Increased Integration of Transplanted CD73-Positive Photoreceptor Precursors into Adult Mouse Retina

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Purpose. Retinal degeneration initiated by loss of photoreceptors is the prevalent cause of visual impairment and blindness in industrialized countries. Transplantation of photoreceptor cells represents a possible replacement strategy. This study determined that identification of cell surface antigens can assist in enriching photoreceptor precursors for transplantation.

Methods. The expression profile of rod photoreceptors at postnatal day 4 was investigated by microarray analysis to identify photoreceptor-specific cell surface antigens. For enrichment of transplantable photoreceptors, neonatal retinas from rod photoreceptor–specific reporter mice were dissociated, and the rods were purified by magnetic associated cell sorting (MACS) with CD73 antibodies. MAC-sorted cell fractions were transplanted into the subretinal space of adult wild-type mice. The number of rod photoreceptors contained in unsorted, CD73-negative, and CD73-positive cell fractions were quantified in vitro and after grafting in vivo.

Results. Microarray analysis revealed that CD73 is a marker for rod photoreceptors. CD73-based MACS resulted in enrichment of rods to 87%. Furthermore, in comparison with unsorted cell fractions, CD73-positive MAC-sorted cells showed an approximately three-fold increase in the number of integrated, outer segment–forming photoreceptors after transplantation.

Conclusions. CD73-based MACS is a reliable method for the enrichment of integrating photoreceptors. Purification via cell surface markers represents a new tool for the separation of transplantable photoreceptor precursors from a heterogeneous cell population, avoiding the need of reporter gene expression in target cells. (*Invest Ophthalmol Vis Sci.* 2011;52:6462–6471) DOI:10.1167/iovs.11-7399

The prime cause of visual impairment or blindness in industrialized countries is the degeneration of photoreceptors located in the outer nuclear layer (ONL) of the retina, as is observed in age-related macular degeneration (AMD) or retinitis pigmentosa (RP). Following up on initial studies by Gouras et al.[1–3] two decades ago, several recent reports have demonstrated that transplantation of photoreceptor precursors into the subretinal space of adult mice results in integration into the host ONL and formation of mature rod and cone photoreceptors.[4–7] Importantly, donor cells correctly integrate and develop, on the one hand, outer segments (OS) that are located in the subretinal space and, on the other hand, axonal terminals (placed in the outer plexiform layer [OPL] in proximity to endogenous bipolar cells), which are indispensable prerequisites for a functional connection to the host neural circuitry. Indeed, first studies suggested some functionality of donor-derived photoreceptors as demonstrated by extracellular field potential recordings from the ganglion cell layer and pupillary reflexes[5] or electroretinography measurements.[8]

To date, most studies have used heterogeneous cell suspensions generated by dissociation of entire retinas. Recent data indicate that cell suspensions from mouse retinas isolated between postnatal day (PN)3 and PN5 have the highest potential for integration.[5,6] Consistent in all published studies in which primary retinal cells were used for transplantation is the relatively low number of donor-derived photoreceptors that integrated into the ONL of the host. Various approaches have been used in an attempt to increase the number and survival of integrating photoreceptors, including disruption of the outer limiting membrane,[9,10] immune suppression,[11] antiapoptotic treatment of donor cells,[12] and enrichment of fluorescence reporter expressing donor cells by flow cytometry.[7]

The purification of transplantable cells based on such ectopic expression of reporter genes, however, is not suitable for therapeutic application in humans. As demonstrated in the stem cell field, it would be more favorable to purify donor cells based on the expression of cell-type–specific cell surface markers. Such selection and enrichment methods are particularly important when photoreceptor precursors are generated from an in vitro expandable cell source, such as pluripotent embryonic stem (ES) or induced pluripotent stem (iPS) cells. Current protocols allow the in vitro generation of rod and cone photoreceptors from mouse and human pluripotent stem cells, albeit still in relative limited quantities.[8,13–16]

In the present study, we showed that ecto-5′-nucleotidase, a 70-kDa glycosylphosphatidylinositol (GPI)-anchored cell surface molecule, also called cluster of differentiation (CD)73, is highly expressed in young rod photoreceptors, confirming recent findings by Koso et al.[17] Furthermore, we demonstrated for the first time that magnetic-associated cell sorting (MACS) using antibodies against CD73 is a reliable and easy method of enriching cells of the photoreceptor lineage. Importantly, on transplantation, positively enriched photoreceptor precursors showed a significantly increased integration rate within the ONL of adult mice when compared to unsorted and/or negative sorted cell fractions.

Materials and Methods

Animals

Retinas were isolated at PN4 from neural retina leucine zipper (Nrl) EGFP[18] or rhodopsin (rho) EGFP[19] reporter mice. In Nrl-EGFP mice, a transgene coding for EGFP driven by the Nrl promoter was introduced.

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Nrl is a transcription factor determining rod fate and is the earliest known marker for rod photoreceptors. Rho-EGFP mice were generated by knocking-in the human rhodopsin gene fused to EGFP at the mouse rhodopsin locus. Expression of EGFP is thus restricted to OS in mature rod photoreceptors. For transplantation studies, rhoEGFP mice were mated with transgenic mice expressing DsRed driven by the ubiquitous active chicken β-actin promoter[20] (actin-DsRed mice; strain B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J; The Jackson Laboratory, Bar Harbor, ME) to generate photoreceptors that are labeled by DsRed in the cell body and by EGFP in the OS. In addition, for microarray analysis some retinas were isolated at PN0 from nestin-EGFP transgenic mice.[21] These reporter mice express GFP under the control of regulatory regions of the nestin gene. Nestin is a class IV intermediate filament expressed in neural/retinal progenitor cells and Müller glia. All animal experiments were conducted in strict accordance with EU and German laws (Tierschutzgesetz) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Isolation for Microarray

Retinas were isolated from PN0 nestin-EGFP and PN4 rhoEGFP mice and dissociated in 0.5 mg/mL trypsin (Invitrogen, Karlsruhe, Germany)/Hanks' balanced salt solution (HBSS; Invitrogen) for 20 minutes at 37°C. Then, 2 mg/mL trypsin inhibitor (Roche Diagnostics, Mannheim, Germany) and 0.1 mg/mL DNase I (Invitrogen) were added. Additional mechanical dissociation was performed by triturating cells 10 times using a 1-mL plastic tip and 10 times through a fire-polished glass Pasteur pipette. The cells were centrifuged 8 minutes at 300*g* and finally resuspended (1×10^7 cells/mL) in FACS-buffer (HBSS) consisting of 5% newborn calf serum (NCS; Invitrogen), 10 mM HEPES (PAA Laboratories, Pasching, Austria), and 2 mM EDTA (Miltenyi Biotec, Bergisch Gladbach, Germany). To prevent RNA degradation, RNaseOut (Invitrogen) was added to recovery tubes (10 μg/mL). The cells were sorted based on GFP expression (FACSAria II; BD Bioscience) and data analysis was performed (FACSDiva Software; BD Bioscience).

