural retina is considered in the context of mechanical stimulation, which may facilitate distribution of ligands (Fig. 1). Finally, we present our recent microarray findings which reveal that *Ap3d1*, *Btrc*, *Snx17* and *Wdr5* are among the few molecules significantly down-regulated in the *Myf5*$^{-/-}$*:Myo*D$^{-/-}$ E18.5 mouse retina.

FOMs and retinal differentiation

To study intrauterine FOMs and as a clinical test for central nervous system (CNS) problems, real-time ultrasound has been employed (reviewed in Horimoto et al., 1993 and the references therein). According to this group, because of the simplicity of extraocular musculature, very fine nervous control is required, thus normal FOMs may reflect proper development of the CNS. Four classes of FOMs have been described (Birnholz, 1981). There are both rapid and slow eye movements in the fetus and these have been quantified as such (Horimoto et al., 1990). Early in gestation (up to 20 weeks), there are several minute-length periods without eye movement, while eye movement periods are approximately 10-12 minutes in length in the later parts of gestation (Nijhuis et al., 1982). In fact, four weeks later (i.e., at 24 weeks on), bursts of movement become more frequent and acquire very high frequency near term (Inoue et al., 1986). Finally, FOMs become less spontaneous and transform into consolidated clusters of eye movement (i.e., sporadic short bursts disappear late in gestation and this disappearance coincides with the stable periods of eye movement). Therefore, more complex and repetitive motions of the eye are more

