Stem Cell Research 21 (2017) 141–147

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Methods and reagents

P-Cadherin is necessary for retinal stem cell behavior *in vitro*, but not *in vivo*

Brenda L.K. Coles *, Derek van der Kooy [1]

Department of Molecular Genetics, University of Toronto, Terrence Donnelly Centre for Cellular & Biomolecular Research, Rm 1130, 160 College Street, Toronto, ON M5S 3E1, Canada

ARTICLE INFO

Article history:
Received 19 February 2016
Received in revised form 3 April 2017
Accepted 1 May 2017
Available online 3 May 2017

Keywords:
Retinal stem cell
Function blocking antibody
Mouse
Sphere assay

ABSTRACT

Adult retinal stem cells (RSCs) are rare quiescent cells within the ciliary epithelium of the eye, which is made up of non-pigmented N-Cadherin^{+ve} inner and pigmented P-Cadherin^{+ve} outer cell layers. Through FACs and single cell analyses, we have shown that RSCs arise from single cells from within the pigmented CE and express P-Cadherin. However, whether the expression of P-Cadherin is required for maintenance of the stem cell *in vivo* or in the formation of the clonal stem cell spheres *in vitro* is not known. Using cadherin functional blocking antibody experiments and a P-Cadherin $^{-/-}$ mouse to test whether the RSC population is affected by the loss of P-Cadherin expression, our experiments demonstrate that the RSCs reside in the pigmented CE layer and express P-Cadherin, which is important to the formation of adherent sphere colonies *in vitro*, however P-Cadherin is not required for maintenance of RSCs *in vivo*.

1. Introduction

Mammalian eyes contain retinal stem cells (RSCs) that are capable of proliferating and differentiating into all of the cell types of the neural retina as well as the retinal pigmented epithelium (RPE) *in vitro* (Tropepe et al., 2000; Ahmad et al., 2000; Demontis et al., 2012; Fang et al., 2013; Ballios et al., 2012; Coles et al., 2004). These stem cells are located at the retinal periphery in the bi-layered ciliary epithelium (CE), which overlays the ciliary body muscles that control the lens. This CE contains an inner non-pigmented layer that is contiguous with the neural retina and a pigmented outer layer that is contiguous with RPE (Xu et al., 2002; Bharti et al., 2006); however, RSCs only reside within the pigmented CE layer. The ciliary marginal zone (CMZ) is a region in the peripheral retina of fish and amphibians that is capable of continuous retinal regeneration (Reh & Levine, 1998). The similar peripheral location of mammalian RSCs suggests there may be some homology in the RSC niche between these animal classes (Reh & Levine, 1998). The two CE layers can be distinguished by the expression of cadherins, in that the non-pigmented CE (and the neural retina) expresses N-Cad (Cdh2) and the pigmented CE (and the RPE) expresses P-Cad (Cdh3) (Xu et al., 2002; Nose & Takeichi, 1986; Inagaki et al., 2005). We have shown through FACS and single cell analyses that RSCs arise from a rare cell within the pigmented CE that expresses P-Cad but not N-Cad (Ballios et al., 2012). These rare prospectively isolated cells proliferate to form clonally derived spheres *in vitro* that are multipotential and self-renewing, arguing that they are stem cells and not cells derived *via* transdifferentiation as suggested by others (Cicero et al., 2009). We sought to distinguish whether P-Cad or N-Cad are simply indicative of the CE tissue of origin or whether they are functionally relevant to the maintenance or proliferation of RSCs *in vivo* and *in vitro*.

2. Material and methods

2.1. Mouse strains

Adult CD1 and C57Bl/6 mice were obtained from Charles River, Canada and P-Cad$^{-/-}$ mice from Jackson Labs (http://jaxmice.jax.org/strain/003180.html) and were then bred in the University of Toronto animal facility. Both sexes of animals were used for the experiments. C57Bl/6 are the appropriate controls for the P-Cad$^{-/-}$ mice. Experimental procedures were performed in accordance with the CCAC and approved by the Animal Care Committee at the University of Toronto.

2.2. Primary cell isolation and culture

Cells were isolated from the CE of the eye. Adult mice were killed by cervical dislocation, and their eyes were harvested in oxygenated artificial cerebral spinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose). The neural retina was first dissected free of the RPE. Then the cornea and iris were cut away on one edge of the CE and the RPE was cut away of the other edge of the CE leaving the sclera over-laid with the ciliary muscle and the bi-layered CE. Each tissue was treated with Dispase (Corning) for

* Corresponding author.
E-mail address: Brenda.Coles@utoronto.ca (B.L.K. Coles).
[1] Co-author: Derek van der Kooy, derek.van.der.kooy@utoronto.ca

