

Cell Based Therapeutics For Retinal Degenerations

by

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Abstract

Vision loss, due to retinal degeneration, is one of the major disabilities in the developed world. Cell based therapeutics showed promising results in preventing retinal degenerations. Here, mesenchymal stem cells have been used to develop therapeutics for two different disorders. First, the innate paracrine activity of the Mesenchymal Stem Cells (MSCs) has been utilized to prevent neurodegeneration in a rodent model (S334ter-4) of Retinitis Pigmentosa. To deliver a significant number of cells to the rodent retina, a novel magnetic targeting approach was applied. The MSCs were pre-labelled with superparamagnetic iron oxide nanoparticles (SPIONs) and targeted to the eye by surgically placing a disc magnet in the orbit after systemic delivery. The magnetically guided MSCs provided better neuroprotection and functional responses (electroretinography and optokinetic tracking response) when compared to control and non-magnetic MSCs. The effect of magnetic nanoparticles on the paracrine secretion profile of MSCs was also analyzed. The concentration of the nanoparticles, used for the magnetic targeting study, had no significant effect on MSC's paracrine activity; however, higher concentrations displayed varying effects for different factors. In the next project MSCs were employed as a vector for an *ex vivo* gene therapy approach for X linked retinoschisis (XLRS). MSCs were genetically modified to secrete an extracellular protein, retinoschisin (RS1), which is either nonfunctional or absent in XLRS. The RS1 expression from the MSCs was controlled in both constitutive (cMSC) and inducible (iMSC) manners. Cells, with both modifications, provided significant structural and functional benefits after intravitreal delivery in a RS1 knock-out mouse model when compared to unmodified MSCs, sham injection and no treatment controls. Among the two genetically modified cell lines, iMSC displayed better response compared to cMSC. These results from the MSC based therapeutic approaches in two different disease models show the potential of MSCs as a resourceful cell type for a number of retinal degenerations as well as diseases of other organs.

Preface

All of the work presented henceforth was conducted in the Retinal Therapeutics Lab at the University of British Columbia, VGH campus.

A part of Chapter 2 (sub section 2.2) has been published as a book chapter in the book *Nanomaterials and Regenerative Medicine* [Bashar E and Gregory-Evans K. Magnetic nanoparticles for cell-based therapies. *Nanomaterials and Regenerative Medicine*. IAPC-OBP 2016:160-181]. Another part (subsection 2.4) has been published in the journal of *Current Gene Therapy* [Gregory-Evans K, Bashar AE and Tan M. Ex vivo gene therapy and vision. *Curr Gene Ther* 2012 Apr 1;12(2):103-15].

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List of Abbreviations

adMSC – Adipose derived mesenchymal stem cells

ANOVA – Analysis of Variance

BDNF – Brain derived neurotrophic factor

BETA2 – Beta cell E box trans-activator2

bFGF – Basic fibroblast growth factor

BRB – Blood retina barrier

CAR – Chimeric antigen receptor

cMSC – Constitutively retinoschisin expressing mesenchymal stem cells

CMV – Cytomegalo virus

CNS – Central nervous system

CNTF – Ciliary neurotrophic factor

COPD – Chronic obstructive pulmonary disease

DAPI – 4',6-diamidino-2-phenylindole

DHA – Docosahexanoic acid

DNA – Deoxy ribonucleic acid

DPBS – Distilled phosphate buffered saline

ECM – Extracellular matrix

EDTA – Ethylene diamine tetra acetic acid

ELISA – Enzyme linked immune sorbent assay

ERG – Electroretinogram

GCL – Ganglion cell layer

GDNF – Glial derived neurotrophic factor

hESCs – Human embryonic stem cells

HGF – Hepatocyte growth factor

HIV – Human immunodeficiency virus

IFN – Interferon

IL10 – Inteleukin 10

iMSC – Inducible retinoschisin expressing mesenchymal stem cells

INL – Inner nuclear layer

iPSCs – Induced pluripotent stem cells

IRES – Internal ribosome entry site

mES – Mouse embryonic stem cell

MMSC – Magnetized mesenchymal stem cells

MNPs – Magnetic nanoparticles

MOG – Myelin oligodendrocyte glycoprotein

MSCs – Mesenchymal stem cells

MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

OCT – Optical coherence tomography

OKT – Optokinetic Tracking

ONL – Outer nuclear layer

PARP – Poly (ADP-ribose) polymerase

PEDF – Pigment epithelium derived factor

PEG – Polyethylene glycol

PFA – Paraformaldehyde

rAAV – Recombinant adeno associated virus

RBC – Red blood cell

RHO – Rhodopsin

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RP – Retinitis Pigmentosa

RPE – Retinal pigment epithelium

RPGR – Retinitis pigmentosa GTPase regulator

RS – Retinoschisin

RT-PCR – Reverse transcription polymerase chain reaction

SCI – Spinal cord injury

SPIONs – Superparamagnetic iron oxide nanoparticles

TRE – Tetracycline response element

tTA – Tetracycline transactivator

VEGF – Vascular endothelial growth factor

XLRS – X-linked retinoschisis

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Chapter 1. Introduction

Vision impairment is one of the commonest disabilities. Developed countries are facing the challenges of combating a number of progressive blinding disorders, such as age-related macular degeneration (more than 14 million people blind or severely impaired), retinitis pigmentosa (~1/3500), diabetic retinopathy (~4.2 million people over the age of 40 in the United States) and X-linked retinoschisis (~1/5000-25000) [1-4]. With retinal degenerations so prevalent worldwide, new approaches of treatment would be widely applicable. Moreover, it will bring significant advancement in improving the quality of life as well as remove pressure from the healthcare systems. Cell based therapy, directed at genetic abnormalities or at modifying pathological processes, holds much promise in treating a wide variety of diseases that lead to vision loss [5]. The major approaches of cell based therapies for retinal degeneration includes cell replacement, providing neuroprotection and *Ex vivo* gene therapy. While cell replacement therapy is facing the challenges of developing functional photoreceptor cells from human embryonic stem cells (hESCs)/induced pluripotent stem cells (iPSCs) and proper integration in the retina, the discovery of mesenchymal stem cells (MSCs) and their versatile characteristics open an attractive field of investigating neuroprotection in dystrophic retina [6]. Moreover, rapid extraction and ease of culture have made MSCs an attractive target for cell-based *ex vivo* gene therapy. Genetic manipulation of cells is done more safely in *ex vivo* gene therapy since it is outside the body and hence this approach is particularly well suited when targeting a specific organ rather than treating a whole organism [7]. The eye and the visual pathways therefore make an attractive target for this approach. Despite being introduced only recently, an *ex vivo* approach for the treatment of blindness has already achieved significant advances in pre-clinical trials [7].

1.1 Overreaching goal

Given the above promising role of MSCs and *ex vivo* gene therapy in preventing retinal degeneration, I proposed to investigate two key interventions. **Firstly, to investigate whether magnetically guided MSCs can provide neuroprotection in dystrophic retina and, secondly, assess MSC-based *ex vivo* gene therapy to rescue retinal structure and function in a X-linked retinoschisis model.**

1.2 Project 1: Neuroprotection from magnetically guided MSCs

Apoptotic cell death is a central mechanism in many blinding retinal diseases, such as retinitis pigmentosa (RP) [8] and the dry (atrophic) age related macular degeneration [9]. Therefore therapeutic approaches targeting apoptotic cell death could play a major role in preventing vision loss. The concept of neuroprotection by inhibiting cell death has emerged over the last decade and a range of factors, such as CNTF, BDNF, GDNF, and bFGF, have been identified as potential neuroprotectants [10]. Major concerns with the neurotrophic factors include their large size for which they may not cross the blood–retinal barrier (BRB) and also the risk of undesirable systemic complication [10]. Cell-based therapies, with their paracrine effects, have overcome the complexities associated with systemic delivery of these molecules. However there are issues, such as targeting cells to disease tissue, potential for collateral damage, breaching local tissue barrier, ease of delivery method and repeatability, which still require significant considerations [11]. In our recent *in vivo* study, we showed that intravenous, magnetic nanoparticle (fluid MAG-D)-labeled MSCs can be magnetically targeted to a specific locus in the dystrophic rodent retina [11]. We also found that the guided MSCs produced enhanced amount of neurotrophic factors (GDNF and CNTF) and anti-inflammatory factors (IL-10 and HGF) in the retina. These results provide encouraging evidence that magnetically guided MSCs will prevent retinal degeneration by secreting these paracrine effector molecules.

1.2.1 Specific hypothesis

The hypothesis was that systemically administered and magnetically guided SPION labeled MSCs are capable of providing neuroprotection in the dystrophic eye of the rodent model (S334ter-4).

1.2.2 Rationale

MSCs are multipotent non hematopoietic stromal cells found within many tissues. Interest in the therapeutic potential of MSCs was originally focused on their tissue regenerative properties but more recently their paracrine effects, in terms of neurotrophic and immuno-modulatory abilities have become increasingly recognized [12]. MSCs are known to protect injured neurons,

stimulate angiogenesis in stroke, promote wound healing and inhibit fibrosis through their paracrine effectors [6]. In addition, their remarkable homing ability allows MSCs to migrate to the area of tissue damage and integrate into the tissue's extracellular compartment [6]. Currently there are three clinical trials investigating MSCs as a therapy for retinal degenerations [13]. The remote delivery of magnetized MSCs, via the systemic circulation, to sites of disease has recently been the focus of work for a number of preclinical research groups [11, 14, 15]. The use of superparamagnetic iron oxide nanoparticles (SPIONs) to magnetize cells has a number of advantages which includes control on the cells via external magnetic field, MRI visualization and insignificant toxicity of SPIONs at the physiological level [12].

1.2.3 Experimental design

We used line 4 of S334 rats which is one of the prominent experimental models for retinitis pigmentosa. Mesenchymal stem cells have been harvested from adipose tissue of syngeneic animals. The *in vitro* cultured MSCs were labelled with magnetic nanoparticles (SPIONs). Animals received multiple doses both labelled and unlabeled MSCs via tail vein injections. A gold plated disc magnet was placed in the orbit to attract the magnetic cells. The initial injection and magnet implantation was done at p21, followed by two more injections at p50 and p75. The eyes were analyzed at p110 through electroretinogram (ERG), optokinetic tracking response (OKT), histology and optical coherence tomography (OCT). All of these assays indicated that rodents with multiple magnetic injections have better preservation of their retina and have higher visual acuity. As a part of this study the effect of SPIONs on the paracrine activity of the MSCs have also investigated. The study showed some inhibitory effects on secretion of innate and genetically modified molecules from adipose-derived rodent MSCs when exposed to increasing concentrations of the magnetic nanoparticles [12].

1.3 Project 2: MSC based *ex vivo* gene therapy for X-linked retinoschisis

X-linked retinoschisis (XLRS) generally affects young males, where progressive abnormal splitting of retinal tissue leads to retinal atrophy early in life and progressive vision loss by the second decade of life [4]. It is caused by a number of mutations in the *RS1* (retinoschisin) gene, which encodes an extracellular multi-subunit protein involved in retinal cell adhesion [16-19].

Retinoschisin is normally secreted by the photoreceptor and bipolar cells of the retina as a soluble homo-octamer non-glycosylated membrane protein [4]. *RS1* mutations, often missense, can lead to secretion of non-functional proteins resulting in schisis cavities within the retina. As loss-of-function mutations are more amenable to gene therapy than gain-of-function mutations, which require gene silencing, XLRS is an excellent candidate for *ex vivo* gene therapy.

1.3.1 Specific hypothesis

The hypothesis was that genetically modified MSCs capable of secreting RS1 can restore structural integrity and functional aspects of retina in RSIKO mouse model.

1.3.2 Rationale

A number of approaches of *in vivo* gene therapy have been reported to ameliorate the condition of XLRS in the pre-clinical trials on animal models [20-23]. However, the limitations of this *in vivo* approach – lack of specificity, low efficiency, and systemic exposure to the transport vector – are considerable [24-27]. An *ex vivo* approach to gene therapy would circumvent these issues. XLRS is a good candidate for *ex vivo* gene therapy for three reasons: firstly, it is a monogenic disorder where the underlying pathophysiology is relatively well understood; secondly, gene therapy techniques to improve wild-type protein production are best suited to loss-of-protein phenotypes such as in XLRS rather than in gain-of-function mutations where pathophysiology has been linked to adverse effects of mutant protein; and thirdly, RS1 is a secreted, extracellular protein, so it can be replaced in the retinal extracellular space through secretion by other cell types (e.g. MSCs genetically modified to secrete RS1). Another particular advantage of an *ex vivo* protocol is the relative ease with which cells can be engineered to vary expression of novel proteins in response to external signals. The tet-On system is a well-known molecular switch for such a purpose, where expression can be induced by exposure to doxycycline.

1.3.3 Experimental design

Two different RS1 secreting mesenchymal stem cells lines were prepared. In one line, constitutively active human RS1 cDNA plasmid was transfected into MSCs. Another line was

transfected with a Tet-On inducible expression system that is controlled by doxycycline. RS1 expression by these cells lines was assayed *in vitro*. For the inducible system, the induction by Dox was also assayed. Two different sets of experiments were performed with these cell lines. In the first experiment, the RS1 KO mouse model received only single injections of these cells (including control groups) and the results were analysed 2, 4 and 8 weeks post injection. In the second set of experiments the animals received multiple injections, 1 per month for 3 months. In both experiments cells were delivered through intravitreal route. Serial analysis (ERG, OKT and histology) were performed to assess the benefit of the therapy.

Chapter 2. Literature Review

2.1 The retina

The retina is the specialized tissue that converts the information on the incident light to suitable electric impulses that can be transmitted to the brain. The retina is a thin, diaphanous tissue that can be divided into neurosensory retina and retinal pigment epithelium. Four types of cells are present in the neurosensory part of the retina: 1) Photoreceptors (rod and cone); 2) Intermediate (bipolar, horizontal and amacrine); 3) Ganglion cells; and 4) Muller cells. These cells and their nerve fibre laminate the retina in a distinct pattern of three nuclear and three nerve fibre layers. The nuclear layers are outer (containing photoreceptors), inner (containing intermediate cells) and ganglion cells. The layers are outer plexiform (containing photoreceptor axons synapsing with intermediates' dendrites), inner plexiform (consists of axons of intermediates synapsing with dendrites of ganglion cells) and nerve fibre layer (containing ganglion cells axons passing to the optic nerve head) (Figure 2.1) [28, 29].

On the sagittal plane, the neurosensory retina can be divided into a central (macula) and peripheral region. There is a higher cone to rod ratio in the macula region compare to the peripheral retina. The centre of the macula is known as fovea and the central 0.57 mm of fovea is entirely composed of cone photoreceptors. A characteristic difference between human and rodent retina is that the rodent retina does not have the fovea region. Approximately 0.4 mm of this region is free of retinal capillaries. Except for this region, the retina gets its supply by two vascular systems: outer retina by choriocapillaries and inner retina by central retinal artery (branch of ophthalmic artery) [29].

All photoreceptors respond to incident light and convert the light energy into a molecular signal through a complex process called phototransduction. The outer segment of the photoreceptors that conduct the phototransduction contains different photopigments. The cones have three types of photopigments (cone opsins: blue, green and red), whereas the rods have only one type

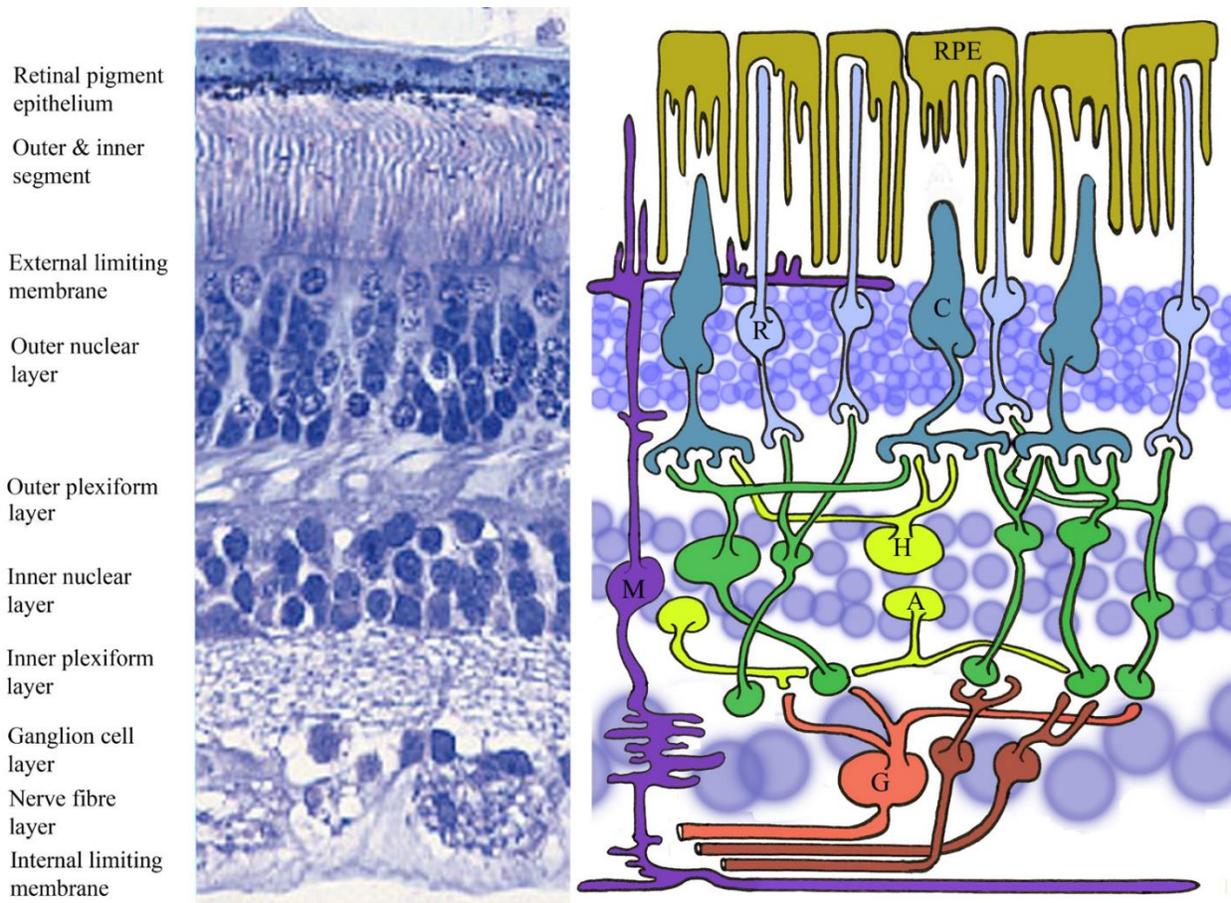


Figure 2.1: **Histological and graphical representation of human retina.** A- low power micrograph of normal human mid-peripheral retina stained with methylene blue. B- corresponding cellular components that result in the stratified appearance of retinal sections. **RPE:** retinal pigment epithelial cell; **R:**rod photoreceptor; **C:** cone photoreceptor; **H-** horizontal cell; **B:** bipolar cells; **A:** amacrine cell; **M:** muller cell; **G:** ganglion cell. Image reconstructed with permission from Kevin Gregory-Evans’s PhD thesis titled “Functional and Molecular Studies in Genetic Retinal Disease” [29].

(rhodopsin). Incident light induces conformational changes in these photopigments which in turn activate a molecular cascade leading to change in transmembrane potential.

The initial signal from the photoreceptors is transmitted through glutamate, an excitatory amino acid neurotransmitter. After the neurotransmitter releases from the photoreceptors, the flow of information is not unidirectional to the bipolar cells. The direction of flow involves centripetal, centrifugal, lateral and reciprocal pathways. At the end of the retinal circuit, the axons of the ganglion cells (nerve fibre layer) carry the signal to the optic disc, which travels all the way through the optic nerve to the to the lateral geniculate nucleus.

2.2 Retinal diseases

A large number of proteins and their pathways are involved in maintaining the normal retinal integrity and complex biochemistry of vision. Some of these proteins are housekeeping (found in other tissues), whereas others are retina specific. Mutations in any of these proteins can lead to retinal dystrophy. Dystrophies can be defined as primary aberrations of mature tissue which undergo atrophy due to an inherited defect. Thus far, 240 genes have been found to be associated with inherited retinal disorders (<https://sph.uth.edu/Retnet>). These disorders can show autosomal dominant or recessive or X-linked inheritance patterns. As a comprehensive description of inherited retinal diseases is beyond the scope of this work, focus will be given on two diseases, retinitis pigmentosa and X-linked retinoschisis, that I investigated for potential intervention development.

2.2.1 Retinitis pigmentosa

Retinitis pigmentosa (RP) is a set of hereditary retinal diseases where primary or secondary loss of rod and cone photoreceptors occurs due to gene abnormalities. The worldwide prevalence of RP is about 1:3000-7000 with more than 1 million affected individuals. The inheritance pattern of the disease can be autosomal-dominant (about 30–40% of cases), autosomal-recessive (50–60%), or X-linked (5–15%) [30]. Although RP is generally confined to the eye, 20-30% of the patients show non-ocular disease association. Thirty different syndromes have been reported so

far for such patients. One of the most common is the Usher's syndrome (accounting for 20-40%), in which RP is associated with hearing impairment [31].

2.2.1.1 Symptoms

The symptoms and/or disease progression vary widely among patients of retinitis pigmentosa. Some patients show symptomatic visual loss in early childhood whereas others do not develop symptoms until mid adulthood. The classic pattern, which many patients develop, includes problem with dark adaptation and night blindness in early life and severely decreased mid-peripheral visual field between age 20 to 30. With age and advancement of the disease, they lose far peripheral vision and eventually develop tunnel vision. By age 60, they can lose central vision. However, individuals with advance RP can have normal visual acuity with a small portion of the central visual field [30]. The rate of the progressive nature of RP can vary. Several factors, such as stage of disease, environmental and dietary factors, primary gene defects, and possible modifier genes can contribute to this variation.