RNA Preparation and PCR

RNA from FAC- and MAC-sorted (see below) or unsorted cells were isolated (RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed with reverse transcriptase (SuperScript II; Invitrogen), according to the manufacturer's instructions. PCR from first-strand cDNAs was performed (HotStarTaq Plus Master Mix Kit; Qiagen) according to the manufacturer's protocol, with the following primers: β-actin sense, 5′-TGGTACGACCAGAGGCATACAG-3′, and β-actin antisense, 5′-GCTGGACTTACGGCTTGTCACC-3′; CD73 sense, 5′-CAGGAAATCCACCTTCCAAA-3′, and CD73 antisense, 5′-TCAGGAATGCTGCTGTTGAG-3′; and rhodopsin sense, 5′-CTCTGCCAGCTTTCTTTGCT-3′, and rhodopsin antisense, 5′-ACAGTCTCTGGCCAGGCTTA-3′.

Microarray and Data Analysis

FAC-sorted retinal cells of rhoEGFP PN4 and nestin-EGFP PN0 mice were analyzed on mouse genome arrays (430 2.0 GeneChip; Affymetrix, Santa Clara, CA). Three replicates of each rhoEGFP PN4-positive and -negative fractions as well as nestin-EGFP PN0-positive fractions were performed. Subsequent steps including synthesizing of probes, hybridization, washing, and scanning of microarray chips were performed at the microarray facility of the Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG, Dresden, Germany) according to the manufacturer's instructions (Affymetrix). Data were analyzed (GeneSpring GX 11.0.1 software; Agilent Technologies, Santa Clara, CA). The normalization algorithm robust multiarray averaging (RMA) was used to reduce the false discovery rate and exclude probe sets with low intensity. Additionally, the lower 20% of entities were filtered out. ANOVA ($P < 0.05$) was performed to show that the entities changed significantly between the three sample types.

Enrichment of Cells by MACS

Retinas were isolated from 4-day-old Nrl-EGFP-, rhoEGFP-, or rhoEGFP/actin-DsRed mice, dissociated (as described above), and centrifuged for 8 minutes at 300*g*. The cells (up to 5×10^7) were resuspended in 0.5 mL MACS buffer (phosphate-buffered saline [PBS; pH 7.2], 0.5% BSA, 2 mM EDTA; Miltenyi Biotec), incubated with 10 μg/mL rat anti-mouse CD73 antibody (BD Biosciences) for 5 minutes at 4°C, washed in MACS buffer, and centrifuged for 8 minutes at 300*g*. The cell pellet was resuspended in 0.4 mL MACS buffer and 0.1 mL goat anti-rat IgG magnetic beads (80 μL buffer and 20 μL MicroBeads per 10^7 total cells; Miltenyi Biotec). The suspension was incubated for 15 minutes at 4°C followed by a washing step with MACS buffer and centrifugation. Before magnetic separation (according to the manufacturer's instructions; Miltenyi Biotec), the cells were resuspended in MACS buffer and filtered through a 30-μm pre-separation filter. Briefly, the cell suspensions were applied onto a pre-equilibrated LS column fixed to a MACS separator. The column was rinsed with 3×3 mL MACS buffer and the flow through was collected (CD73 negative cells). The column was removed from the magnet and placed in a new collection tube. The CD73-positive fraction was eluted by loading 5 mL MACS buffer and immediately applying the plunger supplied with the column. A portion of each retina isolation round was kept on ice during the entire sorting procedure and was used later as an unsorted fraction.

Flow Cytometry Analysis

Unsorted, CD73-negative, and CD73-positive cell fractions were analyzed by flow cytometry (LSRII flow cytometer; BD Bioscience), and the acquired data were evaluated (FACS Diva software; BD Bioscience). The effectiveness of CD73-based MACS enrichment was evaluated by labeling cells with allophycocyanin (APC)-conjugated secondary donkey anti-rat IgG antibodies (eBioscience, Frankfurt, Germany) and quantification by flow cytometry. The number of rod photoreceptors in unsorted, CD73-negative, and CD73-positive cell fractions were identified due to the expression of EGFP (Nrl-EGFP or rhoEGFP, respectively). Cells isolated from wild-type retinas served as negative controls to define the gates for separating EGFP-positive and -negative cells.

For live/dead cell discrimination, propidium iodide labeling (0.1 μg/mL) was performed on MAC- or flow-sorted or unsorted cells before flow cytometry analysis.

In Vitro Cultivation and Quantification

Unsorted, CD73-negative and CD73-positive cells from retinas of Nrl-EGFP or rhoEGFP mice were cultured in vitro in DMEM/F12 containing 5 mM HEPES, 2 mM L-glutamine, 2% B27 supplement, 100 units/100 μg/mL penicillin/streptomycin, 1% NCS (all from Invitrogen), 20 ng/mL FGF2 (Miltenyi Biotec), and 5 μg/mL heparin (Sigma-Aldrich, Munich, Germany). For 4 hours of cultivation, 2×10^5 cells were plated on 12-mm diameter poly-L-lysine-coated coverslips (PLL; Sigma-Alrich) in 24-well culture plates. For 4-day cultures, 2×10^6 cells were plated onto PLL/laminin (5 μg/mL; Invitrogen) coated coverslips. After cultivation, the cells were fixed in 4% paraformaldehyde (PFA) in PBS and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The samples were examined using a fluorescence microscope (Z1-Imager with ApoTome; Carl Zeiss Meditec, Jena, Germany). At least four independent experiments were performed in which GFP-positive cells from four to eight randomly chosen areas of at least two coverslips were quantified.

Immunohistocytochemistry

For immunostaining samples were 4% PFA fixed (10 min); blocked (30 minutes) in PBS containing 5% goat serum (Sigma-Aldrich), 1% bovine serum albumin (BSA; Sigma-Aldrich), and 0.3% Triton X-100 (TU-Dresden Pharmacy, Dresden, Germany), and incubated (2 hours) with the following primary antibodies: mouse anti-rhodopsin (rho4D2; 1:100; kind gift of Robert Molday, University of British Columbia, Vancouver,

BC, Canada) or rabbit anti-recoverin (1:3000; Millipore, Schwalbach, Germany) antibodies. For immunostaining with rat anti-CD73 antibodies (1:500; BD Biosciences) living cells were incubated for 10 minutes followed by fixation with 4% PFA. Primary antibodies were visualized by incubation (1 hour) with Cy3-conjugated goat anti-mouse, anti-rabbit, or anti-rat secondary antibodies, respectively. Samples were finally counterstained with DAPI and mounted on glass slides.

Transplantation

For transplantation of cell suspension into the subretinal space, adult (i.e., 2–4 months old) wild-type (C57BL/6J) mice were anesthetized by an intraperitoneal injection of medetomidine hydrochloride (0.01 mg/10 g body weight; Dormitor; Pfizer, Berlin, Germany) and ketamine (0.75 mg/10 g body weight; Ratiopharm, Ulm, Germany) and fixed in a head holder. Pupils were dilated by drops of 1% tropicamide (Mydrum; Dr. Mann Pharma GmbH, Berlin, Germany) and 2.5% phenylephrine (TU Dresden Pharmacy). A syringe (Hamilton, Reno, NV) with a blunt, 34-gauge needle was inserted tangentially through the conjunctiva and sclera and placed under visual control in the nasal half of the retina into the subretinal space (i.e., between the retina and retinal pigment epithelium). One microliter suspension containing 400,000 cells was injected to produce a bullous retinal detachment. The same person performed all transplantation experiments. For recovery, experimental animals received an injection of atipamezole hydrochloride (0.1 mg/10 g body weight; Antisedan; Pfizer) for reversal of the medetomidine.

