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Transplantation of human bone marrow mesenchymal stem cells as a thin subretinal layer ameliorates retinal degeneration in a rat model of retinal dystrophy

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ABSTRACT

Vision incapacitation and blindness associated with retinal degeneration affect millions of people worldwide. Cell based therapy and specifically transplantation of human adult bone marrow-derived stem cells (hBM-MSCs) present possible treatment strategy. Subretinal transplantation of human or rat BM-MSCs was shown previously to improve retinal function in Royal College Surgeons (RCS) rats. In those studies cells were transplanted via a transscleral-transchoroidal approach, creating a localized subretinal bleb. Limited number of cells could be injected and photoreceptor rescue was restricted to areas in proximity to the injection site. Here we describe a new surgical method for subretinal transplantation that facilitates uniform distribution of transplanted cells as a thin layer along most of the subretinal space. We assessed the therapeutic effect of hBM-MSCs on RCS rats when transplanted either subretinally or intravitreally. We also examined whether a second transplantation can prolong the therapeutic effect. A cell suspension of 2.5×10^6 cells in 5 μl was injected subretinally or intravitreally in RCS rats at 28 days postnatal. In the subretinal group, hBM-MSCs were transplanted posterior to the limbus in the superotemporal part of the eye through a longitudinal triangular scleral tunnel reaching the choroid. In the intravitreal group, the cells were injected into the superotemporal part of the vitreous cavity. In cross sections of subretinally transplanted eyes, removed 2 h following transplantation, hBM-MSCs were distributed as a near-homogenous thin layer along most of the subretinal space. In some animals the cells were also detected in the choroid. In the intravitreal injection group, hBM-MSCs were clustered in the vitreous cavity. Transplanted cells could be detected up to 2 weeks after transplantation but not at later time points. Retinal function and structure were assessed by electroretinogram (ERG) and histology analysis, respectively. Six weeks post transplantation, the mean maximal scotopic ERG b-wave amplitude response recorded in RCS control eyes was 1.2 μV. By contrast, in transplanted eyes mean responses of 56.4 μV and 66.2 μV were recorded in the intravitreally and subretinally transplanted eyes, respectively. In the subretinal group, retinal function was significantly higher in transplanted compared with control eyes up to 20 weeks following transplantation. By contrast, in the intravitreal group, rescue of retinal function persisted only up to 12 weeks following transplantation. Histological analysis revealed that 8 weeks following subretinal transplantation, the retinas of control eyes were dystrophic, with outer nuclear layer (ONL) containing a single cell layer. An extensive photoreceptor rescue was demonstrated in transplanted eyes at this time point, with 3–4 cell layers in the ONL along the entire retina. A second subretinal transplantation at 70 days postnatal did not enhance or prolong the therapeutic effect of hBM-

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MSCs. No immunosuppressants were used and long-term safety analysis demonstrated no gross or microscopic adverse effects. Taken together our findings suggest that transplantation of hBM-MSCs as a thin subretinal layer enhances the therapeutic effect and the safety of cell transplantation.

1. Introduction

Retinal degeneration diseases affect millions of patients worldwide and are the leading cause for blindness in the industrial world. Retinitis pigmentosa (RP), an inherited retinal degeneration, and age-related macular degeneration (AMD) affect the photoreceptors directly or the adjacent supporting retinal pigmented epithelial (RPE) cells (Hartong et al., 2006; Nowak, 2006). No effective therapy is available for majority of these progressive diseases except for neovascular AMD (Miller et al., 2013). Studies in animal models raised the possibility of using cell-based therapies for ameliorating retinal degeneration. The retina is an ideal tissue for cell-based therapy due to the relative immune privileged properties of the eye, relative ease of surgical access and the ability to observe the transplanted cells directly (West et al., 2009; Rodriguez and Vecino, 2011). Transplantation of differentiated embryonic stem cells to the subretinal space decelerates retinal degeneration in animal models of retinal dystrophies (Lund et al., 2006; Idelson et al., 2009; Lu et al., 2009). Recently, the first clinical trial of embryonic stem-cell (hESC)-based therapy was reported in which hESC-derived RPE cells were subretinally transplanted in two patients suffering from macular degeneration (Schwartz et al, 2012). This trial was preceded by studies in rodent models of RP and AMD, where subretinal transplantation of hESC-derived RPE resulted in photoreceptor rescue and improved visual function (Lund et al., 2006; Idelson et al., 2009; Lu et al., 2009). Although this treatment is promising, the use of hESC cells raises ethical and safety issues as well as a potential problem of graft rejection (Lo and Parham, 2009).

Human adult bone marrow mesenchymal stem cells (hBM-MSCs) are attractive candidates for retinal treatment because they are safer to use than hESC, are easily expandable and show a broad differentiation potential, including neuronal and microglia cells, and may offer the possibility of autologous transplantation (Ferrari et al., 1998; Kopen et al., 1999; Kicic et al., 2003; Vandervelde et al., 2005; Oh et al., 2008). These cells produce and secrete various cytokines and neurotrophic factors that could promote the survival of retinal cells and activate resident retinal stem cells (Ferrari et al., 1998; Kopen et al., 1999; Kicic et al., 2003; Levkovitch-Verbin, 2004; Vandervelde et al., 2005; Oh et al., 2008). Moreover, hBM-MSCs display immunosuppressive capacities and can reduce local inflammation by secretion of anti-inflammatory paracrine factors (Chen et al., 2004; Caplan and Dennis, 2006; Park et al., 2006; Caplan, 2009; Oh et al., 2008).

In previous studies that examined bone marrow stem cell-based therapies for ocular diseases, two means of cell delivery were used: intravitreal injection and subretinal injection. Intravitreal injection of adult bone marrow mesenchymal stem cells rescued retinal ganglion cells in different models of glaucoma (Levkovitch-Verbin, 2004; Yu et al., 2006; Li et al., 2009; Johnson et al., 2010). Furthermore, intravitreal transplantation of adult bone marrow hematopoietic stem cells rescued retinal function in two mouse models of retinal dystrophy (Otani et al., 2004). When rat BM-MSCs were transplanted intravitreally in a model of injured rat retina, the cells incorporated into the retina and expressed retinal cell markers (Tomita et al., 2002). To the best of our knowledge, the effect of intravitreal transplantation of hBM-MSCs on retinal function in retinal dystrophy rodent models has not been reported.

