Characterization of early retinal progenitor microenvironment: Presence of activities selective for the differentiation of retinal ganglion cells and maintenance of progenitors

Ganapati V. Hegde, Jackson James, Ani V. Das, Xing Zhao, Sumitra Bhattacharya, Iqbal Ahmad*

Department of Ophthalmology and Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA

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Abstract

The maintenance and differentiation of retinal progenitors take place in the context of the microenvironment in which they reside at a given time during retinal histogenesis. To understand the nature of the microenvironment in the developing retina, we have examined the influence of activities present during the early stage of retinal histogenesis on enriched retinal progenitors, using the neurosphere model. Early and late retinal progenitors, enriched as neurospheres from embryonic day 14 (E14) and E18 rat retina, respectively, were cultured in embryonic day 3 (E3) chick retinal conditioned medium, simulating the microenvironment present during early retinal histogenesis. Examination of the differentiation and proliferation of retinal progenitors revealed that the early microenvironment contains at least three regulatory activities, which are partitioned in different size fractions of the conditioned medium with different heat sensitivity. First, it is characterized by activities, present in heat stable <30 kDa fraction, that promote the differentiation of retinal ganglion cells (RGCs), the early born neurons. Second, it contains activities, present in heat-sensitive >30 kDa fraction, that regulate the number of early born neurons and maintain the pool of retinal progenitors. Third, it possesses activities, present in heat-sensitive <30 kDa fraction, that prevent the premature differentiation of early retinal progenitors into the late born neurons. Thus, our observations demonstrate the regulatory influence of microenvironment on the maintenance and differentiation of retinal progenitors and establish neurospheres as a viable model system for the examination of such influences.

Keywords: microenvironment; niche; extrinsic factors; RGC; rod photoreceptor; retina; progenitor; stem cells

1. Introduction

It is becoming increasingly evident that the regulation of stem cells/progenitors takes place in the context of their microenvironment. Therefore, understanding the biology of stem cells/progenitors and their successful clinical use will require information about the properties of their microenvironment. The developing retina, which has been successfully used to provide insights into cellular and molecular mechanisms of neurogenesis, can serve as a model for characterization of the microenvironment because retinal cell types, which are limited in number, are known to be generated in response to changing environment. For example, different experimental approaches like lineage analysis and cell ablation studies have demonstrated that retinal progenitors are multi-potential, and the decision taken by these cells to differentiate into a particular retinal cell type depends on local cell—cell interactions (Altschuler et al., 1991). Several in vitro studies have suggested that some of the known and unknown factors may mediate cell—cell interactions during retinal neurogenesis (Yang, 2004). Also, evidence emerged that, besides diffusible factors, cell—cell interactions mediated by membrane-bound receptors...
and ligand interactions, such as those exemplified by Notch signaling, play a critical role in retinal neurogenesis (Livesey and Cepko, 2001; Ahmad et al., 2004).

These cell-extrinsic cues, elaborated by progenitors, precursors, differentiated neurons and glia, constitute the milieu of the microenvironment. However, how the activities within the microenvironment maintain retinal progenitors and influence the outcomes of their differentiation remain unknown. Given the evolutionarily conserved temporal nature of cell-fate specification during the two stages of retinal histogenesis (Rapaport et al., 2004), it is likely that at any given time of retinal histogenesis, activities within the microenvironment will be permissive for the differentiation of progenitors into predominant cell types of that stage, and inhibiting for those that are born later, and yet maintain progenitor pool. To test this premise, we examined the influence of microenvironment, created by the presence of cells in the early stage of retinal histogenesis, on the differentiation of retinal progenitors into early and late born retinal neurons, using the neurosphere model (James et al., 2003; Reynolds and Rietze, 2005).

We have used the neurosphere model of retinal progenitors for the following reasons: although not entirely homogeneous, ~90% of cells in neurospheres are proliferative, as demonstrated by their ability to incorporate BrdU and express pan neural (i.e., Nestin and Musashi) as well as retinal progenitor (i.e., Rx, Pax6 and Chx10) markers (Ahmad et al., 2004). Examination and interpretation of results regarding cell proliferation and differentiation in neurosphere are less ambiguous than in explant culture due to limited cellular heterogeneity. The reason for using E3 chick retinal cells to simulate the environment representing the stage of early histogenesis included the fact that stages E3–E5 in chick retina represent a period characterized by the birth of early born neurons such as the RGCs (Prada et al., 1991). Since RGCs are the first retinal cell types to differentiate regardless of species, cell-extrinsic cues that regulate their differentiation are likely to be conserved and read by rat retinal progenitors (James et al., 2003). Besides, tissues are less of a limitation with E3 chick retina than with rat retina at E13–E14, the peak of RGC generation.