2.2.1.2 Pathophysiology

Retinitis pigmentosa causes photoreceptors degeneration that starts with rods and is followed by cones. Mutations in a number of proteins in the rods can cause their death. It has not been established yet why a mutation in a rod specific gene causes the death of cone cells. In some other cases, detached retinal pigment epithelial (RPE) cells migrate to inner retina and induce abnormal vessel formation. The vessel's endothelial cells cause extracellular matrix deposition near the detached RPE, which closely resembles Bruch's membrane in situ [30].

RP is generally monogenic but the disease itself is very heterogeneous genetically. So far over 3000 mutations in more than 60 genes have been identified with variable inheritance pattern that are associated with photoreceptor degenerations [32]. Of those gene mutations, mutation in *RHO*, *USH2A*, and *RPGR* genes cause about 30% of all cases of RP. These mutations cause cellular toxicity in a number of ways such as by interfering with metabolism, by forming intracellular protein aggregation, by disrupting intercellular transport and by destabilizing the structure of the photoreceptor outer segment [30].

2.2.1.3 Current treatments

Currently there is no cure for retinitis pigmentosa. Most of the existing treatment regimens focus on slowing down the disease progression. Patients also receive treatment for other complications like cataract or macular edema, and need support to cope with social and psychological impact of vision loss [33]. Nutritional or neuroprotective treatments are widely used since they are less dependent on the disease causing mutations. These therapeutic interventions mainly affect the secondary biochemical pathways [30]. Therefore, a number of dietary supplements have been suggested for RP, including vitamins (mainly vitamin A), docosahexanoic acid (DHA), fish oil containing omega 3 fatty acid, lutein and ganglioside [33]. Nonpharmacological interventions are based on healthy lifestyle and strategies to prevent bright light exposure. Healthy living reduces the oxidative stress which is responsible for photoreceptor damage [34] and some pigmentary retinopathies are due to exposure to bright light [35].

2.2.2 X-Linked retinoschisis

The name of the disease, X-linked retinoschisis (XLRS; MIM number 321700), derives from the location of the causative gene (X chromosome) and a characteristic internal splitting of the retina. It is one of the most common juvenile onset retinal degenerations [36]. With a prevalence ranging between 1:5000 to 1:20000, this disease mainly affects males early in their life [37]. Homozygous females show similar disease progression as hemizygous males; however, female carriers are mainly asymptomatic with occasional minor retinal abnormalities [38].

2.2.2.1 Symptoms

XLRS patients display a remarkably broad spectrum of phenotypes with a high clinical variability. In some cases, disease progression and severity showed high variability even within a family. In general, the symptoms can appear as early as the first year of age. Foveal retinoschisis occurs in all of the patients, where it is associated with moderate visual abnormality. The peripheral involvement is present in less than 50% affected individuals. The change in visual acuity can vary and decrease to 20/100 (normal 20/20). Although it is a congenital disorder, the

affected individuals generally do not get diagnosed before the school age because they can perform every day activity with their moderate vision [36, 39]. The disease condition can exacerbate by secondary complications including retinal detachment and vitreal hemorrhage. These additional complications can cause asymmetric expression of the disease which is usually symmetrical in both eyes.

2.2.2.2 Pathophysiology

The major clinical pathology of XLRS includes vitreo-retinal degeneration characterized by radial streaks arising from foveal schisis, peripheral retinal schisis by splitting of inner retinal layers and a negative electroradiogram (ERG) arising from a selective b-wave reduction [36, 39]. The gene responsible for XLRS has been identified and located on X chromosome (Xp22.13) [17]. The human *RS1* gene encodes a 24 kDa protein that is mainly expressed in photoreceptors and bipolar cells in the retina. The monomeric form assembles to organize into a homo-octameric configuration before secreting from the cell. The cell surface attached protein provides structural support to retinal architecture and also maintains the synapse between photoreceptors and bipolar cells.

The protein expression can be disrupted in multiple ways by a wide spectrum of mutations in the *RS1* gene. These include pre-RNA transcript processing, initiation of translation, maturation through the secretory pathway, and the ability to fold and form the biologically active octamer. These point mutations can be either missense or null, which result in misfolded protein or no expression. While some misfolded proteins (Leu12His and Leu13Pro) are disposed by cellular machinery, others (Glu72Lys, Arg102Trp, Arg213Trp, Asn179Asp, and Pro192S) form high molecular weight aggregates in the cell. These aggregates can cause cellular stress which can lead to cell death [40]. In terms of clinical significance, the missense mutations seem to have less severe phenotypes than nonsense mutations in male patients [41, 42].

2.2.2.3 Experimental model

Three experimental mouse models of XLRS have been generated so far, which show a lack of expression of the endogenous murine ortholog of human retinoschisin and manifest striking phenotypic similarities with human retinal phenotypes. Morphological features like disruption of retinal layers, schisis formation in the inner nuclear layer and disruption of synaptic structure, were all evident in these models. They also display an “Electro-negative ERG” in scotopic (dark adapted) ERG, with an attenuated B-wave but near normal a-wave [43-45].

2.2.2.4 Current treatment

Currently there is no cure for XLRS and treatment options are mostly limited to visual aids. Recently, topical application of the carbonic anhydrase inhibitor (Dorzolamide) showed reduction of disease progression and improvement of visual acuity in some patients [46]. The effect of this drug, however, varied widely among the patients and is genotype independent [47]. Some of the complications associated with retinoschisis, such as hemorrhage within a large schisis cavity, a dense vitreous hemorrhage and obstruction of the macula by an overhanging inner wall of a large schisis cavity, can be managed by vitreoretinal surgery [48]. This type of surgery, however, can be associated with long term complications including retinal detachment in XLRS patients [36].

2.3 Magnetic nanoparticles in cell therapy

The use of magnetic nanoparticles in medicine can be broadly sub-classified into four themes. Firstly, the use of nanoparticle-bound antibodies to magnetically separate or guide small molecules and drugs; secondly, imaging techniques based on the local magnetic field created by the nanoparticles (magnetic resonance imaging, magnetic particle imaging, and magnetoresistive biosensing); thirdly, nanoparticle-guided delivery of therapeutic cells to different organs using applied magnetic field; and fourthly, magnetic thermotherapy where magnetic fluids are used to heat up a local environment through heat dissipation triggered by an AC magnetic field [49, 50].

Cell based therapy has the potential to overcome the lack of regenerative capacity of the conventional drug. A major challenge in the field of regenerative medicine lies in developing methods of cell delivery to restore tissue function in the injured and/or disease site. The essential strategies for effective implementation of cellular transplantation include less invasive procedures, adequate cell retention and integration of the cells in the tissue of interest. Moreover, the number of targeted cells, the site of integration and the route of administration are critical parameters that can also influence the process of transplantation. Therefore, an efficient delivery model is desirable that would concentrate and facilitate the homing of cells to the site of lesion.

Magnetic nanoparticles have been used to label the grafted cells for both targeting and tracking purposes [51]. The intrinsic magnetic property of these particles render them useful for targeted therapy, where cells loaded with magnetic carriers can be directed or guided by means of a magnetic field gradient toward tissue targets. This nanoparticles guided method is based on the fact that there is a negligible amount of iron present in human cells and accordingly there is no actuation by a magnetic field. Hence the labelled cells can be targeted using an appropriate magnetic field to a region of interest. Recent studies have shown that magnetic labelling of cells is relatively easy and safe. This model of magnetic targeting has been investigated clinically for drug delivery and preclinically for cell and drug targeting [15].

2.3.1 Types of nanoparticles

Magnetic nanoparticles (MNPs) can be classified as superparamagnetic, paramagnetic, ferrimagnetic or ferromagnetic depending on their magnetic cores, and contain manganese, gadolinium or, most commonly, iron oxide [12]. As iron oxide based nanoparticles have been used most widely for cellular interventions, we will discuss only iron based particles in the rest of the chapter. Super paramagnetic iron oxide nanoparticles (SPIONs) are the most widely used magnetic nanoparticles among other iron oxide particles. The core is usually made of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$). To prevent agglomeration of the colloidal suspension and to enhance biocompatibility the surface of the SPION is usually covered with a compatible coating. There are various types of SPIONs with different kinds of coatings; however the most appropriate type for a specific cell labelling remains to be determined. A number of synthetic and

natural coatings have been used to date, such as dextran and carboxymethylated dextran, alginate, starch, polyethylene glycol(PEG), poly(D,L-lactide-co-glycolide) (PLGA) and organosilane. Small molecules with charged surface such as citrate, amino acids, hydroxamate, and dimercaptosuccinic acid have also been used as SPION coatings [52].

2.3.2 Mechanism

Cell migration plays a critical role not only in therapy but also in development, angiogenesis, immune response, wound healing and cancer metastasis. During these processes, the presence of motogenic stimuli act as an external guidance cue to promote the directed migration of the cells [53]. The basic motility machinery coupled to this steering mechanism has been exploited by sensing aligned fibers or gradients in concentration, mechanical properties or electric field [54]. It has been well established that cells undergo electrotaxis in response to electric fields, durotaxis in response to mechanical signals, haptotaxis in response to graded adhesion in the underlying substrate, and chemotaxis in response to soluble cues in the environment [55, 56]. While the signal transduction, cytoskeleton rearrangements and cell migration pattern elicited by other directional cues have been largely explored, mechanisms to magnetic directional cues are much less understood.

The general mechanism of magnetic nanoparticles based cell migration involves confinement of the MNPs inside endosomes upon internalization of the nanoparticles inside the cell. These endosomes are submicrometric vesicles of the endocytotic pathway. Their movement can be modulated in response to an external magnetic field. The endosomes behave as small magnets and attract each other via dipole–dipole interactions with the aid of the magnetic field. Eventually they will form small chains in the direction of the magnetic field and provide the cell a magnetic moment toward the field (Figure 2.2) [57].

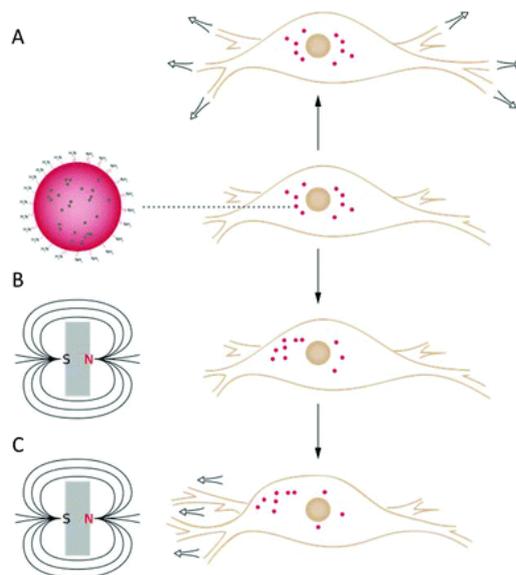


Figure 2.2: **Schematic representation of magnetic nanoparticle labelled cellular migration toward the magnetic field.** Internalized nanoparticles do not direct cellular migration there is an external magnetic field. In presence of the external magnet, the cellular magnetic particles lined up towards the field and provide cells a mechanical cue to migrate. Image reproduced from Bradshaw et al 2015 with the permission from the Royal Society of Chemistry (License #3866581271870) [57].

2.3.3 Therapeutic cell delivery

Developing new ways of delivering cells to target tissue is a major challenge in translating cell therapeutics research into clinical use. The number of transplanted cells, the site of transplantation and the route of administration are the important parameters that influence the effective execution of cellular transplantation strategies in regenerative medicine. To achieve a significant therapeutic benefit, less invasive cell transplantation coupled with sufficient cell retention and integration in the tissue of interest, are essential. Magnetic force based targeting enables delivery of significant numbers of therapeutic cells to key areas of specific organs. Here we report some of the preclinical studies where magnetic targeting has been used to deliver therapeutic cells to diseased tissue.

2.3.3.1 Eye disorder

Vision impairment is one of the most common disabilities. Developed countries are facing the challenges of combating a number of progressive blinding disorders, such as age-related macular degeneration (more than 14 million people blind or severely impaired), retinitis pigmentosa (~1/3500), and diabetic retinopathy (~4.2 million people over the age of 40 in the United States)[1-4]. With retinal degenerations so prevalent worldwide, new treatment approaches would be widely applicable. Moreover, it will bring significant advancement in improving the quality of life as well as removing pressure from the healthcare systems. Cell based therapies, directed at genetic abnormalities or modifying pathological processes, hold much promise in treating a wide variety of diseases that lead to vision loss [5]. The major approaches of cell based therapies for retinal degeneration includes cell replacement and neuroprotection. While cell replacement therapy is facing the challenges of developing functional photoreceptor cells from hESCs/iPSCs and their proper integration in the retina, the discovery of mesenchymal stem cells (MSCs) and their versatile characteristics open an attractive field of investigating neuroprotection in dystrophic retina [6].

Apoptotic cell death is a central mechanism in a number of blinding retinal diseases, such as retinitis pigmentosa (RP) and the dry (atrophic) form of age-related macular degeneration [8, 9]. Therefore, therapeutic approaches targeting apoptotic cell death could play a major role in

preventing vision loss. The concept of neuroprotection by inhibiting cell death has emerged over the last decade and a range of factors, such as CNTF, BDNF, GDNF and bFGF, have been identified as potential neuroprotectants. Major concerns with the neurotrophic factors are either that they are too large to cross the blood–retinal barrier (BRB) or are associated with undesirable systemic complication [10]. Cell-based therapies with their paracrine effects, have overcome the complexities associated with systemic delivery of these molecules. However, there are issues, like targeting cells to disease tissue, potential for collateral damage, breaching local tissue barrier, ease of delivery method and repeatability, which still require significant considerations [11]. Targeting cells to specific loci is particularly important in diseases of central nervous system. For instance, regional differences in tissue function in human retina can make randomly targeted therapy suboptimal. In age-related macular degeneration, cell therapy needs to be targeted specifically at the macular (cone rich central) region of the retina since cells delivered into the peripheral retina will have no functional benefit and might even disrupt normal tissue function. The direct injection of cells into the macula is feasible; however there is significant risk of complications, which increases with repeat injections. In a recent study, intravenous fluidMAG-D-labeled MSCs were magnetically targeted to upper hemisphere of the dystrophic rodent retina by placing a disc magnet in the orbit (Figure 2.3). These magnetized MSCs produced enhanced amount of neurotrophic factors (GDNF and CNTF) and anti-inflammatory factors (IL-10 and HGF) in the retina which, in turn, provides better neuroprotection [11].

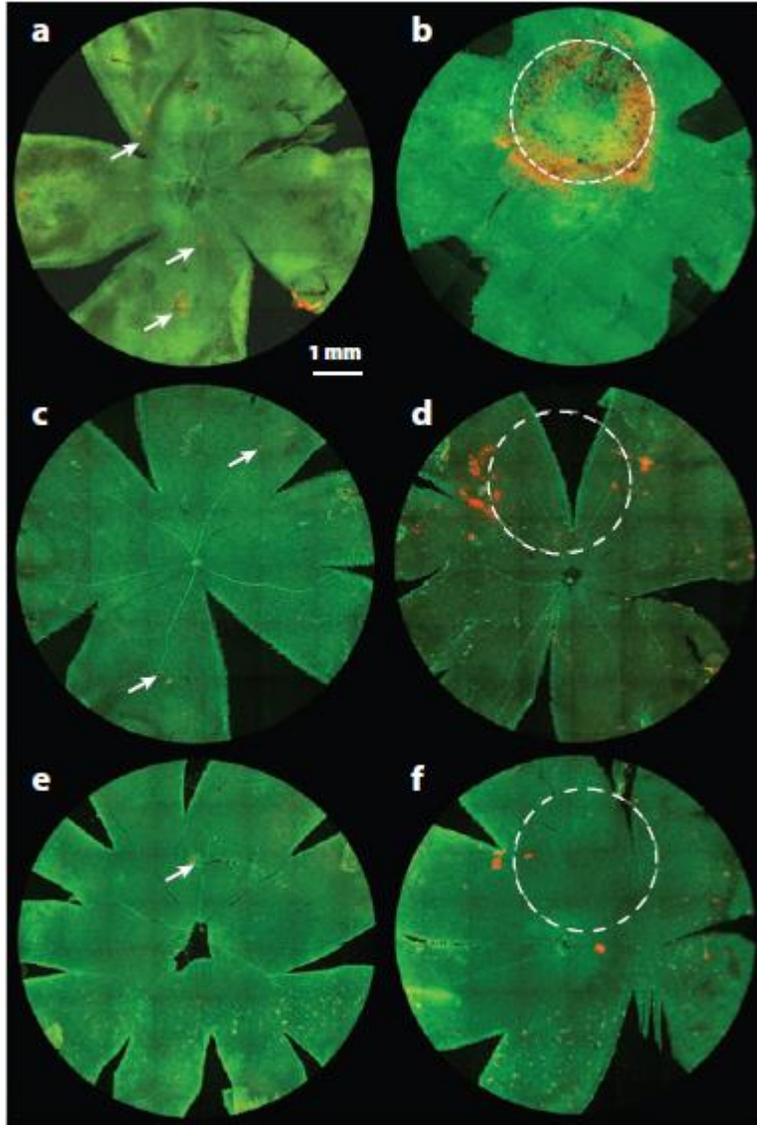


Figure 2.3: **Flat-mount images of rat retinas (a,c,e) in control animals and (b,d,f) after magnetic targeting of mesenchymal stem cells (MSCs).** Arrows indicate small areas of magnetic mesenchymal stem cells in the retinas without a magnet applied (a,c,e). The dashed white circle shows the position of the orbital disc magnet. MSCs have been labeled with Qtracker 655 and appear red/orange on the image. Image reproduced from Yanai A *et al* 2012 with permission from Cognizant Communication Corporation (License # 3865520843005) [11].

2.3.3.2 Spinal cord injury

Spinal cord injury (SCI) is a devastating traumatic injury that can lead to serious neurological deficit and permanent invalidity. The sensory deficits occur due to tissue damage, loss of neurons, axonal degeneration and the poor ability of axons to regenerate across the lesion [58]. This limited regeneration capability of the central nervous system poses the greatest challenge in developing an effective therapy for SCI. Among the potential therapies that have been tested in preclinical and clinical studies, transplantation of stem cells showed promising results. Stem cells can replace lost neurons, provide permissive growth environment and thus enhance regeneration [59]. Despite the potential, a stem cell approach has some serious limitations like low efficiency in delivery, retention and engraftment. A significant therapeutic benefit can be achieved from minimally invasive but highly effective delivery strategy. For SCI model, intrathecal delivery is such a technique where there is more cell retention and survival with higher delivery efficiency than intravenous route. Magnetic targeting can achieve higher efficacy by promoting the homing of cells to the site of injury. A number of groups have reported that a significant number of nanoparticles labeled cells can be accumulated at SCI lesion via magnetic targeting [51, 60, 61]. With magnetic targeting improved behavioral response was recorded compare to conventional cell delivery.

Inappropriate focusing ability is one of the common disadvantages of the magnetic delivery strategies. Magnetic field gradient is at its highest value at the pole where maximum cell capturing occurs. However, most of the time the poles cannot be placed near or at the target site, which renders the system rather inefficient and limited. To overcome these problems, a Czech group has developed a minimally invasive magnetic targeting strategy for SCI model that enables efficient cell retention at a lesion site after intrathecal delivery. This system consists of a ring shaped holder with two cylindrical magnets facing their same poles toward each other. This arrangement creates a focusing zone (named trapping area) where the vertical and the horizontal magnetic force components nullify each other and prevent cells from further movement. The system can be manipulated to place the focusing zone exactly on the lesion site. The cells can be guided to a lesion site in a rat model within two hours of intrathecal delivery with this system which is significantly higher than the usual 10-12 hours time (Figure 2.4) [60].

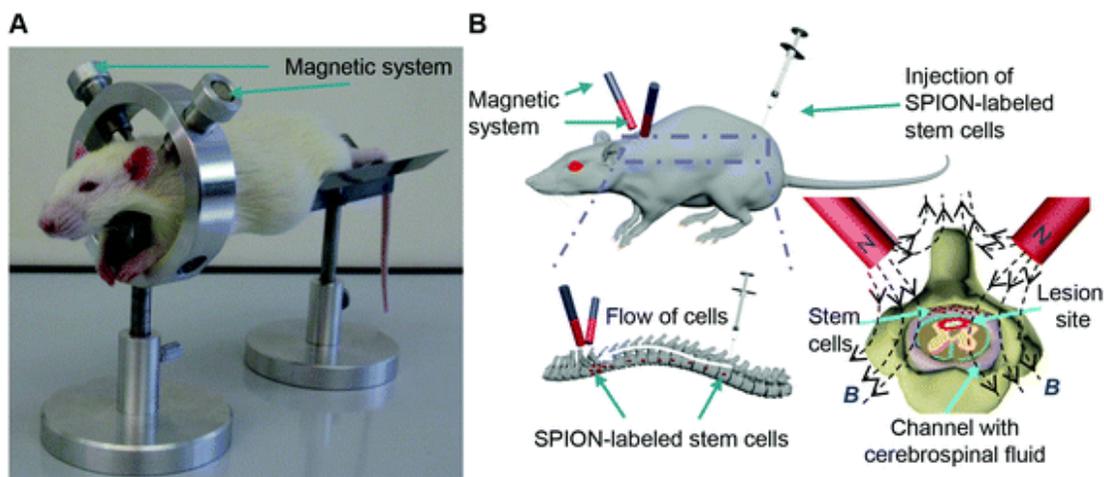


Figure 2.4: **Magnetic targeting of MSCs to the site of SCI. (A) *In vivo* model of the non-invasive magnetic targeting model.** (B) Schematic representation of the magnetic targeting mechanism. A ring shaped holder holds two cylindrical magnets facing their same poles toward each other. This positioning creates a trapping zone where the vertical and the horizontal magnetic force components nullify each other and prevent cells from further movement. The system can be manipulated to place the focusing zone exactly on the lesion site. The cells can be guided to a lesion site in a rat model within two hours of intrathecal delivery. Images reconstructed from Tukmachev et al 2015 with permission from the Royal Society of Chemistry (License # 3866590347013) [60]

2.3.3.3 *Cancer:*

A major difference in the cell based therapeutic approach between cancer and regenerative medicine is that in cancer, the cells generally cause necrosis rather than regeneration in the target site. For cancer therapy, a wide variety of cells, including erythrocytes, bacterial ghosts, genetically engineered stem and dendritic cells, have been manipulated as novel drug-delivery systems. The goals of all sophisticated drug delivery systems, which are currently being investigated, are to be nontoxic and biocompatible, have long half-life, better targeting and less remote accumulation, multi-drug loading and tracking capability. Cell-based systems have been proved to be a better drug carrier considering all these facts compare to the synthetic nanoparticulate system. Red blood cells (RBCs) have been extensively investigated as a vector for cell-based drug-delivery system because of their easy access, inherent bio-compatibility, flexibility, in vivo stability and long systemic half-life. However, in some cases the small size of RBCs limits their chance to directly approach the tumor cells as well as their extravascular diffusion. To circumvent this problem a chimeric RBC, called RBC-IONP-Ce6-PEG, has been developed by a group of scientist where they attached iron oxide nanoparticle coated with chlorine e6 (Ce6- a clinically used photodynamic agent) on the membrane of mouse RBCs and loaded the cells with doxorubicin (DOX- a chemotherapy drug). For further stability the nanoparticles were coated with polyethylene glycol (PEG). Intravenous delivery of these cells showed higher stability, longer systemic circulation and high efficient tumor penetration when an external magnetic field is applied. This strategy also demonstrated low remote organ retention and a synergistic tumor growth inhibition effect after performing a combined photodynamic and chemotherapy (Figure 2.5) [62].