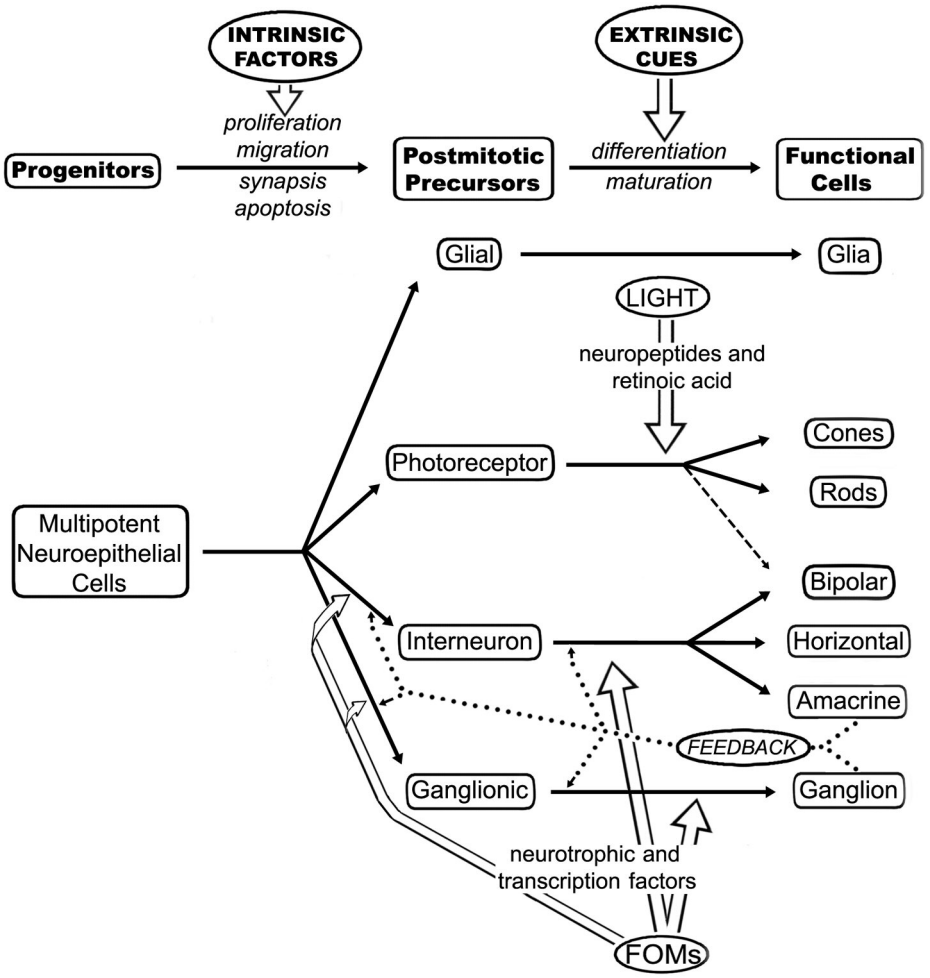

Fig. 1. Hypothesized model of FOMs influence on retinal cell differentiation. Whereas the basic organization of the retina (e.g., number of cell layers and number of cells per layer) appears to be determined by intrinsic genetic factors, the individual progenitor cell fate (i.e., competence to make different cell types) and the ratio of resulting cell types (i.e., cones, rods, bipolar, horizontal, amacrine and ganglion cells) are influenced by extrinsic environmental cues. In our model, we are particularly interested in the light and FOMs, possibly acting via various mediators (e.g., transcription and neurotrophic factors, etc.) to influence the competence of progenitor cells and ratios of resulting cell types.

consistently observed later in fetal development (Birnholz, 1981). On the other hand, slow eye movements result from the inability of the tonic system (i.e., the extraocular muscles) to hold the eyes, and thus they drift back to the original or resting position, where the tensions of the six extraocular muscles are in balance. Thus, the slow eye movements reflect decreased muscle tone and the appearance of slow eye movements coincides with muscle hypotonia (Aserinsky and Kleitman, 1955; Bridgeman, 1983). In addition, binocular eye movements of two types have been described: rapid and in phase (i.e., the same for each eye) and slow and out of phase (i.e., the eyes are not moving in the same fashion). However, only preliminary work has been done on conjugate versus disjunctive eye movements (N.B., probably about 5.4% of all eye movements are the latter) (Birnholz, 1981; Takashima et al., 1991).

FOMs in mice, and possibly humans, are more than just warm-up for looking around at birth. Apparently, they are essential to the development of specialized cells in the retina (Kablar, 2003). Expectant mothers are familiar with their babies' uterine acrobatics involving kicks and turns. Ultrasound images reveal that by the third trimester fetuses are also moving their lungs, as if breathing, and moving their eyes. The movements are currently used as indicators of fetal health. But there is ongoing debate as to the biological role of these movements in fetal development. It is true that we are practising those movements in order to be able to perform them better when we are born. But, it also appears that we need them to stimulate the creation of certain cell types that will be needed as soon as we are born. The ability to isolate the role of eye movements was made possible by using mutant knockout fetuses that lack eye muscles and the associated genes. As mentioned earlier, the cellular development in their unmuscled, non-moving fetal eyes was compared to that in the eyes of healthy mouse fetuses. Although the total number of cells and the basic laminar organization of the neural retina are essentially normal in amyogenic fetuses, it does appear that extraocular muscle activity is important for the differentiation of certain cell types. For example, cholinergic amacrine cells are absent in the double-mutants, whereas other amacrine cell types are present in increased numbers along with decreased amacrine cell precursors (Kablar, 2003). This suggests that the broad organization of the eye is intrinsically controlled by genetic factors (and not Myf5 or MyoD transcription factors) (Kablar, 2004), whereas cell subtype ratios are influenced by extrinsic cues (e.g., mechanical stimuli from the extraocular muscles). In fact, surprisingly, the non-moving eyes did not develop a type of retinal cells involved in motion detection. It appears that the intra-uterine fetal eye movements prepare the retina and make cells differentiate so that the newborn is able to capture motion in their surroundings. If the eye does not move at all in the embryo it appears these cells do not develop. It could be that it was the absence of some known or unknown genes in the mutant fetuses, together with the lack of movements that determines the retinal cells' fate. Furthermore, proliferation and apoptosis appear to be unaffected in amyogenic embryos (Kablar, 2003). Double-mutant retinas contained less amacrine precursors and more of most other amacrine cell types (except, of course, the absent cholinergic type) and at the same time more ganglion cell precursors and logically less differentiated ganglion cells. Thus, it could be that the mechanical stimuli from the extraocular muscle allow competence of progenitors to form CACs, without direct influence on cell cycle kinetics, even though cell cycle kinetics are known to affect the distribution of the amacrine cell population (Dyer and Cepko, 2000).