http://dx.doi.org/10.1016/j.scr.2017.05.001

10 min at 37 °C to facilitate the removal of the RPE from the underlying basement membrane. Tissue was dissected and placed in a trypsin solution at 37 °C for another 10 min [artificial CSF modified to contain high Mg^{2+} (3.2 mM $MgCl_2$) and low Ca^{2+} (108 μM $CaCl_2$), 1.33 mg/ml trypsin (Sigma), 0.67 mg/ml hyaluronidase (Sigma), and 0.2 mg/ml kynurenic acid (Sigma)]. The CE was removed from the muscle and sclera, dissociated and then the cells were centrifuged at 150*g* for 5 min, and the enzyme solution was removed and replaced with serum-free media (described in Tropepe, et al., 2000) containing trypsin inhibitor (1 mg/ml ovamucoid; Boehringer-Mannheim). Cells were triturated until a single cell suspension was achieved and then centrifuged again as above. The cells were centrifuged again and then re-suspended in serum-free media (SFM) at clonal densities (either 5 or 10 cells/μl in 0.5 ml) with the addition of FGF2 (Sigma) and heparin (Sigma) as described previously (Tropepe, et al., 2000; Coles & van der Kooy, 2010). For each mouse the CE from both eyes was combined, except in the P-Cad$^{-/-}$ experiments where each eye was plated separately. At least 4–6 wells were plated for each mouse for each condition to control for sphere formation variability, and the average of the wells was used as a single "n". In the case of the P-Cad$^{-/-}$ experiments, all of the cells were plated in order to count absolute sphere number when plated at 10 cells/μl (5000 cells). Spheres that were >80 μm on day 7 were counted. We use this cut-off since we have found it hard to passage any sphere under this size which makes it hard to assess for self-renewal capability. For cadherin function-blocking antibody experiments, the P-Cad (Cdh3) antibody at 5–50 ng/ml (Lifespan Biosciences, LS-C21057) and the N-Cad antibody (Cdh2) at 5–50 ng/ml (Invitrogen/Zymed NCD-2) were employed. R-Cadherin blocking antibody (5–50 ng/ml) (US Biological, C0108-38) was used as a control cadherin antibody, since it is expressed in the Müller glia cells of the developing mouse eye, but not in retinal precursor cells (Miyawaki, et al., 2004).

2.3. In vitro antibody function blocking

To tease apart the stages of sphere development during which the function-blocking antibodies were having their effects, three timing experiments were performed. First, the primary dissected CE cells were incubated with the function-blocking antibodies for 45 min, washed twice and then plated without the antibodies present in order to test whether the effect is on the initial stem cell divisions. Second, the antibodies were added to the plating media with the cells from the beginning of plating and then diluted out on day 3 *in vitro* during the early phase of sphere formation, from when the cells are initially adherent to the bottom of the plate to the point when the proliferating colony becomes free-floating. Third, the cells were plated without the antibodies and then the antibodies were added on day 3 *in vitro,* at which point the clonal colonies are still very proliferative and are free-floating.

2.4. Live imaging

Imaging of single primary ciliary epithelial cells was done using the IncuCyte live-cell imaging system (Essen Bioscience, Ann Arbor, MI, USA). Nunc (Thermo Scientific) 6-well plates were first coated in Poly-D-Ornithine for 30 min, rinsed three times with DPBS (Invitrogen) and then the primary cells were plated at 60 cells/μl (2.5 mL of SFM + FGF2 + Heparin). The plate was kept in a CO_2 incubator for 16 h and then transferred to the IncuCyte system for 3 days. The videos were analyzed using Quicktime Player on an Apple MacBook Pro connected to a 40 in. Sony television screen.

2.5. Immunostaining

Immunostaining was performed as previously described on 14 μm eye or RSC sphere sections (Tropepe, et al., 2000). Immunofluorescence was reviewed by epifluorescence (Zeiss AxioObserver.D1) as well as confocal image analysis (Olympus Fluoview FV1000) to confirm localization of indicated protein products. The following antibodies were used: Mouse anti-P Cadherin at 1:100 (Lifespan Biosciences, LS-C21057), Rabbit anti- P-Cadherin at 1:50 (Santa Cruz Biotech, sc-1501), Rabbit anti- N-Cadherin at 1:100 (Abcam, ab12221) and Mouse anti- N-Cadherin at 1:250 (Abcam, ab98952). Hoechst at 10 ng/ml (Sigma B1155) was used as a nuclear marker.

2.6. Eye measurements

The diameters of P-Cad$^{-/-}$ and wild-type eyes were measured at their widest point in sagittal sections, and the CE length within these sections was measured from the edge of the Neural Retina to the beginning of the iris, including all folds along the pigmented layer of the CE using ImageJ software.

2.7. Statistics

ANOVA and *t*-test statistics were carried out using *Prism 5* statistical software (GraphPad Software Inc., La Jolla, CA, USA). Where multiple comparisons are being made, Bonferroni post-hoc analyses were employed.

3. Results

3.1. Cadherin expression in the adult mouse eye

The RSC is a rare cell found in the pigmented CE (Fig. 1A) within the eye. In order to confirm the pattern of expression in adult mouse eyes and to determine if there was any co-localization of the two cadherins, we used non-pigmented CD1 mouse eye sections and double-stained with both P- and N-Cad antibodies (Xu et al., 2002; Nose & Takeichi, 1986). Using confocal imaging analysis, the outer CE cells express P-Cad and the inner CE cells express N-Cad, and there does not appear to be any co-localization of the cadherin antibodies (Fig. 1B: P-Cad: red, N-Cad: green) within the same cell. Furthermore, no outer CE cells expressed N-Cad and no inner CE cells expressed P-Cad, which concurs with the findings of Inagaki et al. (2005). In addition, using the data from FACs experiments (Ballios, et al., 2012) we can demonstrate that there is no overlap of P-Cad and N-Cad immunostaining of the isolated primary CE.