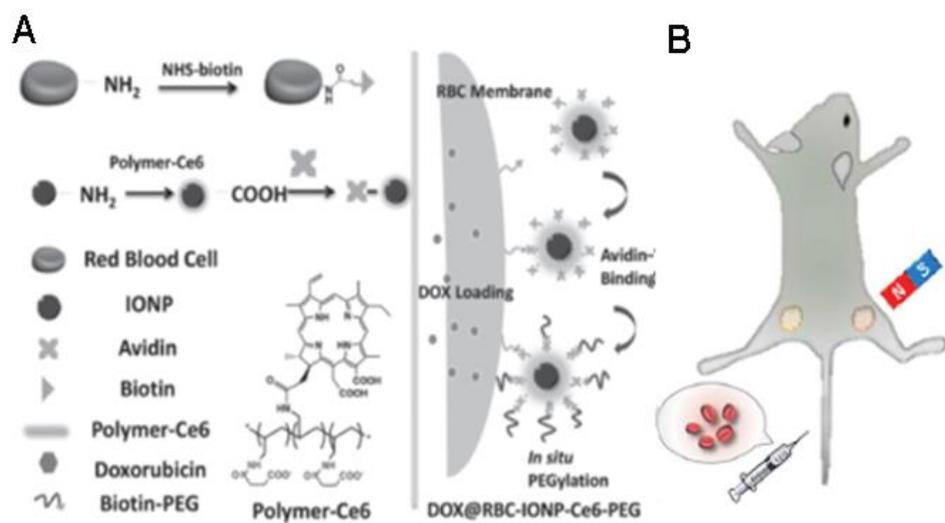


Figure 2.5: **Preparation and delivery of magnetic RBC.** Schematic diagram showing (A) the preparation steps of theranostic RBCs modified with magnetic nanoparticles, Ce6, DOX, and PEG; and (B) in vivo magnetic tumour targeting. Image Reconstructed from Wang C et al with permission from John Wiley and Sons (License #3858491404359) [62].

2.3.3.4 Heart diseases

Cardiovascular diseases, such as myocardial infarction, are the leading causes of death and disability worldwide [63]. Myocardial infarction (MI) is the irreversible damage of the healthy and contractile myocardium. The tissue becomes akinetic and fibrotic and the heart cannot pump blood at its full potential. Although the current available treatments have greatly impacted the trajectory of patient health following a MI, the rate of mortality and morbidity still remains very high [63, 64]. In recent years, stem cell based regenerative therapy has emerged as a potential therapy. A number of preclinical studies have reported improved cardiac function after administering cells to treat MI through direct myocardial injection. However, this cell delivery method causes needle-mediated tissue damage and rapid cell attrition due to leakage of the transplanted cell suspensions. Thus alternative cell delivery approaches have been explored. A Japanese group has recently developed a 300 um thick cell sheet, comprised of 10-15 piled-up magnetized cells, using magnetic force. This cell sheet, comprised of either mesenchymal stem cells or induced pluripotent stem cells, induced angiogenesis when transplanted into an ischemic mouse heart [65, 66]. They termed this nanoparticles aided system as magnetic force based tissue engineering system (Mag-TE). Lipid coated iron oxide nanoparticles labeled free cells in a small plate with media, forms a sheet-like structure by piling up according to the magnetic force after 24 hrs of incubation. In this approach magnetic nanoparticles and force have been used for structural purpose rather than directional.

2.3.3.5 Respiratory disease:

The airway epithelium is the primary environmental barrier and has a number of important physiological functions which includes humidifying the air, regulating the airway smooth muscle, eliminating inhaled pathogens and particulates, and recruiting immune cells in response to injury. Chronic airway diseases, for instance COPD (chronic obstructive pulmonary disease), cystic fibrosis and lung cancer, cause major morbidity and mortality worldwide [67]. Although lung transplant is considered as a viable treatment option for these patients, its use is severely limited by donor availability and post surgical complications [68]. A rapidly growing number of investigations of stem cells and progenitor cells based therapies as well as *ex vivo* lung bioengineering have offered exciting new avenues for providing novel potential therapeutic

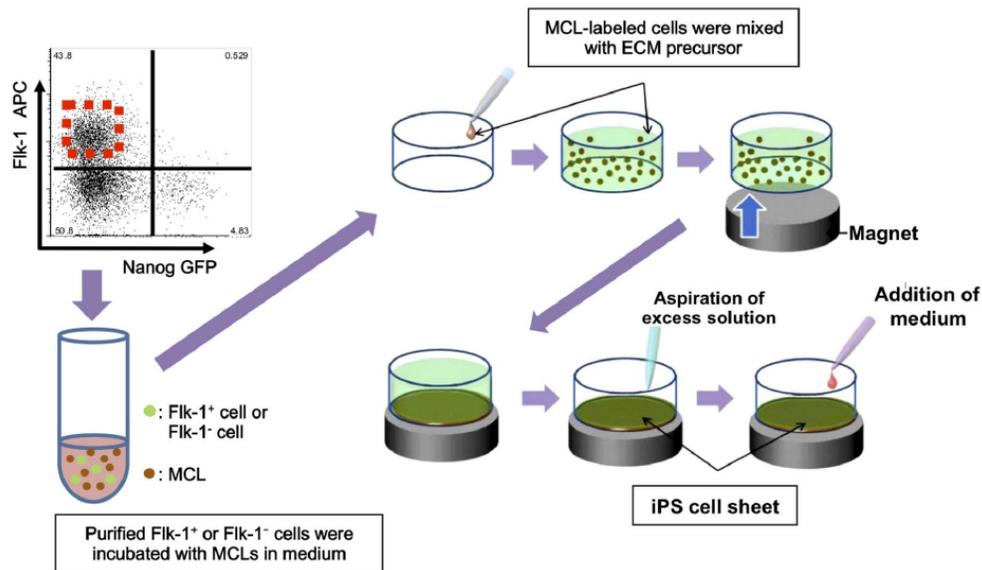


Figure 2.6 : **Development of iPS cell-derived cell sheet by combining magnetic nanoparticle and ECM precursor embedding systems.** FACS sorted, purified cells were mixed with magnetic nanoparticles and incubated for 2 hours. 10^6 labeled cells were mixed with ECM precursor and seeded onto ultra-low-attachment plates. When these plates were placed on a cylindrical neodymium magnet, a vertical magnetic force was applied to the plate. After washing and media replacement, the incubation was continued for an additional 24 hours. Image reconstructed from Tetsutaro K et al (2013) with permission [64].

approaches for respiratory diseases [67]. Like other fields, failure to deliver significant number of cells to the target site severely undermines the utility of airway repair by cell therapy.

Magnetic nanoparticles based cell targeting has showed promising results so far in long term cell engraftment. Labelling endothelial progenitor cells with magnetic nanoparticles and targeting them with Halbach cylinder magnet has increased the number of retained cells in a tubular structure, which in principle, is compatible with airway epithelium [69]. In this technique the tubular scaffold, positioned inside the cylindrical Halbach magnet, faced an intense magnetic field that draws the nanoparticles labelled cells and facilitates an efficient and uniform delivery. In an early work, dynamic rotational cell seeding has been used to seed autologous cells onto the lumen of decellularized trachea, prior to transplantation into a human recipient [70].

Although the technique was clinically successful, the continuous movement between the cell suspension and scaffold surface over a period of 96 hours made it inefficient. Compare to this technique, magnetic targeting produces a uniform cellular distribution on the graft luminal surface in only 2 hours [69].

2.3.4 Safety

Unlike other forces such as light, electric fields and ultrasound for manipulating therapies, magnetic fields have a very minimal effect on biological systems. Moreover it can reach and penetrate safely and deliver cells deep in to the tissue targets [49]. Nonetheless there are negative effects of these particles and they can be divided into direct (immediate) and indirect (mediated). The direct effects are conferred by the influence of nanoparticles on cells – their accretion in the cytoplasm, docking on membranes, mechanical destruction of cell membranes, conformational changes of cytoskeleton and biopolymers followed by their functional impairment, etc.

The indirect influence of nanoparticles can be mediated by a number of factors, like peroxide modification of molecules with alteration of their properties, changes in homeostasis parameters, activation of endogenous damage factors, coagulating and kallikrein–kinin systems. These features appear in response to the exposure of nanomaterial into the cell. Nanoparticles could

also be involved in stimulation of the immune system through hapten or adjuvant activity, as well as mediated by cytokines secreting activated phagocytes.

2.3.4.1 Toxicity

A number of studies have investigated the cytotoxic potential of several different magnetic nanoparticles on different cell types (Table 2.1) and generally have found no or low cytotoxicity at lower concentration (>100ug/ml). Figure 2.7 shows schematic representation of different potential pathways through which MNPs can confer cytotoxicity. This cytotoxicity is mostly conferred by the fact that the core of the particle contains a transition metal [71]. Other factors such as composition of the coating or its breakdown products, cell-media composition etc. can also play a role in conferring cytotoxicity. The core generally has high dispersion rate and the large specific surface area allows a large number of transition metal atom, such as Fe atoms, to interact with the cellular environment by accepting or donating electrons, thus stimulating the formation of ions and radicals [71]. Most commonly formed radicals are reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radicals and the non-radical hydrogen peroxide. Higher dose or greater reactive transition metal leads to increased production of ROS. A higher ROS level has been associated with significant toxic effects such as disrupting DNA, altering gene transcription, peroxidizing lipids, modulating proteins that result in decreasing physiological function and apoptosis. Aside from producing directly from the nanoparticles' core, ROS can also be generated from leaching of iron molecule, altering mitochondrial function and inducing cell signaling pathway [72]. Besides ROS mediated cytotoxicity, several other nanoparticle-induced cell injury mechanisms have been proposed, such as genotoxicity, actin cytoskeleton disruption [73] and loss of mitochondrial membrane potential. The exposure of nanoparticles can lead to deleterious DNA damage that may initiate carcinogenesis. It has been reported that intramuscular injection of iron dextran complex is associated with spindle cell and pleomorphic sarcoma in rat [74]. Exposure to iron oxide nanoparticles can also induce actin stress fiber formation. Actin has many important cellular functions, such as providing morphological stability, motility and permeability and also modulating adhesion. One or more of these functions may be declined due to actin stress fiber formation and increased cell elasticity.

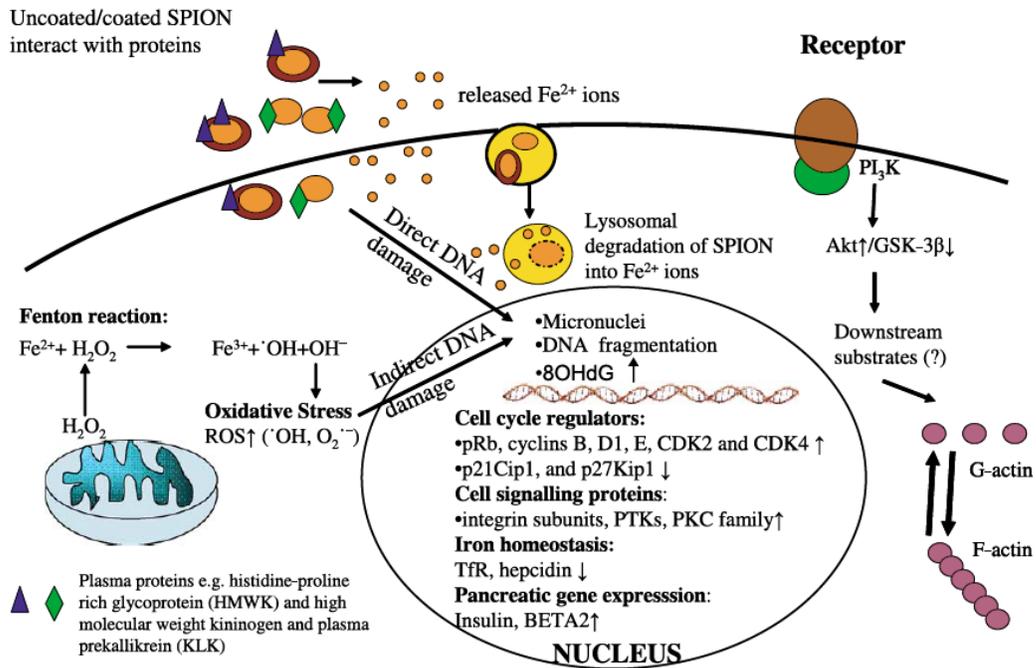


Figure 2.7: Schematic representation of potential SPION induced cellular toxicity. Image reproduced from Singh et al 2010 with permission [74].

These stress fiber formation can also be a secondary effect of ROS accumulation in response to nanoparticles. A study showed that ROS scavengers reduced actin stress fiber formation and cell death in endothelial cells after nanoparticles exposure [73].

Mitochondrial membrane potential loss increases with nanoparticle exposure in a dose dependent manner in human mesenchymal stem cells. Mitochondrial membrane potential ($\Delta\psi$) plays an important role in ATP synthesis, the redox system, and cell defense mechanisms. The loss of $\Delta\psi$ may activate oxidative stress response, which can lead to neurodegenerative disorder, metabolic diseases, aging and cancer. Downregulation of SOD, GSTM3, GPX, and TNFRSF1A expression in hMSC may be attributed to nanoparticle induced loss of mitochondrial membrane potential. TNFRSF1A is associated with increase production of ROS, mitochondrial membrane damage and onset of apoptosis [75].

Although the dose of nanoparticle administered for cellular therapy is only 1.25-5% of the total stored iron in the body, magnetic targeting can result in higher localized iron concentration in the target site. This excessive accumulation of nanoparticles can lead to imbalance of iron homeostasis and can cause aberrant cellular responses. The nanoscale size of the magnetic nanoparticles also allows them to diffuse across biological membrane and tissue barrier and thereby can potentially induce cytotoxicity by impairing the functions of the major components of the cell, like mitochondria, nucleus and DNA [72].

So far a large number of apparently contradicting results on toxicity of nanoparticles have been reported due to various cell types tested and the difference in concentrations used (Table 2.1). Therefore, it would be ideal to generate a standard which is more representative for a number of primary cells and established cell lines to represent different physiological conditions.

Table 2.1: Toxicity study of nanoparticles on different cell types.

Coating	Size	Concentration	Cell	Incubation Time	Toxicity
Dextran	100–150 nm	0.1 mg/mL	macrophages (human)	7 days	20% viable [76]
	35 nm	10 mg/mL	macrophages (human)	14 days	Mildly toxic [77]
	30 nm	10 mg/mL	malignant mesothelioma cells (human)	3 days	Mildly toxic
	15 nm	0.05 mg/mL	hTERT-BJ1 (human fibroblasts)	3 days	Mildly toxic [78]
	10–100 nm	0.2 mg/mL	GL261 (mouse brain tumor cells)	1 day	Mildly toxic [79]
poly(vinyl alcohol)	82 nm	0.2–20 mM	L929 (mouse fibroblasts) and K562 (human leukemia)	2 days	toxicity was dependent on nanoparticle shape and size [80, 81]
silica	50 nm	4 mg/mL	A549 (human lung adenocarcinoma epithelial cells)		IC ₅₀ = 4 mg/mL [82]
	30–120 nm	10 µg/mL	macrophages and dendritic cells (human)	2 days	dose- and size-dependent damage [83]
amine-surface	61–127 nm	0.03 µg–3 mg/mL	HepG2 (human liver carcinoma cells)	5 days	high positive charge causes severe cytotoxicity [84]
	10–20 nm	36 mM	human heart (HCM), brain (BE-2-C), and kidney (293T) cell lines	1 day	cells were viable (< 40%) after 1 day [85]
Tetraheptyl ammonium	30 nm	2.5 µg/mL	K562 (human leukemia)	3 days	cells were viable (< 60%) after 2 days [86]

Coating	Size	Concentration	Cell	Incubation Time	Toxicity
Tween 80	30 nm	25–500 µg/mL	macrophages J774 (mouse)	1–6 h	dose- and time-dependent damage [87]
1-hydroxyethylidene-1,1-bisphosphonic acid	20 nm	0.1 mg/mL	mesenchymal stem cells (rat)	2 days	cells were viable (70%) [88]
Chitosan	13.8 nm	123.52 µg/mL	SMMC-7721 (human hepatocellular carcinoma cells)	12 h	cells were viable (10%) [89]
Human like collagen	35.5 nm	25 to 250 µg/mL	NIH3T3 cells	24 h	Dose and coating dependent [90]
Starch	200 nm	0.05-0.5 mg/mL	Mesenchymal stem cells (rat)	24 h	>95% cells survived [11]
None	20–40 nm	1.0 mg/mL	aortic endothelial cells (porcine)	24 h	Dose dependent [73]
	50-75 nm	25-400 µg/mL	Mesenchymal stem cells (human)	24-72 h	Dose and time dependent [75]

2.3.4.2 Biochemical effects

The biochemical effects of the magnetic nanoparticles have been studied extensively. The major type of nanoparticle, SPION, as well as its different surface coatings, may result in physiological changes in cells. This includes alteration in actin cytoskeleton, modulation of gene expression profiles, imbalance of iron homeostasis, activation of signaling pathways and impairment of cell cycle [72]. As speculated, the effects vary depending on the cell and particle type. For instance, SPION does not affect the viability, proliferation, multiple differentiation and membrane antigen of bone marrow derived MSCs [91] but adipose derived MSCs showed higher osteogenic differentiation capability when incubated with SPION in vitro [92].

As discussed earlier, some cells showed increased ROS production when incubated with nanoparticles. Higher ROS activity can lead to a number of biochemical and physiological changes in the cells. For instance, in endothelial cells iron oxide nanoparticles induce ROS formation which disrupts the actin cytoskeleton and alters cell morphology, locomotion, chemotaxis and mechanics [73]. While not cytotoxic, a high dose of nanoparticle interferes with actin cytoskeleton, which decreased the cell proliferation in neural progenitor cells and human blood outgrowth endothelial cells [93]. In another study, exposure to nanoparticles rearranges the dynamic cortical meshwork of F-actin in human microvascular endothelial cells and increased permeability on the membrane [94]. The mechanism that changes actin dynamics involved ROS induced GSK-3 β inhibition through activation of the Akt signaling pathway. Akt also plays an important role in connecting growth factor signaling via PI 3-kinase and in insulin signaling to basic metabolic functions, including carbohydrate metabolism, protein and lipid synthesis and gene transcription. Therefore, iron oxide nanoparticles could potentially lead to perturbation of these normal cellular and physiological pathways through Akt signaling [74, 94].

The change in actin cytoskeleton architecture also leads to modification of actin-associated genes. Microarray analysis of human fibroblast cells showed higher expression of genes associated with actin remodeling after 48 h exposure to nanoparticles. Genes of other pathways have also been influenced by nanoparticles in different cells. For instance signal transduction

pathway genes such as integrin subunits, tyrosine kinases and several members of the protein kinase C family showed increase expression in presence of SPION. Nanoparticles can also upregulate the expression of genes that are involved in cell motility and cell-cell interactions, such as ion channels, growth hormones and Ras-related proteins. SPION exposure also induce reorganization of fibroblast's matrix material by significantly increasing the expression of ECM proteins and matrix metalloproteinases [95].

Expression of endocrine hormones, such as insulin, glucagon and somatostatin, were observed in pancreatic islet cells after labeling them with Resovist (carboxydextran-coated SPION). Among the hormones, only insulin showed higher expression along with its transcription factor Beta-cell E-box trans-activator (BETA2). Higher expression of insulin in response to nanoparticles has two clinical implications. First, islet cells labeled with magnetic nanoparticles can produce more insulin for a patient undergoing cell transplant; second, higher insulin induces iron uptake by fat cells by increasing ferritin synthesis and localizing transferrin protein on the membrane. Under pathological conditions, such as cancer, atherosclerosis, hypertension and arthritis, iron can leave its ferritin bound steady state condition. Thus, higher insulin can initiate a vicious cycle where higher iron uptake results in more insulin production and iron overloading in the cell. As we discussed before, this could lead to accumulation of highly toxic ROS [96, 97]. Secretion of other paracrine factors and secreted proteins can also be modified by MNPs. Glial derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) from mesenchymal stem cells showed opposite secretion profile in presence of increasing SPION concentration. While higher concentration of SPION has an inhibitory effect on GDNF, it has a stimulatory effect on CNTF [98].

Exposure to magnetic nanoparticle augments cell cycle progression via upregulating cell cycle controlling proteins. For instance, mesenchymal stem cells showed higher accumulation of hyperphosphorylated retinoblastoma tumour suppressor protein pRb, cyclins and cyclin-dependent kinases (cyclins B, D1, E, CDK2 and CDK4), when labelled with Resovist (Ferucarbotran). At the same time negative regulators of the cell cycle, such as p21Cip1, and p27Kip1, members of the CIP/KIP family as well as tumor suppressing p53 showed decreased

expression. Since iron oxide nanoparticles can also activate Akt pathway, it can be speculated that nanoparticle exposure induces proliferation and survival by PI3/Akt mediated escaping apoptosis mechanism [94, 99]. Cell-cycle analysis in another study indicated that high dose of Fe₃O₄ nanoparticles altered the cell-cycle progression in human mesenchymal stem cells. Although no significant arrest in the S-phase and the G2/M compartment was evident, a dose dependent increase in the sub-G0–G1 population was observed [75].