Quantification of Integrated Photoreceptors

Three weeks after transplantation, experimental animals were killed, and the eyes were removed and fixed in 4% PFA in PBS for 12 hours. Retinas were dissected and embedded in 4% agarose to produce 30-μm-thick serial sections using a vibrating microtome (model 1200; Leica, Wetzlar, Germany). Beside morphologic criteria like a cell body located in the ONL and the generation of spherule synapses and/or inner segments integrated donor photoreceptors were identified by their red (DsRed in the cell body) and green (EGFP in the OS) fluorescence. For quantification seven retinas that received unsorted cell fractions, six retinas that received CD73-negative fractions, and seven retinas that received CD73-positive fractions were analyzed. The number of integrated donor cells per retina was calculated after quantification of every fourth serial section, with a total of 18 sections per experimental retina, by fluorescence microscopy (Z1-Imager with ApoTome; Carl Zeiss Meditec).

Statistics and Graphics

Results of data analyses are presented as ± SEM from at least three independent experiments (ImageJ, National Institutes of Health, Bethesda MD; AxioVision; Carl Zeiss Meditec; Excel, Microsoft, Redmond, WA; or iWork Numbers, Apple Computer, Cupertino, CA) software. Significance was calculated by unpaired, two-tailed, Student's *t*-test and is represented in figures by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Increased Expression of CD73 in Rod Photoreceptors

Recent studies demonstrated that cell suspensions isolated from the mouse retina at PN3 to PN5 have the highest integration rate after transplantation into the adult mouse retina.[5–7] Therefore, murine retinas used in this study for isolation of photoreceptors were dissected at PN4. To identify genes that are specifically upregulated in young rod photoreceptors, microarray analysis was performed to compare gene expression of rhoEGFP-positive and -negative cells isolated from PN4 mice. To further specify the profile of rhoEGFP positive photoreceptors their gene expression was additionally compared to nestin-EGFP-positive cells isolated at PN0. Nestin, an intermediate filament protein, is highly expressed during retinal development and marks retinal progenitor cells. Microarray analysis revealed 8354 significantly changed entities (ANOVA; $P = 0.05$); 1125 entities showed more than a twofold higher expression in rhoEGFP-positive cells in comparison to rhoEGFP-negative sorted cells (Fig. 1A) and nestin-EGFP-positive sorted cells (Fig. 1B). These 1125 entities represent 744 genes. The microarray data generated in this study have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Bethesda, MD)[22] and are accessible through GEO Series accession number GSE29318 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29318).

Several genes specific for the rod photoreceptor lineage—rhodopsin, *Pde6b* (phosphodiesterase subunit 6b), *Gngt1* (gua-

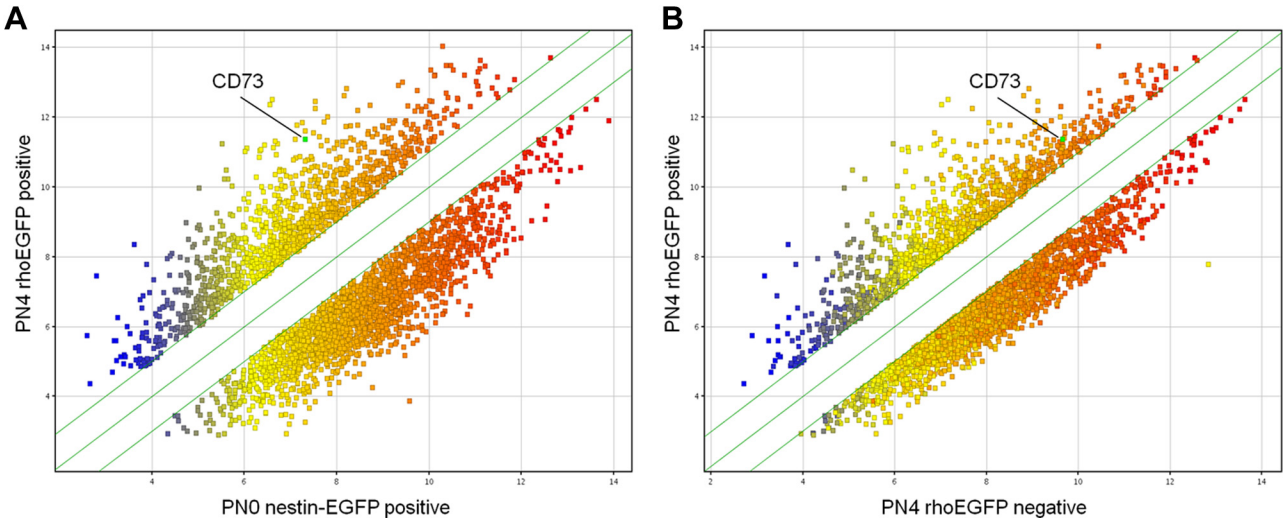

Figure 1. Comparison of the global expression profile of rhoEGFP-positive cells versus rhoEGFP-negative and nestin-EGFP-positive cells. Scatterplots of differentially expressed genes in PN4 rhoEGFP-positive cells versus PN0 nestin-EGFP-positive cells (**A**) and PN4 rhoEGFP-positive versus PN4 rhoEGFP-negative cells (**B**) revealed twofold gene expression differences (*green lines*) of 1125 upregulated and 1948 downregulated entities. CD73 is indicated by a pointer and a *green* symbol (**A**, **B**).

nine nucleotide binding protein (G protein) gamma transducing activity polypeptide 1), or recoverin—showed a more than 10-fold higher expression in rhoEGFP-positive cells, indicating the high selectivity of FACS enrichment for rod photoreceptors (Table 1). Microarray analysis also revealed genes encoding proteins located on the cell surface that may serve as antigens for antibody-mediated MACS. For example, CD73 (Nt5e, ecto-5′-nucleotidase), Cnga1 (cyclic nucleotide gated channel alpha 1), Gje1 (gap junction protein, epsilon 3), and Pcdh15 (protocadherin 15) showed more than twofold higher expression in rhoEGFP PN4-positive cells compared with the other fractions (Fig. 1, Table 1). Altogether, 120 entities representing 79 different transcriptional units with GO-Term plasma membrane were identified and showed a more than twofold higher expression in PN4 rhoEGFP-sorted cells (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental).

Since the CD73 antibody was commercially available, we further focused on it in the present study. Indeed, PCR analysis confirmed the expression of CD73 transcripts in PN4 mouse retina (Fig. 2C) and immunocytochemistry, using CD73-specific antibodies on dissociated and cultured rhoEGFP retinas showed CD73-immunoreactivity in the majority of GFP-positive rod photoreceptors (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental). In line with our results, CD73 was recently identified as the first cell surface marker for early stages of the photoreceptor lineage after screening of several CD antigens.[17] Because of these findings we evaluated CD73 as the first candidate for proof-of-principle experiments investigating the transplantation of cell surface marker–enriched photoreceptors.