Three different activities were discerned in the early retinal progenitor microenvironment. These were partitioned into different size fractions with differential heat sensitivity and influence on cell differentiation and proliferation. These constituted (1) activities that promoted the differentiation of retinal progenitors into early born neurons, such as RGCs (2) activities that regulated the number of early born neurons and maintained the pool of retinal progenitors and (3) activities that prevented the premature differentiation of early retinal progenitors into late born neurons, such as rod photoreceptors, before the onset of the late stage of histogenesis. Together, these cell-extrinsic activities are likely to regulate the efficiency and fidelity of cell differentiation during the early stage of retinal histogenesis while maintaining a pool of retinal progenitors for subsequent rounds of differentiation. In addition, our observations present the neurospheres as a viable model for examining the influence of microenvironment on stem cells/progenitors in controlled conditions.

2. Materials and methods

2.1. Enrichment and culture of retinal progenitors as neurospheres

Timed-pregnant E14 and E18 Sprague–Dawley rats were obtained from Sasco (Wilmington, MA), and the gestation day was confirmed by the morphological examination of embryos (Christie, 1964). Fertilized hen eggs (Hoovers, Omaha, NE) were incubated in a humidified incubator at 38 °C for 3 days, and embryonic stages were confirmed by morphological examination (Hamburger and Hamilton, 1951). Embryos were harvested at appropriate stages, and eyes were enucleated. Retina was dissected out, and dissociated by trypsin digestion, as previously described (Ahmad et al., 1999). Rat retinal dissociates were cultured in retinal culture medium (RCM; DMEM/F12, 1× N2 supplement, 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with either the combination of EGF (20 ng/ml; Collaborative Research), FGF2 (10 ng/ml; Collaborative Research) and 0.1% FBS for E14 cells, or with EGF (20 ng/ml) for E18 cells, at a density of 10^5 cells/cm^2 for 4–6 days to generate neurospheres. Neurospheres were exposed to 10 μM 5-bromo-2’-deoxyuridine (BrdU) (Sigma) for the final 48 h of culture to tag the dividing cells. BrdU-tagged neurospheres, obtained from E14/E18 retina, were plated on poly-d-lysine-(250 μg/ml) and laminin-(5 μg/ml) coated 12 mm glass cover slips, and co-cultured either across a 0.4 μm pore size semi-permeable membrane (Millipore, Bedford, MA) with E3 chick retinal/forebrain cells, or in the presence of E3CM/RCM supplemented with 1% FBS for 5–6 days. To ascertain the effects of E3CM on the generation of neurospheres, E14 retinal dissociates were cultured in the presence of 1% FBS or E3CM fractions in a 96-well plate at a density of 10,000 cells/well for 2–3 days. The number of neurospheres generated/cm^2 was quantified.

2.2. Preparation, fractionation and treatment of conditioned medium

The dissociated E3 chick retinal cells were cultured in RCM supplemented with 1% FBS for 3 days. The supernatant containing enriched activities was separated by centrifugation (5000 rpm, 4 °C, 10 min), and defined as E3 chick retinal conditioned medium (E3CM). E3CM and RCM containing 1% FBS were fractionated into >30 kDa and <30 kDa fractions using 30 kDa centrifuge tubes according to the manufacturer’s instruction (Millipore). The fractionated E3CM was reconstituted by adding the missing fraction from fractionated RCM containing 1% FBS as shown in the respective flow charts (Figs. 4A and 7A). To determine the heat stability of activities, E3CM fractions were subjected to heat treatment (70 °C for 30 min), and the heat-treated E3CM fractions were
reconstituted as shown in the respective flow charts (Figs. 5A, E and 8A).

2.3. Explants culture

E14 retinal explants were cultured in 1% FBS or E3CM on a 0.4 μm pore size semi-permeable membrane for 5 days. Explants were either fixed for immunohistochemical or frozen for RT-PCR analyses as previously described (James et al., 2003).