In addition, several studies demonstrated improved retinal function following subretinal transplantation of hBM-MSCs in rodent models of laser injury, light damage and retinal dystrophy (Arnhold et al., 2006; Castanheira et al., 2008; Lu et al., 2010; Zhang and Wang, 2010). In those studies, cells were transplanted subretinally via a transscleral- transchoroidal approach, creating a subretinal bleb that limited the number of cells that could be injected (5×10^4 cells or less). The cells formed large clumps at the injection site, and photoreceptor rescue was confined to the injection area. Hence, this approach may not be efficient in treating AMD or RP, in which large areas of the retina are damaged.

In an attempt to overcome this limitation we developed a new method of subretinal transplantation in which the cells are transplanted as a thin layer along the entire retina. Transplantation of hBM-MSCs using this method resulted in photoreceptor rescue along most of the retina and significantly enhanced retinal function for up to 5 months following cell transplantation.

2. Materials and methods

2.1. Animals

RCS rats carry a deletion mutation in the gene encoding the MerTK which leads to retinal degeneration and complete sight loss by the age of three months (Bourne et al., 1938; Edwards and Szamier, 1977; D'Cruz et al., 2000). The pigmented RCS strain was used in this study. Rats were born and bred in the Sheba Medical Center animal facility under dim cyclic light (12 h at <5 lux, 12 h in the dark). No immunosuppression was used in this study. All animal procedures and experiments were conducted with approval and under the supervision of the Institutional Animal Care Committee at the Sheba Medical Center, Tel-Hashomer, and conformed to recommendations of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Cell preparation

Fresh human adult bone marrow mesenchymal stem cells (hBM-MSCs) cultures were derived from seven healthy volunteers (ages 35–50 years). Bone marrows were collected in the operating room under sterile conditions. The research was approved by the institutional review board at the Sheba Medical Center. All ex-vivo processes were done under sterile conditions, in designated laminar flow hoods. Bone marrow mononuclear cells were separated by Ficoll gradient (1.077 g/dl) according to the manufacturer instructions and seeded in tissue culture flasks with culture media containing low-glucose Dulbecco's Modified Eagle's Medium supplemented with 15% FCS, 100U/ml penicillin, 100ug/ml streptomycin and 2 mM L-Glutamine. Tissue culture media was changed after 48 h and then twice a week until 70–80% confluence was reached. Cells were monitored for viability by trypan blue staining before transplantation. For immunophenotype analysis, cells were labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) - conjugated monoclonal antibodies in 100 μl phosphate buffer for 15 min at room temperature or 30 min at 4 °C. Antibodies used were anti- CD14, CD34, CD45, CD90 and CD105 (from eBioscience) and anti-CD73 (from R&D Systems).

Cultured cells were extensively washed and resuspended in PBS with 0.5% FBS, passed through a 70 μm filter and analyzed for surface marker profile by flow cytometry (FACSCalibur -Becton, Dickinson). Proliferation Index was tested within the first 72 h after seeding by XTT reagent according to the manufacturer instructions (Biological industries − Beit Haemek, Israel). For Colony Forming Unit-Fibroblasts (CFU-F) assay, the number of colonies produced in each passage per 100 cells was calculated. Cells were seeded in 24 wells plate and incubated in a humidify 5% CO2 incubator. Colonies were formed, analyzed and counted within 7–14 days after first seeding. Cells were washed to remove non adherent colonies. Colonies were fixed with 1 ml methanol, stained with Giemsa stain, and manually counted. Cells were routinely tested for Mycoplasma contamination by using EZ-PCR Mycoplasma Test Kit (Biological industries − Beit Haemek, Israel).

2.3. Subretinal transplantation

Human BM-MSCs were transplanted at 28 days postnatal (P28), an age preceding major onset of retinal degeneration. Separate transplantation experiments were performed using different batches of cells and rat litters in order to ensure that results were repeatable. 0.25×10^6 cells in 5 μl were subretinally injected in RCS rats ($n = 69$). When indicated in the text, cells were prelabeled with the lipophilic membrane stain 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate ('DiI'), according to manufacturer instructions (Invitrogen). Rats were under xylazine (10 mg/kg) and ketamine (75 mg/kg) intraperitoneal anesthesia. Cell transplantation was performed under a surgical microscope (Leica Wild M690; Wild Herring, Herring, Switzerland). For subretinal transplantation, a peritomy was made 2.0 mm posterior to the limbus in the superotemporal quadrant of the eye. A sideport knife Beaver blade (#376620,BD Beaver-Visitic International, Inc.) was used to perform a longitudinal triangular scleral incision starting 2 mm away from the limbus at about 5° axis toward the choroid until minimal blood reflux appeared. At this point an additional tract through the choroid toward the RPE was created by a 30-gauge needle (Supplementary Fig. 1). The cells were injected through the scleral tunnel and its extension using a Hamilton 10 μl blunt syringe with a 30-gauge needle. Immediately after cell injection, the syringe was pulled back, allowing a backflow of the excess of cell suspension and preventing prolonged increase in intraocular pressure (IOP).

2.4. Intravitreal transplantation

Five μl of 0.25×10^6 hBM-MSCs were injected intravitreally in 20 RCS rats using a Hamilton syringe with a 30-gauge needle in the superotemporal part of the eye. The needle was introduced 2 mm posterior to the limbus to avoid touching the lens and was passed through the retina. A surgical microscope was used to observe the needle in the vitreous, and the injections could thus be visually controlled.

2.5. Electroretinogram (ERG) recording

For dark-adapted ERG, rats were kept in total darkness for 12 h prior to testing. Animals were anesthetized with intraperitoneal injection of 75 mg/kg Ketamine and 10 mg/kg Xylazine, pupils were dilated with topical 1% tropicamide and the corneas kept moist with 2.5% hydroxypropyl methylcellulose. Body temperature was kept at 37 °C with a heating pad. ERGs were recorded from both eyes simultaneously using golden wire loops on the corneas. A chloride silver reference electrode was placed subcutaneously near the temporal canthus. The ground electrode was placed on the tail. Responses were amplified at 10,000 gain at 0.1–1000 Hz, filtered to remove 60 Hz noise, and digitized at a 10-kHz rate. For dark-adapted ERG, responses were averaged with stimulus intervals of 1–30 s depending on the stimulus luminance level. For light-adapted ERG, the animals were light-adapted for 10 min prior to testing and responses were averaged with stimulus intervals of 1 s.

2.6. Histology

Rats were euthanized with CO_2 and the eyes were removed, fixed in formalin and embedded in paraffin. Retinal cross sections (4 μm) were cut along the vertical meridian of the eye through the optic nerve and were stained with hematoxylin and eosin. Light microscopic evaluation of the position of transplanted cells and any changes in the eye was preformed.