2.3.4.3 Metabolism

The metabolism of the magnetic nanoparticles can be subdivided into cellular and physiological level. At the cellular level, different types of cells handles the nanoparticles in more or less similar fashion- nanoparticles enter the cells via endocytosis, accumulate in the endosomes and ultimately fuse with lysosomes. The acidic environment of the endosome accelerates the dissolution of the nanoparticles, which is gradually released to the cytoplasm and ultimately contributes to the overall cellular iron deposition [71]. At the physiological level, cell death due to iron overload or any other reasons lead to accumulation of the nanoparticles in the extracellular matrix (ECM). The absorption, metabolism and excretion of the particles in the ECM are actively conducted by the reticuloendothelial system and its component like the mononuclear phagocytes. Any nanoparticles not engulfed by the RES system or leaked to the blood get picked up by the iron binding proteins. Transferrin, ferritin and lactoferrin are the major iron-binding proteins in the circulatory system of the vertebrate organisms. Iron released from the nanoparticles, carried by these proteins to various organs and tissues. The classic pattern of iron oxide nanoparticles' biodistribution is: 80–90% in liver, 5–8% in spleen and 1–2% in bone marrow. The majority of the nanoparticles are carried to the liver for metabolism and subsequently used by RBC or excreted by kidney. Extravasation and renal clearance generally rapidly remove the small iron nanoparticles (>10 nm). The large particles (>200 nm) are removed slowly by getting sequestered in the spleen via mechanical filtration [71].

Blood compatibility is very important for the physiological metabolism of any nanoparticles. To assess the safety of the nanoparticles blood compatibility and blood contact characteristics should always be evaluated before clinical trials. There are several clinical assays available, such

as prothrombin time, activated clotting time, activated partial thromboplastin time and thrombin time, which can determine the coagulation properties of the particles. Effective protein adsorption actively influences nanoparticles fate and biodistribution inside the body. Generally, nanoparticle size, surface charge, surface coating, shape, and stability contribute to the interaction of the nanoparticles with proteins. Sometimes the preliminary modifications of coating the nanoparticles with lipids or polysaccharides make them more amenable for biodistribution [72].

2.3.5 Conclusion

In summary, magnetic nanoparticles demonstrate unique properties which provide various scopes and advantages in biomedical applications including targeted cellular therapy. Locating cells using magnetism and directing their passage along magnetic fields is evolving as a particularly useful, non-contact approach in solving targeting and tracking problems in regenerative medicine. Theoretical adverse effects have yet to be realized in preclinical studies showing large-scale, serious adverse effects at MNP doses that would be applicable in cell therapeutics. The consensus however is that while some nanoparticles are safe for certain biomedical applications, they need to be considered more carefully for other uses. Surface coatings and particle size seem to be crucial parameters for the observed MNP-induced adverse effects, as they are critical determinants of cellular responses and intensity of effects. There is an urgent need for more rigid, standardized nanoparticle toxicology research because many of the published toxicology studies report conflicting results. In addition, longer preclinical and clinical prospective studies that are sufficiently powered are needed to establish the most important therapeutic targets for this emerging technology.

2.4 *Ex vivo* gene therapy and vision

Vision impairment is one of the commonest disabilities. With widely diverse causes for visual loss, new approaches to treating incurable diseases are needed as well as significant improvements in health delivery. Gene therapy, directed at primary genetic abnormalities or at modifying pathological processes such as inflammation, neovascularization and cell death, holds much promise in treating a wide variety of blinding diseases. Numerous *in vivo* gene therapy

trials, both in animal models [100, 101] and patients [102-104] have already shown significant benefits with this approach.

Ex vivo gene therapy is described by the American Society of Gene and Cell Therapy as a type of gene therapy where patient's cells are harvested, cultivated in the laboratory, and incubated with vectors carrying a corrective or therapeutic gene. These cells, with new genetic information, are then transplanted back into the patient from whom they were derived.

(http://www.asgct.org/about_gene_therapy/terminology.php#E).

2.4.1 *Ex vivo* verses *in vivo* gene therapy

Limitations associated with *in vivo* gene therapy include: lack of specificity; low efficiency; and systemic exposure to the transport vector (e.g. possibly leading to neoplastic and inflammatory complications).[24-27, 104] In addition, suboptimal responses leading to re-treatments increasing the likelihood of complications.[105, 106] An *ex vivo* approach to gene therapy may resolve some of these issues. In general, to date the most successful *ex vivo* gene therapy studies have been directed to produce:

2.4.1.1 *Novel function within targeted cells.*

Ex vivo gene therapy is commonly used to confer a novel function on cells. Examples include inducing drug resistance to allow for high-dose anti-tumor chemotherapy regimens, [107] and induction of interferon- α expression in tumor-homing macrophages which then seek out and can kill tumors.[108]

2.4.1.2 *Augmented therapeutic gene dosage.*

Genetic manipulation can be used to increase target cell gene dosage to supra-normal levels.[109, 110]

2.4.1.3 Enhanced immune targeting

Very recent studies in leukemia have demonstrated the power of immunotherapy in cancer [111-113]. Allogenic T cells can recognize and kill cancer cells but recognition of healthy tissues can also result in detrimental ‘graft-versus-host disease’. The transfer of a suicide gene into these donor lymphocytes can reduce these toxic effects [111]. Autologous human T lymphocytes have also been genetically modified using a lentiviral vector to express chimeric antigen receptors to target CD19 and contain a co-stimulatory domain from CD137 and the T cell receptor z chain. These cells have been infused into three patients with advanced chronic lymphocytic leukemia. After transplantation, the engineered T cells trafficked to bone marrow and expanded more than 1000-fold *in vivo*. They also continued to express functional CARs at high levels for 6 months resulting in complete remission of disease for at least 10 months [112]. Toxic side effects such as hypogammaglobulinemia were however seen [113].

Such *ex vivo* gene therapy approaches are already proving advantageous in preclinical [114, 115] and even clinical trials [112, 116, 117]. These studies have highlighted a number of principles important in construct design and delivery. In this review we will evaluate the techniques emerging to optimize this approach and review results targeted at diseases affecting the visual pathways (Figure 1).

2.4.2 Techniques and methods

2.4.2.1 Design and manufacture of gene constructs

As well as deciding which gene to transfect into target cells, a number of other issues need to be considered at the stage of gene construct design. The principle components of vector constructs traditionally include: a suitable promoter capable of constitutive or inducible transcriptional activity [118]; transcription initiation and post transcriptional modification signals such as a Kozak sequence, translation termination, mRNA splicing, mRNA modification, polyadenylation and a transcription terminator [119]; antibiotic selection genes for maintaining stable expression and amplification of the gene of interest[120]; and bacterial origin of replication and selection markers for vector cultivation in bacteria [121]. Other important factors include considering the

dosage of resulted protein that needs to be delivered [122] and the time-course over which one wishes to deliver treatment (e.g. short-term or lifelong) [123].

Design involves critical selection of promoters. In work on *ex vivo* gene therapy in the CNS, ubiquitous promoters have mostly been used although future work may well involve tissue specific or composite promoters. The most common ubiquitous promoters in use are β -actin [124, 125], cytomegalovirus [126, 127], phosphoglycerate kinase [126], Rous sarcoma virus [127, 128] and ubiquitin C promoter [129]. Specific promoters for neurological tissues could include synapsin I [130], platelet-derived growth factor beta [131] and opsin [132]. In addition, it is possible to have expression regulation systems made up by a combination of different promoters and enhancers, such as ‘CAGG’ [133]. An interesting and innovative approach has been control of expression using pharmacological adjuvants. Tetracycline-mediated transgenic green fluorescent protein expression has been achieved *in vivo* in photoreceptors and ganglion cells after rAAV transduction [134]. Other drugs that have been used to control gene expression including rapamycin, mifepristone and ecdysone [120].

2.4.2.2 Gene construct and delivery to target cells

A major challenge for gene therapy, be it *in vivo* or *ex vivo* however, is how best to transfect these genetic constructs into therapeutic cell lines for stable, non-toxic expression of the desire gene. A key issue determining the best approach for transport across the cell plasma membrane, but also when long-term expression is needed, transport across the nuclear membrane also needs to be considered. Another issue, particularly when the nucleus is targeted is avoiding disruption of endogenous gene expression [117]. Finally; the type of the cell to be transfected can also influence the method of gene transfer [135]. Two broad themes have emerged: viral and non-viral constructs.

2.4.2.3 Viral constructs

Numerous viral vectors have been used to deliver gene constructs in *ex vivo* work [136], however today most gene therapy work that targets the central nervous system involves using recombinant

adeno associated virus (rAAV) or lentiviral vectors. The rAAV vector overcomes many of the problems associated with adenoviral vectors and can express transgenes stably for long periods both in a constitutive and regulated fashion. Various rAAV serotypes have been exploited to transfect different ocular cells [120, 137, 138], and to some extent the usefulness of rAAV has been improved of late through increases in the amount of foreign DNA that can be packaged within the vector [118, 119, 121, 139]. Lentiviruses vectors, especially equine infectious anemia virus and human immunodeficiency virus-1, have been shown to have a greater transgene capacity (8-10 kb) than most rAAV and thus have been the vector of choice in many applications requiring expression of multiple proteins in single cells. Other advantages in using lentivirus vectors are that they can transfect both dividing and non-dividing cells and host cell genome integration provides long term expression [136, 140]. Importantly, proof of principle work has been undertaken on how best to manufacture HIV-1-derived lentiviral vectors on a large scale and under the 'good medical practice' conditions that would be needed for clinical applications [141].

Examples of lentivirus in human *ex vivo* gene therapy clinical trials include work on adrenoleukodystrophy [142] and β thalassemia [143]. Both studies showed clear therapeutic benefits and suggested that lentiviral vectors provide efficient and safe gene delivery. Other work however suggests that such benefits may be specific to certain cell-types. *In vitro* work in ovine corneal endothelial cells for instance has shown that when cells are transfected with either adeno- and lenti-viral based vectors carrying the ovine IL-10 gene, mRNA expression was 10^3 times higher with the adenoviral vector [144].

2.4.2.4 Non-viral constructs

Non-viral techniques for integrating new genetic material into cells have clear advantages over viral methods in that they raise less concern about side effects such as infectious contamination of target cells and subsequently the host. Such approaches should also elicit less inflammation. To some extent though, their inefficiency and transient transgene expression profile detracts from their usefulness [135]. However, there may be some circumstances in which short-term gene expression is all that is required, e.g. when targeting cancer cells.

Ex vivo gene therapy studies have employed numerous non-viral techniques to get DNA cross the plasma membrane. These including the ‘gene gun’ [145], electroporation [146], dendrimers [147], cationic liposomes [148], antibody or peptide-targeting vector systems [149] and nanoparticles [150]. Of these techniques electroporation has so far proven the most efficacious and simple method to transfect cells *ex vivo* [135, 144, 151]. Electroporation makes cell walls transiently permeable by using a high field-strength, square wave electric pulse.[135, 151] This approach allows for larger sized DNA construct transfection [119, 152], although the technique is associated with cell death in large percentages of cells and also insertional genotoxicity [153].

Liposomes, synthetic phospholipid vesicles, carry charged-particles like DNA through lipid bilayers of the cell by direct fusion with the membrane and endocytosis, which can be receptor mediated via the clathrin-coated pit pathway.[154, 155] Liposomes have been used in the *ex vivo* transfection of primary chondrocytes in cartilage repair experiments in rabbits.[156] A transfection efficiency of over 70% was achieved. A liposome approach has also been proposed for the *ex vivo* genetic manipulation of corneal endothelial cells.[157] Using a number of lipid-based gene transfer reagents, lipofectin has been shown to be the most efficacious at transfecting immortalized human corneal endothelial cells (approximately 20%) with a plasmid encoding for fibroblast growth factor-1[148] Human clinical trials using liposome delivery showed only moderate success,[158] however, recent advances have increased interest in this technique: cationic polymers have been designed to improve transgene expression and reduce toxicity [159, 160]; and polymers (polyethylenimines) have been combined with liposome to form “lipopolyplexes” [155].

Once genetic material has been transported through the plasma membrane for long-term expression a range of non-viral methods have been developed to integrate genetic material into the host genome. In the **Cre-loxP recombination system** the therapeutic transgene is flanked by two *loxP* sites and a Cre recombinase enzyme engineered into a separate plasmid. The therapeutic transgene is integrated into the genome by Cre catalyzed reciprocal recombination between the two *loxP* sites.[119] Lack of directionality can be corrected through the use of

directional lambda integrase.[161] A Cre/loxP-dependent gene recombination system has been used to introduce a retinoic acid receptor gene into hematopoietic stem cells in *ex vivo* culture to transiently block differentiation and hence enhance self-renew and engraft into host tissue.[162] A major caveat however is the requirement of bacterial host proteins.[161] A Cre-*loxP* recombination system may not therefore be suitable in human clinical trials where therapeutic cells need to be free from exposure to viral or bacterial material (xeno-free). Other non-viral genome integration systems that are applicable in future *ex vivo* gene therapy work include the **bacteriophage C31 integrase system** [163] which consists of recombination sites and an integrase enzyme. Here reciprocal recombination occurs between *attB* and *attP* where the plasmid contains the *attB* site and the *attP* site is found in the target genome [164]. This system is unidirectional and does not utilize bacterial protein. Although there are no endogenous *attB* or *attP* sites in human DNA, there are pseudo recombination sites albeit with reduced integration efficiency [119]. Also, there is the **transposon integration system** which avoids the use of bacterial recombination sites [165]. This system is also designed to direct integration to a specific region of the genome by targeting TA dinucleotides. Since TA dinucleotides occur commonly throughout the genome however this specificity has been questioned and therefore the system's ability to prevent insertional genotoxicity might be limited [166]. Many transposon systems have the general structure of one transposase gene flanked by two inverted terminal repeat sequences [167]. The most common strategy in transposon system involves a co-transfection of two plasmids where one plasmid ensures the recombinase expression, and a second plasmid carries the therapeutic gene flanked by the appropriate recombination signals [168]. A clear advantage is an almost unlimited cargo size. However, the rate of transposition is inversely related to the transposon length, making it less efficient with larger transgenes [119]. More recently, enzymatic integration systems, such as zinc finger nucleases (ZFNs) [169], meganucleases [170] and transcription activator-like (TAL) effectors [171] have been used to achieve more precise site specific integration of the target gene. All these systems include an endonuclease that is attached to a DNA binding protein and induces homologous recombination to insert target DNA into the genome. The DNA binding domain can be modified to attain the site specificity [169, 170].

2.4.2.5 Efficiency of transfection

Transfection efficiency can vary quite markedly between different delivery systems. There is therefore critical need to assess transfection efficiency in all studies. Fluorescent microscopy, is commonly used to undertake this work. A qualitative and to some extent quantitative technique (e.g. through flow cytometry studies) [118], a green fluorescent protein gene is often included in DNA constructs. Fluorescent microscopy has been used for example to compare transfection efficiency using either lipofection or nucleofection, in ARPE-19 cells [151]. Western blot analysis can also be performed if an antibody is available for the protein of the target gene. After *ex vivo* transfection of a plasmid through nucleofection into primary bovine retinal and iris pigment epithelial (RPE, IPE) cells, expression of a fusion protein, EGFP-PEDF was detected for 60 days and 90 days, respectively, through western blot [151]. More commonly though, enzyme-linked immunosorbent assay (ELISA) is employed because of the small amounts of protein being expressed [142]. ELISA has also been used to detect the expression of viral proteins in transgenic cell culture [172]. In addition, RT PCR can be used to detect mRNA expression to quantify transgene expression in the transcriptome of target cells [172].

2.4.2.6 Target cell types

A range of cell types are being assessed for therapeutic potential in eye disease. These include embryonic [146], fetal [173] and adult stem cells e.g. corneal limbal cells [174] as well as hematopoietic [175] and mesenchymal stem cells [6]. All these cell types can be genetically manipulated to enhance clinical effectiveness. Here, the use of mesenchymal stem cells in *ex vivo* approaches will be discussed along with other cell types which have been used for similar purpose.

2.4.2.6.1 Mesenchymal stem cells

MSCs may be especially suitable for *ex vivo* gene therapy since viral and non-viral engineering does not appear to impair the ability of MSC to proliferate, self-renew, differentiate and most importantly to migrate into damaged tissues [176-179]. This has led to their use in numerous studies. An interesting approach to *in vitro* manipulation has been developed in mesenchymal

stem cells (MSCs) to change their secretion profile. One such study modified the mouse mesenchymal stem cells *ex vivo* to produce neurotrophin-4, a neurotrophic factor. When these cells are injected into a mouse model with acute retinal injury, they were able to protect damaged retinal tissue. It was also found that NT-4 modulates the expression of several genes that are important for the retinal cell survival [180]. In another study, MSCs have been genetically modified to treat sodium iodide (SI) induced retinal degenerations in rat. Similar to our study, they also used constitutive (EPO-rMSC) and inducible cell lines (Tet-on EPO-rMSC). Both cell lines improved retinal morphology and function by rescuing RPE and retinal neurons [181].

Epo-producing MSC's [182, 183] alone or combined with insulin-like growth factor I (IGF-I)-overexpressing MSC [184] for example have been used for long-term correction of renal failure-induced anemia. Xu and co-workers examined the efficacy of murine MSC-based angiopoietin-1 gene delivery for acute lung injury [185]. Primary human bone marrow-derived MSCs have been transduced *ex vivo* using a lentiviral vector expressing lysosomal enzyme beta-glucuronidase (MSCs-GUSB). One million cells were transplanted intraperitoneally into separate groups of mice with neonatal, non-obese, diabetic, severe combined immunodeficient mucopolysaccharidosis type VII (NOD-SCID MPSVII). These MSCs were found to secrete high levels of protein in the recipients, raising circulating serum levels of GUSB to nearly 40% of normal. In addition, at least one physiologic marker of disease, retinal function, was normalized following transplantation [179, 186]. Another interesting type of MSC has been produced through modification of culture conditions so as to augment MSC neurotrophic factor secretion (GDNF, BDNF, VEGF) [187]. Some neuroprotective effect has been shown with these cells in animal models of traumatic optic neuropathy, Parkinson's disease, Huntington's disease and 6-hydroxydopamine (6-OHDA)-induced lesion [187-190].

2.4.2.6.2 Other cell sources

Prominent examples include work on IPE cells which have been genetically modified to over-express neuroprotective pigment epithelium-derived factor (PEDF) [191, 192]. In another study these cells were also transfected with recombinant adeno-virus carrying the brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and fibroblast growth factor

(bFGF) [193]. RPE cells have also been modified genetically to secrete PEDF [151]. In other retinal neuroprotection work, mouse embryonic stem cell line E14TG2a has been genetically modified to constitutively express and secrete glial derived neurotrophic factor (GDNF) [10, 146].

A particularly important class of *ex vivo* gene therapy is very recent work on induced pluripotent stem (iPS) cells, adult, differentiated cells genetically reprogrammed *ex vivo* to regain pluripotency [194]. In a rapidly evolving field, iPS cells originally generated by transduction of somatic cells with retroviruses or lentiviruses containing various transcription factors [195], are now successfully reprogrammed with proteins and small molecules [196, 197]. The efficiency of such approaches however can be variable [198, 199]. Early work using cells reprogrammed with transcription factors *OCT4*, *SOX2*, *NANOG*, and *LIN28* [200] has been successful in generating retinal progenitors using differentiation protocols very similar to those used with human ES cells [201]. Optimizing the efficiency of retinal cell production using iPS cells has proven difficult and to some extent depends on the cell line used [202, 203]. Future work using iPS cells to generate neurons for various CNS diseases will also be relevant to blindness attributable to optic nerve and visual pathway abnormalities [204].

An interesting innovation has been to transfect whole explanted tissue rather than a specific cell type. In one study Stout and co-workers successfully transduced whole cornea explants with a lentivirus vector containing a fusion protein that improved corneal allograft survival in subsequent transplantation into a rabbit eye [172]. In similar studies, *ex vivo* lentivirus transfer of interleukin-10 (IL-10) to endothelial cells in explanted corneal buttons improved allograft survival in sheep eyes [144].

2.4.2.7 *In vivo* cell delivery

Various routes for therapeutic delivery of cells to the eye have emerged. Of these, topical application to the ocular surface would be easy and relatively safe to perform although limited cell migration means that such an approach would be confined to therapeutics targeted at the cornea and conjunctiva [174]. For retinal disease, intravitreal injection has emerged as relatively simple

and associated with minimal risk of complications. Mouse ES cells genetically modified to express GDNF have shown neuroprotective potential via this route [146]. In this study, cells were seen to penetrate only through the retinal nerve fiber layer [146]. Other work is emerging on improving retinal penetration of injected cells [205], although to some extent tissue barriers are already compromised in retinal disease [206]. Subretinal injection has also been undertaken. Technically more difficult (particularly in humans) and much more prone to adverse local complications (especially if repeat injections are needed) this approach has been used to deliver genetically modified human neural progenitor cells [207] and modified iris pigment epithelial cells to the retina [193].

A very recent innovation has showed that cells injected into the systemic circulation can accumulate in the eye opening up systemic injection as a safe and repeatable route for *ex vivo* gene therapy in the eye. A single mesenchymal stem cell injection into the tail vein of dystrophic rats produced a neuroprotective effect for at least 60 days in degenerating retina [208]. Mesenchymal stem cells are known to be amenable to genetic modification [6] and thus a systemic approach to delivering such modified cells seems feasible. For central nervous system diseases relevant to blindness, direct injection into brain tissue by lumbar puncture and by intrathecal injection have been studied [209-211].