2.4. Immunofluorescence analysis

Immunocytochemical analysis was carried out for the detection of cell specific markers and BrdU as previously described (Ahmad et al., 1999). Briefly, 4% paraformaldehyde-fixed cells were incubated in PBS containing 5% normal goat serum (NGS) and 0.2/0.4% Triton X-100 followed by an overnight incubation in Brn3b (1:300, BabCo)/Nestin (1:4, DSHB) and BrdU (1:100) antibodies at 4 °C. Since BrdU immunocytochemistry interfered with fluorescence specific for opsin-immunoreactivity, immunocytochemistry to detect opsin was carried alone. TUNEL staining was carried out as per the manufacturer’s instruction (Promega). Immunohistochemical analysis of explants was carried out on paraformaldehyde-fixed, 10-μm cryostat sections as previously described (Ahmad et al., 1998), and counterstained with DAPI. Cells were examined for epifluorescence following incubation in species specific IgG conjugated to Cy3/FITC. Images were captured using cooled CCD-camera (Princeton Instruments) and Open lab software (Improvision Inc.). To determine the percentage of specific cell types in a particular condition, number of BrdU+ and cell specific antigen positive cells were counted in 10–15 randomly selected fields in two–three different cover slips. Each experiment was repeated at least three times. Values are expressed as mean ± SEM. Statistical analysis was done using Student’s t-test to determine the significance of the differences between various conditions.

2.5. RT-PCR analysis

Total RNA was isolated from neurospheres in differentiating conditions using RNA isolation kit (Qiagen RNeasy mini kit), and the genomic DNA was removed by RNase-free DNase I digestion as per the manufacturer’s instruction (Ambion DNA free kit). RNA (2 μg) was transcribed to cDNA using superscript reverse transcriptase as previously described (James et al., 2003). The cDNA was normalized against a house keeping gene, β-actin, whose primers span an intron, which was used to detect the presence of traces of genomic DNA even after DNase digestion. To detect any cDNA contamination in other reagents in the PCR mix, the amplification was carried out using water instead of cDNA. We were unable to do a negative RT because of limited amount of samples. Specific transcripts were amplified with gene specific forward and reverse primers (Table 1) using a step-cycle program as previously described (James et al., 2003). PCR amplification was carried for 25–30 cycles. Products were visualized by ethidium bromide staining after electrophoresis on a 2% agarose gel. The bands corresponding to specific transcripts, were scanned and normalized against those corresponding to β-actin, amplified from the same batch of cDNA. Mean fold change for the respective genes in the experimental groups

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank accession number</th>
<th>Primer sequences</th>
<th>Temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
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<tr>
<td>β-Actin</td>
<td>XM_037235</td>
<td>(F) 5'-GTGGGCGCCACAGCAAC-3'</td>
<td>50</td>
<td>548</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(R) 5'-CTCCTAAATGTCACGCACGTTC-3'</td>
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<td>Ath5</td>
<td>AF071223</td>
<td>(F) 5'-GGGGCACTGATACAGACGCAAGCAAG-3'</td>
<td>52</td>
<td>231</td>
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<tr>
<td></td>
<td></td>
<td>(R) 5'-CTCATGAGAAGGCGCTGCTGAGAC-3'</td>
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<tr>
<td>Brn3b</td>
<td>AF390076</td>
<td>(F) 5'-GGGCTGAGGGAAGCAGAAAACAC-3'</td>
<td>60</td>
<td>141</td>
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<tr>
<td></td>
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<td>(R) 5'-TTTGCTGGATGGGGAAGTAGCC-3'</td>
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<tr>
<td>RFP1</td>
<td>XM_344604</td>
<td>(F) 5'-TTTCCAGGGATTCTTGTTGTC-3'</td>
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<td></td>
<td>(R) 5'-CGTTTTTTGGATGGCTTACCT-3'</td>
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<td>Thy1</td>
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<td>(F) 5'-TCAGTTCTGGAAGCAAGATT-3'</td>
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<td>(R) 5'-CTCACAGAGAAAATGAGTCCGTCGTGAC-3'</td>
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<td></td>
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<td>Rhodopsin kinase</td>
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<tr>
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<td>(R) 5'-TGTTGATATGAGTAGGGAGCAAGATG-3'</td>
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<tr>
<td>Arrestin</td>
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<td>(R) 5'-CGTCTGCTTCTTCTTCTGTCG-3'</td>
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<tr>
<td>Chx10</td>
<td>L34808</td>
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<td>56</td>
<td>350</td>
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<tr>
<td></td>
<td></td>
<td>(R) 5'-GACTTCAGGATGACTCTGAGCAG-3'</td>
<td></td>
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</table>

F: forward primer, R: reverse primer.

Table 1: List of primers used for RT-PCR analyses
was calculated with respect to controls (fold value = 1). Values are expressed as mean fold change ± SEM from three different experiments. Statistical analysis was done using Student’s t-test to determine the significance between different conditions.