The intrinsic (genetic) control of cellular competence for differentiation in the neural retina

Recent evidence suggests that extrinsic cues influence the ratios of cell types, whereas changes in the intrinsic properties of retinal precursors determine their competence for differentiation (Cepko, 1999). The vertebrate retina consists of six major classes of neurons and Müller glial cells, arising from a multipotent precursor cell population. Marking mitotic precursor cells can end up labeling two very different cell types (i.e., rod and amacrine cells produced from the same retinal precursor) (Turner and Cepko, 1987). There are two models proposed for the cell fate specification process. The first involves cell fate depending entirely on the environment while the second involves complete dependence on intrinsic (genetic) factors. In late embryonic rat retina there are at least 2 types of retinal precursor cells present. One expresses markers of mature horizontal and amacrine cells (i.e., the syntaxin epitope recognized by HPC-1 antibody and sugar epitope recognized by VC1.1 antibody). The latter cells produce horizontal and amacrine cells in the embryonic and postnatal period. In late embryonic development, an increasing number of rods is also produced by these cells. This is consistent with the second model (i.e., intrinsic control of cell fate). However there is some flexibility. The VC1.1/syntaxin-positive horizontal/amacrine-producing cells give rise to post-mitotic cells which do not have these markers and thus the VC1.1/syntaxin-cell type is transient and does not persist, meaning that extrinsic cues can still be involved. As mentioned earlier, placing an E16 progentior cell in an *in vitro* P0 cell environment reduces its production of amacrine cells and increases production of cone photoreceptors (Belliveau and Cepko, 1999), suggesting that extrinsic information can also play a role in cell fate determination (N.B., normally P0 is a period of rod production, but the induced E16 cells did not produce extra rods). It is therefore possible that there is a signal that prevents amacrine cell genesis in these E16 cells. Most amacrine cells are produced by P0 and only 10% of P0 cells have amacrine fate, possibly because of the

feedback inhibition on cell fate by amacrine cells themselves. Amacrine-enriched P0 cell populations have the same amacrine-inhibiting effect when co-cultured with E16 cells, but depletion of P0 amacrine cells allows the E16 cells to produce more amacrine cells in co-culture (Belliveau and Cepko, 1999). In fact, even more than normal, suggesting that the E16 environment also contains amacrine cells which feedback inhibit their production. Indeed, we have observed that many amacrine cell subtypes are up-regulated in amyogenic fetuses but that one particular subtype, the CAC, is completely absent (Kablar, 2003). Perhaps an early elevation in the level of many amacrine populations feedback inhibits the production of CACs. For example, with insufficient FOMs, attenuating signals (i.e. ligands or cell-surface interactions) may become more concentrated than in the normally stimulated eye. As Cepko (1999) notes, the co-culture experiments also suggest that there is a photoreceptor-inducing signal since cones are not induced to form by the amacrine-rich P0 population, but they are by the "regular" P0 population. Inhibition of E16 cells from adopting the amacrine fate only occurs if co-cultured prior to M phase of the cell cycle (Belliveau and Cepko, 1999). The same applies to treatment with P0 extract (<10 kDa), suggesting the presence of a diffusible ligand as an inhibitor of the amacrine cell fate. Thus, amacrine cell commitment likely occurs in late G2 phase. Notch signaling affects the development of most retinal cell types (reviewed in Henrique et al., 1997). Mutant forms of fibroblast growth factor receptor (FGFR) in *Xenopus* alter amacrine cell production (McFarlane et al., 1998) and, therefore like in our experiment, a loss-of-function approach shows an effect on cell fate. However, evidence for stochastic fate choices also exists. For example, the presence of a uniform progenitor population (from E17) producing distinct cell types at different times in the same *in vitro* culture environment (Jensen and Raff, 1997). Alternatively, one could argue that these cells do have intrinsic genetic differences. In conclusion, it appears that cell fate specification is a fluid process, where the progenitor has a limited number of cell types that can be produced, but with the fate choice also open to environmental control. Thus, specification is influenced by the changing cellular environment during ontogeny, with the possibility of feedback regulation by, for example, amacrine cells.