2.4.3 Applications

Of the numerous applications of *ex vivo* gene therapy, notable success in translation to human clinical trials has been achieved with hematopoietic stem cells [117]; immunotherapy, where *ex vivo* genetically modified T cells have been targeted at cancers [212], infections and autoimmune diseases [213], and work with cardiac stem cells [214]. Significant advances also continue to be achieved with genetically modified neural progenitor cells [215, 216]. The advent of *ex vivo* gene therapy in vision has already started to yield promising results in pre-clinical trials (Table 1). Notable areas where *ex vivo* gene therapy has achieved significant success are in corneal disease, glaucoma and retinopathy. Additionally, therapy targeted at other loci in the central nervous system can have impact on the central afferent visual pathways, in particular work on stroke, multiple sclerosis, and cancer.

2.4.3.1 Retina

Retinal diseases, including age-related macular degeneration, diabetic retinopathy and retinitis pigmentosa are the natural targets for any therapeutic strategy aimed at having a significant impact on blindness in the western World [217]. Hence retinal disease has been the focus of most ocular *ex vivo* gene therapy work.

Early work on reprogramming a variety of ocular-derived cells to replace lost retinal neurons included work to show that retinal replacement cells might be derived from mammalian ciliary body epithelium [218] however this has recently been contested [219]. Other work has included efforts to modify iris-derived cells (excluding cells from the ciliary body) from rodents and primates [220]. Cells were treated with retroviral delivery of gene constructs containing Crx, Nrl and NeuroD and cultured with retinal explants. This resulted in photopigment expression in these cells and electrophysiological signs of responsiveness to incident light [221]. More recently, retinal pigment epithelium from chick embryos has been reprogrammed via retroviral delivery of neurogenin 1 and neurogenin 2 to express photopigment and phototransduction proteins [221]. Other work, *e.g.* through forced expression of *ash-1*, suggests that chick embryo RPE can also be induced to express proteins suggestive of retinal amacrine, horizontal and ganglion cells [222, 223]. It is unclear however how effective such cells will be *in vivo*. Relatively more success and perhaps with more potential for generating sufficient cells suitable for clinical trials involves reprogramming of non-ocular cells (such as fibroblasts) for retinal replacement [200, 201]. Refinement has led to small molecule retinal progenitor differentiation protocols from ‘three-factor’ (OCT3/4, SOX2 and KLF4) and ‘four-factor’ (OCT3/4, SOX2, KLF4 and MYC) human iPS cells [224, 225]. More recently, work has begun on generating non-photoreceptor cell types such as ganglion cell precursors [225] and retinal pigment epithelial cells [226].

Work on *ex vivo* gene therapy to neuroprotect degenerating retina has also achieved significant success. Subretinal injection of autologous iris pigment epithelial cells genetically modified to express neurotrophic factors such as BDNF, Axokine, CNTF and bFGF has suggested some neuroprotection [192, 193]. Neural stem cells in tissue derived from embryonic rat cerebellum

sub-ependymal zone have also been modified to secrete BDNF using a recombinant retrovirus (pLXSN-BDNF). Subretinally injected cells were shown to continually secrete BDNF despite differentiation [227]. To improve safety, recent work has emphasized non-viral approaches [151] and this has been refined further in work on rat retinal pigment epithelial cells that have undergone cationic liposome transfection with a plasmid containing a tetracycline-responsive element along with BDNF cDNA. These cells were injected subretinally into littermates who underwent phototoxic retinal degeneration. Treated animals further dosed with topical doxycycline showed histological and electrophysiological preservation of retinal tissue [228].

With significant potential for collateral damage (e.g. retinal tears and detachment) it is unclear whether a subretinal route for injection of neuroprotective cells is the best proposition for clinical use. Intravitreal injection, being simpler and less prone to complications, might be preferable if found to be effective. Mouse embryonic stem cells have been genetically modified to over-secrete GDNF [146]. These modified cells were shown to be neuroprotective after intravitreal injection in a rodent model of retinal degeneration. Very recently, this approach has been shown to be augmented by adjuvant therapy with gentamicin to inhibit gain-of-function mutant protein expression blocking aberrant transcription [10].

2.4.3.2 Cornea and ocular surface

Despite significant advances in treatment (e.g. penetrating keratoplasty), corneal disease is still the second commonest cause of blindness in the world [229, 230]. The cornea is an attractive target for *ex vivo* gene therapy for a number of reasons. Firstly, whole corneal buttons, either normal donor tissue or diseased tissue can readily be removed and cultured *in vitro* (and thus be amenable to *ex vivo* manipulation) [231]. Secondly, the ocular surface can be directly observed for therapeutic effects [232].

A particular theme has been the genetic manipulation of explanted corneal buttons to improve their survival once surgically implanted back into the recipient [233, 234]. For example, one very recent study used a lentivirus vector containing bcl-xL (an antiapoptotic agent) to inhibit corneal

endothelial cell death in murine donor tissue [235]. Treated allografts showed a 90% survival after eight weeks (compared with only 30-40% in controls) despite a transduction efficiency of only 16% in endothelial cells. A similar study in human corneal tissue also enhanced corneal endothelial survival using a lentiviral vector to transduce cells with either baculoviral p35 or mammalian Bcl-xL [236]. In another study, allograft median survival was improved to 55 days (compared to 22 days in controls) using an adenoviral vector encoding an IL-10 transgene (an anti-inflammatory cytokine) [237]. It has been suggested that in corneal allografts, adenoviral vectors may be more efficient at IL-10 expression than lentiviral vectors [144]. This may however reflect the influence of vector design. For instance it has been shown in human corneal explants that the choice of AAV serotype has significant influence on transduction efficiency (AAV9 > AAV8 > AAV6) [238].

Non-viral vectors have also been employed although these are generally considered to be less efficient than viral vectors and prone to only short-term expression [239]. Electroporation has been used to transfect endothelial cells in human corneal explants with a construct containing a CMV promoter to drive reporter genes eGFP and beta-galactosidase [240]. Also, a polylysine-molossin/fusogenic peptide has been used to deliver DNA to cultured rabbit corneal endothelium [241]. An interesting ballistic technique ('gene gun'), targeting corneal epithelium used a minimalistic, immunologically defined gene expression vector containing IL-10 and CTLA4 [145]. This resulted in improved corneal allograft survival in mice. It has more recently been suggested though that this is through an effect on the host rather than on donor tissue, possibly by modifying lymphatic drainage from the cornea [242, 243]. Neovascularization, a common cause of graft rejection, has also been targeted using a non-replicative lentiviral vector encoding a fusion transgene of the human endostatin gene and the kringle-5 domain of the human plasminogen gene. Allogenic transplants in rabbits demonstrated a marked decrease in corneal neovascularization with continuous fusion gene protein transcription and allograft survival in all ten treated animals for up to 39 days post-operatively [172].

Other genetically manipulated cells hold great promise in ocular surface disease. For instance, areas of interest could include long-term delivery of anti-inflammatory agents for chronic disease

(e.g. keratoconjunctivitis sicca, Steven's Johnson's disease). In addition, induced pluripotent stem cell methodology may be a useful strategy in manufacturing new cornea [244].

2.4.3.3 Uveal tract and glaucoma

Uveitis is a common and difficult to treat inflammatory disease of the eye. Chronic forms, especially those affecting the posterior segment, often require prolonged systemic immunosuppression with many adverse systemic effects. Many *in vivo* gene therapy approaches are under investigation. In particular, adenoviral vectors have been used to deliver: hIFN- α to attenuate uveo-retinitis in mice [245]; IL-10 to attenuate uveitis in rodents [246]; and IL-1 receptor agonist to attenuate uveitis in rabbits [247]. *Ex vivo* delivery of these gene constructs may offer significant advantages. Chronic uveitis, unlike many chronic eye diseases is rarely active lifelong but is more a relapsing and remitting disease [248]. The design of any therapeutic approach therefore needs to include the ability to titrate the therapeutic construct. The ability to include large gene constructs containing regulatory elements that can be controlled via systemic or possibly topical drugs is therefore particularly advantageous in *ex vivo* approaches in uveitis. In addition, introducing suicide genes would allow for therapeutic cells to be easily cleared from the eye. This would clearly be more efficient than needing lifelong suppression therapy to suppress *in vivo* gene constructs [111, 249].

The diagnosis of glaucoma covers a wide range of disorders that have a distinct type of optic nerve impairment in common, with irreversible defects in the visual field. As a leading cause of blindness, many medical, surgical and laser-based therapies have been developed. Despite this, glaucoma continues to be a leading cause of worldwide blindness prompting the need for novel therapeutic strategies [250].

Remarkable work has suggested that impaired ganglion cell retrograde transport of neurotrophic factors is a contributory mechanism in the pathogenesis of glaucoma [251, 252]. It has therefore been proposed that augmenting the concentration of neurotrophic factors within ganglion cells will impede the cell death seen in glaucoma. Mesenchymal stem cells genetically modified to

secrete BDNF [6] have been injected into the vitreous cavity of rats with ocular hypertension (a model of glaucoma). Subsequent pupillometry and electroretinography showed a significant preservation in ganglion cell number and cell function [253]. In other work, Levkovitch-Verbin and co-workers [190] have shown that MSC can be induced to over-secrete BDNF and GDNF without direct genetic manipulation but through incubation in novel culture conditions. They report significant neuroprotective benefits in ganglion cells after optic nerve transection [190]. It has however been suggested that the effectiveness of BDNF may be transitory. To overcome this, it has also been proposed that the effectiveness of BDNF-secreting cells can be enhanced through co-expression of a LINGO-1 antagonist [254]. Other *ex vivo* gene therapy work relevant to glaucoma includes iPS techniques to manufacture cells to replace lost retinal ganglion cells [255] and to replace cells in the trabecular meshwork [256]. The towering interest in applying cell-based neuroprotection in glaucoma management warrants advanced work on neuroprotection in glaucoma models [257]. It should be noted that the true value of these novel approaches in glaucoma patient care may be through combination with intraocular pressure control rather than as an alternative strategy to it.

Other areas of future research in glaucoma might include control of intraocular pressure through anterior chamber injection of cells modified to secrete COX-2, a regulator of prostaglandin production and known to increase uveal scleral outflow [258]. It has also been suggested that intraocular pressure control might be augmented in some types of glaucoma through trabecular meshwork extracellular matrix modification. This could be approached through an anterior chamber injection of cells genetically modified to overexpress matrix metalloproteinase I [259]. Secretion in such cells could be controlled by including an antibiotic-inducible promoter in the construct and topical application of antibiotics.

2.4.3.4 Central nervous system afferent visual pathway disease

Diseases such as stroke, trauma, multiple sclerosis and neoplasia can cause visual deficit through damage along afferent pathways. *Ex vivo* gene therapy has undergone extensive evaluation in such diseases. This has been particularly true for strategies aiming at delivering therapeutic molecules to the brain [122]. Key examples include cells secreting: BDNF [260]; neurotrophin-3

and neurotrophin-4/5 [261]; GDNF [262, 263]; nerve growth factor [264]; the neurotransmitters such as DOPA (tyrosine hydroxylase) [265, 266]; and GABA (glutamine decarboxylase) [267].

Stroke is a common and debilitating disease affecting a large percentage of people. Of the many consequences of stroke, vision loss in the form of varying degrees of hemianopia, is amongst the most prevalent. Numerous *ex vivo* gene therapy studies have shown benefits in model systems of CNS ischaemia. Stem cells, genetically modified to secrete genetically modified vascular endothelial growth factor [268], GDNF [269], angiopoietin-1 [270], and hepatocyte growth factor [271], has all demonstrated neuroprotection and some enhanced recovery of neurological function in model systems. A particularly interesting neuroprotective molecule has been erythropoietin. With neurotrophic [272], anti-oxidant and anti-inflammatory effects, its value in ischaemic stroke models [273, 274] has been limited because erythropoietin does not cross the blood brain barrier [275]. Human MSCs have however been transduced with lentivirus vector-containing a human erythropoietin construct. These cells were then injected intra-cerebrally into rats in which a middle cerebral artery had been surgically occluded. Motor and sensory function decline was significantly inhibited in treated animals. Infarct volume, as measured by MRI scan was also limited by treatment. However, it was noted that these erythropoietin-secreting cells also had enhanced secretion of other neurotrophic factors and that this probably contributed to the measured benefits [276].

Traumatic brain injury is a leading cause of disability in young adults [277] with visual impairment and blindness an important contributor to this disability [278]. Specifically targeting traumatic brain injury, undifferentiated human neuronal precursor cells (NTera2) have been transduced *in vitro* with a lentiviral vector to release neuronal growth factor, differentiated by exposure to retinoic acid and transplanted into the medial septum of mice 24 hours after controlled cortical impact injury (using a rigid impounder driven by a pneumatic piston). At 1 month post-transplantation, animals engrafted with NGF-expressing NT2N neurons showed significantly improved learning ability (using a water maze) compared to brain injured mice receiving either vehicle or un-transduced cells. However, no benefit was seen with motor function [279]. In addition, neural progenitor cells have been engineered to secrete GNTF [280].

Injected into the peri-lesional area of a rat brain which had undergone lateral fluid percussion injury, treatment with these cells was shown to improve cognitive function. Importantly though, this was attributed mainly to improved survival of these neural progenitor cells that migrated and differentiated into neurons [280].

Multiple sclerosis is another CNS disease often associated with visual symptoms with visual loss a common presenting feature for the condition [281]. CD34+ positive bone marrow stem cells have been transduced with a retroviral vector containing a BDNF gene construct. These cells were injected intravenously into mice with experimental autoimmune encephalomyelitis (EAE), a model that shows many immunological and histological features in common with human MS [282, 283]. Treated mice demonstrated a delay in onset and reduction in overall clinical severity of disease compared to mice receiving cells transfected with an empty vector lacking the BDNF gene. The study also showed improved remyelination, a reduction of pro-inflammatory cytokines TNF-alpha and IFN-gamma and enhanced expression of the anti-inflammatory cytokines IL-4, IL-10, and IL-11 in CNS tissue from treated mice [284].

An intriguing and possibly far-reaching approach in the treatment of MS is the use of hematopoietic stem cells to promote immune tolerance to the disease [285, 286]. Ko and co-workers have genetically manipulated mouse hematopoietic (bone marrow) stem cells with a retroviral vector to express myelin oligodendrocyte glycoprotein (MOG), a target auto-antigen in EAE [287]. These cells were injected into young irradiated mice and resulted in chimeras with dendritic cells (a major class of antigen presenting cell and derived from the injected bone marrow cells) that expressed MOG. These mice proved to have lifelong tolerance and were completely resistant to induction of EAE [286, 287]. In addition, treatment with these cells induced remission and immune tolerance even in mice with established EAE [287]. It should be noted though that human trials using autologous hematopoietic stem cells that have not been genetically modified have also demonstrate some rescue in human clinical trials of MS (but not to the degree seen with MOG expression) [288].

Finally, advances in the treatment of brain tumors are also relevant to blindness. Neural stem cells have significant tumor-homing ability making them ideal cells to deliver therapeutic molecules to CNS neoplastic lesions [289]. This useful property has been exploited for targeted delivery of a range of cytotoxic molecules at tumor foci [290-294]. This property is also found in neural stem/progenitor cells derived from embryonic stem cells and iPS cells [295]. Such cells have been transduced with a baculoviral vector containing the herpes simplex virus thymidine kinase suicide gene and injected into the cerebral hemisphere of mice contralateral to the site of an induced glioma lesion. In the presence of ganciclovir, such treated animals demonstrated effective inhibition of glioma growth [295].

2.4.4 Conclusion

Exciting times approach as cell-based therapies to neuroprotect and regenerate dystrophic tissue in the eye and brain gain momentum. However, key challenges remain to be solved. Transgene and vector safety, particularly viral vector safety are issues for *ex vivo* as well as *in vivo* gene therapy protocols. In addition, availability of directly relevant animal models for preclinical testing continues to be an issue for common eye diseases such as age-related macular degeneration. On a more positive note, an ever larger variety of techniques to transfect cells with therapeutic molecules and the emergence of new types of cells for use in *ex vivo* gene therapy bode well for future work.

Chapter 3. Retinal Neuroprotection Using Magnetic Mesenchymal Stem Cells

3.1 Introduction

Retinitis pigmentosa (RP) is a group of hereditary retinal diseases where primary or secondary loss of rod and cone photoreceptors occurs due to gene abnormalities. The worldwide prevalence of RP is about 1:3000-7000 with more than 1 million affected individuals [30]. Although the symptoms and disease progression vary widely, in most cases, photoreceptor degeneration generally starts with rods followed by cones. The current therapeutic regimen focuses on slowing down disease progression with no complete cure available [33].

As cell-based medicine evolves, it is becoming increasingly clear that cellular therapeutics encompass beyond the ability to solely replace lost cells. Useful trophic and other paracrine effects of transplanted cells are emerging as viable approaches in treating diseases [6, 296]. Amongst others, an increasingly important issue is how best to target these cells to the diseased tissue. Critical considerations include the potential for collateral damage within that tissue, systemic side effects, local tissue barriers, simplicity of delivery method, repeatability and whether specific loci within the tissue need to be targeted. Direct injection of cells is feasible in the eye. However, direct injection of cells into the vitreous cavity or sub-retinal delivery is associated with uncommon but significant risk of serious complications (such as retinal detachment, hemorrhage, and endophthalmitis) [297]. These complications are even more common if treatments need to be repeated [6]. More indirect methods of cell delivery, for instance via the systemic circulation, would therefore be advantageous.

Mesenchymal stem cells (MSCs) are remarkable and versatile components of the body's innate regenerative capacity [6, 298, 299]. A range of therapeutic roles for MSCs including neuroprotection and immune modulation as well as tissue regeneration are becoming increasingly recognised, particularly in pre-clinical studies of graft-versus-host disease, cardiovascular disease and neurological disorders such as stroke [299-302]. There are currently more than 400 clinical trials involving MSCs are listed by the US National Institutes of Health trial database (www.clinicaltrials.gov), which reflects on the broad applicability of MSC therapy

on diverse range of diseases. Although no human clinical trials have yet used MSCs in eye diseases, a broad role for MSC therapy is emerging in pre-clinical trials in the eye, especially in corneal disease [303], glaucoma [304] and retinopathy [208].

Recently we have shown that MSCs can be preferentially targeted to specific loci in the rodent retina after injection into the vitreous cavity or intravenously, via the tail vein [11]. The introduction of superparamagnetic iron oxide nanoparticles (SPIONs), with their ability to adhere to therapeutic agents and become incorporated within cells is emerging as a useful strategy for using magnetism to selectively direct therapeutics around the body [305]. We have now designed experiments that show that remotely targeted magnetic cells have therapeutic, neuroprotective effects in a rodent model of retinal degeneration.

3.2 Methods

3.2.1 MSC culture

MSCs were isolated from adipose tissue. Briefly, adipose tissue from rat inguinal canal of a single littermate of test animals was harvested and placed at 5g per well in a 6-well dish. The tissue was perfused with 3ml of 1 mg/ml Collagenase-1 (Sigma-Aldrich, St Louis, USA) and macerated by the use of surgical scissors. After an hour's incubation at 37°C, the collagenase-dissociated tissue was filtered through 40 µm cell strainers and centrifuged at 1000g for 10 minutes. The supernatant was removed and the pellet re-suspended in Mesencult MSC Basal Media (Stemcell Technologies Inc, Vancouver, Canada) supplemented with 1% penicillin/streptomycin and 15% heat-inactivated fetal bovine serum (both from ThermoFisher, Carlsbad, CA USA). Cells were seeded on 6-well plates and allowed to adhere for a period of 48 hours before media change. After the cells reached confluency, they were passaged by trypsinization (0.05% Trypsin-EDTA; ThermoFisher) to a 75cm flask for further culturing. Cells of passage number 4-9 were used in all experiments. To confirm MSC identity, osteogenesis and adipogenesis were induced in an aliquot of cells as previously described [11] using the MesenCult[®] Adipogenic Stimulatory kit and the MesenCult[®] Osteogenic Stimulatory kit (both from Stemcell Technologies Inc).

3.2.2 Magnetic nanoparticle and fluorescent labeling

FluidMAG-D nanoparticles (Chemicell GmbH, Berlin, Germany) are ferrofluid, 200nm diameter particles composed of a magnetite core covered by hydrophilic starch to protect against aggregation. Nanoparticle uptake and the viability of such fluidMAG-D labeled MSCs (magnetic MSCs: MMSCs) has been verified previously [11]. MSCs (at passage 4-9) cultured in T75 flasks were incubated for 24 hours with 10 μ L fluidMAG-D. Just before use, cells were also incubated with 2.5 μ L Q-Tracker[®] 655 (ThermoFisher), as described by the manufacturer, a fluorescent marker for identifying labeled cells in tissue sections and retinal flatmounts. Cells were washed 8 times with Dulbecco's phosphate-buffered saline (DPBS; ThermoFisher) and then harvested with 0.05% Trypsin- EDTA (ThermoFisher). MSCs were stained with 0.4% Trypan Blue Stain (ThermoFisher), counted and then 1.0×10^6 cells were suspended in 0.5 mL DPBS for injection.

3.2.3 Animal studies

The S334ter-4 heterozygous transgenic rat line (obtained from Professor Matt LaVail, University of California at San Francisco, CA, USA) was used since it is a well characterized model of retinal degeneration [306, 307]. Throughout all experiments rodents were given free access to food, water and an enriched environment e.g. polymer cylinders, as a hiding place to allow a sense of safety. Animals were maintained on a 12 h light/dark cycle. After all ocular procedures, eyes were treated with topical anaesthetic and antibiotics. All work was carried out with adherence to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Under 3% isoflurane anaesthesia, a 3 mm diameter x 1.5 mm thick gold plated neodymium iron boron (NdFeB) disc magnet with a maximum energy product of 43.2 MGOe (D0301G, Gaussboys Super Magnets, Portland, OR USA), was placed within the orbit, but outside the eye. This was achieved by making a 4 mm limbal incision in the superior fornix and inserting the magnet back into the orbital apex with a non-magnetic blunt probe. Eighteen heterozygous S334ter-4 animals were divided into three separate cohorts that were treated with either: 1- a tail-vein injection of vehicle (DPBS supplemented with cell media); 2- a tail-vein injection of MSCs or 3- had a magnet placed in the orbit and underwent a tail vein injection of magnetized MSCs

(MMSCs). Initial injections were performed at post-natal day 21 (P21) and the study period was completed at P110. Results were compared to data obtained from age-matched wild-type, Sprague-Dawley albino animals (same strain of S334ter-4). Each data point represents results from six eyes of six animals (n=6).