2.7. Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated. Following antigen retrieval (in citrate buffer pH 6.0), sections were incubated in 30% hydrogen peroxide solution for 10 min at RT, followed by blocking in PBS containing 5% normal goat serum, 1% BSA and 0.3% Triton-X-100 for 30 min at RT. Then sections were incubated with anti-human nuclei antibody, clone 3E1.3 (20 ng/μl, Millipore) for 20 h at 4 °C followed by secondary antibody staining protocol following manufacturer's instructions (Histostain®-Plus Kit, Invitrogen).

2.8. Immunofluorescence

Eyes were removed and immersed in 4% PFA for 24 h, after which they were infiltrated with a solution containing 2% PFA and 5% sucrose for 1 h. Then eyes were incubated in sucrose solutions (5%, 10%, 12.5%, and 15% sucrose) for 30 min each followed by incubation in 20% sucrose for 16 h. Eyes were embedded in 20% sucrose in optimum cutting temperature (O.C.T, Sakura). Ten micrometer sections along the vertical meridian of the eye through the optic nerve were cut using a cryostat. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) for 3 min, washed with PBS and checked under a fluorescent microscope to identify DiI positive cells. For detection of rod photoreceptor cells, paraffin sections were incubated with anti-rhodopsin antibody (RET-P1, Abcam) following manufacturer instructions, and Alexa Fluor 488-conjugated anti-mouse antibody. Sections were counterstained with DAPI for 3 min, washed with PBS and examined by confocal microscopy (Zeiss LSM 510).

2.9. Safety analysis

RCS rats ($n = 7$) were transplanted subretinally with hBM-MSCs, and monitored for six months for general health. At six months, animals were sacrificed and examined by a pathologist for gross and microscopic pathological changes in the eyes and major organs.

2.10. Statistical analysis

MANOVA analysis was performed using SPSS for windows version 20.0. We conducted a 14 (time in weeks) × 5 (amplitude) × 2 (transplanted versus control) MANOVA with time in weeks and stimuli amplitude defined as within-subjects factors, transplanted versus control serving as a between-subjects factor, and the b-wave recordings serving as dependent variables. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Characterization of human Bone Marrow Mesenchymal Stem Cells (hBM-MSCs)

A reproducible, quality-controlled process with specific release criteria was used for the ex-vivo expansion of hBM-MSCs. The ex-vivo expanded cells were characterized at each passage by different parameters, including growth index, morphology, cells viability, proliferation, colonogenic potential and surface marker profile and underwent routine testing for microbial contamination. The mean percentage of viable cells in all experiments was 91 ± 6.2, indicating that growth conditions were optimal and suitable for this type of cells. As shown in Supplementary Fig. 2, hBM-MSCs maintained plastic adherence and spindle shape fibroblast like cell morphology throughout the expansion procedure. No contaminating hematopoietic stem cells were identified by cyto-fluorimetric analysis with antibodies against CD14, CD31, CD34 and CD45 antigens (Fig. 1). A typical mesenchymal stem cell phenotype was demonstrated by expression of CD73, CD90 and CD105 and lack of expression of the surface human leukocyte antigen DR (HLA-DR, Fig. 1). Lack of incorporation of the 7AAD peptide indicated high cell viability (Fig. 1). Proliferation index, growth index and the ability to form colonies showed a decrease after passage four (Table 1). Hence, only cells at passages1–4 were used for transplantation.

3.2. Subretinal transplantation of hBM-MSCs as a thin cell layer

We developed a new method for transplanting hBM-MSCs as a thin subretinal layer. The cells were transplanted through a scleral longitudinal triangular tunnel, as detailed in the "Material and Methods" section. Cells were subretinally transplanted in the right eye of RCS rats. The left eye was injected with medium as control, using the same surgical procedure. There was no bleeding throughout or following the surgical procedure. Cross sections of eyes removed 2 h following transplantation of hBM-MSCs prelabeled with DiI, revealed that transplanted cells were distributed as a thin subretinal layer along most of the retina. In some cases the cells were also found in the choroid (Fig. 2A–C). Histological examination with human specific antibodies showed that transplanted cells were immunoreactive for anti-human nuclei, thus confirming the presence of human cells in the subretinal space (Fig. 2D). DiI-labeled cells were clearly detected in a thin cell layer in the subretinal space and in the choroid along most of the retina two weeks following transplantation (Fig. 2E and F, respectively).

3.3. Intravitreal transplantation of hBM-MSCs

The vitreous cavity is easily accessible and considered a drug reservoir for medical treatment of intraocular diseases. As the therapeutic effect of hBM-MSCs is believed to be mainly mediated by the paracrine secretion of cytokines, we also examined the therapeutic effect of intravitreal transplantation, which is technically easier to perform. Histological examination of eyes 2 h following intravitreal transplantation demonstrated a large cluster of cells in the vitreous cavity that were immunoreactive for anti-human nuclei (Fig. 2G).

3.4. Subretinal transplantation of hBM-MSCs as a thin layer significantly enhances retinal function

Retinal degeneration in RCS rats leads to complete loss of retinal function by 2–3 months of age. The effect of hBM-MSC transplantation on retinal function was assessed by ERG that measures