3. Results

3.1. Activities elaborated by cells representing the early stage of retinal histogenesis regulate the differentiation of retinal progenitors into early born neurons

To demonstrate the influence of the microenvironment on retinal progenitors during the generation of specific retinal cell types, BrdU-tagged E14 neurospheres were cultured in RGC differentiating conditions, defined by the presence of E3CM and absence of mitogens. Controls include E14 neurospheres, similarly cultured in the presence of 1% FBS, minus mitogens. In this condition, neurospheres, characterized by BrdU+ and Nestin+ cells (Fig. 1A–D), spread out on the surface and differentiate (Fig. 1E–H). The differentiation of early retinal progenitors along RGC lineage was examined by the expression of Brn3b, a Pou domain transcriptional factor, known to regulate RGC differentiation and maturation (Xiang et al., 1993). In addition, levels of transcripts corresponding to Ath5, a bHLH transcription factor that plays a role in the specification of RGCs (Brown et al., 2001), RPF1, another Pou domain regulator of RGC differentiation (Zhou et al., 1996), and Thy1, a marker of matured RGCs (Barnstable and Drager, 1984) were examined. We observed a ~2-fold increase in the proportion of BrdU+ and Brn3b+ cells when E14 neurospheres were cultured in E3CM, compared to those in FBS controls, suggesting a positive influence of E3CM on the differentiation of early retinal progenitors along RGC lineage (Fig. 1E–M). RT-PCR analysis revealed that levels of Ath5, Brn3b, RPF1 and Thy1 transcripts were increased in neurospheres exposed to E3CM as compared to those in controls (Fig. 1N, O), corroborating results obtained by immunocytochemical analysis, and suggesting that RGC differentiation in neurospheres involved normal mechanism.

The specificity of E3CM influence on retinal progenitors was determined as follows: we examined the effects of E3CM on the expression of s-cone opsin and syntaxin 1, markers for S-cone photoreceptors and amacrine cells, respectively (Fig. 1N, O). As expected, levels of s-cone opsin transcripts were higher in E3CM-exposed neurospheres than in controls, given the fact that cones birth date closely parallels that of RGCs (Rapaport et al., 2004) and E3CM may contain cone-promoting activities as it is obtained from cone-rich retina. A slight difference (~0.9-fold) in levels of syntaxin 1 transcripts was observed between the two groups, suggesting that E3CM may not influence the differentiation of amacrine cells whose temporal generation lags that of RGCs and cone photoreceptors (Rapaport et al., 2004). To ascertain that the influence on the proportion of Brn3b+ cells was attributed directly to E3 chick retinal cells, E14 neurospheres were co-cultured in the presence of these cells and cells derived from E3 chick forebrain across a semi-permeable membrane. We observed a similar increase in the proportion of Brn3b+ in the presence of E3 chick retinal cells as observed with E3CM, compared to FBS controls (Fig. 1P). No significant change in the proportion of Brn3b+ cells was observed when E14 neurospheres were co-cultured with E3 chick forebrain cells (Fig. 1P). Together, these observations suggested a positive influence of these cells and factors elaborated by them on the differentiation of E14 retinal progenitors into RGCs.

To address the issue that the observed effects of extrinsic factors on cell differentiation could be a function of dissociated cell culture, we examined the effects of E3CM on RGC differentiation in E14 retinal explants. In retinal explants, the dimensionality and cell interaction of the intact retina are maintained and cells are born in a spatio-temporal order, comparable to that in vivo (Sparrow et al., 1990). The proportion of Brn3b+ cells and the relative levels of Brn3b transcripts were higher in explants in the presence of E3CM than in those cultured in the presence of FBS only (Fig. 2A–K), thus corroborating the results obtained using the neurosphere culture paradigm (Fig. 1).

3.2. Activities elaborated by cells representing the early stage of retinal histogenesis regulate the differentiation of retinal progenitors into late born neurons