MACS Enrichment of Photoreceptor Precursors

We investigated whether CD73 antibodies are reliable tools for purification of cells of the photoreceptor lineage. Nrl-EGFP and rhoEGFP transgenic mice were used to identify rod photoreceptors. In unsorted (see the Materials and Methods section), MACS-negative, or MACS-positive cell fractions the number of EGFP-positive rod photoreceptors was analyzed using flow cytometry and fluorescence microscopy after in vitro culturing. To test whether CD73-based MACS indeed results in enrichment of CD73-positive cells, we first analyzed the number of CD73-positive cells in unsorted, CD73-negative and -positive sorted fractions by flow cytometry with APC-conjugated secondary antibodies. The results showed that unsorted cells contained 52% ± 9.89% CD73-positive cells (Figs. 2Ai, 2B) whereas negative fractions contained only 16% ± 10.76% (Figs. 2Aii, 2B). As anticipated, a strong increase—that is, 83% ± 4.04%, of CD73-positive cells was detected in the CD73-positive MAC-sorted fractions, confirming their successful enrichment by MACS (Figs. 2Aiii, 2B). Semiquantitative RT-PCR revealed also a strong expression of CD73 transcripts in CD73-positive sorted cells, whereas unsorted fractions and CD73-negative fractions showed moderate or low expression, respectively (Fig. 2C). Interestingly, RT-PCR analysis using rhodopsin-specific primers revealed a high expression of rhodopsin transcripts in CD73-positive sorted fractions in contrast to CD73-negative ones, demonstrating the enrichment of rod photoreceptors (Fig. 2C).

The most common techniques for the enrichment of specific cell-types from heterogenous cell populations are either MACS or flow cytometry. In our hands, CD73-based MACS of retinas from one litter (6–10 pups) typically resulted in 20 to 30 million rhoEGFP-positive cells in ~60 minutes. In contrast, 4.4 to 7 million rhoEGFP-positive cells were isolated by flow cytometry with green fluorescence in ~180 minutes. To evaluate why MACS resulted in a higher number of cells, we compared the effect of both techniques on the survival of the photoreceptor precursors. The number of dead cells was quantified by propidium iodide incorporation followed by flow cytometry. Whereas MAC-sorted cell fractions contained an average of 15% propidium iodide–positive cells, their number increased up to ~37% in the FAC-sorted fractions (Fig. 2D). Given that the time for high yield enrichment by flow cytometry was considerably longer, we investigated whether the prolonged periods in suspension, rather than the sorting method itself, might be the underlying cause of the reduced number of cells obtained by FACS. Indeed, the analysis of unsorted cell suspensions 60 or 180 minutes after tissue dissociation revealed similar amounts of propidium iodide–positive cells (Fig. 2D). Thus, the increased cell death observed on flow cytometry appears to be due to the prolonged incubation periods, rather than the procedure itself. Consequently, all further experiments were performed with the fast and easy method, MACS.

By using retinas from Nrl-EGFP and rhoEGFP mice, we next quantitatively investigated the enrichment of cells of the rod photoreceptor lineage by means of CD73-based MACS. FACS quantification showed that unsorted cell fractions contained 53% ± 5.25% Nrl-EGFP-positive (Figs. 3Ai, 3B) and 41% ± 3.35% rhoEGFP-positive (Figs. 3Ci, 3D) cells. In contrast, the CD73-positive cell fractions showed a significantly increased num-

Table 1. Subset of Genes with Higher Expression in PN4 rhoEGFP-Positive Cells in Comparison to PN4 rhoEGFP-Negative and PN0 Nestin-EGFP-Positive Cells

Gene Symbol	Gene Title	GO (Avadis)	Fold Change*
Photoreceptor Marker			
Rho	Rhodopsin	Photoreceptor activity	+++
Gngt1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1	Signal transducer activity	+++
Pde6b	Phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide	3′,5′-Cyclic-GMP phosphodiesterase activity	++
Pde6g	Phosphodiesterase 6G, cGMP-specific, rod, gamma	cGMP binding	++
Rcvrn	Recoverin	Calcium ion binding	++
Cell Surface Marker			
Cnga1	Cyclic nucleotide gated channel alpha 1	cGMP binding	+++
Gje1	Gap junction protein, epsilon 1	Cell morphogenesis	++
Pcdh15	Protocadherin 15	Protein binding	+
Nt5e (*CD73*)	5′ Nucleotidase, ecto	Metal ion binding	+

* +, 2–10-fold; ++, 10–20-fold; +++, ≥20-fold upregulated, respectively.