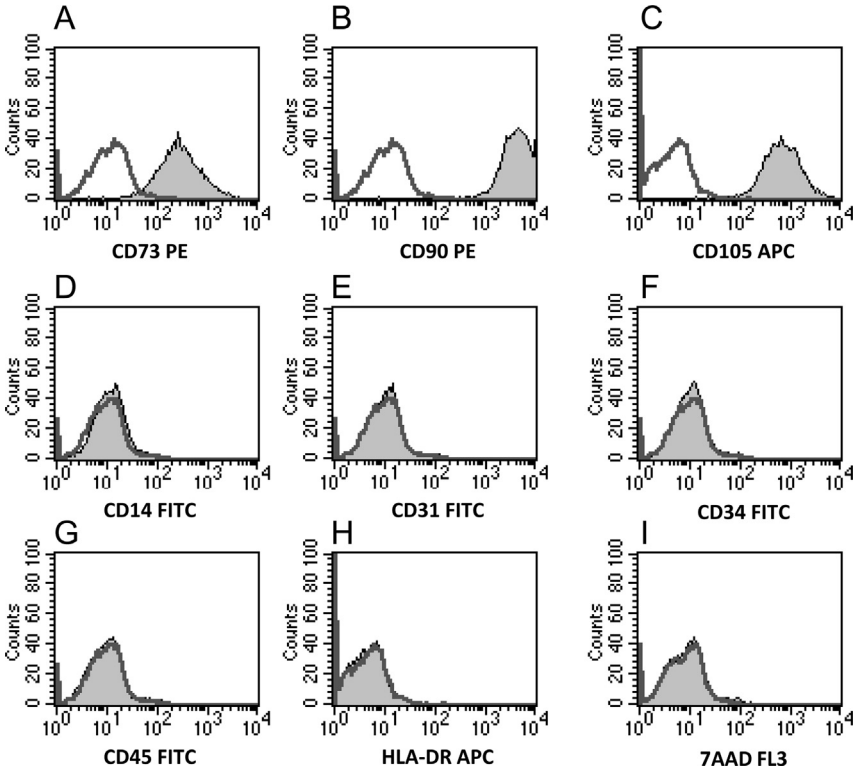

Fig. 1. Cell-Surface markers of hBM-MSCs. Human BM-MSCs express CD73(A), CD90(B) and CD105(C) but lack expression of CD14(D), CD31(E), CD34(F), CD45(G), HLA-DR(H) and 7AAD(I) by flow cytometry. Isotype-matched IgG controls (non-shaded curves) and hBM-MSC curves (shaded) are shown. A minimum of 10,000 events was recorded. FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC – allophycocyanin.

Table 1
Growth parameters index of ex-vivo expanded human BM-MSCs.

Passage no.	Growth index[a]	Proliferation index	Colony forming unit-fibroblasts – (CFU-F)[b]
0	0.055 ± 0.07	ND	ND
1	3.19 ± 2.02	1.32	10
2	3.49 ± 2.17	1.42 ± 0.85	7
3	3.19 ± 2.12	1.81 ± 0.78	18
4	3.12 ± 1.19	1.83 ± 0.53	14
5	2.16 ± 0.69	2.03 ± 0.34	2
6	2.21 ± 0.49	1.41 ± 0.38	2.5
7	1.83 ± 1.13	0.94 ± 0.19	2

ND – Not determined.
[a] Growth index was calculated by dividing the number of collected cells with the number of seeded cells in each passage.
[b] The number of colonies produced in each passage per 100 seeded cells. Results are presented as mean from 4 wells at each passage.

the mass electrical response of the retina to photic stimulation and is widely used for objective determination of the functional status of the retina. Specifically, we measured the amplitude of the b-wave which is generated by the bipolar cells of the inner retina and is most commonly used parameter in clinical studies and animal research and the a-wave which is generated by the cones and rods in the outer photoreceptor layer (Penn and Hagins, 1969; Kofuji et al., 2000; Young et al., 2012). The responses of cones and rods can be isolated when the test is performed under light or dark adaptation, respectively (Wurziger et al., 2001; Young et al., 2012).

Human BM-MSCs were subretinally transplanted into the right eye of RCS rats and ERG analysis was performed in both eyes simultaneously prior to and every other week following cell transplantation to monitor retinal function. In preliminary experiments, the left eye was untreated or injected with medium as control. There were no significant differences between ERG b-wave amplitudes recorded in untreated or medium injected eyes (Supplementary Fig. 3, $n = 10$, all p values >0.05). The mean maximal b-wave amplitudes obtained in response to different stimulus luminance levels under dark- and light-adaptation conditions are shown in Fig. 3. A slight and transient reduction in ERG b-wave response was recorded in transplanted eyes at 35 days postnatal, one week following cell transplantation. The reduced ERG response was non significant under dark-and light-adaptation (all p values >0.157) except for the response to a stimulus of 23.5 cd-s/m^2 under dark-adaptation conditions ($p = 0.023$). At subsequent time points b-wave amplitude recordings under dark-adaptation conditions were significantly higher across amplitudes in transplanted eyes compared to control eyes (all p values ≤ 0.05, Fig. 3). Thus, at 70 days postnatal, ERG responses became non recordable in control eyes, while in transplanted eyes the mean maximal ERG b-wave amplitude response was 66.2 μV ± 6.5 (mean ± SE, Fig. 3) The maximal ERG b-wave amplitude response from normally sighted rats at 70 days postnatal was 410.8 μV ± 24.5 (data not shown). B-wave recordings under light-adaptation were significantly higher across amplitudes in the transplanted compared to the control eyes starting at 3 weeks post-engraftment (all p values ≤ 0.05, Fig. 3). Treatment benefit was lost by 22 weeks post engraftment, as evident by loss of b-wave ERG responses in transplanted eyes (Fig. 3).

The mean maximal a-wave amplitudes were significantly higher in transplanted compared with control eyes between 1 and 14 weeks following transplantation (Fig. 4A). Maximal a-wave amplitudes were 3–4 fold higher in transplanted compared with control eyes between 6 and 14 weeks post transplantation ($p < 0.004$).

3.5. *Treatment benefit of intravitreal transplantation of hBM-MSCs persists up to 12 weeks following transplantation*

Similar to subretinal transplantation, intravitreal transplantation of hBM-MSCs resulted in a transient and non significant