Microarray findings in the search for molecules characteristic of CACs

It is reported that the synthesis of acetylcholine (i.e. the incorporation of labeled choline into acetylcholine) is four times accelerated by *in vitro* exposure of an isolated rabbit retina to light (Masland and Livingstone, 1976). The electrophysiological function of the retina can be maintained because it is simple to simulate vascular diffusion from the choroid. Furthermore, increasing the concentration of Mg^{2+} and Ca^{2+} in the perfusate blocks the light-induced release of acetylcholine from the retina. Therefore, as expected, high concentrations of divalent cations are able to block the neurotransmitter secretion, suggesting that the release of acetylcholine occurs at a synapse and implying the presence of cholinergic cells in the retina (Masland and Livingstone, 1976). Indeed, the choline acetyltransferase staining reveals two distinct populations of cholinergic amacrine cells. One is located where most amacrine cells localize, at the inner nuclear layer (INL), but the other population is at the ganglion cell layer (GCL), (Voigt, 1986).

Cyclin kinase inhibitors (CKIs) of the Cip/Kip family regulate cell cycle progression. Three Cip/Kip family members, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, have been identified in mammals. $p57^{Kip2}$, in addition to its role in cell cycle exit, is also expressed in a subset of amacrine cells and is required to produce the correct number of cells for this amacrine subtype (Dyer and Cepko, 2000). This is the first reported mutation (i.e., the lack of $p57^{Kip2}$ in postnatal mouse retinal cells) that affects the distribution of amacrine cell subtypes. In embryonic development $p57^{Kip2}$ is important for cell cycle exit of retinal progenitors, and without it the excessive proliferation appears to be precisely balanced by increased apoptosis. In fact, whether a progenitor cell exits the cell cycle via $p27^{Kip1}$ or $p57^{Kip2}$ may affect the available fates of daughter cells (i.e., competency). The balancing apoptosis retains the majority of cell-type ratios of the mouse retina, suggesting the existence of a built-in cellular mechanism to maintain the basic laminar organization of the eye. Indeed, we have observed in amyogenic fetuses that the basic laminar organizations are retained even though certain cell-type ratios are out of balance (i.e., reduced differentiation of ganglion cells, up-regulated amacrine cell differentiation, absence of CACs) (Kablar, 2003). Thus, amacrine subpopulation ratios are influenced not only by genetic factors (lack of CKI) but also by extrinsic factors (as occurs in amyogenic fetuses). However, it is intuitive that proteins regulating cell cycle exit would also be involved in the terminal differentiation of a cell, or at least the initiation of this process. Consistently, $p57^{Kip2}$ appears to be expressed during the period of amacrine cell differentiation (i.e., P2-P10) in a restricted subpopulation of amacrine cells (and not in the calbindin-expressing subpopulation) (Dyer and Cepko, 2000). Furthermore, calbindin-expressing subpopulation of amacrine cells is larger in $p57^{Kip2}$-deficient mice, which is consistent with our observation that certain amacrine cell subpopulations are larger in amyogenic fetuses. In conclusion, this is some of the first evidence for a connection between two different amacrine cell subpopulations (Dyer and Cepko, 2000), while we provide evidence for connections among multiple amacrine cell subpopulations (Kablar, 2003).

At least 22 morphologically distinct amacrine cell subtypes are described in the adult vertebrate retina and each subtype is believed to play a unique role in visual

signal processing (MacNeil and Masland, 1998). Only a small population of CACs (6.8%, according to Dyer and Cepko, 2000) exists in the retina. These cells synthesize and release acetylcholine, necessary for generating spontaneous bursts of activity in immature ganglion cells, resulting in waves of correlated activity across the retina (Wong et al., 1995). These spontaneous bursts are found to play a role in refinement of early synaptic connections between the ganglion cells and extra-retinal areas of the brain (Penn et al., 1998). Therefore, well-described starburst cholinergic amacrine cells execute their role in directional selectivity (Moran and Schwartz, 1999 and references therein) by potentiating the response of ganglion cells to moving stimuli (He and Masland, 1997). Their dendritic processes make synaptic connections with OFF- and ON/OFF-center ganglion cells, directly shaping how visual stimuli are processed (Nguyen et al., 2000). Recently, starburst amacrine cells were found to be the key element that discriminates the direction of stimulus movement and stabilizes image motion (Yoshida et al., 2001).