To know whether or not the early retinal microenvironment is selective for early born neurons, we examined the effects of E3 chick retinal cells on the differentiation of the retinal progenitors along rod photoreceptor lineage. Birth dating analyses have shown that a small subset of rod photoreceptors is generated at E13/14 (Morrow et al., 1998; Rapaport et al., 2004). We hypothesized that the environment of early retinal neurogenesis may not be conducive for the generation of late born neurons. We assumed that the full extent of the early microenvironment’s inhibitory influences on rod photoreceptor differentiation may not be apparent in the context of early retinal progenitors due to the relative lack of their competence to generate late born neurons. Therefore, we first tested the hypothesis on late retinal progenitors, enriched from E18 rat retina as neurospheres (E18 neurospheres), that preferentially generate rod photoreceptors (Ahmad et al., 1999; Bhattacharya et al., 2003). We observed a significant decrease in the proportion of opsin+ cells in E18 neurospheres, cultured in the presence of E3CM, compared to those in FBS controls (6.5 ± 0.4% vs. 9.8 ± 0.4%; p < 0.001, Fig. 3A–I). Similarly, levels of transcripts corresponding to photoreceptor regulator, Mash1 (Ahmad et al., 1998) and phenotype specific markers, opsin, arrestin and rhodopsin kinase were also observed to be decreased in E18 neurospheres in the presence of E3CM, compared to those in controls (Fig. 3J, K). No difference in levels of transcripts characteristic of bipolar cells (Chx10) was observed between the two groups, demonstrating the cell type-specific influence of E3CM. Together, these observations suggested that E3CM or activities present in early microenvironment were inhibitory to rod photoreceptor differentiation.
To address the issue if these activities influenced the nascent rod differentiation during early histogenesis (Morrow et al., 1998), E14 neurospheres were cultured in the presence of E3CM or 1% FBS (controls) for 5 days followed by immunocytochemical and RT-PCR analyses of cell type-specific regulators and markers. BrdU-tagged E14 neurospheres contained significantly more BrdU$^+$ and Brn3b$^+$ cells (arrows) in the presence of E3CM (E–H, M) than in controls (I–L, M). Levels of transcripts corresponding to RGCs (Ath5, Brn3b, RPF1 and Thy1) and S-cone photoreceptors (s-cone opsin) were higher in E14 neurospheres, cultured in the presence of E3CM (N, lane 2; O) than in controls (N, lane 1). A slight decrease in levels of syntaxin 1 transcripts between the two groups was observed. There were significantly more BrdU$^+$ and Brn3b$^+$ cells when E14 neurospheres were co-cultured across the membrane with E3 chick retinal cells, as compared to those co-cultured with either E3 chick forebrain (FB) cells or FBS (P). Data are expressed as mean ± SEM from triplicate cultures of three different experiments. ×100 for A–D and ×200 for E–L (**p < 0.001).

3.3. Activities present in heat stable <30 kDa and heat labile >30 kDa E3CM fractions promote and inhibit the differentiation of retinal progenitors into RGCs, respectively

To characterize the regulatory activities present during early retinal histogenesis, we fractionated E3CM into two different size fractions (i.e., <30 kDa and >30 kDa), based
on the fact that many reported factors that influence cell differentiation have molecular weight less (e.g. FGF1, FGF2, CNTF) or more (e.g. Shh, EGF, GDF11, Wnt2b) than 30 kDa (Fig. 4A). E14 neurospheres were cultured in reconstituted fractions as described in Fig. 4A. The proportion of BrdU+ and Brn3b+ cells increased significantly in <30 kDa E3CM fraction, compared to those in >30 kDa E3CM fraction or FBS controls (Fig. 4B–N, Table 2). Levels of Ath5 and Brn3b transcripts were similarly higher in the presence of <30 kDa E3CM fraction compared to those in >30 kDa E3CM fraction or FBS controls (Fig. 4O, P). Together, these observations suggested that the RGC promoting activities of the E3CM reside in <30 kDa E3CM fraction.

Both immunocytochemical and RT-PCR analyses of the reconstitution of fraction experiments revealed that >30 kDa E3CM fraction contained activities that inhibited RGC differentiation. For example, the proportion of BrdU+ and Brn3b+ cells was significantly lower in the presence of >30 kDa E3CM fraction than in FBS controls (Fig. 4F–I, Table 2). Similarly, levels of Ath5 and Brn3b transcripts were relatively lower in the presence of >30 kDa E3CM fraction as compared to FBS controls (Fig. 4O, P). The presence of RGC inhibiting activities in >30 kDa E3CM fraction explained the observation that the proportion of BrdU+ and Brn3b+ cells was relatively higher in <30 kDa E3CM fraction than in un-fractionated E3CM (47 ± 1.4% vs.

Fig. 2. Activities present in E3CM promote RGC differentiation in explants culture. E14 retinal explants were cultured in the presence of E3CM or 1% FBS (controls) followed by immunohistochemical and RT-PCR analyses of Brn3b expression. There were significantly more Brn3b+ cells when retinal explants were cultured in presence of E3CM (A–D, I) than in controls (E–H, I). There was a relative increase in the levels of Brn3b transcripts when retinal explants were cultured in presence of E3CM (J, lane 2; K), compared to those in controls (J, lane 1). OR = outer retina; GCL = ganglion cell layer. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. ×100 (**p < 0.001).
40.1 ± 0.8%; p < 0.001) (Figs. 4B–N; 1E–M). To ensure that the observed results were not due to differential cell survival in different conditions, TUNEL assay was carried out which demonstrated no difference in the number of apoptotic cells between the groups (Fig. 4Q–V).