3.2.4 Histological assessment

Animals were sacrificed using carbon dioxide euthanasia. Eyes were enucleated and marked at the 12 o'clock position, using black India ink for orientation. For cryostat sectioning, eyes were immersed in 4% paraformaldehyde (PFA, EMS, Hatfield, PA USA) for 1 hour, cryoprotected in 30% sucrose at 4° C overnight, snap frozen on dry ice and then embedded in optimal cutting temperature compound (OCT, Tissue-Tek). Sagittal sections of 12 µm were stained with 4',6-diamidino-2-phenylindole (DAPI) and were visualized with confocal laser scanning microscope (Zeiss LSM 510 META). Montages were made in Photoshop CS2 (Adobe). Eyes for retinal flatmounts were immersed for 1 hour in 4% PFA. The anterior segment was then removed and the eyecup immersed for a further 1 hour in 4% PFA. The orientation mark was transferred to the retina by cutting a notch and then radial cuts were made toward the optic nerve using sharp scissors. The retina was gently teased off the eye cup as a single piece of tissue, and transferred to a microscope slide. Several drops of Fluoromount-G (SouthernBiotech) were placed on the tissue and a coverslip added. Samples that were used for counting photoreceptor nuclei were immersed in half strength Karnovsky's fixative (VWR) for 1 hour, and the anterior segment was removed by excision at the ora serrata. The remaining posterior segments were embedded in paraffin, cut sagittally to a thickness of 5 µm, and counterstained with hematoxylin and eosin. Nuclei within the outer nuclear layer were analyzed as previously described [10]. To standardize photoreceptor nuclei counting from different eyes, we only used sagittal sections that included the optic nerve. Images were overlaid with a 100 µm-length rectangular template using Photoshop CS2 software. This template was positioned at four predetermined coordinates to give counts from two central (behind the equator) and two peripheral (in front of the equator) coordinates [10]. These four images for each eye were then imported into Photoshop, and photoreceptor nuclei in the outer nuclear layer were labeled with overlaying spots. An exported JPEG of this layer of spots was then counted in ImageJ v 1.37 software to give the total number of outer nuclear layer nuclei in each grid.

3.2.5 Optical coherence tomography (OCT)

A noninvasive prototype spectrometer-based frequency domain-OCT system was used to acquire the retinal images as previously described [308]. The OCT system operated at line rate of 36 kHz, and utilized a superluminescent diode (SLD) source with an effective center wavelength of ~863 nm and a full width half maximum bandwidth of ~50 nm. Spectrally resolved detection was performed using a commercially available spectrometer from Wasatch Photonics (Durham, NC, USA), and processing of the interferometric signal to a real time image was performed using custom software developed at Simon Fraser University.

Rodent imaging was undertaken in the test eye only, whilst under isoflurane general anaesthesia. The OCT sample arm was mounted on a fixed stage, and the specimen was held in a customized 5-degree of freedom slit lamp to facilitate alignment of the eye to the optical beam. The pupils were dilated using topical atropine sulphate 1% (Alcon, Fort Worth, TX). A converging beam was used for imaging with refraction at the cornea cancelled using a plano-concave lens coated with a generic artificial tear gel. The optic nerve head was used as a landmark to align the optical system to the rat retina. Imaging was undertaken at four specific loci approximately ~1.5 mm from the optic nerve head in the superior nasal, superior temporal, inferior temporal and inferior nasal quadrants. Measurements were made at P110.

3.2.6 Spatial frequency thresholds

Optokinetic tracking (OKT) was undertaken under photopic (light adapted) conditions to examine spatial frequency thresholds [309]. A vertical sine wave grating (100% contrast) was projected as a virtual cylinder in three-dimensional space on computer monitors arranged in a quadrangle around a testing arena (OptoMotry; CerebralMechanics, Lethbridge, Alberta, Canada). Unrestrained rats were placed on the elevated platform at the center of the equipment. An observer used a video monitor to ensure that the virtual cylinder was kept centred on the animal's head and recorded head movements in response to cylinder rotation. The highest spatial frequency that resulted in a consistent response was documented.

3.2.7 Electoretinographic (ERG) assessment

The animals that were tested were put in a dark room for overnight for dark adaptation. They were anesthetized with xylazine (4 mg/ml) and ketamine (54 mg/ml) and maintained on a heating pad. The corneas were locally anesthetized with 0.5% proparacaine hydrochloride and the pupils were dilated with 2.5% phenylephrine and 1% atropine. Electoretinograms were recorded using Espion V5 Electoretinogram Console plus ColorDome (Diagnosys LLC, Lowell, MA, USA) with corneal gold loop electrodes. Scotopic ERGs (reflecting rod function) were recorded using white flashes of intensity 2.25 cd.s/m². Photopic ERGs (mixed rod and cone function) were recorded with the background illumination set at 30 cd/m² and white flashes of 3.0 cd.s/m². In addition 30 Hz flicker (cone function) responses were recorded with white flashes of 2.25 cd.s/m². Averages of 15 recordings were taken for scotopic and photopic responses and averages of 50 for 30Hz flicker responses. Average amplitudes are presented as means ± SEM. For each datapoint n=6.

3.2.8 Statistical analysis

Results were assessed for statistical significance using an IBM SPSS Statistics software package (version 21.0). We performed ANOVA one-tailed assay and multiple comparisons were made using Dunnett's test. Statistical significance was set as $p \leq 0.05$.

3.3 Results

To confirm that an orbital magnet was able to target intravenously-delivered magnetized cells to the retina, flatmounts and cryosections of retinal tissue were analyzed by confocal microscopy (Figure 3.1A-C). In the presence of a magnet, accumulation of MMSCs in the retina was apparent (Figure 3.1B) compared to control, where no cells were injected (Figure 3.1A). Retinal cryosections showed that most fluorescently labeled MMSCs accumulated within the outer retina

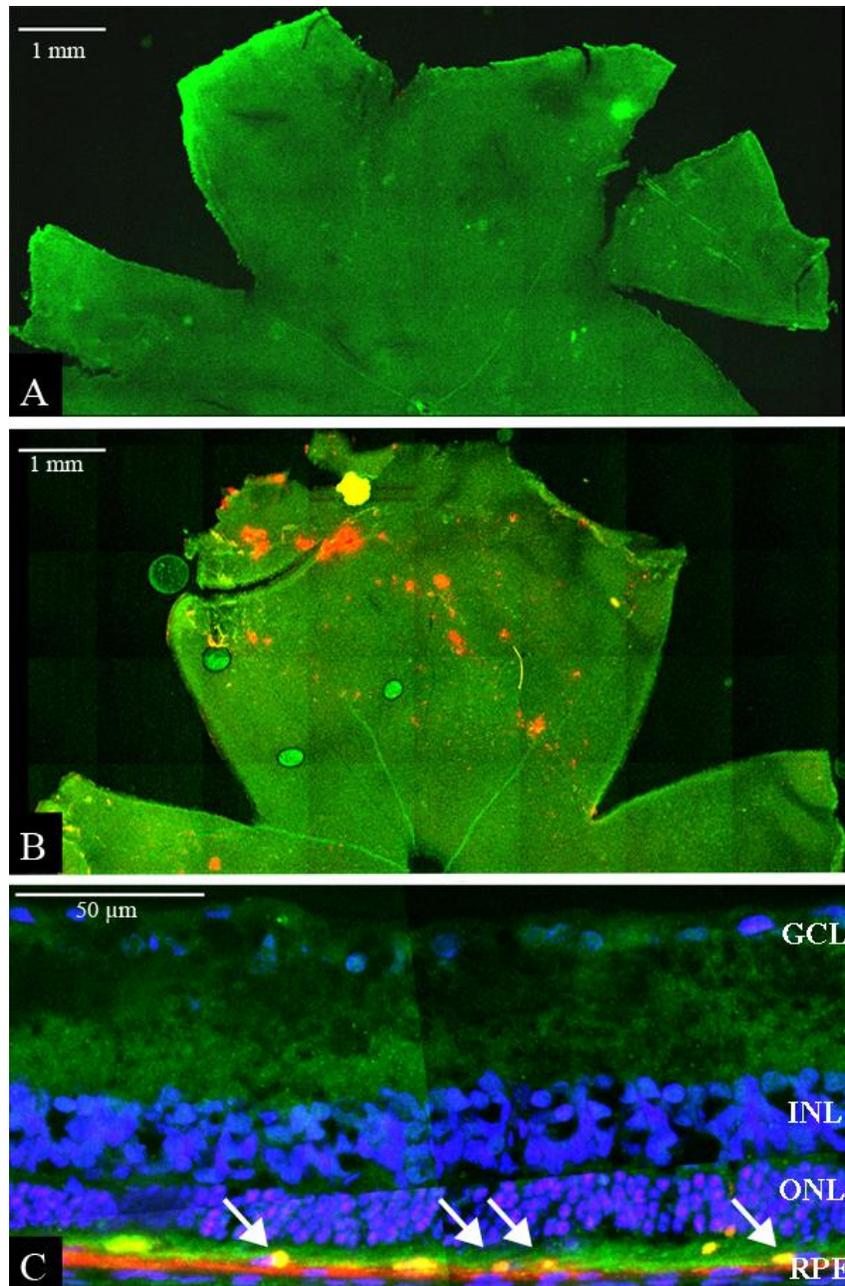


Figure 3.1: **Retinal flatmounts and a cryosection of S334ter-4 rats.** **A:** lower hemisphere flatmount from control (intravenous vehicle-treated) animal; **B:** corresponding area from an animal treated with intravenous MMSCs and a magnet in the orbit showing numerous QTracker labelled cells (red). **C:** A cryosection from retina treated with MMSCs accumulating within the outer retina (arrows).

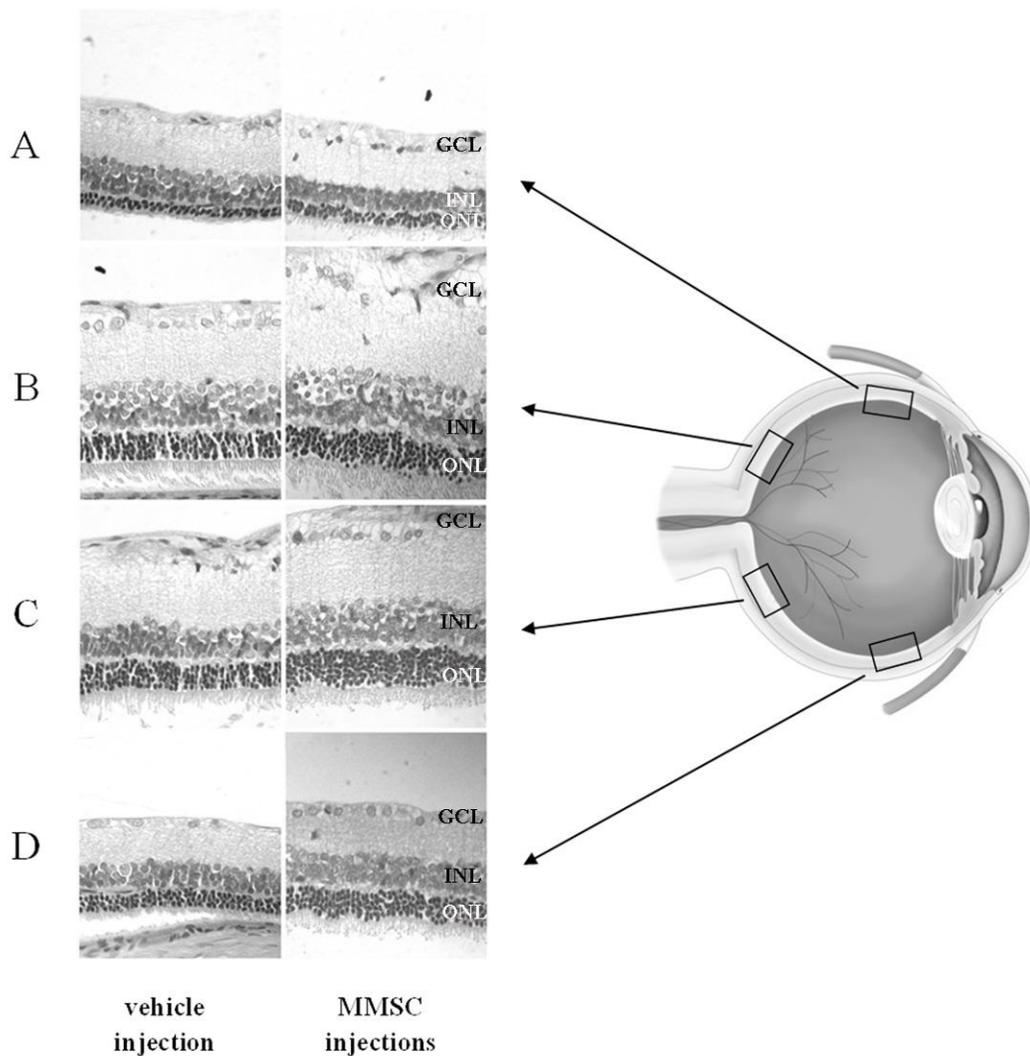


Figure 3.2 **Neuroprotection with MMSCs in the S334ter-4 rodent model of retinal degeneration at P110.** Retinal wax sections stained with H&E from **A:** upper retinal periphery; **B:** upper central retina; **C:** lower central retina; and **D:** lower peripheral retina from an animal treated with either sham intravenous injection of vehicle or with intravenous injections of MMSCs. The retinal outer nuclear layer was analyzed in rodent eyes sectioned vertically and through the optic nerve.

(Figure 3.1C, arrows). Wax retinal sections were also examined to qualitatively assess the effect of MMSC injection on retinal morphology. At P110 the average outer nuclear layer (ONL) thickness in all 4 areas was greater in rodents that had received MMSC injections relative to vehicle-treated controls, with most benefit in the central retinal areas (Figure 3.2B-C). More detailed assessment of the neuroprotective effect of the MMSCs was undertaken by counting the number of photoreceptor nuclei in the outer nuclear layer. Independent counts were performed in all four of the different retinal areas (Fig 2) and were averaged to give regional counts and an average count for the whole retina (Figure 3.3). Vehicle treated animals (group 1) had an average count of only 62 nuclei/100 μm of retinal length. Average cell counts for animals treated with a MMSC injection were 98 nuclei/100 μm of retinal length. An ANOVA assay was performed among the groups ((i) vehicle injection only, (ii) MSC injections with no orbital magnet; and (iii) magnetized MSC injections with an orbital magnet in place (MMSCs)). ANOVA was statistically significant ($P = 0.019$) and post hoc tests (Dunnett's) showed that only treatment with MMSCs was significantly different from treatment with vehicle alone ($P = 0.011$). The results were even more significant for nuclei counts restricted to the upper central retina (ANOVA $P = 0.003$, Dunnett $P = 0.004$). Histological benefits with MMSC treatment were also reflected in OCT imaging of the outer nuclear layer thickness (Figure 3.4).

We carried out electroretinography and OKT assessments to determine if histological preservation correlated with *in vivo* functional preservation of responses to visual stimuli. Preservation of electroretinographic responses was statistically significant only with MMSC treatment (Table 3.1). ANOVA assay showed a significant effect of MMSC treatment on flicker amplitudes ($P = 0.00002$). While post hoc testing showed that MMSC injection had a significant effect only on the flicker response ($P = 0.029$), a higher response was observed in photopic and scotopic amplitudes relative to vehicle control.

The spatial frequency threshold response for wild-type, albino, Sprague-Dawley rodents is 0.25-0.4 cycles /degree (c/d) [309]. In vehicle treated animals the mean spatial threshold frequency was 0.22 c/d (Figure 3.5). Although a standard ANOVA assay found no significant difference, both MSC and MMSC therapy showed higher OKT values compared to the vehicle treated

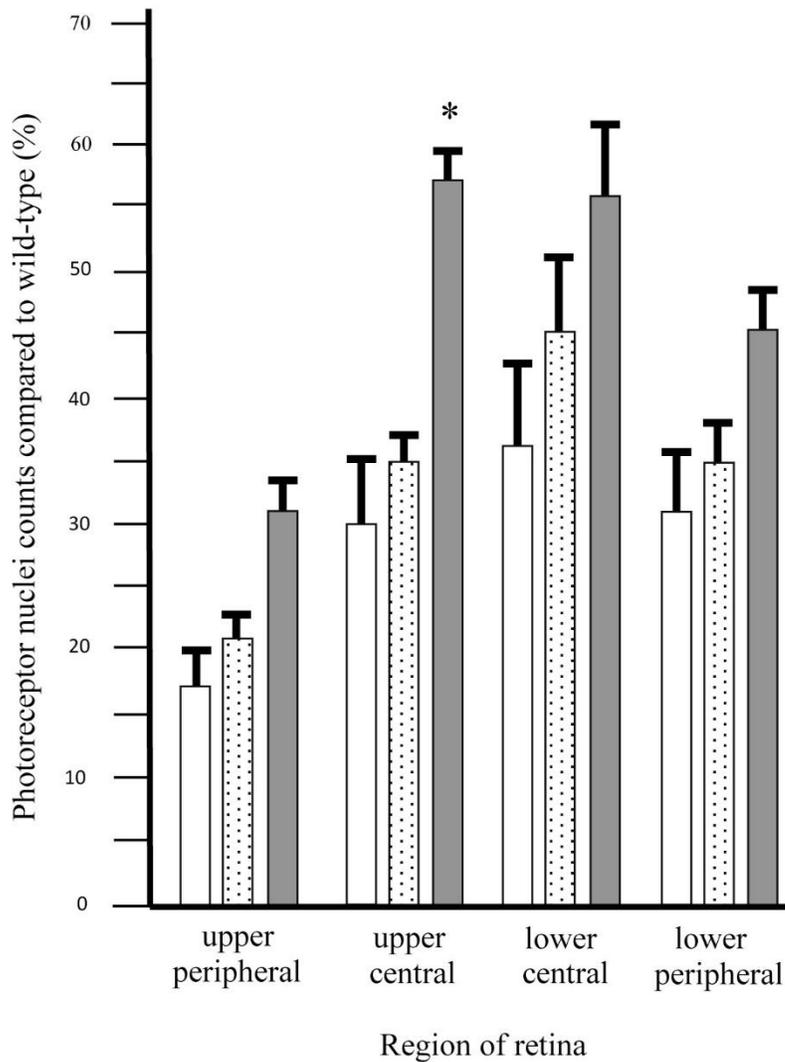


Figure 3.3: **Outer nuclear layer cell counts in the S334ter-4 rodent model of retinal degeneration at P110.** Photoreceptor nuclei in the outer nuclear layer were counted over a 100 μm length of retina. Counts were undertaken in regions A-D as described in Figure 3.2. \square intravenous vehicle injection; \square intravenous injection of MSCs; \blacksquare intravenous injection of MMSCs plus magnet. Data presented as means \pm SEM. For each data point n=6. * = P<0.05.

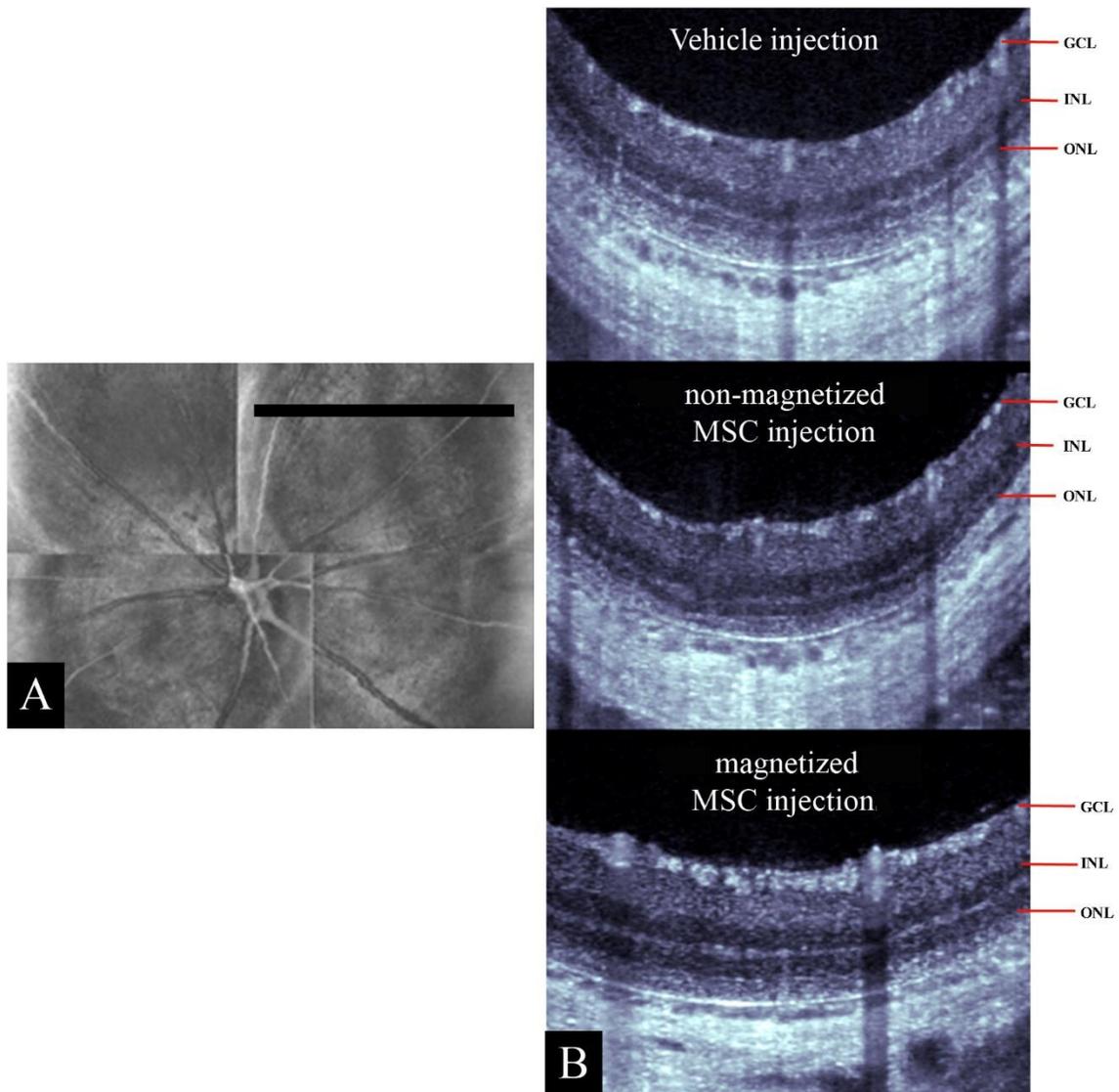


Figure 3.4: **OCT imaging in the S334ter-4 transgenic rat at P110.** **A:** fundus image reconstruction was used to localize OCT imaging (left eye) **B:** OCT images captured from the upper temporal quadrant (black bar in A, 1.5 mm) approximating to histology sections obtained from the upper central quadrant.

group. The mean spatial frequency threshold of 0.28 of MMSC treated group is within the normal range for Sprague-Dawley rodents [309].