We decided to further our understanding of the recent data on amyogenic embryos by taking advantage of the retinas that specifically lack the CACs and employing cDNA microarray analysis that efficiently allows the isolation of differentially expressed mRNAs (Hubank and Schatz, 1994; Pastorian et al., 2000; Gilbert et al., 2003). Using this approach, we were able to perform molecular comparisons between the mutant and control retinas. Molecules that were not present in the mutant retinas were assumed to be specific for the lacking neuronal cell types. Taken together, this approach might allow identification of genes that are involved in the development of CACs and, importantly, are specific markers for CACs. In turn, that would provide the basis for numerous follow-up experiments, such as expression studies at different developmental stages to specifically address the development of CACs. Generation of a knockout mouse or a conditional mouse mutant could be used to study *in vivo* and in the whole animal the role of the gene of interest.

There are some obvious pitfalls of using cDNA microarrays for this study. Using this technique one can only detect gene expression patterns of genes that are represented on the slide. Also, it is not very sensitive given that our target cells are only 4% of the total population. This is where Representation Difference Analysis (RDA) comes in. RDA allows for the selective production of cDNA only from mRNA that is present in one sample but absent in the other, through a subtraction step. That cDNA is then amplified by PCR and sequenced. Genbank is then used to identify the cDNA

Table 1. Genes down-regulated in Myf5-/-:MyoD-/- mouse embryonic retina.

GENE	GENE TITLE	BIOLOGICAL FUNCTION	CHARACTERISTICS OF DELETION MUTANTS
Ap3d1	adaptor-related protein complex 3, delta 1 subunit	Vesicle-mediated transport; antigen presentation	Pigment dilution, platelet defects, lysosomal abnormalities, blindness, deafness and neurological defects, increased perinatal mortality (1, 2)
Btrc	beta-transducin repeat containing	Ubiquitin-mediated protein catabolism; signal transduction	Mammary, uterine and ovarian tumorigenesis and male infertility (3, 4)
Snx17	protein sorting nexin 17	Receptor-mediated endocytosis and protein trafficking	NA[a]
Wdr5	WD repeat domain 5	G-protein coupled signal transduction; skeletal development	NA
BG063010[b]	NA	NA	NA

1. Kantheti et al., 1998; 2. Qiao et al., 2003; 3. Guardavaccaro et al., 2003; 4. Kudo et al., 2004. [a]. NA, not applicable, or data not available; [b]: expressed sequence tag (EST), i.e., nucleotide sequence clustering near known genes, but itself not yet annotated to code for a characterized gene.
Note: Retinas from embryonic day (E) 18.5 old mouse embryos that were either wild-type (WT) or Myf5-/-:MyoD-/- double-mutants (DM) were isolated. Total RNA from three WT retinas was extracted and pooled and the same was done with three DM retinas. 15K mouse cDNA microarray slides from the Microarray Centre at the Ontario Cancer Institute were used, as previously reported (Gilbert et al., 2003). Cy3/Cy5 labeled cDNA was produced from total RNA isolated from the WT and DM retinas, using SuperScript II Reverse transcriptase (Invitrogen) and hybridization reactions were carried out essentially as described previously (Wigle et al., 2002). The reverse transcription reaction mixture contained less of dTTP relative to the other dNTPs. It was replaced with aminoallyl-dUTP, which allowed for the labeling of the cDNA strands with Cy3 and Cy5 fluorescent dyes. Using this process we produced WT cDNA that was labeled with the fluorescent dye Cy3 (which fluoresces green) and the DM cDNA was labeled with Cy5 (which fluoresces red). These were then mixed and allowed to hybridize onto the microarray slide. If a gene was equally expressed in both WT and DM retina, there would have been mRNA present for that gene in both the DM and WT retina. Thus relatively equal red and green labeled cDNA binding would make that spot appear yellow. Accordingly if a gene was only expressed in the WT retina or had higher expression in the WT retina compared to the DM retina, the spot for that gene would have appeared green. While in the opposite case, when a gene is expressed only in the DM or has higher expression in the DM, the spot for it would appear red. To confirm any observations, the experiment was repeated, but with reciprocol labeling (i.e. the WT cDNA was labelled with Cy5 and DM cDNA with Cy3) to rule out the binding efficiency of the dyes as a factor in the results. A total of two such microarray experiments were performed according to the detailed instructions previously reported (Gilbert et al., 2003) and in the very same facility (kindly provided by Dr. H. A. Robertson at Dalhousie University).