These results suggested that the early retinal microenvironment possesses both RGC promoting and RGC inhibiting activities. We were interested in knowing the nature of these different regulatory activities that are partitioned in different fractions. To begin with, we examined the temperature sensitivity of these activities by incubating <30 kDa E3CM and >30 kDa E3CM fractions at 70 °C for 30 min, a condition known to inactivate proteins and used for the characterization of factors (Waid and McLoon, 1998). E14 retinal progenitors, cultured either in presence of reconstituted heat-treated or untreated <30 kDa E3CM, showed no significant difference in the proportion of BrdU⁺ and Brn3b⁺ cells (Fig. 5A, B, Table 2). Also, levels of Ath5 and Brn3b transcripts remained the same between the two groups (Fig. 5C, D), suggesting that the RGC promoting activities, present in the <30 kDa E3CM fraction, were heat stable. The reconstitution protocol for examining the heat sensitivity of >30 kDa E3CM fraction was modified since it contained inhibitory activities, whose absence can be more pronounced against the presence of differentiation-promoting activities (Fig. 5E). Therefore, controls included fractionated E3CM, reconstituted without heat treatment. The experimental group was similarly reconstituted except that >30 kDa E3CM fraction was heat-treated. The proportion of BrdU⁺ and Brn3b⁻ cells increased significantly in reconstituted fraction that contained heat-treated >30 kDa
Fig. 4. Activities that promote and inhibit the differentiation of retinal progenitors into RGCs are present in <30 kDa and >30 kDa E3CM fractions, respectively. BrdU-tagged E14 neurospheres were cultured in presence of reconstituted >30 kDa or <30 kDa E3CM fractions (A) or 1% FBS (controls), followed by immunocytochemical and RT-PCR analyses for RGC-specific markers and regulators. There was a significant increase in the proportion of BrdU\(^+\) and Brn3b\(^+\) cells (arrows) when neurospheres were cultured in presence of <30 kDa E3CM (B–E, N), compared to those in >30 kDa E3CM (F–I, N) or controls (J–M, N). There was a significant decrease in the proportion of BrdU\(^+\) and Brn3b\(^+\) cells (arrows) when neurospheres were cultured in the presence >30 kDa E3CM, compared to those in controls (J–M, N). There was a relative increase in levels Ath5 and Brn3b transcripts in neurospheres cultured in the presence of <30 kDa (O, lane 3; P), compared to those in controls (O, lane 1). TUNEL analysis revealed only few TUNEL positive cells and their number remained similar in different conditions (Q–V). Square symbols in the reconstitution scheme correspond to conditions described in the graph. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. \(*p < 0.001\); **p < 0.01).
3.4. Activities present in RGC lineage.

The regulated differentiation of retinal progenitors along RGC promoting and inhibiting activities, respectively, for the microenvironment is characterized by three non-overlapping cell regulatory activities, partitioned into different fractions of the CM, derived from cells in the early stage of early retinal progenitor microenvironment. For example, developing retina, unlike stem cell niche in adult tissues like bone marrow (Arai et al., 2005), intestinal crypt (Leedham et al., 2005), hair follicle (Christiano, 2004) and brain (Wurmser et al., 2004), is ever changing and therefore, relatively more complex due to the demand of the ongoing histogenesis. Here, we demonstrate a complex regulatory nature of early retinal progenitor microenvironment. For example, the microenvironment is characterized by three non-overlapping cell regulatory activities, partitioned into different fractions of the CM, derived from cells in the early stage of retinal histogenesis. The activities that promote the

<table>
<thead>
<tr>
<th>E3CM fraction</th>
<th>E14 neurosphere (%Brn3b&lt;sup&gt;+&lt;/sup&gt; cells)</th>
<th>E18 neurosphere (%opsin&lt;sup&gt;+&lt;/sup&gt; cells)</th>
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<tr>
<td></td>
<td>Fraction</td>
<td>Control</td>
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<tr>
<td>&gt;30 kDa</td>
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<td>20.8 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>47 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>&gt;30 kDa (HT)</td>
<td>51.8 ± 0.9***</td>
<td>40.6 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup> RCM + 1% FBS.
<sup>b</sup> Untreated <30 kDa E3CM fraction.
<sup>c</sup> Untreated >30 kDa E3CM fraction reconstituted with <30 kDa E3CM fraction.