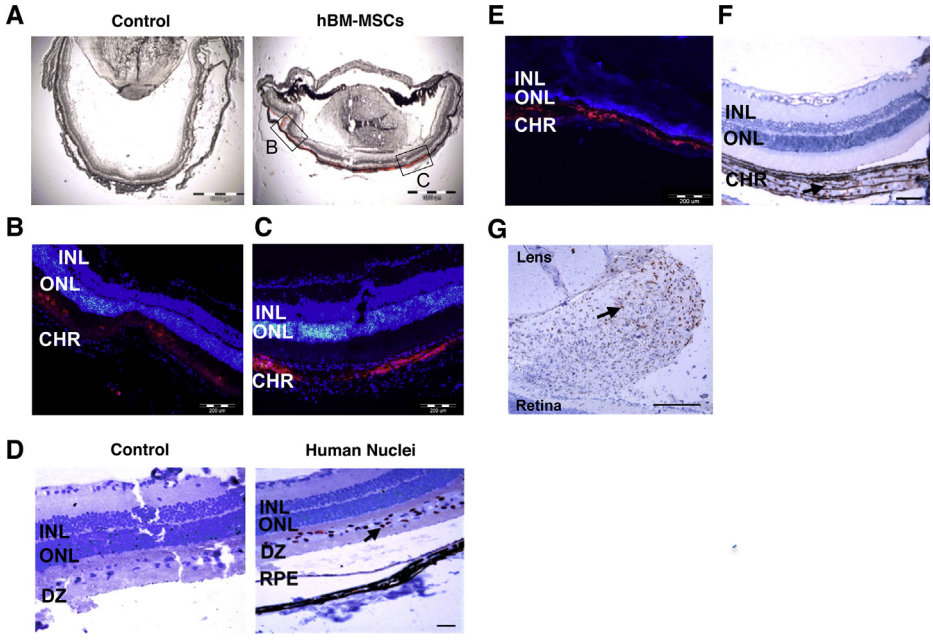

Fig. 2. A new method for subretinal transplantation of hBM-MSCs. Cross sections of eyes removed 2 h (A–D) or 2 weeks (E, F) following subretinal transplantation of DiI-labeled hBM-MSCs (red) or medium (Control, A). High power images of indicated areas in panel A demonstrate cells transplanted in the subretinal space (B) or choroid (C). Nuclei were counterstained with DAPI (B, C, E). D&F – Sections were stained with anti-human nuclei antibody and counterstained with hematoxylin. Arrows point to positively stained cells in the subretinal space (D) or choroid (F). D- Control, no primary antibody. G- Eyes were removed 2 h following intravitreal transplantation of hBM-MSCs. Arrow points to cells in the vitreous cavity positively stained with anti-human nuclei antibody. Scale bar: 1000 μm (A), 200 μm (B, C, E-G) and 100 μm (D). INL – inner nuclear layer; ONL – outer nuclear layer; CHR – choroid; DZ – debris zone; RPE – retinal epithelial cells.

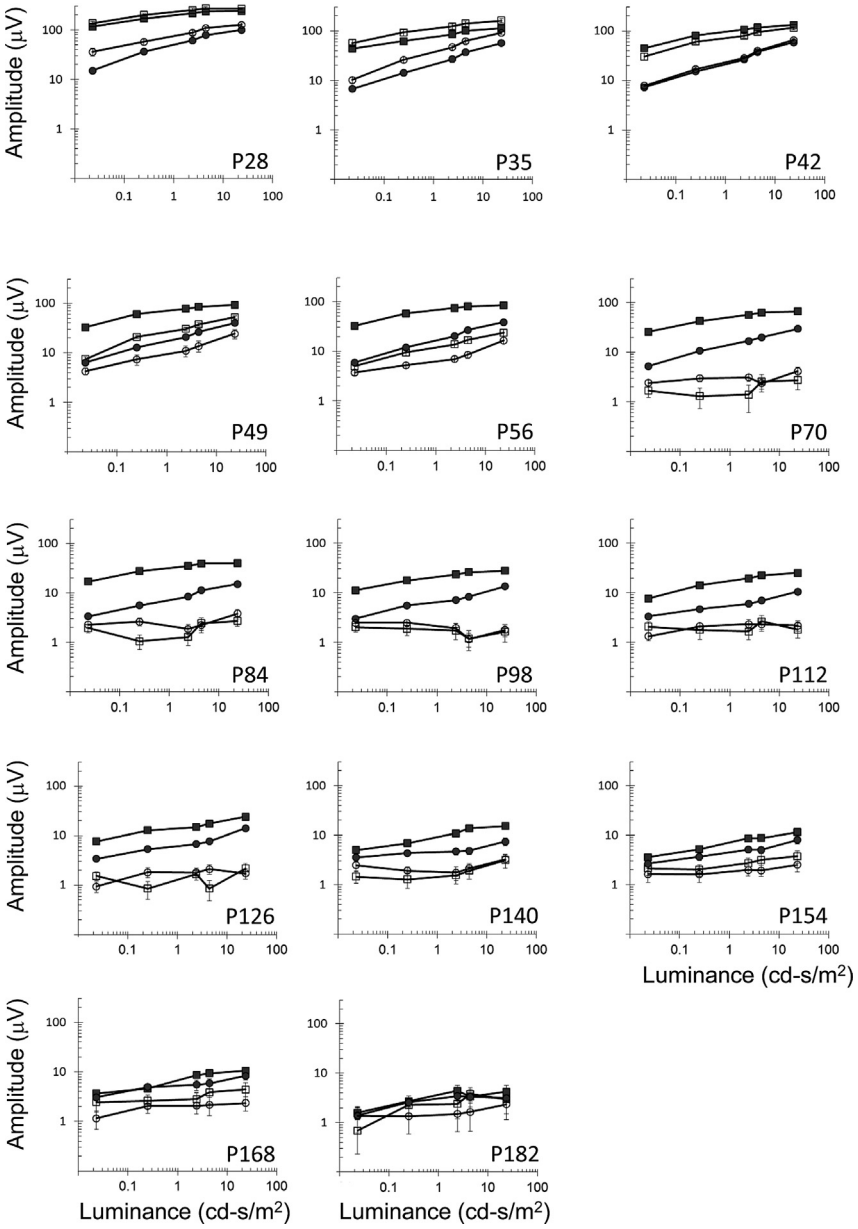

Fig. 3. – Long-term rescue of retinal function by subretinal transplantation of hBM-MSCs. Mean maximal b-wave amplitude ERG responses to light flashes in increasing luminance levels were recorded following dark- (squares) or light (circle)-adaptation, in transplanted (closed) or control (open) eyes at indicated days postnatal. Data is presented as mean ± SE.

reduction in b-wave ERG responses one week following cell transplantation (all *p*-values >0.131, Supplementary Fig. 4). At subsequent time points b-wave recordings were significantly higher across amplitudes in transplanted eyes compared to control eyes up to 12 weeks following transplantation (all *p* values ≤0.05). At 14 weeks post transplantation, eyes with intravitreal transplantation demonstrated significantly enhanced dark-adapted ERG response only to the strongest stimulus (23.5 cd-s/m^2, Supplementary Fig. 4). The mean maximal b-wave ERG responses were lower in the intravitreal compared with the subretinal transplanted eyes, but the difference between the groups was not statistically significant (all *p* values >0.05, Fig. 4B).

The mean maximal a-wave amplitudes in eyes following intravitreal transplantation were significantly higher compared with control eyes only at 6, 10 and 14 weeks following transplantation, and were consistently lower compared with subretinally transplanted eyes (Fig. 4A). Since treatment benefit of subretinal transplantation was higher and lasted 6 weeks longer than the intravitreal transplantation (Figs. 3 and 4), we proceeded with in-depth analysis of the therapeutic effect and safety of the new subretinal transplantation method.