3.2. Inhibition of P- and/or N-cadherin decreases the formation of RSC spheres

Retinal stem cell spheres express both P-Cad and N-Cad, and although most sphere cells stain only for P-Cad or only for N-Cad, in a few rare cases (three cells total from 10 spheres cut into 14 μm sections, two cells found in the same sphere) both P- and N-Cad appear to be co-localized on the same cell *in vitro* (Fig. 1C), To test whether P-Cad and N-Cad were important to the formation of RSC sphere colonies, function-blocking cadherin antibodies were used. The CE was isolated from mice, dissociated into single cells and plated at a clonal density of 10 cells/μl (Tropepe et al., 2000), and then treated with function-blocking antibodies at 5 and 10 ng/ml, 50 ng/ml was used in one experiment but was not significantly different from 10 ng/ml therefore was not continued due to cost. A two-way ANOVA comparing function-blocking antibodies and dose concentration revealed a significant main effect of antibodies ($F_{3,16} = 21.64$, $p < 0.05$), a significant main effect of antibody concentrations ($F_{2,16} = 18.01$, $p < 0.05$), but no significant interaction of antibodies and concentrations (nor any additive effect of the P- and N-Cad antibodies), demonstrating significant decreases produced by P- and N-Cad antibodies in sphere formation compared to both the control R-Cad antibody and non function blocking antibody controls. In most experiments, two controls were used, one with no functional antibody added and one with the R-Cadherin function blocking antibody which is found in the neural retinal Müller glia.

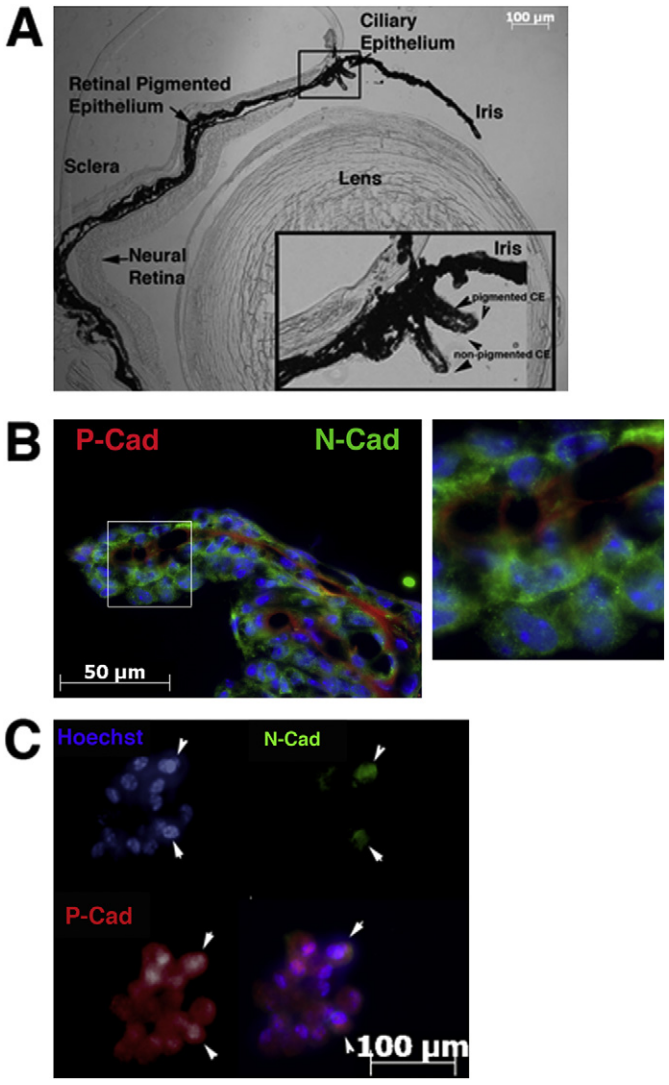

Fig. 1. RSC Niche. A. Section of the mouse eye showing the location of the CE. The inset shows a magnified view of the ciliary margin area that is boxed in the lower magnified panel (scale bar = 100 µm). B. Confocal pictures of the mouse eye stained with Hoechst (blue), P-Cad Antibody (Alexa 568 goat anti-mouse: red), and N-Cad Antibody (Alexa goat anti-rabbit: green). The image on the right is an enlargement of the boxed region in the image on the left showing P-cad staining of the outer layer of the CE and N-cad staining of the inner layer of the CE (scale bar = 50 µm). C. Fluorescent images of 10 µm thick, sectioned retinal stem cell derived spheres, Hoechst (blue) P-Cad Antibody (Alexa 568 goat anti-rabbit: red), and N-Cad (Alexa 468 goat anti-mouse: green) (scale bar = 100 µm). The arrowhead shows a rare cell double-labeled with P- and N-Cad. The vast majority of the cells stain only for one of these two markers.