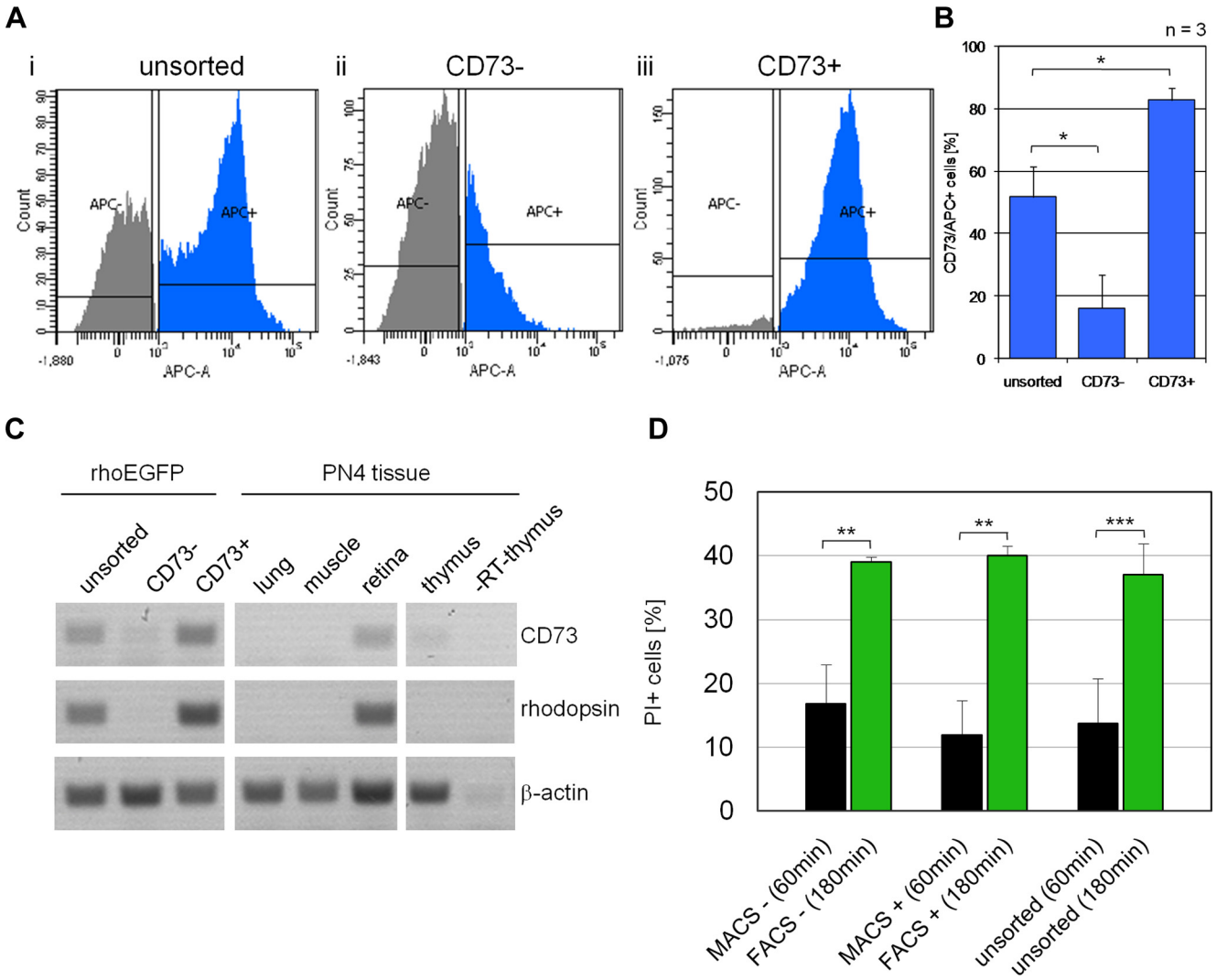

Figure 2. Analysis of unsorted, CD73-negative, and CD73-positive cells isolated from PN4 mouse retinas on MACS procedures. APC-conjugated secondary antibodies were used to detect CD73-positive cells after MACS. Unsorted cell fractions contained 52.0% ± 9.89% CD73-positive cells (**Ai**, **B**). After MACS, the number of CD73-positive cells was reduced to 16.0% ± 10.76% in CD73-negative sorted fractions (**Aii**, **B**) and increased to 83.0% ± 4.04% in positive sorted fractions (**Aiii**, **B**). Semiquantitative RT-PCR analysis using CD73-specific primers revealed CD73 expression in the PN4 mouse retina and CD73-positive cells (**C**). Using rhodopsin-specific primers, a strong expression of this rod photoreceptor marker was also detected in CD73-positive cells. Actin was used as internal control. Weak CD73 expression was also detected in the thymus (positive control), whereas lung and muscle served as negative controls beside thymus preparations without reverse transcriptase (−RT-thymus). Quantification of propidium iodide incorporation in PN4 retinal cells from rhoEGFP retinas after CD73 MACS or GFP FACS (**D**). In MACS propagated cells (MACS− and MACS+) an average of 15% showed incorporation of propidium iodide (PI), representing dead cells. The number of PI-positive cells was significantly increased in cells after flow cytometry (~37%; FACS− and FACS+). In comparing PI incorporation of unsorted cells after 60 or 180 minutes, similar proportions of PI-positive cells were detected (unsorted 60 minutes: 15% and unsorted 180 minutes: 37%). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ber of Nrl-EGFP- and rhoEGFP-positive cells, to 87% ± 6.33% (Figs. 3Aiii, 3B) and 74% ± 9.95% (Figs. 3Ciii, 3D), respectively. Furthermore, in CD73-negative cell fractions the amount of Nrl-EGFP- and rhoEGFP-positive cells was strongly reduced (25.0% ± 2.96%; Figs. 3Aii, 3B; 6.0% ± 2.3%; Figs. 3Cii,3 D, respectively). In addition to the quantification of Nrl-EGFP- and rhoEGFP-positive cells from freshly isolated cells, the three separated cell fractions were further cultivated in vitro for either 4 hours or 4 days. Immunocytochemistry on cultured cell fractions from rhoEGFP retinas after 4 days revealed a large number of CD73-immunoreactivity events in positive sorted cells, whereas their number in negative fractions was substantially reduced (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental). Importantly, virtually all GFP-positive cells (rod photoreceptors) showed CD73 immunoreactivity, irrespective of the cell fraction analyzed (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental). Quantification of in vitro propagated cell fractions showed also a significant increase in the number of Nrl-EGFP- and rhoEGFP-positive cells in the CD73-positive fractions by comparison to unsorted or negative ones (Fig. 3; Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental): 4 hours after MACS, the CD73-positive fractions contained 62.0% ± 4.56% Nrl-EGFP-positive (Fig. 3Ei) or 66.0% ± 3.42% rhoEGFP-positive (Fig. 3Fi) cells in comparison to unsorted cells (Nrl-EGFP, 23.0% ± 2.62%; rhoEGFP, 18% ± 0.99%) or CD73-negative cells (Nrl-EGFP, 2.0% ± 0.41%; rhoEGFP, 4.0% ± 1.21%; Figs. 3Ei, 3Fi). After 4 days in culture, similar proportions of EGFP-positive cells were detected for unsorted (Nrl-EGFP, 19.0% ± 0.62%; rhoEGFP, 10.0% ±