3.6. Subretinal transplantation of hBM-MSCs as a thin layer ameliorates photoreceptor degeneration along most of the retina

Retinal degeneration in RCS rats is characterized by loss of photoreceptor cell bodies in the outer nuclear layer (ONL) and thinning of this layer (Bourne et al., 1938). To further assess the therapeutic effect of the subretinal transplantation, retinal sections were analyzed for ONL thickness. Four weeks following cell

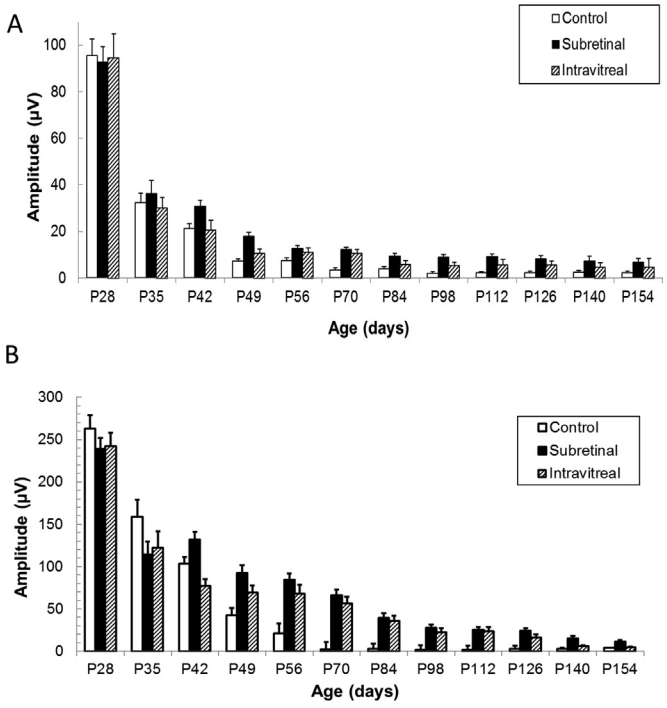

Fig. 4. The therapeutic effect of subretinal transplantation lasts longer than intravitreal transplantation. Mean maximal a-wave (A) and b-wave (B) amplitude ERG responses to light flashes of 23.5 cd-s/m^2 were recorded following dark-adaptation, before (P28) and at different time points following subretinal or intravitreal transplantation. Data is presented as mean ± SE.

transplantation, the ONL in control eyes contained only 2 cell layers. By contrast, in transplanted eyes the ONL was 4–5 cells thick along most of the retina, with rescued photoreceptors covering an average length of 6815 ± 250 μm (Fig. 5A–D and 6). At this time point the ERG b-wave responses were 4 fold higher in transplanted compared with control eyes at all stimulus luminance levels tested (all p values <0.001, Fig. 3). At 84 days postnatal, retinas of control rats were dystrophic, with ONL containing a single cell layer and no measurable ERG b-wave responses. In transplanted eyes at this time point, extensive photoreceptor rescue was observed, with 3–4 layers of cells in the ONL along most of the retina, covering an average length of 6275 ± 301 μm (Fig. 5E–H and 6) and mean maximal ERG b-wave responses reached 40 μV ± 5 (mean ± SE, Fig. 3). The mean number of photoreceptor cell layers present in the ONL was significantly higher along most of the retina in transplanted eyes compared to control eyes up to 20 weeks following transplantation ($p < 0.05$, Fig. 6). At P182 the ONL in transplanted eyes contained only a single cell layer and full field ERG was non-detectable (Fig. 3).

Next we examined rod cell structure by immunostaining for rhodopsin. Rhodopsin-stained outer segments were demonstrated in retinas from 28 days old RCS rats (Fig. 7A). At 84 days postnatal the debris zone in control eyes became more prominent, outer segments were no longer seen and a strong aberrant accumulation of rhodopsin in rod cell bodies was observed (Fig. 7B). By contrast, in transplanted eyes at P84, rhodopsin-stained outer segments were clearly evident along most of the retina, and there was no aberrant staining of rod cell bodies (Fig. 7C).

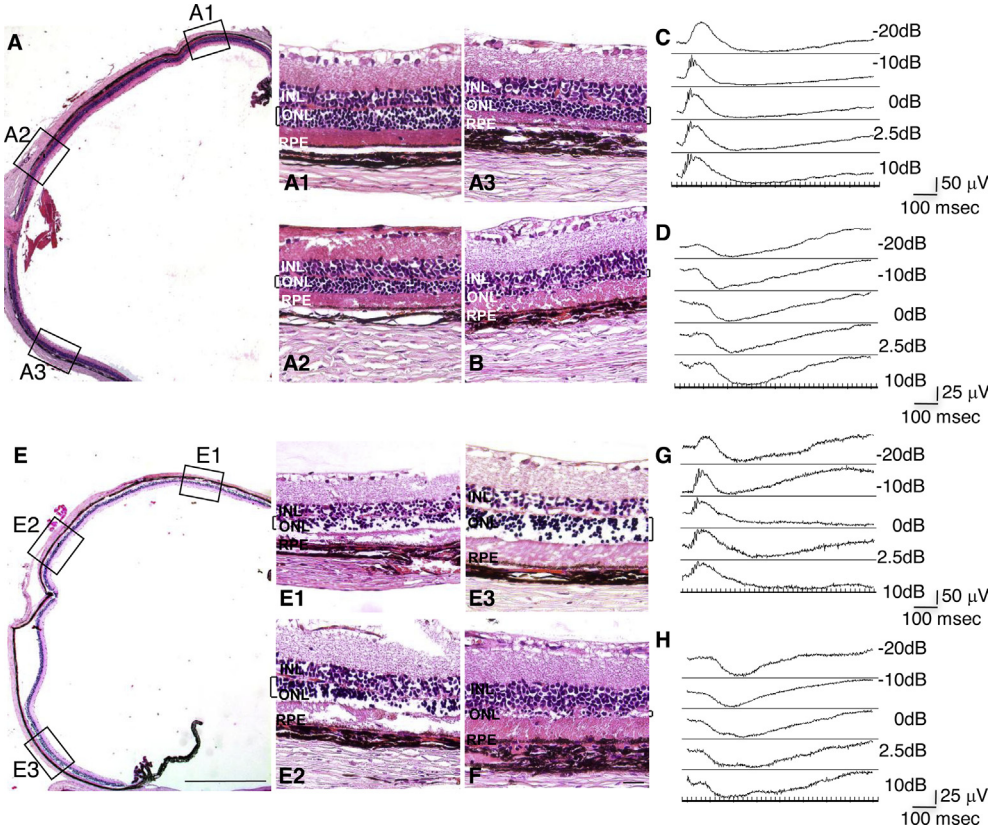

Fig. 5. Photoreceptor rescue along the entire retina following subretinal transplantation of hBM-MSCs. H&E stained sections and ERG responses to flashes at indicated luminance levels at P56 (A–D) and P84 (E–H) in control (B, D, F, H) and transplanted (A-A3, C, E-E3, G) eyes. Black lines emphasize ONL depth. A1-A3, E1-E3: high power images of indicated areas in panels A and E, respectively. Scale bar: 1000 μm (A, E), 100 μm (A1-B, E1-F). INL – inner nuclear layer; ONL – outer nuclear layer; RPE – retinal epithelial cells.