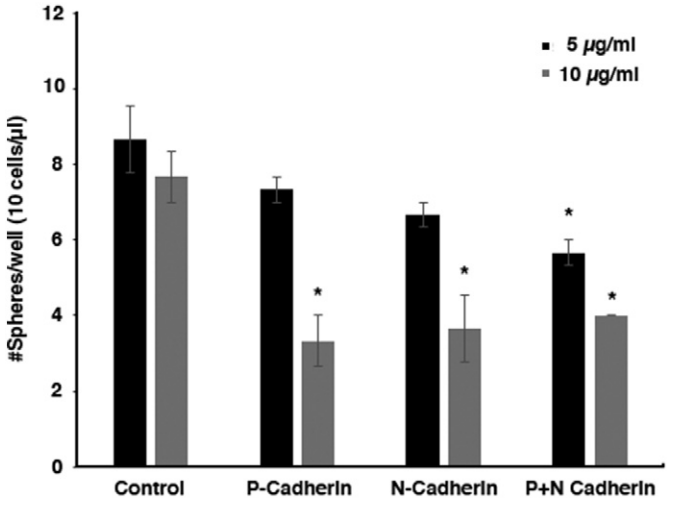

Fig. 2. Function Blocking Antibody Dose Response. Primary CE cells were plated at 10 cells/µl to generate dose responses for the function blocking antibodies, P-Cad, N-Cad, and P-Cad + N-Cad and no antibody vehicle controls. Data represent means ± SEMs.

However, there were no significant differences between these two controls in any of the experiments (*t*-tests demonstrating this are listed in the appropriate figure captions), and therefore the two controls were combined into one control group for graphing purposes.

The combination of the P- and N-Cad antibodies did not have additive effects on clonal sphere formation compared to each antibody alone, and this suggests that the two antibodies might be acting on similar cell populations, which would be surprising given their lack of co-localization *in vivo* (Fig. 2). Since there is a small percentage of the cells within each RSC spheres *in vitro* that do appear to have both N- and P-Cad within the same cell, these data can not rule out the idea that the two antibodies are acting on a few of the same cells during sphere formation (Fig. 1C). These results do suggest that P- and N-Cad may facilitate the adherence of the cells in the formation of the clonally derived spheres. Most interesting, the spheres that do arise in both antibody conditions are similar in size and morphology to the control spheres, and very few spheres below the 80 µm diameter criterion were present in any of the treatment groups. This may indicate that the primary effects of the P-Cad and N-Cad function blocking antibodies are only on the initial formation of clonal spheres, potentially due to disrupting the adherence of the cells during their first few divisions (see below). Since the RSC spheres are made up of both pigmented (RPE progenitors and stem cells) and non-pigmented (neural retina progenitors) cells, the losses of spheres in both P- and N-Cad function-antibody conditions may have been expected.

To address at what point during sphere formation *in vitro* that the cadherins are acting we used function blocking antibodies. In the first condition, the primary cells were pre-exposed to the antibodies and then plated so only the early stem cell divisions should be affected since they are the only proliferative sphere forming cells in the CE. A one-way ANOVA ($F_{2,25} = 7.83$, $p < 0.05$) revealed that there was a main effect of antibody treatment, and multiple comparison tests demonstrated that there was a significant decrease in sphere number only in the condition where the spheres were pre-exposed to P-Cad (7.5 ± 1.28, $p < 0.05$) and no effect with N-Cad antibodies (N-Cad = 11 ± 1.51; Control = 11.5 ± 1.12). Nevertheless, there was no noticeable change in sphere diameters or any increase in small spheres below our cut-off of 80 µm in diameter after P-Cad antibody treatment (Fig. 3A).

When the function-blocking antibodies were added at the same time as the cells on the day of plating (day 0) and then diluted out on day 3, again there was a main effect of antibody treatment ($F_{2,17} = 5.976$, $p < 0.05$), but now the sphere numbers (but not sphere diameters) were decreased by 50–60% in both the P-(3 ± 0.68) and N-Cad (3 ± 0.58) antibody conditions ($p < 0.05$) compared to control (5.33 ± 0.33) (Fig. 3B). During this phase of sphere formation, the cells start out attached to the bottom of the plate and appear to only disengage from the bottom of the plate after dividing enough to reach the 8–16 cell stage, and it is possible that the stem cells may rely on N- and P-Cad to keep the cells together in order to form the initial clonally derived spheres.

There were no changes in sphere numbers (Fig. 3C) or diameters when the function blocking antibodies were added on day 3 and kept in until they were counted on day 7. This might be attributed partially to the ability of the antibodies to penetrate the developing larger spheres. However, since the spheres were not smaller than controls at days 3 or 7, this seems unlikely to account for the lack of effect from the late addition of the antibodies.