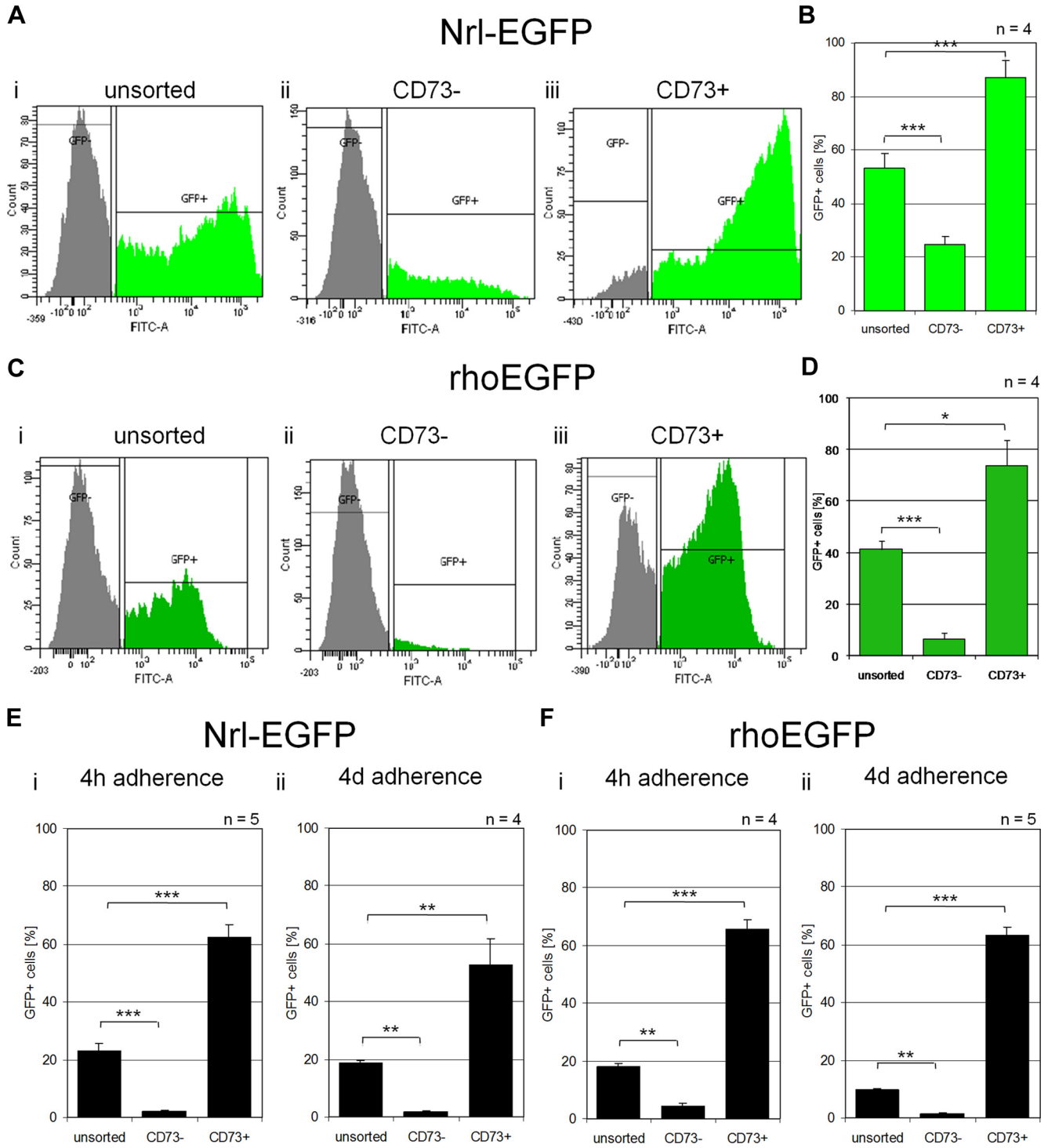

FIGURE 3. Analysis of unsorted, CD73-negative, and CD73-positive cells isolated from PN4 retinas after MACS. Quantification of dissociated retinal tissue from Nrl-EGFP mice by flow cytometry, directly after CD73-based MACS revealed a significant increase in GFP-positive cells in CD73-positive fractions (**Aiii**, **B**, CD73+) in comparison to unsorted cell fractions (**Ai**, **B**), whereas the number of GFP-positive cells significantly decreased in CD73-negative fractions (**Aii**, **B**, CD73−). Similar results were obtained after quantification of cells isolated from rhoEGFP transgenic retinas (**C**, **D**). Note the higher number of GFP-negative cells in CD73-positive fractions sorted from rhoEGFP (**Ciii**) in comparison to Nrl-EGFP (**Aiii**) retinas. The number of GFP-positive cells was additionally quantified after in vitro culturing of CD73-sorted fractions on coverslips for 4 hours (**Ei**, **Fi**) or 4 days (**Eii**, **Fii**). CD73-positive fractions contained significantly higher amounts of GFP-positive cells in comparison to unsorted fractions from Nrl-EGFP (**E**) and rhoEGFP (**F**) transgenic retinas, whereas CD73-negative cells contained a significantly reduced number of GFP-positive cells (**E**, **F**) at both time points. *n*, number of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

0.41%), negative (Nrl-EGFP, 2.0% ± 0.28%; rhoEGFP, 1.0% ± 0.34%), and positive (Nrl-EGFP, 53.0% ± 9.01%; rhoEGFP, 63.0% ± 2.71%) fractions (Figs. 3Eii, 3Fii), although with a general slight decrease in all fractions in comparison to the 4-hour culturing time. Taken together, we could demonstrate by using two independent EGFP-reporter lines that

CD73 is a useful marker for enriching rod photoreceptor precursors via MACS.

Transplantation of Enriched Photoreceptor Precursors

Promoter-driven expression of fluorescence reporters (as in Nrl-EGFP mice) results in cytoplasmic located reporter protein and strong labeling of the cell body of photoreceptors, including axonal terminals and inner segments. However, OS are only faintly stained in such reporter mice, and thus detailed analysis of OS formation of donor cells in high resolution is difficult to perform.[6] The fusion of EGFP to rhodopsin in rhoEGFP mice, in contrast, allows the detection and investigation of OS in more detail, as rhodopsin and thus EGFP are exclusively located in the OS disc membranes in mature photoreceptors.[19] Consequently, the cell body in mature photoreceptors of rhoEGFP mice are not labeled by the reporter (Fig. 4B). Thus, for all transplantation experiments, donor cells were isolated from rhoEGFP mice that additionally carried a ubiquitously expressed actin-DsRed transgene (Fig. 4) allowing simultaneous identification of integrated donor cells and formation of OS. Although the DsRed transgene was virtually expressed in all retinal cell types, the fluorescence signal intensity was highly variable. Of note, before the formation of OS during development (Fig. 4A) or after dissociation (Fig. 4C) young photoreceptors of rhoEGFP mice show EGFP expression located in the entire cell as could be also shown by immunocytochemistry using rhodopsin-specific antibodies (Supplementary Figs. S3A–C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental). In addition, all GFP-positive cells from rhoEGFP retinas expressed recoverin, another photoreceptor marker (Supplementary Figs. S3D–F, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7399/-/DCSupplemental).

After CD73-based MACS unsorted, negative and positive cell fractions were transplanted into the subretinal space of adult wild-type mice. Three weeks after transplantation, experimental retinas were analyzed for the integration of donor cells by fluorescence microscopy. Several DsRed-positive donor cells were found correctly integrated into the ONL of host retinas resembling the morphology of mature photoreceptors: a round, condensed nucleus containing a cell body located in the ONL, a synaptic terminal placed in the OPL, and an inner segment above the ONL (Figs. 5A–D). Furthermore, several integrated donor cells generated an EGFP-positive OS (Figs. 5A–D). Besides showing the characteristic morphology of mature photoreceptors and expression of rhodopsin-fused EGFP, all integrated donor cells expressed recoverin (data not shown; see also Ref. 6). Quantification of integrated donor photoreceptors revealed a significantly higher integration rate of cells from the CD73-positive sorted fractions in comparison to negative or unsorted fractions (Fig. 5E). Whereas transplantation of unsorted or CD73-negative cells resulted in integration of 680 ± 410 cells/retina or 144 ± 102 cells/retina, respectively, CD73-positive cells showed an integration rate of 2199 ± 1006 cells/retina (Fig. 5E). Thus, the number of donor-derived photoreceptors that integrated into the ONL of adult hosts was increased by approximately threefold. We also found few integrated rhoEGFP-positive cells after transplantation of CD73-negative fractions. Beside some cells that may have escaped the sorting process, these cells may have been also originated from early photoreceptor precursors that had not developed further than the Crx or Nrl state and thus were CD73-negative when sorted (see the Discussion section).

Collectively, our data demonstrate that MACS based on CD73 antibodies is a reliable method for the enrichment of transplantable photoreceptors resulting in an increased num-