Table 2

Summary of the differentiation of E14 and E18 retinal progenitors into RGCs (Brn3b<sup>+</sup> cells) and rod photoreceptors (opsin<sup>+</sup> cells), respectively, in the presence of different E3CM fractions

As described in Fig. 7A. We observed that the proportion of opsin<sup>+</sup> cells was decreased significantly in the presence of <30 kDa E3CM fraction, compared to those in >30 kDa E3CM fraction or FBS controls (Fig. 7B–N, Table 2). There was no significant difference in the proportion of opsin<sup>+</sup> cells when E18 neurospheres were cultured in the presence of >30 kDa E3CM or FBS (Fig. 7B–N, Table 2). Results obtained by immunocytochemical analysis were corroborated by RT-PCR analysis; levels of Mash1 and opsin transcripts were lower in cells in neurospheres that were cultured in the presence of <30 kDa E3CM fraction, compared to those cultured in the presence of >30 kDa E3CM fraction or in FBS controls (Fig. 7O, P). These observations suggested that the rod photoreceptor inhibiting activities of the E3CM were localized in <30 kDa E3CM fraction. To determine the temperature sensitivity of activities present in <30 kDa E3CM fraction, we carried out heat treatment and reconstitution experiments as described above (Fig. 8A). We observed a significant increase in the proportion of opsin<sup>+</sup> cells in the presence of heat-treated <30 kDa E3CM, compared to those in untreated <30 kDa E3CM (Fig. 8B, Table 2). Consistent with the immunocytochemical results, levels of Mash1 and opsin transcripts were increased in the presence of heat-treated <30 kDa E3CM (Fig. 8C, D). These observations suggested that rod photoreceptor inhibiting activities in <30 kDa E3CM fraction were heat labile and therefore, distinct from RGC promoting activities that reside in the same fraction but are heat stable.

4. Discussion

The stem cell or progenitor microenvironment in the developing retina, unlike stem cell niche in adult tissues like bone marrow (Arai et al., 2005), intestinal crypt (Leedham et al., 2005), hair follicle (Christiano, 2004) and brain (Wurmser et al., 2004), is ever changing and therefore, relatively more complex due to the demand of the ongoing histogenesis. Here, we demonstrate a complex regulatory nature of early retinal progenitor microenvironment. For example, the microenvironment is characterized by three non-overlapping cell regulatory activities, partitioned into different fractions of the CM, derived from cells in the early stage of retinal histogenesis. The activities that promote the
Fig. 5. Heat sensitivity of activities present in E3CM fractions that influence the differentiation of retinal progenitors into RGC differentiation. BrdU-tagged E14 neurospheres were cultured in the presence of reconstituted untreated or heat-treated <30 kDa E3CM fractions (A), followed by immunocytochemical and RT-PCR analyses for RGC-specific markers and regulators. There was no significant difference in the proportion of BrdU+ and Brn3b+ cells between the two groups (B). Similarly, there was no difference in relative levels of Ath5 and Brn3b transcripts between the two groups (C–D). There was a significant increase in the proportion of BrdU+ and Brn3b+ cells when neurospheres were cultured in the presence of heat-treated >30 kDa E3CM fraction as compared to those in untreated fraction (E–F). Similarly, there was an increase in the relative levels of Ath5 and Brn3b transcripts when progenitors were cultured in the presence of heat-treated >30 kDa E3CM fraction (G, lane 2; H), compared to those in untreated fraction (G, lane 1). Square symbols in the reconstitution scheme correspond to conditions described in the graph. Data are expressed as mean ± SEM from triplicate cultures of three different experiments (***p < 0.001).
differentiation of early retinal progenitors into RGCs are localized in <30 kDa fraction of the E3CM and appeared to be heat stable. Amongst the known factors that could contribute to such activities may include FGFs, that are known to promote RGC differentiation (Zhao and Barnstable, 1996; Yang, 2004), and whose molecular weight is <30 kDa. However, the relative heat sensitivity of FGFs may exclude them as a part of RGC promoting activities in the <30 kDa heat-stable fraction. We observed that the >30 kDa E3CM fraction harbors heat labile activities that are inhibitory to the differentiation of early retinal progenitors into RGCs. Similar RGC inhibiting activities have been observed in >10 kDa fraction of conditioned medium obtained from E9 chick retinal cells (Waid and McLoon, 1998). These activities, present in the microenvironment along with those that promote progenitor differentiation, suggest that the microenvironment fine-tunes the outcomes of differentiation process by striking a balance between differentiation-promoting and differentiation-inhibiting activities. The activities present in the heat labile >30 kDa E3CM fraction may play an important role in the maintenance of early retinal progenitors pool by promoting their proliferation. Amongst the known factors that could contribute towards such activities may include Wnts and Shh, that are heat labile >30 kDa proteins and observed to regulate proliferation of retinal progenitors (Kubo et al., 2003; Yang, 2004; Ahmad et al., 2004; Das et al., 2006). Such activities, which can positively influence proliferation, will ensure the maintenance of retinal progenitors for subsequent cell generation.