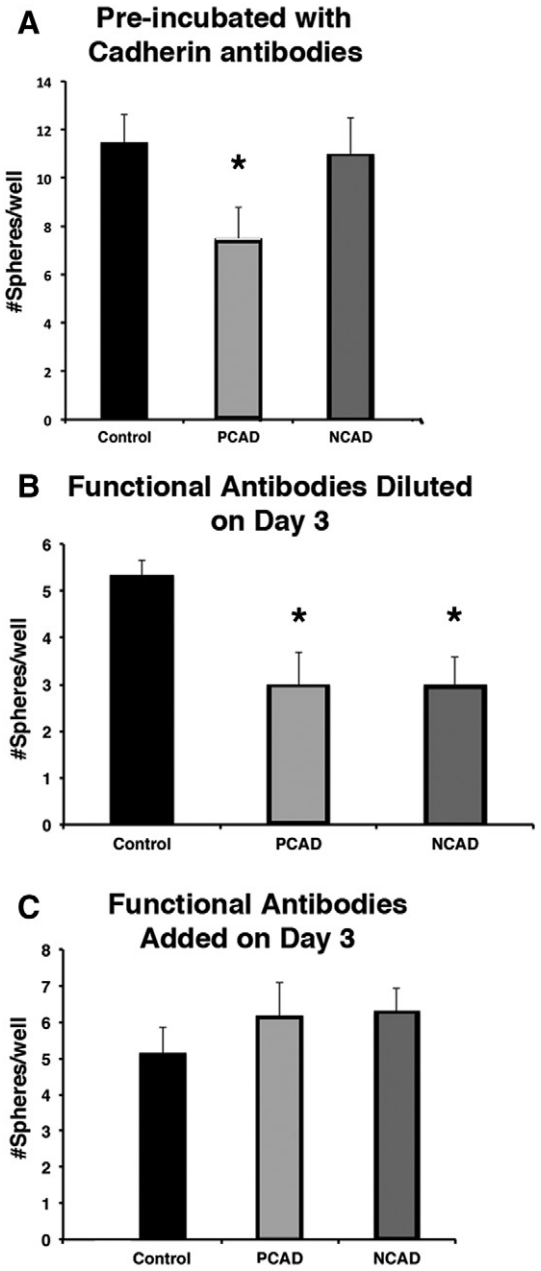

Fig. 3. P-Cadherin Is Important for RSC Sphere Initiation. Primary CE cells were plated at 10 cells/μl. A. P-Cad and N-Cad function blocking antibodies were pre-incubated and labeled with the function blocking antibodies for 30 min, centrifuged to wash off the excess antibody and then plated. B. Function antibodies were added at time of plating and diluted out on day 3 *in vitro*. C. Function blocking antibodies were added on day 3 *in vitro* and left until the end of the experiment. At day 3, the plated cells either had not proliferated or the small clusters were up to 8–16 cells in size. At this stage the colonies were still at the bottom of the well and most were not free floating. R-Cad and no antibody control groups were combined in all graphs, since they were not different significantly from each other (A:($t_{7,6} = 1.029$), B: ($t_{3,3} = 1.625$), C: ($t = {}_{5,5} = 1.00$)). Data represent means ± SEMs.

3.3. Rare pigmented primary ciliary epithelial cells form clones

Using the IncuCyte live-cell imaging system, dissociated single adherent primary adult ciliary epithelial cells were followed from their first division through 3 days of divisions and each cell was followed to identify whether pigmented and/or non-pigmented cells were dividing to form clones in adherent cultures. The cells were very motile and some of the cells moved outside of the field of view, and thus only clones where the all progeny could be followed from the primary cell were used for the analyses. In all cases, only pigmented cells divided to form clones consisting of 2 or more cells (100% of 42 clones followed), and the average clone size was 12.52 ± 1.53 cells after 3 days. Only 3/42 clones gave rise to a non-pigmented by the 4th division *in vitro*.

3.4. Loss of P-cadherin in vivo does not affect the survival of clonal sphere forming cells

Wild type (C57Bl/6) and P-Cad$^{-/-}$ mouse eyes were sectioned and immunostained for N-Cad and P-Cad, which confirmed the loss of P-Cad in the knock-out mouse and the normal expression of N-Cad (Fig. 4 A, B). The CE then was dissected from P-Cad$^{-/-}$ and wild type mice to assess the total numbers of clonal RSC spheres and their frequencies of formation. The spheres that arose were indistinguishable from wild type controls in pigmentation (Fig. 4E) and in sphere diameter ($t_{20,13} = 1.533$). The P-Cad$^{-/-}$ mouse CE produced 2.5 times more RSC spheres on average than the wild type (P-Cad$^{+/+}$) mouse CE per eye ($t_8 = 6.229$, $p < 0.01$) (Fig. 4C), but the frequency of sphere formation from P-Cad$^{-/-}$ mice (number of spheres per cells plated) was not significantly different from the wild type controls ($t_{10} = 0.6936$, $p > 0.05$) (Fig. 4D). The eyes were similar in diameter (Wild type: Ave 2.95 mm ± 0.03; P-Cad$^{-/-}$: Ave 2.96 ± 0.01), and the lengths of the pigmented CE in the P-Cad$^{+/+}$ and P-Cad$^{-/-}$ retinas were not different significantly (Wild type: 1600 ± 82.24 μm; P-Cad$^{-/-}$: 1681.4 ± 71.35 μm). To test for the specificity of the P-Cad antibody used in the experiments above, P-Cad$^{-/-}$ adult CE cells were exposed to the P-Cad or N-Cad function-blocking antibodies. The P-Cad function-blocking antibody did not decrease the number of CE P-Cad$^{-/-}$ clonal RSC spheres compared to the number of untreated CE P-Cad$^{-/-}$ clonal RSC spheres (Control = 17.83 ± 1.242, P-Cad = 18 ± 1.08; $t_{10} = 0.101$, p = ns), but the N-Cad function blocking antibody still decreased significantly the number of P-Cad$^{-/-}$ spheres compared to the number of untreated P-Cad$^{-/-}$ spheres (Control = 17.83 ± 1.242, N-Cad = 12.75 ± 1.109; $t_{24} = 3.053$, $p < 0.05$) (Fig. 4F).