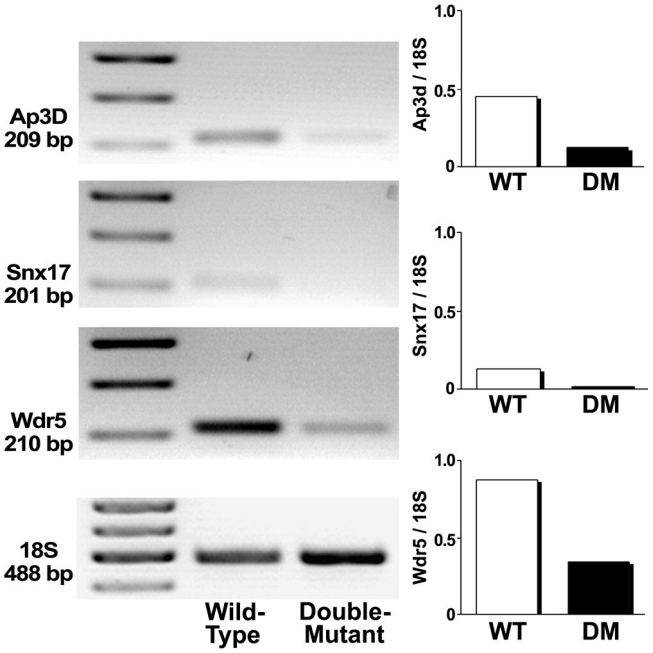

Fig. 2. RT-PCR verification of differential gene expression in the retina of wild-type (WT) and *Myf5*$^{-/-}$:*Myo*D$^{-/-}$ double-mutant (DM) embryos. Total RNA was pooled from the retinas of three fetuses in each group. Graphs plot expression relative to 18S rRNA.

Table 2. Conditions for PCR of transcripts used to verify gene expression differences.

Target	Primer Sequence, 5' to 3'	Product Size (bp)
Ap3d1	forward: CCT GAA GAC ACA CCC CAA GT reverse: TCT TGG TGA GCA GTT CAT CG	209
Snx17	forward: GCA GCT TCG GAA GGA GTA TG reverse: ACC TGT TGG GTC TCC TGT TG	201
Wdr5	forward: CGT TCA TTT CAA CCG TGA TG reverse: CCC TTG CTG TAG TCC CAG AG	210

Note: To confirm the differential mRNA expression, total RNA was isolated from the retinas of E18.5 mouse embryos using the RNeasyTM kit from Qiagen, Mississauga, Ont., Canada. RNA from three fetuses in each group (wild-type or *Myf5*$^{-/-}$:*Myo*D$^{-/-}$ double-mutant) was pooled, reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) and amplified using the primers listed in Table 2. DNA levels were normalized against the QuantumRNA™ 488 bp 18S ribosomal RT-PCR product (Ambion, Austin, TX) amplified from the same RT reaction, as previously described (Baguma-Nibasheka et al., 2005). The results (Fig. 2) confirmed the down-regulation of the tested genes (Ap3d1, Snx17 and Wdr5) in the double-mutant embryonic retina.

and its source gene (Hubank and Schatz, 1994; Pastorian et al., 2000). In the meantime, the results from our cDNA microarray analysis have pointed towards a few genes as being expressed in the wild-type (WT) and not (or at very low levels) in the double-mutant (DM) fetal retina (Table 1). These results have been routinely confirmed by RT-PCR (Fig. 2 and Table 2). Surprisingly, we did not obtain a large number of candidate molecules, but instead only one unknown gene (its gene ID is: BG063010) and just four known genes (i.e., Ap3d1, Btrc, Snx17 and Wdr5). Therefore, we obtained a set of genes of interest, which we relate to the CACs. Since we are hoping to find a marker for these cells, we will have to provide evidence that the gene in question is actually expressed by CACs and that its expression is selective enough to be used as a marker. One way to do that can be through tissue *in situ* hybridization or immunohistochemistry experiments using sections of WT and DM retina. This technique can potentially indicate whether a gene of interest is expressed by CACs or not, and whether they express this gene selectively in the retina. This approach may also indicate whether its expression is absent in the DM or is simply at a lower level compared to the WT. In that case of course the gene may not be specifically expressed by CACs, but its expression may be important for the differenciation of CACs, or may be a product of these cells. Finally, we will analyze the development of the retina and especially the CACs of the existing Mocha mice (available from the Jackson Laboratory) which result from a spontaneous mutation in Ap3d1, one of the genes identified by our microarray analysis. Mocha mice are found to be blind two weeks after birth (Qiao et al., 2003). Similarly, we are going to analyze the development of the retina and CACs of the Btrc$^{-/-}$ mice (Guardavaccaro et al., 2003; Kudo et al., 2004).