3.4 Discussion

In a previous short-term study we showed that MSCs, magnetized with SPIONS could be preferentially targeted to the dystrophic retina if a small magnet was placed in the orbit to overlie the area of degenerate retina intended for treatment [11]. After tail vein injection, a ten-fold increase in MSC delivery to the retina could be achieved [11]. In this study, we further assessed the functional benefit of targeting MSCs to the dystrophic retina over a longer study period of three months. Overall, significant benefits for MSC therapy were only achieved through magnetic targeting. This is contrary to another study where anatomical and functional benefits were achieved with intravenous non-magnetic MSCs [208]. However, this previous study differed from that presented here in that bone marrow-derived MSCs were used which may have different neuroprotective effects to adipose tissue-derived MSCs [310]. In addition, a different animal model of retinal degeneration was used (the Royal College of Surgeons rat) and this may indicate that the retinal neuroprotective effects of MSCs could be disease specific.

Optokinetic tracking results, and to a greater extent outer nuclear cell count and electrophysiological analysis, suggested that the benefits of MMSC therapy are enhanced with magnetic targeting. This might be explained in a number of ways. Firstly, this could reflect an increasing neuroprotective benefit with exposure to larger numbers of MSCs. It has been suggested that systemic delivery of MSCs does not result in accumulation of large number of cells in the retina [11, 208]. But with magnetic guidance, significantly higher number of cells can be directed to the retina [11]. It has also been shown that intravenous delivery of cells can penetrate retina both from the RPE side and the ganglion cell side, which is better than one sided penetration (ganglion side) of intravitreal delivery [11]. We did, however, observe a better dark adapted electrophysiological response from MSC injections compare to the MMSC injected animals. This could be due to the targeted accumulation of the MMSCs rather than spreading throughout the retina. Another reason could be that the magnetic nanoparticles alter the secretion pattern of the MSCs, so that they provide better protection to the cone cells, as evident from the

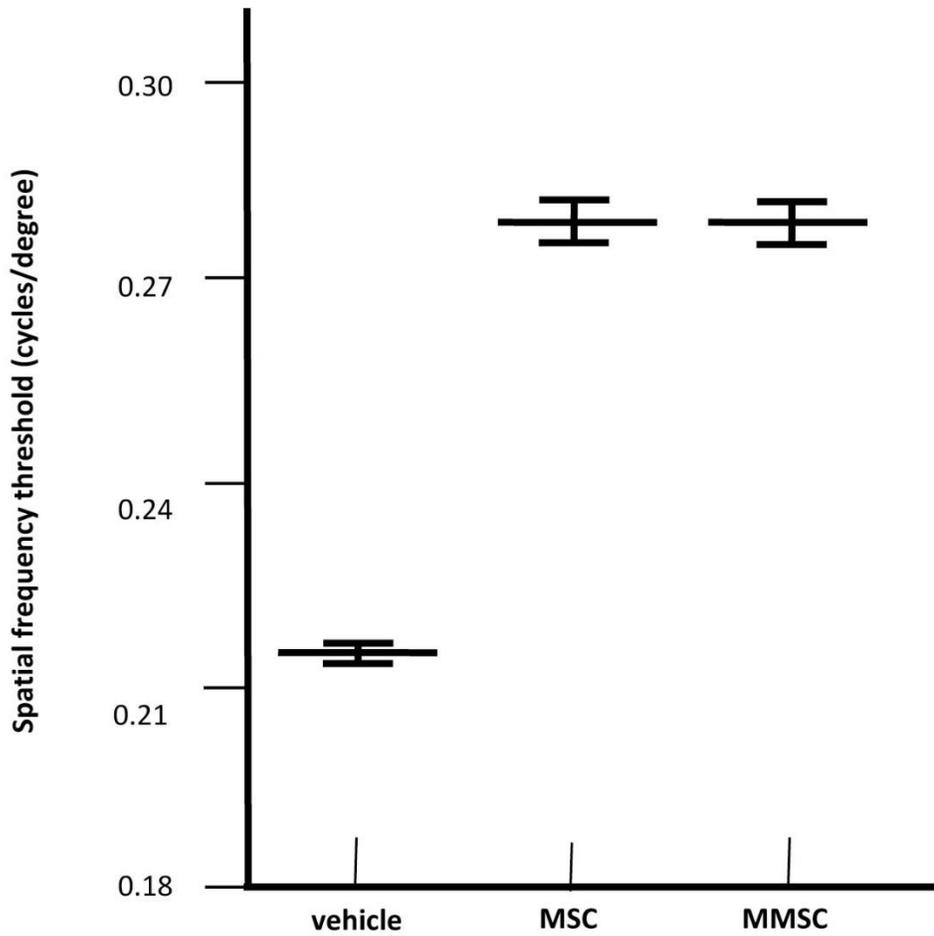


Figure 3.5: **Optokinetic tracking responses to moving gratings in rodents at P110.** Data are presented for 3 groups of animals: tail-vein injection of vehicle; tail-vein injection of MSCs; and tail-vein injection of MMSCs (with orbital magnet in place). In each cohort n=6. Normal range: 0.25-0.39 cycles/degree.

Table 3.1: **Electroretinographic responses.** In each treatment cohort plus wild-type controls, rod-predominant (scotopic), cone-predominant (photopic) and cone-dependent (30 Hz Flicker) responses were quantified. Data presented as mean \pm standard error of the mean. For each datapoint N=6. Results suggest preservation of cone function in animals treated with intravenous MMCS injections.

	Scotopic B wave		Photopic B wave		30 Hz Flicker	
	Amplitude μ V	Implicit time, ms	Amplitude μ V	Implicit time, ms	Amplitude μ V	Implicit time, ms
Vehicle injection	129 \pm 10	76 \pm 3	39 \pm 2	53 \pm 3	10 \pm 2	29 \pm 1
MSC injection	174 \pm 13	75 \pm 5	48 \pm 6	51 \pm 2	14 \pm 2	34 \pm 2
Magnetic MSC injection	154 \pm 14	80 \pm 2	49 \pm 4	51 \pm 1	17 \pm 2	31 \pm 2
Wild-type controls	425 \pm 41	73 \pm 6	130 \pm 15	50 \pm 2	21 \pm 1	30 \pm 0

photopic ERG responses. This functional and histological data taken together could be interpreted as suggesting that the enhanced functional benefits of MMSC therapy are due to a more efficient preservation of photoreceptors (a neuroprotective effect).

It is as yet unknown how MSCs in the retina can inhibit neurodegeneration. Previous work has suggested an up regulation of neurotrophic factors: BDNF and ciliary neurotrophic factor (CNTF) [208], and anti-inflammatory factors: interleukin-10 and hepatocyte growth factor [11]. A number of potential cell death mechanisms have been proposed in the S334ter-4 rat. One explanation has been that cell death is triggered by a caspase-independent mechanism involving activation of *poly (ADP-ribose) polymerase* (PARP) and calpain [311] and it has been shown for example that BDNF can inhibit PARP cleavage [312]. More recently, endoplasmic reticulum stress leading to an unfolded-protein response has also been proposed as a prominent caspase-dependent cell death mechanism in the S334ter-4 rat [313]. Further work will be needed to more precisely identify the molecular consequences of MSCs in the dystrophic retina.

A substantial advantage for magnetic targeting could be repeated injections without the risk of collateral damage associated with repeated intraocular injections. The ‘top-up’ effect of repeated injections could be significant and could circumvent the problem of finite life span of MSCs in the central nervous system [190, 314]. It has also been shown that different clonal populations of MSCs can have quantitatively different neuroprotective effects [310]. Multiple injections could therefore be advantageous because this increases the likelihood of being exposed to a particularly effective MSC clonal population.

A key issue in the magnetic targeting of cells has been SPION safety [315]. SPIONs are based on magnetite (Fe_3O_4), or maghemite ($\gamma\text{Fe}_2\text{O}_3$) molecules encased in polysaccharide, polymer, or monomer coatings and range in size from 10-180nm [316]. Currently SPIONs are under investigation as drug delivery molecules [317], as contrast enhancing media in MR imaging [318], and in cancer thermotherapy [319]. In all these studies, SPIONs have been found to be

biocompatible and very safe [320]. We have however previously shown that SPIONs do alter the expression profiles of MSCs [98] suggesting further work is needed in this area.

In summary, significant histological and functional preservation of the outer retina was achieved in a rodent model of retinitis pigmentosa with targeted intravenous MMSCs injection. The magnitude of retinal protection however was limited. Longer studies are therefore needed to determine whether retinal protection is sufficiently maintained to be biologically as well as statistically significant. This may also imply that MMSCs therapy might be more useful clinically as an adjuvant, augmenting other therapeutic approaches to synergistically achieve greater therapeutic benefits [10, 321, 322].

Chapter 4. Influence of SPIONs on MSCs

4.1 Introduction

Mesenchymal stem cells (MSCs) are multipotent non hematopoietic stromal cells found within many tissues. They are characteristically able to differentiate into osteocytes, chondrocytes, and adipocytes, and have a major role in regenerating mesenchymal lineages in both physiologic and pathologic conditions [6, 323]. Interest in the therapeutic potential of MSCs was originally focused on their tissue regenerative properties but more recently their paracrine effects, in terms of neurotrophic and immunomodulatory abilities have become increasingly recognized. MSCs are known to protect injured neurons, stimulate angiogenesis in stroke, promote wound healing, and inhibit fibrosis through their paracrine effectors. MSCs have been shown to secrete neuroprotective molecules such as: brain-derived neurotrophic factor, ciliary neurotrophic factor (CNTF), insulin-like growth factor 1, nerve growth factor, vascular endothelial growth factor, and basic fibroblast growth factor, as well as immunomodulatory molecules: matrix metalloprotease-9, tumor necrosis factor-alpha, interleukin 10 (IL-10), and hepatocyte growth factor (HGF) [6].

Ex vivo expanded MSCs have, therefore, been investigated as a potential cell based therapy in a wide range of clinical applications with over 100 MSC clinical trials currently listed by the U.S. National Institutes of Health trial database (www.clinicaltrials.gov). The remote delivery of magnetized MSCs, via the systemic circulation, to sites of disease has recently been the focus of work for a number of research groups [11, 14, 15]. The use of superparamagnetic iron oxide nanoparticles (SPIONs) to magnetize cells is based on a number of advantages: 1) the degree of magnetization possible means that external magnetic fields can be used to target the cells to specific locations; 2) such cells can be visualized by magnetic resonance imaging; 3) *in vivo*, SPIONs are converted into nontoxic ions; and 4) such biodegradability would avoid long-term toxicity as a consequence of long term storage [324]. Both *in vitro* [320] and *in vivo* studies [325, 326] have reported that such particles are nontoxic. However, it has been suggested that SPIONs do have some effect on MSC physiology. For instance it has been shown that SPIONs might also disrupt normal iron-oxide homeostasis. It has also been shown that they can cross the blood-

brain-barrier accumulate in other organs and that cell agglomeration in the presence of an external magnetic field might cause harmful vascular embolization [327].

In our recent *in vivo* study, we showed that intravenous, magnetic nanoparticle (fluidMAG-D)-labeled MSCs can be magnetically targeted to a specific locus in the dystrophic rodent retina [11]. Specifically to assess toxicity, we undertook a MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay) using bone-marrow derived MSCs in the presence of three different concentrations (0.05, 0.25 and 0.5 mg/mL) of SPION fluidMAG-D. We observed at least 95% cell survival at each of these concentrations. ELISA measurements from rat retinal tissue showed that this magnetic cell therapy resulted in enhanced tissue concentrations of neuroprotectant molecules: glial cell derived neurotrophic factor (GDNF) and CNTF; and anti-inflammatory factors: IL-10 and HGF. In this present study we proposed to determine whether exposing MSCs to fluidMAG-D influenced the cells ability to secrete these four molecules. In addition we proposed to determine whether fluidMAG-D had any effect on MSCs genetically altered to release a novel therapeutic molecule. For this latter work we elected to use MSCs genetically modified to secrete retinoschisin, an extracellular matrix molecule deficient in x-linked retinoschisis, the commonest cause of blindness in young children [328].

4.2 Experiment

4.2.1 MSC harvest and culture

MSCs were isolated from inguinal adipose tissue based on our previously described methods (where MSCs were isolated based on their phenotypic characteristics of adherence to plastic surfaces and ability to differentiate into adipocytes, chondrocytes or osteocytes) [11]. Postnatal day 21 Sprague-Dawley rat tissue was used for work on innate MSC secretion (of GNTF, CNTF, IL-10 and HGF). Postnatal day 20 C57BL/6 mouse tissue was used for work on MSCs genetically modified to secrete retinoschisin. Briefly, fresh tissue was harvested and distributed at 5 g per well of a 6-well dish. The tissue was macerated with surgical scissors in 3 mL of 1 mg/mL Collagenase 1 (Sigma) in DPBS (Life Technologies) and incubated at 37 °C for 1 h. Collagenase-dissociated tissue was filtered through a sterile 40 µm cell strainer into a 50 mL tube and centrifuged at 1000 g for 10 min. The supernatant was removed and the cell pellet was

resuspended in MesenCult MSC Basal Medium (Stemcell Technologies) supplemented with 15% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). The cells were plated in a 6-well dish and allowed to adhere for 48 h in a 37 °C incubator with 5% CO₂. Following the initial incubation, medium was replaced and upon reaching confluency the cells were trypsinized and transferred to a T-75 cell culture flask.

4.2.2 MSC transfection

For stable transfections, the human retinoschisin 1 (RS1) cDNA (gift from Dr. R. Molday) was cloned into pIRES2-DsRed2 (Clontech) under the control of the CMV promoter. This plasmid carries the neomycin-resistance gene for the selection of transfected cells with the antibiotic G418. Mouse MSCs were electroporated using the Gene Pulser Xcell system (BioRad). Briefly, MSCs from an early passage (3–5) were trypsinized, counted and collected by centrifugation. One million cells were re-suspended in 500 µL electroporation buffer, transferred into a 0.4 cm electroporation cuvette (BioRad) and mixed thoroughly with 100 µL plasmid DNA (1 µg/µL). A square wave pulse of 220 V for 25 ms was applied to the cells, which were then quickly plated in a 6-well dish in 2 ml MSC medium and incubated in a 37 °C incubator. Twenty four hours later, the medium was replaced with selection medium 200 µg/ml G418 (Life Technologies) in MSC medium and selection continued for two weeks. Expression of RS1 was confirmed by visualization of the reporter gene DsRed2, using a Zeiss LSM 510 META confocal laser scanning system attached to a Zeiss Axiovert 200 M inverted microscope.

4.2.3 Incubation with magnetic nanoparticles

Rat MSCs and RS1-expressing mouse MSCs were collected from confluent plates and cultured overnight at a density of 10,000 cells per well of 24-well plates. FluidMag-D particles, superparamagnetic iron oxide nanoparticles coated with starch of average size 2000 nm (ChemiCell GmbH, Germany), were diluted in MSC medium at concentrations of 0.05 mg/mL, 0.25 mg/mL and 0.5 mg/mL and 350 µl from each dilution was distributed to designated wells. These concentrations were chosen to correspond with previous studies using 0.05 mg/mL [11] and 0.5 mg/mL [14]. Cells incubated without fluidMag-D served as control. The secretion of GDNF, CNTF, IL-10, HGF and RS1 was examined at 24 and 96 h after the addition of

fluidMag-D. Experiments were done in duplicates. To determine the iron load carried by MSCs treated with different fluidMag-D concentrations, 50 mL aliquots containing 250,000 cells from all four groups were suspended in distilled water and underwent inductively coupled plasma atomic emission spectroscopy (Exova, Surrey, Canada). These experiments were done in triplicate.

4.2.4 Cell lysis

Cells were lysed by the addition of 250 μ L lysis buffer (10 mM Tris-HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.875% Brij-96, 0.125% NP-40, 1 μ g/ml Aprotinin, 1 μ g/ml Leupeptin and 174 μ g/ml PMSF) to each well of a 24 well plate. Lysates were collected in 0.5 mL tubes and were subjected to 3 freeze-thaw cycles. After a brief centrifugation for 1 min at 10,000 g, the supernatant was collected and used in ELISA.

4.2.5 Enzyme-linked immunosorbent assay (ELISA)

Following 24 or 96 h incubation with the respective concentrations of fluidMAG-D, 100 L of cell lysate or culture medium were used to determine the concentration of the following molecules using sandwich ELISA kits. ELISA kits for GDNF (Emax ImmunoAssay System, Promega; Madison, WI), CNTF (RayBiotech; Norcross, GA), IL-10 (Abnova; Taipei City, Taiwan), HGF (B Bridge; Cupertino, CA), and Retinoschisin (Uscn Life Sciences Inc., Wuhan, China) were each used according to the manufacturer's instructions. Visualization of the chromogenic substrates was measured at 450 nm using a POLARstar Omega plate reader (BMG LABTECH, GmbH, Germany). For innate protein secretion assays (GDNF, CNTF, IL10 and HGF) three technical repeat assays were undertaken from each of two biological replicate samples (n = 6). Data was analyzed as mean values \pm standard error of the mean. For retinoschisin assays, two separate biological assays were undertaken at each data-point.

4.2.6 Statistical analysis

In order to determine whether a significant positive or negative effect of fluidMAG-D was present in the results of each set of the ELISA assays, we fit the data to a linear curve using

standard simple regression analysis (Sigma Plot versus 10 Systat software, San Jose, CA, U.S.A.), and determined values for the coefficient of determination (R^2) and for the F statistic and corresponding P value in order to test the hypothesis that the regression coefficient (i.e. slope) $\neq 0$.

4.3 Results and discussion

4.3.1 Innate secretion of MSC

4.3.1.1 Neuroprotective molecules

After 24 h of incubation in the presence of iron-oxide nanoparticles, regression analysis suggested that GDNF concentration in culture media significantly increased with increasing fluidMAG-D dose ($R^2= 0.75$; $p < 0.0001$). However, this had reversed by 96 h incubation ($R^2= 0.82$; $p < 0.0001$) (Figure 4.1 (a)). This contrasted with CNTF concentration in culture media which suggested a trend of reduced concentration with increasing fluidMAG-D dose at both time-points (Figure 4.1(b)). However, these changes were not statistically significant suggesting no effect on CNTF secretion (at 24 hour timepoint $R^2= 0.04$; $p = 0.35$ and at 96 h timepoint $R^2= 0.04$; $p = 0.33$). These conflicting results between GDNF and CNTF at different time-points suggest that different effects are being triggered by exposure to fluidMAG-D.

4.3.1.2 MSC Secretion of anti-inflammatory molecules

For anti-inflammatory molecule IL-10, no significant secretion could be detected at either the 24 or 96 h time-points for untreated cells or cells treated with fluidMAG-D. For HGF also, little could be detected at the 24 h time-point. However, significant concentrations were detectable in culture media at the 96 h time-point suggesting a statistically significant suppression of HGF with increasing fluidMAG-D dosage ($R^2= 0.49$; $p = 0.0001$) (Figure 4.1(c)). These results are in

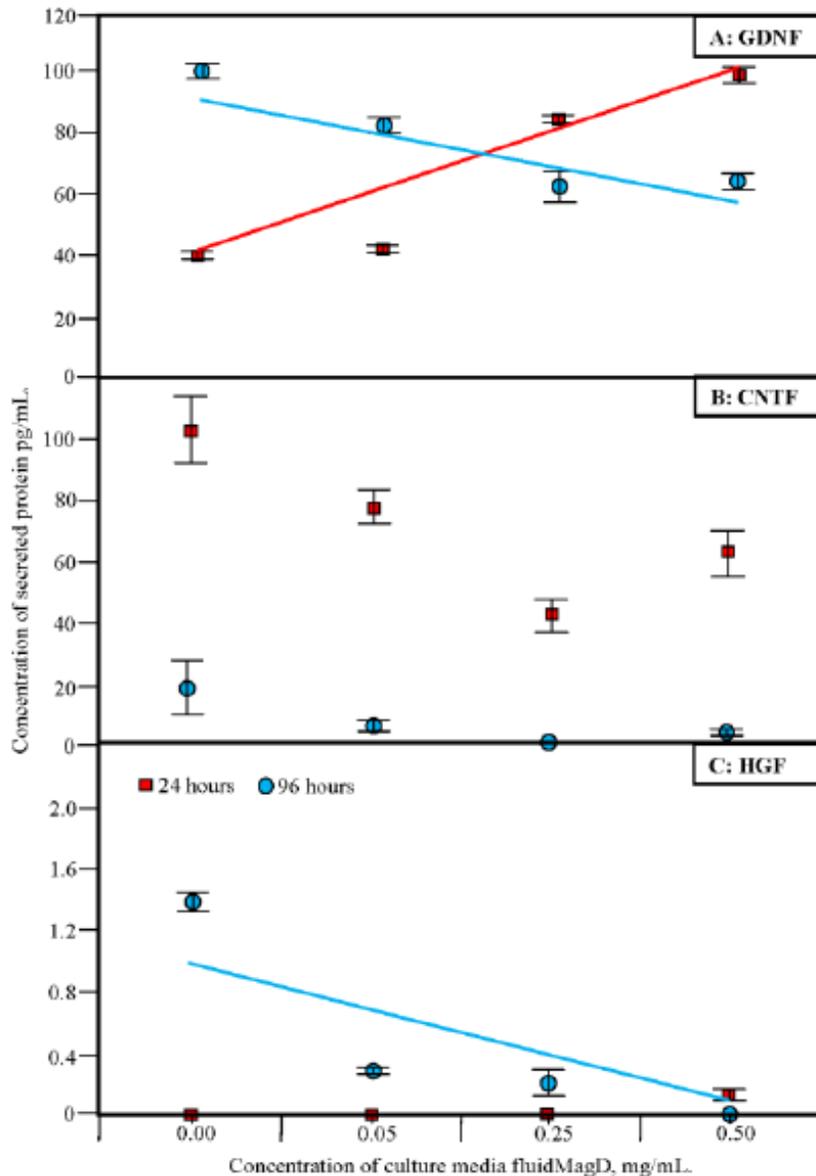


Figure 4.1: **ELISA assay results from culture media of MSCs.** Untreated cells were compared with cells cultured with either: 0.05 mg/mL; 0.25 mg/mL; or 0.5mg/mL fluidMAG-D. Measurements were undertaken after 24 hours and 96 hours. Measurements obtained for – (a): GDNF (glial-derived neurotrophic factor); (b): CNTF (ciliary-derived neurotrophic factor); and (c): HGF (hepatocyte growth factor). Each data point represents the mean value (n=6) \pm standard error of the mean. Lines correspond to standard simple regression analysis.

contrast with results observed *in vivo* where significant levels of both IL-10 and HGF were detected in retinal tissue containing fluidMAG-D treated cells [11].