During the primary dissections, it was noted that the CE of the P-Cad$^{-/-}$ mice was much easier to dissociate and required less mechanical dissociation using the small borehole glass pipette in order to get single cell suspensions, and therefore most likely leading to less cell death during dissociation of the P-Cad −/− CE. Indeed, many more viable cells were plated from the P-Cad$^{-/-}$ mouse CE (average total plated cells): WT = 60,000 ± 15,000 cells; P-Cad$^{-/-}$ = 125,000 ± 20,000 cells, ($t_6 = 13.0$, $p < 0.05$). However, the frequencies of sphere forming cells (1 in 400) were not different between the P-Cad$^{+/+}$ and P-Cad$^{-/-}$ mice. Given that there were no differences in eye diameter or in total length of the pigmented CE between the P-Cad$^{-/-}$ and the wild type mouse and the fact that their sphere forming frequencies were not significantly different from one another, the most likely explanation for the 2.5 times increase in sphere formation in the P-Cad$^{-/-}$ mouse is less cell death of the primary CE cells during the dissociation process. Indeed, the greater survival of all cells after CE dissociation can account quantitatively for the greater numbers of absolute clonal spheres isolated from each P-Cad$^{-/-}$ mouse than from each wild type control.

4. Discussion

Adhesion molecules have been implicated in forming the stem cell niche in many systems (Raymond et al., 2009), including germ cells in Drosophila testis (Voog et al., 2008), murine mammary stem cells (Stingl et al., 2006) and murine neural stem cells in the brain (Shen et al., 2008). For example, in the fly testis if there is a loss of adhesion of the stem cells to the hub, then the stem cells will lose their ability to maintain their stemness (Raymond et al., 2009; Voog et al., 2008). In mammalian mammary cells if there is a loss of B1 Integrin, then the stem cells appear to lose proliferative ability (Taddei et al., 2008). In the present study, P-Cad was found to be restricted to the RPE and