An intuitive characteristic of the early retinal stem cell/progenitor microenvironment is to ensure that progenitors
at that stage do not prematurely differentiate into cell types that are predominantly generated in the late stage of histogenesis. This is particularly important with the emerging evidence that progenitors within a tissue are plastic and can respond to conducive microenvironment for generating different cell types (James et al., 2003) and for regeneration (Conboy et al., 2005). For example, it has been demonstrated that the late retinal progenitors, when exposed to conducive microenvironment, can generate early born neurons, demonstrating their inherent plasticity (James et al., 2003). Evidence suggests that the early retinal progenitors may be similarly plastic and may possess some capacity to generate

Fig. 7. Activities that inhibit the differentiation of retinal progenitors into rod photoreceptor are present in <30 kDa E3CM fraction. E18 neurospheres were cultured in the presence of fractionated and reconstituted >30 kDa or <30 kDa E3CM fractions or 1% FBS (controls) (A) followed by immunocytochemical and RT-PCR analyses of the expression of opsin and Mash1. There was a significant decrease in the proportion of opsin+ cells (arrows) when progenitors were cultured in the presence of <30 kDa E3CM fraction (B–E, N), compared to those cultured in the presence of >30 kDa E3CM fraction (F–I, N) or FBS (J–M, N). There was a decrease in the relative levels of opsin and Mash1 transcripts when progenitors were cultured in the presence of <30 kDa E3CM fraction (lane 3) as compared to those cultured in the presence of >30 kDa E3CM fraction (O, lane 2; P) or FBS (O, lane 1). Square symbols in the reconstitution scheme correspond to conditions described in the graph. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. ×200 (***p < 0.001).
cells other than those that are predominantly born during early neurogenesis (Watanabe and Raff, 1990, 1992). That the generation of such cells can be influenced by microenvironment was demonstrated when E15 retinal cells were observed to generate significantly more rod photoreceptors in the presence of cells from postnatal stage than from E15 retina (Watanabe and Raff, 1990, 1992). Based on these observations it could be surmised that the early progenitors are competent to give rise to early born neurons by virtue of the selective expression of the genome (James et al., 2004). However, this competence is unlikely to be absolute, considering the fact that few rod photoreceptors are born during early histogenesis (Morrow et al., 1998). But the odds are heavily against such specification as the early progenitors, besides being partial towards the generation of early born neurons, are surrounded by cues for their specification and the presence of inhibitory activities, present in heat labile <30 kDa, for the generation of late born neurons. It could be suggested that CNTF, a heat labile <30 kDa protein and a candidate regulator of rod photoreceptors, may contribute to such activities. However, based on the observation that the expression of CNTF is detected much later in histogenesis (Kirsch et al., 1997) it may be ruled out from the <30 kDa fraction. As the development progresses, due to differential gene expression (James et al., 2004), progenitors acquire the ability (competence) to respond more favorably to cues that promote the generation of the late born neurons than to those conducive for giving rise to early born neurons. This process is likely to be aided further by changes in the microenvironment which progressively attenuate the inhibitory influences on the generation of the late born. Alternatively, it can be proposed that there could be sub pools of progenitors with variations in levels of competence to generate early and late born neurons during early histogenesis. Examination of these possibilities will require further studies. Taken together, our observations reveal the complex nature of the early retinal progenitor microenvironment for the maintenance and differentiation of retinal progenitors. It is characterized by different activities, the combined actions of which may regulate the efficiency and fidelity of cell differentiation during early retinal histogenesis.

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Fig. 8. Heat sensitivity of activities present in E3CM fractions that influence the differentiation of retinal progenitors into rod photoreceptor. E18 neurospheres were cultured in presence of untreated or heat-treated <30 kDa E3CM fraction (A), followed by immunocytochemical and RT-PCR analyses for the expression of opsin and Mash1. There was a significant increase in the proportion of opsin+ cells when progenitors were cultured in the presence of heat-treated <30 kDa E3CM fraction as compared to untreated fraction (B). Similarly, there was an increase in the relative levels of Mash1 and opsin transcripts when progenitors were cultured in the presence of heat-treated <30 kDa E3CM fraction (C, lane 2; D) as compared to untreated controls (C, lane 2). Square symbols in the reconstitution scheme correspond to conditions described in the graph. Data are expressed as mean ± SEM from triplicate cultures of three different experiments (**p < 0.001).
References