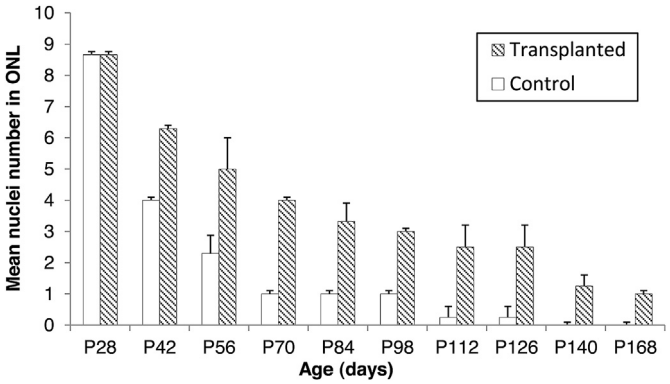

Fig. 6. Subretinal transplantation of hBM-MSCs ameliorates photoreceptor degeneration up to 20 weeks following cell transplantation. Number of cell layers in ONL was evaluated by nuclei counting of 4 fields along the entire retina in H&E stained sections. Data is presented as mean ± SD from 3 eyes at each time point.

3.7. Second transplantation of hBM-MSC does not improve treatment outcome

Our findings that the therapeutic effect of subretinal transplantation was limited to 20 weeks suggested that although the eye is considered immune-privileged, the xenograft donor cells may have been rejected. Therefore we assessed whether a second transplantation could prolong and enhance the therapeutic effect of subretinal transplantation. We chose to perform the second transplantation at P70, when a substantial retinal function was measured in transplanted eyes (Fig. 3). A significant reduction in ERG responses was recorded two weeks after the second transplantation (P84, Fig. 8). At subsequent time points, ERG responses did not significantly differ between eyes that received a single transplantation and eyes that received a second transplantation (Fig. 8 and Supplementary Fig. 5).

3.8. Safety assessment

The safety of hBM-MSC subretinal transplantation was tested in 7 RCS rats. No gross or microscopic changes or inflammatory reaction were observed in any of the animals 6 months following hBM-MSC transplantation in any of the major organs examined, including heart, lungs, liver, spleen, eyes, kidneys, and intestine. In addition, none of the rats transplanted with hBM-MSCs for the function study ($n = 69$), showed any evidence of eye inflammation, tumor formation or changes in body weight compared to non-transplanted animals.

4. Discussion

In this study we show for the first time that transplanting hBM-MSCs as a thin near-homogenous subretinal layer resulted in long-term rescue of retinal function and significantly delayed photoreceptor degeneration throughout the retina in a large cohort of animals. Although subretinal transplantation did not restore ERG responses to normal values, the a- and b-wave amplitudes were 4 and 10-fold higher, respectively, in transplanted as compared with control eyes. Together with our findings that subretinal transplantation of hBM-MSCs had no adverse effect on any organ, our data suggest that subretinal transplantation of hBM-MSCs cells may be used to safely and efficiently treat retinal dystrophies and that transplanting the cells as a thin subretinal layer enhances their therapeutic effect as compared with intravitreal transplantation.

In other studies aimed at photoreceptor rescue by subretinal cell transplantation, neural progenitor cells, hESC-derived RPE cells or hBM-MSCs were injected into the subretinal space via a transscleral-transchoroidal approach, creating a localized bleb (Gamm et al., 2007; Wang et al., 2008; Idelson et al., 2009; Lu et al., 2010). An additional paracentesis was performed to lower the IOP, preventing reattachment of the retina. In those studies, transplanted cells clustered in the injection area and photoreceptor rescue was limited to areas in proximity to the injection site. In the surgical procedure presented here, the triangular scleral tunnel formed a tract parallel to the retina and functioned as a valve, sealing off the opening to the subretinal space immediately after injection termination. The volume of the injected cell suspension increased the intraocular pressure, pressing the cells along the vector of injection in the de-novo created space between the RPE and choroid layers. This led to a nearly homogenous distribution of the cells as a thin subretinal layer along the subretinal space and reattachment of the retina.

Recent studies suggested that the secretion of trophic factors that alter tissue microenvironment plays a key role in the therapeutic effect of hBM-MSCs in different disease models, including the RCS retinal dystrophy model (Phinney and Prockop, 2007; Prockop, 2007; Wang et al., 2010). Our findings that hBM-MSCs can rescue retinal function when transplanted as a thin layer in the choroid or in the vitreous support this possibility. To the best of our knowledge, this is the first demonstration of rescue of retinal function by intravitreal transplantation of hBM-MSCs. Nevertheless, the therapeutic effect of subretinal transplantation was higher and lasted 6 weeks longer than intravitreal transplantation, suggesting that subretinal transplantation, in close proximity to the RPE and photoreceptors, may be a more effective treatment. This may be especially relevant in human eyes with a larger globe and thicker retina, where cytokines secreted from intravitreally

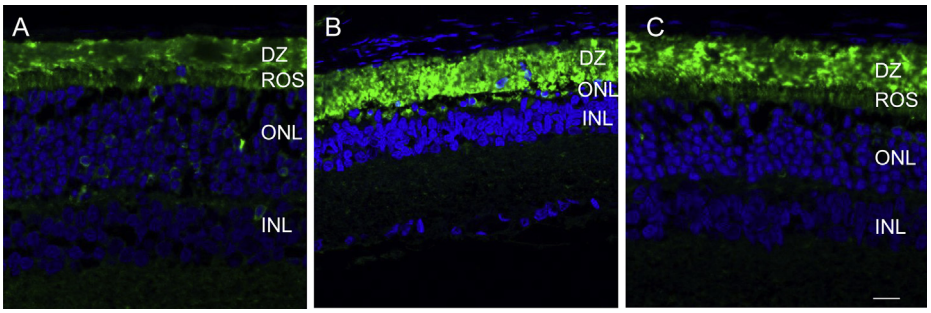

Fig. 7. Subretinal transplantation of hBM-MSCs preserves rod cell structure. Rhodopsin staining (green) and DAPI counterstaining (blue) in P28 (A) and P84 (B, C) retinas injected with medium (B) or transplanted with hBM-MSCs (C). Scale bar: 20 μm. INL – inner nuclear layer; ONL – outer nuclear layer; ROS – rod outer segments; DZ – debris zone.