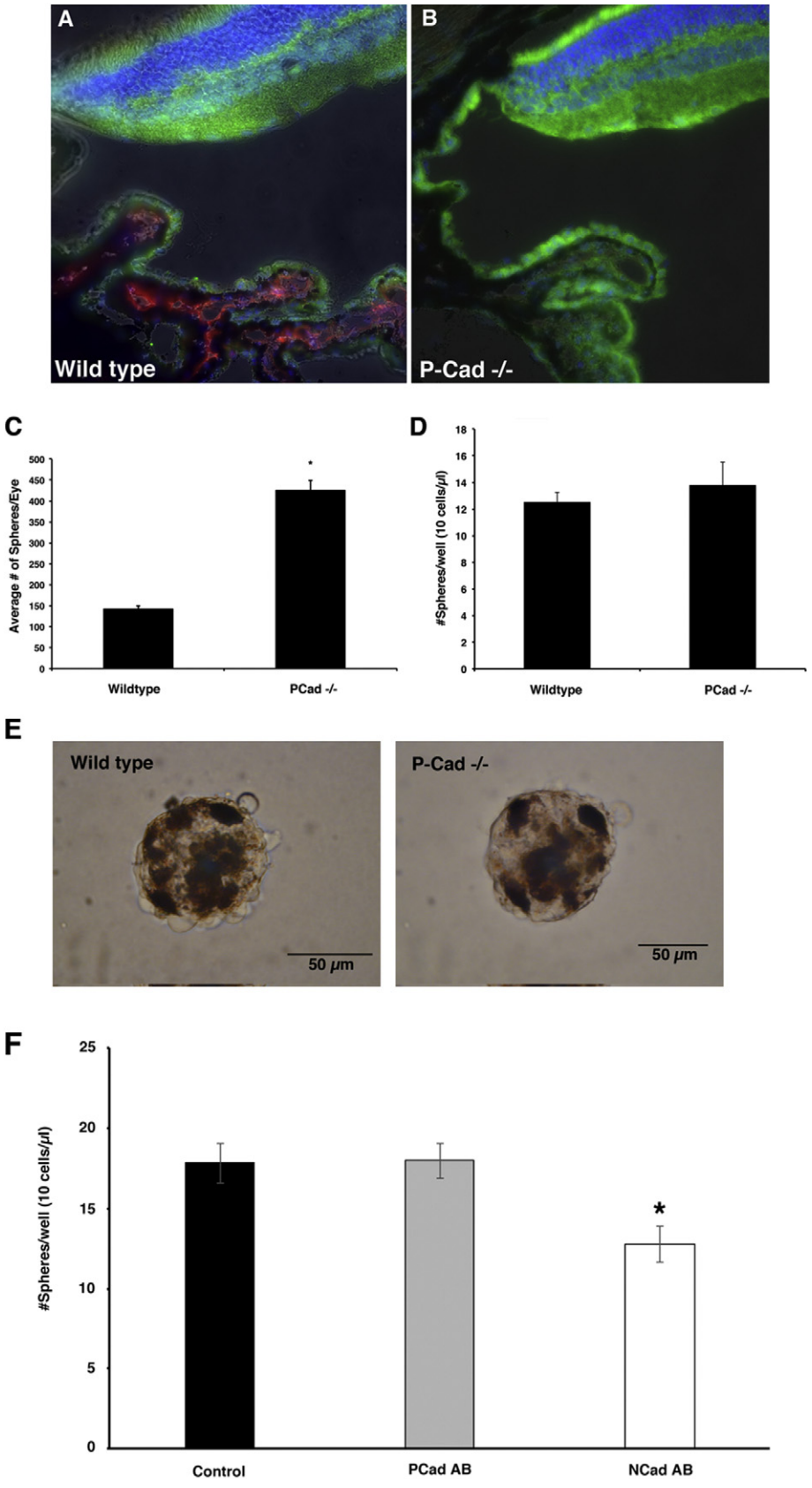

Fig. 4. Loss of P-Cadherin not required for RSC Maintenance. A. Wild type eye and B. P-Cad$^{-/-}$ eye showing CE and NR (Green: N-Cad, Red: P-Cad, Blue: Hoechst). C. Numbers of RSC spheres per CE derived from P-Cad$^{-/-}$ mice compared to C57Bl/6 wild type controls. D. The frequency of sphere formation from P-Cad$^{-/-}$ mice is not significantly different from the frequency from wild type controls. The primary CE from the P-Cad$^{-/-}$ was easier to dissociate than the control and can account for the 2.5× increase in sphere numbers, since the size of the CE *in vivo* was not different. Data represent means ± SEMs. E. Representative spheres from the Wild type and the P-Cad$^{-/-}$ mice, Scale bar = 50 μm. F. Blocking antibody to P-Cad had no effect on P-Cad$^{-/-}$ sphere formation, but the NCad blocking antibody had a significant decrease in sphere numbers.

pigmented CE and N-Cad was restricted to the NR and non-pigmented CE, with no overlap of expression of P-Cad and N-Cad in single cells *in vivo*. We also demonstrated that P-Cad was not required *in vivo* to maintain the RSC niche, and that the CE appeared to be morphologically intact in P-Cad$^{-/-}$ mice. P-Cad is expressed in the cap and myoepithelial cells of the mammary gland; however, in the P-Cad$^{-/-}$ mouse these cells are not affected and maintain their structural integrity (Radice et al., 1997), suggesting that there may be compensatory mechanisms in place that prevent tissue from disorganizing *in vivo* with the loss of one of its main adhesion proteins. A similar compensatory adhesive mechanism may be at work to maintain the structure of pigmented CE and its RSCs *in vivo*. However, the CE from P-Cad$^{-/-}$ mice was easier to dissociate, which appears to have led to more of the RSCs and CE cells in general surviving the combination of enzymatic digestion and harsh mechanical dissociation into single cells. It is possible that we have been underestimating the total number of stem cells in the wild type CE due to cell death during the dissociation of the tissue. Indeed, the total number of clonal spheres obtained per retina was significantly greater in the P-Cad$^{-/-}$ mice compared with wild type controls, but the frequencies of clonal sphere formation were not different significantly. Thus, if there is a compensatory adhesive mechanism, it does not appear to completely rectify the loss of P-Cad mediated adhesion, as evinced by the greater ease of dissociation of pigmented CE cells *in vitro*. In addition, there appears to a low upregulation of NCad expression in a subset of the PCad$^{-/-}$ pigmented CE cells which may suggest that NCad may be compensating for the loss of PCad in these mice. However, blocking NCad in the PCad$^{-/-}$ cells has no affect which leads us to conclude that there may be other compensatory mechanisms in these mice.

In contrast, the *in vitro* studies demonstrate that blocking P-Cad during a pre-incubation period prior to clonal plating had a detrimental effect on the formation of retinal spheres from single RSCs; even though they proliferated in the absence of antibody. However, we were unable to completely block the formation of RSC derived spheres, perhaps due to an insufficient level of P-Cad function blocking achievable with antibodies (Takeichi, 1991). Interestingly, N-Cad also inhibited sphere formation, but only when the antibody was added to clonal cell growth media and not during the pre-incubation period. These effects during initial clonal sphere growth may reflect the mottled retinal spheres that contain both non-pigmented cells that express N-Cad and pigmented cells that express P-Cad (Ballios et al., 2012). The pre-incubation data also may suggest that P-Cad may be required for the initial formation of spheres by P-Cad expressing RSCs; however, once the cells have begun to divide on the bottom of the plate they are making both P-and N-Cad positive cells since both cadherin function blocking antibodies were able to decrease the number of clonal spheres that are formed when added with the cells during initial plating. P-Cad and N-Cad prefer to form homophilic bonds *in vivo*, therefore it is possible that within the initial sphere initiating process some cells do not adhere to one another enough to form the initial clone that can go on to become a free-floating sphere, possibly accounting for the loss when the function blocking antibodies are added from Day 0–3. However, after enough adhering cells have coalesced within the clonal sphere, and the sphere is free-floating, then the addition of P- and N-Cad antibodies to the culture after day 3 no longer inhibited the formation of spheres. This may be due to newly divided cells being surrounded sufficiently by other cells within the sphere to keep the sphere intact and proliferating.

The live imaging experiments demonstrated that only the pigmented primary ciliary epithelial cells proliferate and not until the 4th division of these clonal pigmented RSCs are non-pigmented cells produced. Indeed, in the function blocking antibody experiments only the P-Cad antibody blocked sphere formation in the pre-incubation experiment when presumably only the stem cells themselves would be affected, but both of the cadherin antibodies could decrease sphere numbers when applied over the first 3 days in clonal cultures.