In conclusion, we believe that the results of the follow-up studies will increase our understating of the particular aspects of retinal cell differentiation and of the visual system function, therefore increasing the knowledge in the field and helping in medical practice in application to conditions such as those characterized by inability of gaze stabilization and/or spatiotemporal interactions (e.g., as occurs when driving).

Acknowledgements. Our grateful appreciation to Dr. Harold A. Robertson, Dr. Robert W. Gilbert and Dr. Willard J. Costain for their help in microarray analysis. This work was supported by an operating grant from Natural Sciences and Engineering Research Council of Canada (NSERC) and infrastructure grants from Canada Foundation for Innovation (CFI) and the Dalhousie Medical Research Foundation (DMRF) to B.K. T.R. is the recipient of an NSERC graduate scholarship for this project.

References

Aserinsky E. and Kleitman N. (1955). Two types of ocular motility occurring in sleep. J. Appl. Physiol. 8, 1-10.

Baguma-Nibasheka M., Li A.W., Osman M.S., Geldenhuys L., Casson A.G., Too C.K. and Murphy P.R. (2005). Coexpression and regulation of the FGF-2 and FGF antisense genes in leukemic cells. Leuk. Res. 29, 423-433.

Belliveau M.J. and Cepko C.L. (1999). Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina.

Development 126, 555-566.
Birnholz J.C. (1981). The development of human fetal eye movement patterns. Science 213, 679-681.
Bridgeman B. (1983). Phasic eye movement control appears before tonic control in human fetal development. Invest. Ophthalmol. Vis. Sci. 24, 658-659.
Cepko C.L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr. Opin. Neurobiol. 9, 37-46.
Dyer M.A. and Cepko C.L. (2000). p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Development 127, 3593-3605.
Gilbert R.W., Costain W.J., Blanchard M.-E., Mullen K.L., Currie R.W. and Robertson H.A. (2003). DNA microarray analysis of hippocampal gene expression measured twelve hours after hypoxia-ischemia in the mouse. J. Cereb. Blood Flow Metab. 23, 1195-1211.
Guardavaccaro D., Kudo Y., Boulaire J., Barchi M., Busino L., Donzelli M., Margottin-Goguet F., Jackson P.K., Yamasaki L. and Pagano M. (2003). Control of meiotic and mitotic progression by the F box protein beta-Trcp1 *in vivo*. Dev Cell. 4, 799-812.
He S. and Masland R.H. (1997). Retinal direction selectivity after targeted laser ablation of starburst amacrine cells. Nature 389, 378-382.
Henrique D., Hirsinger E., Adam J., Le Roux I., Pourquie O., Ish-Horowicz D. and Lewis J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signaling in the embryonic chick retina. Curr. Biol. 7, 661-670.
Horimoto N., Koyanagi T., Satoh S., Yoshizato T. and Nakano H. (1990). Fetal eye movement assessed with real-time ultrasonography: are there rapid and slow eye movements? Am. J. Obstet. Gynecol. 163, 1480-1484.
Horimoto N., Hepper P.G., Shahidullah S. and Koyanagi T. (1993). Fetal eye movements. Ultrasound Obstet. Gynecol. 3, 362-369.
Hubank M. and Schatz D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. 22, 5640-5648.
Inoue M., Koyanagi T., Nakahara H., Hara K., Hori E. and Nakano H. (1986). Functional development of human eye movement in utero assessed quantitatively with real-time ultrasound. Am. J. Obstet. Gynecol. 155, 170-174.
Jensen A.M. and Raff M.C. (1997). Continuous observation of multipotential retinal progenitor cells in clonal density culture. Dev. Biol. 188, 267-279.