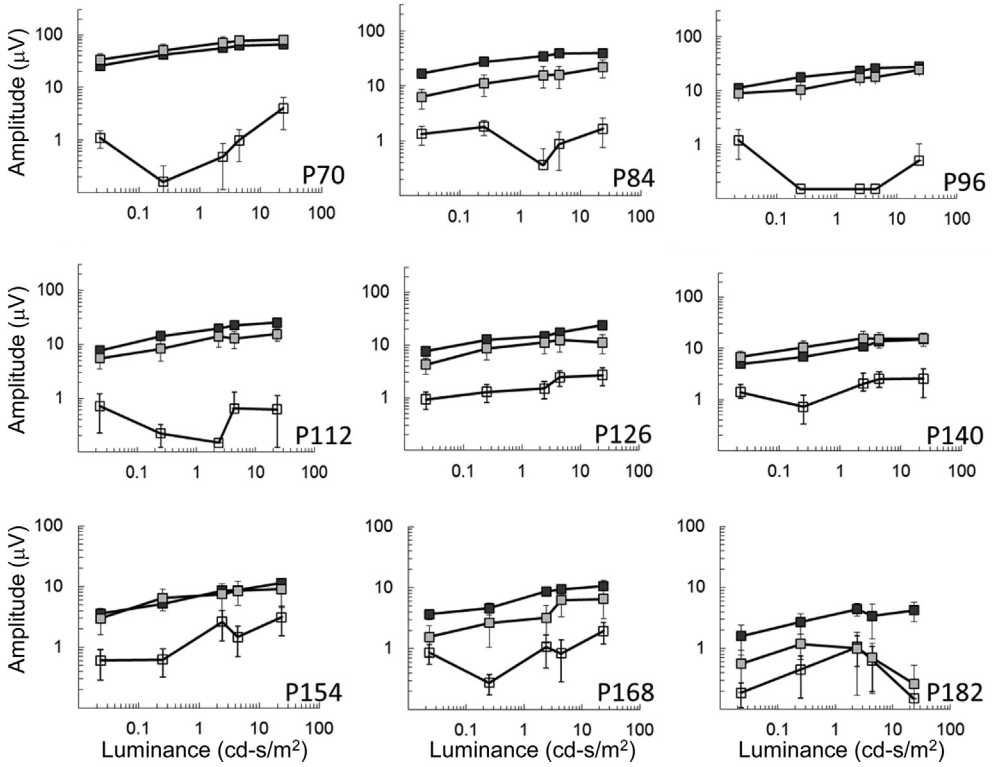

Fig. 8. A second subretinal transplantation of hBM-MSCs does not improve treatment outcome. Rats underwent a second subretinal transplantation at P70. Mean maximal b-wave amplitude ERG was recorded following dark-adaptation, in control (open) eyes and in eyes following a single (black) or second transplantation (gray) at indicated days postnatal. Data is presented as mean ± SE.

transplanted cells will be more diluted in the larger volume and will need to pass through a thicker neuroretina to reach the target cells. Our results are in agreement with those of Park et al. (2012) that showed that subretinal transplantation of brain derived neurotrophic factor (BDNF) secreting hBM-MSCs was more effective for delivery of BDNF to the retina than intravitreous transplantation. It is probable that subretinal transplantation is more effective as it allows more intimate contact between the transplanted cells and the retina. The beneficial effects on photoreceptor degeneration throughout the retina might be explained by the relatively homogenous distribution of transplanted cells all along the retina that may have further facilitated their trophic effect to encompass most of retina. Furthermore, the distribution of transplanted cells as a relatively thin cell layer may have increased the surface area of the graft and could potentially enhance both cell secretion efficiency as well as host–graft interaction.

The eye is considered immune privileged (Foster and Wetzig, 1982), and Lu et al. reported that treatment of rats with cyclosporine A had no effect on hBM-MSC therapeutic benefits (Foster and Wetzig, 1982; Lu et al., 2010). Hence, we did not use immune suppressants in this human-to-rat xenograft study. Although we observed a small and transient reduction in ERG signal shortly after cell transplantation, most likely this reduced retinal response represents an acute trauma to the retina caused by the transplantation procedure. Although nearly whole retinal detachment occurred during cell transplantation, the detachment was shallow and retinal function was significantly enhanced in the following weeks. Furthermore, the shallow retinal detachment that was generated during transplantation facilitated the transplantation of larger amount of cells. No signs of inflammation were detected in transplanted eyes at any time point following transplantation (data not shown). However, as the therapeutic effect persisted up to 20 weeks post transplantation, it is possible that transplanted cell survival may have been compromised due to host rejection of the xenograft. Our attempts to prolong therapeutic effect by a second transplantation did not succeed. Further studies will be needed to establish whether changing different parameters of the second transplantation (e.g. time of transplantation, amount of cells and location) improve treatment outcome. In addition, immunosuppression or scaffolds and slow release of cells may maintain transplanted cell survival for longer periods of time. Nevertheless, as one of the advantages of using hBM-MSCs is the possibility of obtaining these cells from patients' own bone marrow, it is conceivable that autologous transplantation may present enhanced graft survival and prolonged therapeutic effect. We are currently examining the possibility of implementing the new subretinal transplantation method in larger eye models. This may overcome the need for three port pars plana vitrectomy that is currently used in clinical trials (Schwartz et al., 2012).

Taken together, we show for the first time that transplanting hBM-MSCs as a thin sub-retinal layer resulted in long-term protection of retinal function and significantly delayed photoreceptor degeneration along most of the retina. This study further supports the notion that the subretinal space is one of the more favorable organs to evaluate the therapeutic effect of cell therapy of neurodegenerative diseases and can help to determine protocols, cell types and administration techniques. The new transplantation method could potentially be used for other cell-based therapies and may directly lead to clinical trials aimed at development of efficient treatments for incurable blinding diseases such as AMD and RP.

Disclosure of potential conflicts of interest

Dr. Ygal Rotenstreich applied for a patent through the Sheba Medical Center (Patent Application 61/419,160 filed December 2, 2010).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2013.10.023.

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