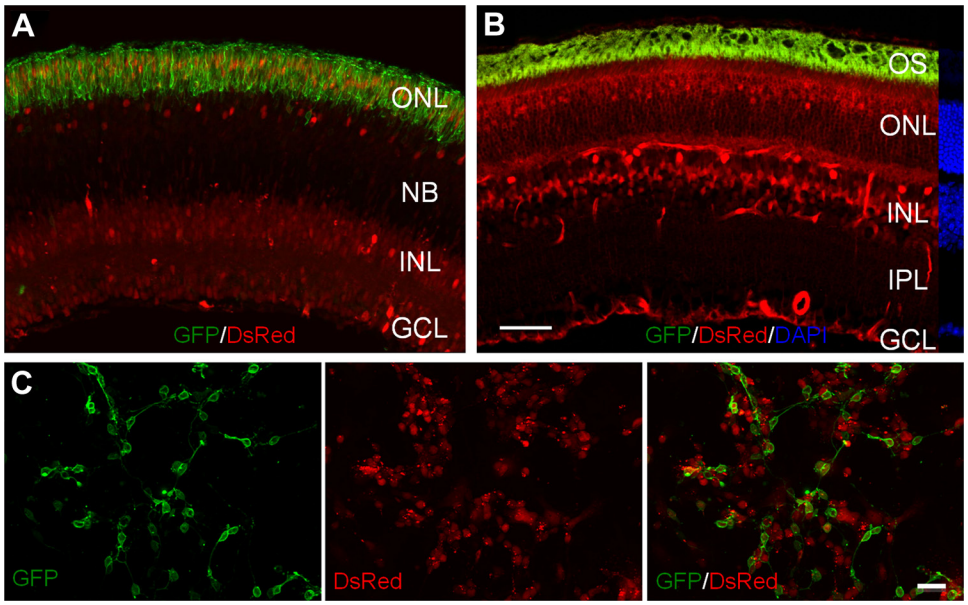

Figure 4. Reporter expression in rhoEGFP/DsRed double transgenic mice. Section of a rhoEGFP/DsRed retina at PN4 (**A**). All retinal cells show *red* fluorescence due to expression of DsRed albeit in different intensities. In the forming ONL, most of the cells show strong GFP expression. Photoreceptors have not generated OS at this developmental stage and express the rhodopsin-EGFP fusion protein in the entire cell. After maturation (a retinal section at PN29 is shown) rhodopsin expression and thus GFP-positivity is restricted to the OS of photoreceptors whereas *red* fluorescence can be detected in all retinal cell types, including endothelial cells albeit in highly variable intensities (**B**). Cells of a dissociated PN4 rhoEGFP/DsRed retina after 4 days in culture. All cells show *red* fluorescence (DsRed), whereas many additionally express GFP indicating them as rod photoreceptors (**C**). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NB, neuroblast layer; ONL, outer nuclear layer; OS, outer segment. Scale bar: (**A**, **B**) 50 μm; (**C**) 20 μm.

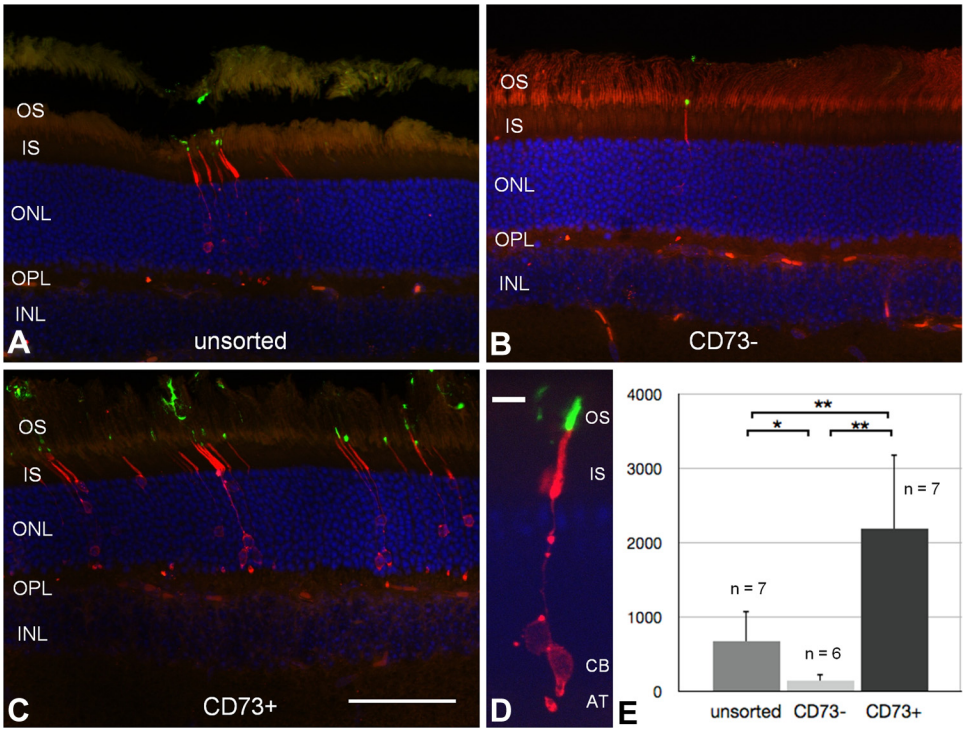

FIGURE 5. Integration of MACS-isolated donor cells into adult retinas. The integration potential of unsorted, CD73-negative, and CD73-positive retinal cells isolated from rhoEGFP/DsRed mice at PN4 was analyzed after transplantation into the subretinal space of adult wild-type mice. Donor cells identified by their expression of DsRed integrated into the ONL of hosts and developed the typical morphology of mature photoreceptors, including a round cell body (**D**, CB) located in the ONL, an axonal terminal (**D**, AT) in the OPL, and an inner segment (IS) above the ONL. In addition, most donor cells generated an OS identified by the distinct expression of GFP at the tip of the donor photoreceptors (**A–C**; a magnification of a donor photoreceptor is shown in **D**). Whereas the integration rate of CD73-negative cells was low (144 ± 102 cells/retina; **B**, **E**), many cells from unsorted fractions were found in the ONL (680 ± 410 cells/retina; **A**, **E**). Transplantation of CD73-positive cell fractions significantly increased the number of integrated donor photoreceptors (2199 ± 1006 cells/retina; **C**, **E**). AT, axonal terminal; CB, cell body; INL, inner nuclear layer; IS, inner segment; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bar: (**A–C**) 50 μm; in (**D**) 10 μm.

ber of donor photoreceptors that successfully integrate into the adult mouse retina.

DISCUSSION

To date, there is no treatment available to regain visual function after degeneration of photoreceptor cells besides approaches using artificial retinal protheses.[23] Recent advantages in cell transplantation strategies for the replacement of photoreceptors in inherited forms of retinal degeneration have encouraged further research to pave the way toward clinical applications.[5–7] These preclinical studies provide evidence that predifferentiated photoreceptors rather than stem and/or progenitor cells resemble a suitable cell type that has the potential to integrate into the adult mammalian retina and generate mature photoreceptors. Interestingly, initial data imply that donor-derived photoreceptors form functional connections to the endogenous neural circuitry.[5,8] One of the problems that limit a possible therapeutic use in patients is the low integration rate of transplanted cells into the ONL of hosts. Some improvements for donor cell integration were recently shown including short time disruption of the outer limiting membrane[9,10] and transplantation of purified cell suspensions enriched for photoreceptor precursors.[7] However, photoreceptor enrichment in the latter study was achieved by flow cytometry using reporter mice expressing GFP under control of the Crx promoter. Such genetic manipulation of cells before transplantation might impede their use in clinical applications.

In this study, we showed that cells within the rod photoreceptor lineage can be efficiently purified by MACS with antibodies against the cell surface molecule CD73. Enriched CD73-positive cell fractions contained ~87% rod photoreceptors (i.e., Nrl-EGFP-positive cells), an increase of approximately 40% the amount found in unsorted fractions. Indeed, our microarray analysis demonstrated a more than threefold upregulation of CD73 transcripts in rod photoreceptors in comparison to other retinal cells including retinal progenitors. These data confirm the recent findings[17] that CD73 is a reliable marker for photoreceptors in the mouse retina. Of note, CD73 is also a marker of rod photoreceptors in primates, as demonstrated in FACS-purified cells from the common marmoset retina[17] implying that CD73 is a promising candidate for the enrichment of rods from human cell sources.