Kablar B. (2003) Determination of retinal cell fates is affected in the absence of extraocular striated muscles. Dev. Dyn. 226, 478-490.
Kablar B. (2004). MyoD-lacZ transgenes are early markers in the neural retina, but MyoD function appears to be inhibited in the developing retinal cells. Int. J. Dev. Neurosci. 22, 215-224.
Kantheti P., Qiao X., Diaz M.E., Peden A.A., Meyer G.E., Carskadon S.L., Kapfhamer D., Sufalko D., Robinson M.S., Noebels J.L. and Burmeister M. (1998). Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 21, 111-122.
Kudo Y., Guardavaccaro D., Santamaria P.G., Koyama-Nasu R., Latres E., Bronson R., Yamasaki L. and Pagano M. (2004). Role of F-box protein betaTrcp1 in mammary gland development and tumorigenesis. Mol. Cell. Biol. 24, 8184-8194.
MacNeil M.A and Masland RH. (1998). Extreme diversity among amacrine cells: implications for function. Neuron 20, 971-982.
Masland R.H. and Livingstone C.J. (1976). Effect of stimulation with light on synthesis and release of acetylcholine by an isolated mammalian retina. J. Neurophysiol. 39, 1210-1219.
McFarlane S., Zuber M.E. and Holt C.E. (1998). A role for the fibroblast growth factor receptor in cell fate decision in the developing vertebrate retina. Development 125, 3967-3975.
Moran D.W. and Schwartz A.B. (1999). Motor cortical representation of speed and direction during reaching. J. Neurophysiol. 82, 2676-2692.
Nguyen L.T., De Juan J., Mejia M. and Grzywacz N.M. (2000). Location of choline acetyltransferase in the developing and adult turtle retinas. J. Comp. Neurol. 420, 512-526.
Nijhuis J.G., Prechtl H.F.R., Martin C.B. Jr and Bots R.S.G.M. (1982). Are there behavioural states in the human fetus? Early Hum. Dev. 6, 177-195.
Pastorian K., Hawel L. 3rd and Byus C.V. (2000). Optimization of cDNA representational difference analysis for the identification of differentially expressed mRNAs. Anal. Biochem. 283, 89-98.
Penn A.A., Riquelme P.A., Feller M.B. and Shatz C.J. (1998). Competition in retinogeniculate patterning driven by spontaneous activity. Science 279, 2108-2112.
Qiao X., Pannesi M., Seong E., Gao H., Burmeister M. and Wu S.M. (2003). Photoreceptor degeneration and rd1 mutation in the grizzled/mocha mouse strain. Vision Res. 43, 859-865.
Takashima T., Horimoto N., Satoh S., Maeda H., Koyanagi T. and Nakano H. (1991). Characteristics of binocular movements in the human fetus at term, assessed with real-time ultrasound. Jap. Soc. Ultrason. Med. 59 (Suppl. II), 883-884.
Turner D.L. and Cepko C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature 328, 131-136.
Voigt T. (1986). Cholinergic amacrine cells in the rat retina. J. Comp. Neurol. 248, 19-35.
Wigle D.A., Jurisica I., Radulovich N., Pintilie M., Rossant J., Liu N., Lu C., Woodgett J., Seiden I., Johnston M., Keshavjee S., Darling G., Winton T., Breitkreutz B.J., Jorgenson P., Tyers M., Shepherd F.A. and Tsao M.S. (2002). Molecular profiling of non-small cell lung cancer and correlation with disease-free survival. Cancer Res. 62, 3005-3008.
Wong R.O., Chernjavsky A., Smith S.J. and Shatz C.J. (1995). Early functional neural networks in the developing retina. Nature 374, 716-718.
Yoshida K., Watanabe D., Ishikane H., Tachibana M., Pastan I. and Nakanishi S. (2001). A key role of starburst amacrine cells in originating retinal directional selectivity and optokinetic eye movoemnt. Neuron 30, 771-780.

Accepted May 29, 2006