5. Conclusions

RSCs arise from rare single P-Cad expressing cells. Their immediate downstream progeny are initially P-Cad expressing cells (pigmented RSCs and RPE progenitors), and then only after several divisions do N-Cad expressing cells (non-pigmented neural retinal progenitors) appear. Thus, cell adhesive functions of RSC progeny after the first few RSC divisions would be influenced by inhibition of either P-Cad or N-Cad functions. Nevertheless, after clonal RSC spheres have produced sufficient numbers of P-Cad or N-Cad expressing cells, function-blocking antibodies are no longer able to inhibit the further growth of the spheres. *In vivo*, P-Cad may not be necessary for RSC function, or alternatively other adhesive mechanisms may compensate for the loss of P-Cad function. Nevertheless, the absence of P-Cad function does make the peripheral retina easier to dissociate *in vitro* and ensures that a higher absolute number of clonal RSCs can be isolated in culture.

Funding

The authors declare no competing financial interests.

Acknowledgements

Funded by the Krembil Foundation via the Foundation Fighting Blindness Canada (Int Fund # 492035), the Ontario Institute for Regenerative Medicine (GCS:106737.1), the Medicine by Design CFREF program (C1TPA-201 & CFREF-2015), and the Canadian Institutes of Health Research (CIHR: MOP-115162).

References

Ahmad, I., Tang, L., Pham, H., 2000. Identification of neural progenitors in the adult mammalian eye. Biochem. Biophys. Res. Commun. 270 (2), 517–521.
Ballios, B.G., Clarke, L., Coles, B.L., Shoichet, M.S., van der Kooy, D., 2012. The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. Biol. Open 1 (3), 237–246.
Bharti, K., Nguyen, M.T., Skuntz, S., Bertuzzi, S., Arnheiter, H., 2006. The other pigment cell: specification and development of the pigmented epithelium of the vertebrate eye. Pigment Cell Res. 19, 380–394.
Cicero, S.A., Johnson, D., Reyntjens, S., Frase, S., Connell, S., Chow, L.M., Baker, S.J., Sorrentino, B.P., Dyer, M.A., 2009. Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 106 (16), 6685–6690.
Coles, B.L., Angénieux, B., Inoue, T., Del Rio-Tsonis, K., Spence, J.R., McInnes, R.R., Arsenijevic, Y., van der Kooy, D., 2004. Facile isolation and the characterization of human retinal stem cells. Proc. Natl. Acad. Sci. U. S. A. 101 (44), 15772–15777.
Coles, B.L., van der Kooy, D., 2010. Isolation of retinal stem cells from the mouse eye. J. Vis. Exp. 43 (pii: 2209).
Demontis, G.C., Aruta, C., Comitato, A., De Marzo A. Marigo V., 2012. Functional and molecular characterization of rod-like cells from retinal stem cells derived from the adult ciliary epithelium. PLoS One 7 (3), e33338.
Fang, Y., Cho, K.S., Tchedre, K., Lee, S.W., Guo, C., Kinouchi, H., Fried, S., Sun, X., Chen, D.F., 2013. Ephrin-A3 suppresses Wnt signaling to control retinal stem cell potency. Stem Cells 31 (2), 349–359.
Inagaki, M., Irie, K., Ishizaki, H., Tanaka-Okamoto, M., Morimoto, K., Inoue, E., Ohtsuka, T., Miyoshi, J., Takai, Y., 2005. Roles of cell-adhesion molecules nectin 1 and nectin 3 in ciliary body development. Development 132, 1525–1537.
Miyawaki, T., Uemura, A., Dezawa, M., Yu, R.T., Ide, C., Nishikawa, S., Honda, Y., Tanabe, Y., Tanabe, T., 2004. Tlx, an orphan nuclear receptor, regulates cell numbers and astrocyte development in the developing retina. J. Neurosci. 24 (37), 8124–8134.
Nose, A., Takeichi, M., 1986. A novel cadherin cell adhesion molecule: Its expression patterns associated with implantation and organogenesis of mouse embryos. J. Cell. Biol. 103 (6,pt2), 2649–2658.
Radice, G.L., Ferreira-Cornwell, M.C., Robinson, S.D., Rayburn, H., Chodosh, L.A., Takeichi, M., Hynes, R.O., 1997. Precocious mammary gland development in P-cadherin-deficient mice. J. Cell Biol. 139 (4), 1025–1032.
Raymond, K., Deugnier, M.-A., Faraldo, M.M., Glukhova, M.A., 2009. Adhesion within the stem cell niches. Curr. Opin. Cell Biol. 21, 623–629.
Reh, T.A., Levine, E.M., 1998. Multipotential stem cells and progenitors in the vertebrate retina. J. Neurobiol. 36 (2), 206–220.
Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S.M., Goderie, S.K., Roysam, B., Temple, S., 2008. Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell 3, 289–300.
Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., Eaves, C.J., 2006. Purification and unique properties of mammary epithelial stem cells. Nature 439, 993–997.

Taddei, I., Deugnier, M.A., Faraldo, M.M., Petit, V., Bouvard, D., Medina, D., Fässler, R., Thiery, J.P., Glukhova, M.A., 2008. Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. Nat. Cell Biol. 10, 716–722.

Takeichi, M., 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251 (5000), 1451–1455.

Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R., van der Kooy, D., 2000. Retinal stem cells in the adult mammalian eye. Science 287, 2032–2036.

Voog, J., D'Alterio, C., Jones, D.L., 2008. Multipotent somatic stem cells contribute to the stem cell nice in the Drosophila testis. Nature 454, 1132–1136.

Xu, L., Overbeek, P.A., Reneker, L.W., 2002. Systematic analysis of E-, N- and P-cadherin expression in mouse eye development. Exp. Eye Res. 74 (6), 753–760.