Notably, CD73-enriched cell fractions showed a significantly increased integration potential in comparison to unsorted cells after transplantation into adult mouse retinas. This finding demonstrates that purification of the proper cell type before transplantation is highly important and that young photoreceptors rather than retinal progenitors, which are mainly contained in the CD73-negative fractions, have the potential to integrate into the ONL. For transplantation, we used photoreceptors from double reporter mice that express DsRed ubiqui-

tously as well as rhodopsin-EGFP fusion protein located on mature photoreceptor OSs, resulting in the identification of integrated donor cells without further staining procedures. Furthermore, rhodopsin-fused EGFP allows, for the first time, direct identification of fully mature photoreceptors and the detailed analysis of OS formation down to the ultrastructural level in wild-type mice, as well as mouse models of retinal degeneration after transplantation (Eberle D, Ader M, unpublished observations, 2010).

Technically, the use of cell surface antigens for the purification of young photoreceptors allows the separation of target cells by MACS. Compared to flow cytometry, the former method has the advantage that much more cells can be purified in a shorter period leading to increased photoreceptor survival. Indeed, we demonstrated here that the time-consuming sorting procedure to enrich a high number of photoreceptor precursors (e.g., by flow cytometry), resulted in increased cell damage and eventually death, which obviously negatively affected the survival rate of photoreceptor donor cells after transplantation.[14] However, flow cytometry has numerous advantages, including enrichment with highest purity and the possibility of separating cells by using multiple markers simultaneously. This process may also be necessary for optimizing sorts when specific subpopulations and/or additional combination with vital dyes are needed. Therefore, reducing the procedure time may result at the end in an increase of integration rate as recently suggested.[7]

In the present study, MACS resulted in an average enrichment of rod photoreceptors (Nrl-EGFP-positive cells) to 87% in CD73-positive fractions. Possibly the enrichment of rod photoreceptors by CD73 (the maximum in this study was 92.5% rods) can be further increased, for example, by optimizing the procedure, by direct coupling of paramagnetic microbeads to the primary antibody, multiple sortings, prior negative sorting with cell surface antigens not expressed in photoreceptors, or controlling flow-through rates by the use of automated systems. There was no significant difference between the number of CD73-positive and Nrl-EGFP-positive cells in unsorted or CD73-positive fractions. Thus, rod photoreceptors represent the major CD73 expressing cell population in the retina. Furthermore, the number of rhoEGFP-positive cells was slightly but significantly reduced to Nrl-EGFP-positive cells in unsorted and CD73-positive cell fractions. In line with these findings, the number of rhoEGFP-negative cells was significantly higher than the Nrl-EGFP-negative cells in CD73-positive fractions. During photoreceptor development, Nrl precedes rhodopsin expression, and consequently our findings suggest that CD73 expression parallels that of Nrl and occurs before that of rhodopsin. Many of the rhoEGFP-negative cells in CD73-positive sorted cells may represent photoreceptor precursors at an earlier developmental stage that already express Nrl, but not rhodopsin. These results are in line with the hypothesis that the CD73 expression is downstream of Crx, upstream or parallel to Nrl, and upstream of rhodopsin in the developmental hierarchy of photoreceptors.[17]

The purification of transplantable photoreceptors is of specific interest in light of recent advances in the generation of photoreceptors from pluripotent stem cell sources like ES or iPS cells.[8,13–16] Although very promising, the proportion of cells of the photoreceptor lineage generated from such cultures is still relatively low, thus requiring effective sorting procedures before transplantation. Easy and fast separation methods like MACS, based on cell surface antigens, may be useful tools in this respect. However, the use of antibodies against solely CD73 may not represent the optimal sorting strategy since this antigen is not restricted to photoreceptors but could also be detected in other cell types—for example, T- and B-cell subsets and endothelial cells of capillaries and venules.[24,25] This wider expression profile may somehow impair the specific CD73 enrichment of photoreceptors from heterogenous cell cultures like those differentiating from pluripotent stem cells. Furthermore, during photoreceptor development CD73 expression follows upregulation of Crx and then stays stable until photoreceptor maturation.[17] Thus, early (e.g., Crx-positive) photoreceptor precursors that might have integration potential after transplantation[7] are not included in the sorted cell fraction. In addition, more mature photoreceptors that might have limited integration capacity[5] can still be present after enrichment. This might avert a higher integration rate of sorted cells. As a consequence, new cell surface antigens that specifically recognize photoreceptors at a developmental stage that represents the ideal characteristics for integration have to be identified and evaluated. Furthermore, identification of cell surface markers for the exclusive separation of rods or cones may be an essential prerequisite for the development of cell-based therapies for retinal degenerative diseases characterized by either rod, as in RP, or cone, as in AMD, loss.

Our study demonstrates that the enrichment of photoreceptor precursors from the neonatal mouse retina lead to increased integration into adult hosts. However, the absolute number of integrated donor photoreceptors remains relatively low (up to 3400 cells). In comparison to other published reports, it is obvious that the variability of successful cell integration is relatively high between studies and even within the same study.[5–7,9,11] Such variability may be due to diverse procedures and/or the techniques used, including cell dissociation, transplantation techniques, cell reflux, genetic background, and age of donor and host mice, transgene reporters, and number of transplanted cells. However, enrichment of young photoreceptors could significantly increase the number of integrated cells (Ref. 7 and this study). The highest integration up to now was reported by Lakowski et al.[7] using fluorescence-based sorting of Crx-GFP cells; however, their numbers strongly varied between 700 and up to 15,000 integrated cells after transplantation into the adult wild-type mouse retina. Therefore, the optimization and standardization of the entire transplantation process is of prime importance in bringing photoreceptor replacement closer to a clinical application, keeping in mind that the combined data reveal that the integration of a couple of thousand donor photoreceptors after transplantation into mouse models of retinal degeneration is still inefficient to cause functional changes that can be measured by electroretinogram (Ref. 7; Ader M, unpublished data, 2010). Thus, for the development of an effective cell therapy approach, the purification of transplantable photoreceptors has to be combined with methods that allow higher integration rates. For instance, these might include manipulation of the host tissue by acutely disrupting the outer limiting membrane using RNA interference technology,[9,10] immune modulation,[11,26], or co-injection of factors, allowing increased donor cell migration and synapse formation like MMP-2[27] or chondroitinase ABC.[26,28] Furthermore, it may be important to increase survival rates of transplanted photoreceptors[12,29] or implant donor cells into the subretinal space in a more oriented fashion instead of cell suspensions.[30]

In conclusion, this study demonstrates for the first time the efficient enrichment of transplantable photoreceptors from the developmental mouse retina using cell surface–specific antibodies. Enriched cell fractions integrated with a significantly higher number into the ONL of adult hosts, generating mature, OS-forming photoreceptors. Thus, cell surface antigen–based enrichment of young photoreceptors represents a new and reliable method for future cell transplantation, studies to treat retinal degeneration characterized by photoreceptor loss.

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