4.3.2 Secretion of retinoschisin from genetically modified MSCs

Increasing fluidMAG-D concentration had no statistically significant effect on retinoschisin secretion into culture media (at 24 h timepoint $R^2 = 0.1$; $p = 0.43$ and at 96 h timepoint $R^2 = 0.03$; $p = 0.68$ [Figure 4.2(a)]. However, it was noted that increasing fluidMAG-D concentration was associated with increasing retinoschisin concentration in cell lysate (at the 96 h timepoint $R^2 = 0.83$; $p = 0.01$) [Figure 4.2(b)]. This former result at first might appear unsurprising since the retinoschisin construct is driven by a CMV promoter and is independent of normal cell mechanisms. The latter results, however, suggest that this might be an oversimplification. This may be explained as fluidMAG-D increasing retinoschisin production but with a corresponding inhibitory effect on retinoschisin secretion from the MSCs. An alternative explanation might be that the transport and secretory mechanisms employed by MSCs to secrete retinoschisin are easily saturated and are unable to deliver ever increasing amounts of retinoschisin production.

4.3.3 Iron load

The amount of iron carried by the cells after 24 h of incubation was measured. It was found that increasing fluidMAG-D concentration in culture media was associated with increasing concentration within cultured MSCs. Emission spectroscopy results were averaged ($n = 3$ for each datapoint) and showed that no fluidMAG-D in culture media resulted in nondetectable iron oxide load. Doses of 0.05, 0.25 and 0.5 mg/mL fluidMAG-D resulted in corresponding iron oxide loads in MSCs of 7, 120 or 274 pg iron oxide per cell. These figures correlate with iron oxide levels reported in other studies [11][14, 325].

Although our previous studies [11] had suggested that SPION treatment had little impact upon MSC viability *in vitro*, this present study does suggest some effect on MSC secretion *in vitro*. An initial stimulatory effect on GDNF secretion was seen along with suppression in the longer term (96 h). It is possible that increasing fluidMAG-D dosage initially induces a ‘stress response’ in

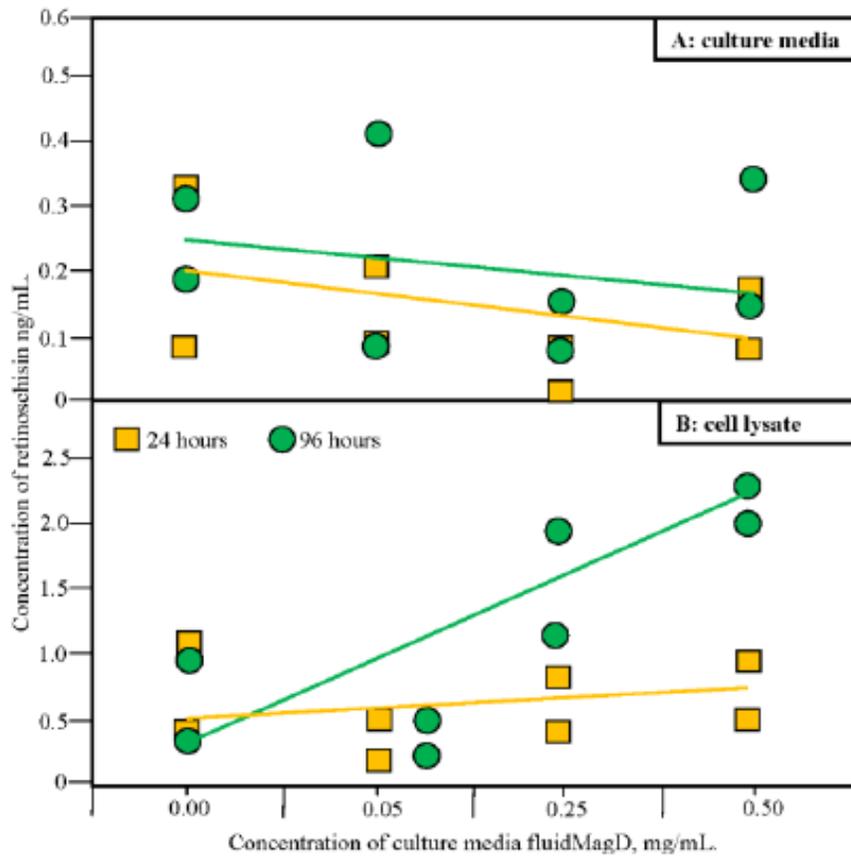


Figure 4.2: ELISA assays of – (a): culture media, and (b): cell lysate, of MSCs genetically modified to secrete retinoschisin. Untreated cells were compared with cells cultured with: 0.05 mg/mL; 0.25 mg/mL; or 0.5mg/mL fluidMAG-D. Measurements were undertaken after 24 hours and 96 hours. Each point represents a separate biological repeat sample. Lines correspond to standard simple regression analysis.

cells, increasing the secretion of protective molecules but that this is eventually overtaken by a toxic effect inhibiting secretion [329]. It is unclear, however, why fluidMAG-D did not have the same effect on CNTF secretion. This suggests that fluidMAG-D has selective effects on cells rather than a general toxic influence affecting many biochemical pathways. This might be explained in two ways. Firstly that fluidMAG-D has an effect on the intracellular manufacture of only some molecules or inhibits their intracellular transport and secretion at the cytoplasmic membrane. Further studies are required to establish the exact mechanism of action; however, effects on retinoschisin secretion in genetically modified MSCs could suggest an inhibition of retinoschisin transport and/or secretion rather than an inhibitory effect on protein production (since lysate concentrations actually increased with fluidMAG-D concentration without increase in culture supernatant). The secretion of retinoschisin from cells is partially controlled by actin polymerization [330]. This may be affected by SPION treatment since at a concentration of 0.5 mg/mL, SPIONs can cause formation of actin stress fibers in endothelial cells [73]. It is, therefore, possible to hypothesize that SPIONs such as fluidMAG-D have some inhibitory effects on cell protein secretion whilst also stimulating or having no effect on intracellular production of these proteins. Given that our *in vitro* results, showing little IL-10 and HGF secretion, are opposite to our observations on secretion of these molecules *in vivo*, this would suggest that extrapolation of *in vitro* results should be done with caution.

In a study similar to that reported here [326], adipose-derived rat MSCs were incubated in the presence of two different concentrations of iron-oxide nanoparticle (0.05 mg/mL and 0.1 mg/mL). Total amounts of VEGF, HGF and insulin like growth factor mRNA were reported as unaffected by nanoparticle incubation. Our study, however, differs from this in key areas. The SPION used in our study, fluidMAG-D, is starch coated whereas in this previous study the magnetic nanoparticle used was dextran coated (ferumoxides). A difference between cellular handling of the dextran and starch coating might result in different metabolic effects.

Interestingly though, we previously compared cell viability after treatment with starch coated and dextran coated nanoparticles and found that, at comparable concentrations, dextran coated SPIONs appeared more toxic (67% viability compared to 95%). In addition, the range of concentrations used (0.05–0.1 mg/mL) was much more restricted than the range used in our study (0.05–0.5 mg/mL). Finally, the total mRNA concentrations measured in this previous study

reflect total cellular concentrations rather than concentrations of these molecules when secreted into the culture media.

4.4 Conclusion

MSCs have demonstrable value in the treatment of neurodegenerative diseases such as retinopathy—specific examples of which include age-related macular degeneration and retinitis pigmentosa, the leading causes of blindness in the western World [331]. In particular, secretion of neuroprotective and immunomodulatory molecules suggest significant therapeutic potential [6]. Most pertinent to the present study, previous reports have already demonstrated a retinal neuroprotective effect with MSCs delivered either by intravenous or intravitreal injection [208, 332]. In addition, *ex vivo* genetic modification of MSCs could extend the range of therapeutic benefits of MSC therapy [7].

A significant problem, however, is how to efficiently deliver such cells to target organs. Systemic delivery has significant advantages, in particular in situations where cell therapy needs to be repeated over many years. Magnetic targeting (after SPION treatment) has already been shown to be a feasible and practical adjunct to systemic cell delivery [11, 14, 15]. However, there are contradictory reports on the effects of SPION treatment on the metabolism of MSCs. A number of studies have reported that SPION treatment does not affect pluripotency of the MSCs [11, 325]. However, it has also been reported that nanoparticle labelling can affect iron metabolism, migration capacity and colony formation although this does not appear to correlate with poorer cell viability [333]. The same study also suggested that SPION treatment enhances the adipogenic differentiation of the MSCs [87, 333]. SPIONs have been shown to accumulate in cell endosomes and lysosomes but with time these nanoparticles are released into the cytoplasm, ultimately increasing the total cellular iron pool. It has been speculated that this could result in a range of effects including stimulation of cell proliferation and accumulation of toxic reactive oxygen species [87, 320].

Our results demonstrate some inhibitory effects on secretion of innate and genetically modified molecules from adipose-derived rat MSCs when exposed to increasing concentrations of fluidMAG-D. This data adds to the accumulating literature suggesting complex effects of SPIONS on cell physiology which may limit their potential clinical effectiveness. However, recommending that lower dosages of fluidMAG-D be used in future studies (which would correspondingly reduce the magnetization of cells) may not always be necessary. For instance whereas increasing dosage does inhibit GDNF secretion, if an intended effect of MSC therapy would be through CNTF secretion then higher fluidMAG-D dosage might have no deleterious effect. Further studies are required to more fully assess the extent to which deleterious effects influence MSCs and whether these *in vitro* results correlate with *in vivo* effectiveness [6, 334].

Chapter 5. An *Ex vivo* Gene Therapy Approach for XLRS

5.1 Introduction

X-linked retinoschisis is one of the most common causes of retinal disease and blindness in young males, with a prevalence of 1:5000 – 1:25000 [4]. The disease is caused by mutations in the retinoschisin gene (RS1) localized in the X-chromosome (Xp22.13) [17, 18]. RS1 expresses a cell surface attachment protein which is exclusively expressed and secreted by photoreceptors and bipolar cells [16]. Absence or non-functional expression of RS1 protein results in significant and progressive structural abnormalities in the outer and inner retina described as schisis cavities with accompanying progressive death of the photoreceptor cells [17].

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic stromal cells found within many tissues. Interest in the therapeutic potential of MSCs was originally focused on their tissue regenerative properties. More recently their paracrine effects, in terms of neurotrophic and immuno-modulatory abilities have become increasingly recognized [12]. MSCs are known to protect injured neurons, stimulate angiogenesis in stroke, promote wound healing, and inhibit fibrosis through their paracrine effectors [6]. In addition, their remarkable homing ability allows MSCs to migrate into areas of tissue damage and integrate into the tissues extracellular compartment [6]. Mesenchymal stem cells have been used in a number of cell based therapeutic approaches to treat retinal degeneration [11, 335-338]. The ability to use MSCs for combined cell (through the paracrine effects) and gene therapy (through expressing novel therapeutic protein) has yet to be assessed.

A number of approaches of *in vivo* gene therapy have been reported to ameliorate the condition of XLRS in the pre-clinical trials on animal models [20-23]. The limitations, however, of this *in vivo* approach are considerable, including lack of specificity, low efficiency, and systemic exposure to the transport vector [24-27]. An *ex vivo* approach to gene therapy would circumvent these issues. XLRS is a good candidate for *ex vivo* gene therapy for three reasons: firstly, it is a monogenic disorder where the underlying pathophysiology is well understood; secondly, gene

therapy techniques to improve wild-type protein production are best suited to loss-of-protein phenotypes such as in XLRS rather than in gain-of-function mutations where pathophysiology has been linked to adverse effects of mutant protein; and thirdly, RS1 is a secreted, extracellular protein, so it can be replaced in the retinal extracellular space through secretion by other cell types (e.g. MSCs genetically modified to secrete RS1). Another particular advantage of an *ex vivo* protocol is the relative ease with which cells can be engineered to vary expression of novel proteins in response to external signals. The Tet-On system is a well-known molecular switch used for such a purpose, where expression can be induced by exposure to doxycycline.

In this study we have therefore hypothesized that an *ex vivo* gene therapy approach in XLRS could be an alternative strategy to *in vivo* gene therapy protocols with the advantages of reduced risks (e.g. associated with integration of genetic material into the human genome) and the added benefits of better control of protein secretion (using an inducible protocol). Additionally we would use MSCs which have inherent neuroprotective abilities. MSCs both constitutively (cMSCs) and inducibly (iMSCs) expressing RS1 were delivered into the Rs1 knockout mouse eye by intravitreal injection. In treated animals both histological and functional outcomes would be measured.

5.2 Methods

5.2.1 Disease model

Homozygous female RS1KO and hemizygous male RS1KO animals were donated from the laboratories of Dr. R. Molday (University of British Columbia). The knock-out status of offspring was verified by PCR using two sets of primers. One set (5'-TGAGGACCCCTGGTACCAGAA-3', 5'-CCATCTCAGGCAAGCCAGG-3') was designed to amplify a 260bp region of the wild-type Rsh1 gene. Secondly, the same 5' primer was used in combination with 3' primer (5'-CAAGGCGATTAAGTTGGGTAAC-3') targeting LacZ to detect the mutant Rs1h gene (product size of 180 bp) [339]. The animals were housed under standard laboratory conditions (22 F 28C, 60 F 10% relative humidity, and a 12-h light–dark cycle) and had free access to food and water throughout the experiment. The conditions of housing and experiments were in accordance with the ARVO Statement for the Use of Animals

in Ophthalmic and Vision Research using protocols approved by the University of British Columbia.

5.2.2 Cloning of RS1 cDNA

For the constitutive expression cassette, the human retinoschisin 1 (*RS1*) cDNA (gift from Dr. R. Molday) was cloned into pIRES2-DsRed2 (Clontech) vector. The cloning site is controlled by a constitutive (CMV) promoter. This plasmid also carries the neomycin-resistance gene for the selection of transfected cells with the antibiotic Geneticin (G418). This plasmid was then transformed into competent DH5 α *E. coli* (New England Biolab) cells. The transformed bacterial cells were cultured overnight at 37 °C in LB media (LB Broth, Miller, Fisher Scientific) containing Kanamycin. Plasmid isolation was performed using the PureYield™ Plasmid Maxiprep System (Promega) according to the manufacturer's instruction. The presence of the RS1 cDNA in the plasmid was confirmed by restriction digestion and subsequent gel electrophoresis.

For the inducible expression cassette, a Tet-On system (Clontech) was used containing two plasmids: pTRE3G-BI-mCherry and pCMV-Tet3G. The human RS1 cDNA was cloned into the pTRE3G-BI-mCherry vector. This plasmid system carries the hygromycin-resistance gene for the selection of transfected cells with the antibiotic Hygromycin (Hyg). The other plasmid (pCMV-Tet3G) carries the neomycin-resistance gene for the selection of transfected cells with the antibiotic Geneticin (G418). Both the plasmids were amplified using DH5 α *E. coli* cells following the above mentioned procedure. The presence of the RS1 cDNA in the pTRE3G-BI-mCherry plasmid was confirmed by restriction digestion and subsequent gel electrophoresis.

5.2.3 Isolation and culture of MSCs

MSCs were isolated from inguinal adipose tissue as previously described [12]. Briefly, the tissue from postnatal day 21 C57BL/6 mice was macerated with surgical scissors in 3 mL of 1 mg/mL Collagenase 1 (Sigma) in DPBS (Life Technologies), and incubated at 37 °C for 1 h. Collagenase-dissociated tissue was filtered through a sterile 40 μ m cell strainer into a 50 mL tube

and centrifuged at 1000 g for 10 min. The supernatant was removed and the cell pellet was resuspended in MesenCult MSC Basal Medium (StemCell Technologies) supplemented with 15% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). The cells were plated in a 6-well dish and allowed to adhere for 48 h in a 37 °C incubator with 5% CO₂. Following the initial incubation, media was replaced and upon reaching confluency the cells were trypsinized and transferred to a T-75 cell culture flask.

5.2.4 MSC transfection with RS1 cDNA containing expression vector

Mouse MSCs were electroporated using the Gene Pulser Xcell system (BioRad). Briefly, MSCs from an early passage (3–5) were trypsinized, counted and collected by centrifugation. One million cells were re-suspended in 500 µL electroporation buffer and transferred into a 0.4 cm electroporation cuvette (BioRad). For the constitutive expression, 100 µL pIRES2-DsRed2-RS1 plasmid (1 µg/µL) was mixed thoroughly with the MSCs containing electroporation buffer. A square wave pulse of 220 V for 25 ms was applied to the cells, which were then quickly plated in a 6-well dish with 2 ml MesenCult MSC Basal Medium (StemCell Technologies) and incubated in a 37 °C incubator. 24 hours later, the medium was replaced with MSC media containing 200 g/ml G418 (Geneticin, Life Technologies) selection agent. The selection was continued for two weeks. Transfection efficiency was confirmed by visualization of the reporter gene DsRed2, using a Zeiss LSM 510 META confocal laser scanning system attached to a Zeiss Axiovert 200 M inverted microscope.

For the inducible system, two rounds of transfection were performed. At first, 100 µL of pCMV-Tet3G (1 µg/µL) plasmid was used to transfect mouse MSCs following the above mentioned protocol. Successfully transfected cells (TetMSC) were selected using G418 (200 g/ml) for two weeks followed by rapid expansion. A second round of transfection was performed using the pTRE3G-BI-mCherry vector with RS1 cDNA (1 µg/µL) on the TetMSC cells following a similar electroporation process. Transiently transfected cells were observed by imaging the reporter gene mCherry using Zeiss LSM confocal microscope. Hygromycin selection media (200 g/ml) was used to select the successfully transfected cells (iMSC) for two weeks.

5.2.5 Intravitreal injection

Two different studies were performed to analyse the effect of the *ex vivo* gene therapy approach on the RS1KO mouse model. In one study, the animals received only one injection (single), whereas multiple injections were administered in the second study. For the single injection study, expanded adMSCs, cMSCs and iMSCs were resuspended in PBS to give a final concentration 50,000 cells/ μl . A Hamilton syringe was used to inject 2 μl of the cell suspension (1×10^5 cells) into the vitreous cavity of the left eye of p21 RS1KO mice. The injection site was ~1 mm behind the limbus. Animals were anesthetized using 3% Isoflurane. In total, five groups of animals were treated; first with constitutive RS1 secreting MSCs (cMSC); second with inducible RS1 secreting MSCs (iMSC); the third with unmodified MSCs (MSC); the fourth with cell culture media (vehicle) and the fifth was sham (untreated). Immunosuppression was not used in any animals. Data was collected at 2-, 4-, and 8-weeks post injection time points.

For the multiple injection study, the first injection was done at p30, followed injections at p60 and p90. There were three cohorts in this study, iMSC, cMSC and sham. Data were collected at p120.

5.2.6 Topical Doxycycline administration

Selected RS1KO mice were treated with topical doxycycline hyalate (Dox) (Sigma Life Sciences) for seven consecutive days. Three different concentrations of Dox (0, 5 and 10 mg/ml) were prepared using phosphate buffered saline (PBS) (Life Technologies). 50 μL of Dox were applied to the left eye of each animal using a dropper. For each group $n=3$. For the intravitreal iMSC study, 50 μL of 5 mg/mL Dox was topically administered to all the animals one day after the injection and continued until the sampling date.

5.2.7 Electroretinography (ERG)

The animals that were tested were put in a dark room for overnight for dark adaptation. They were anesthetized with Xylazine (16 mg/kg) and Ketamine (120 mg/kg) and maintained on a heating pad. The corneas were locally anesthetized with 0.5% proparacaine hydrochloride and

the pupils were dilated with 2.5% phenylephrine and 1% atropine. Electroretinograms were recorded using Espion V5 Electroretinogram Console plus ColorDome (Diagnosys LLC, Lowell, MA, USA) with corneal contact lens electrodes. Scotopic ERGs (reflecting rod function) were recorded using white flashes of intensity 2.25 cd.s/m². Photopic ERGs (mixed rod and cone function) were recorded with the background illumination set at 30 cd/m² and white flashes of 3.0 cd.s/m². Average scotopic b/a wave ratio were measured and noted with standard error of the means. Sample size (n) were 6 for all the cohorts for both single and multiple injection studies.

5.2.8 Optokinetic tracking (OKN)

In order to examine optokinetic tracking, the spatial frequency thresholds in experimental animals was measured under photopic (light adapted) conditions [309]. A vertical sine wave grating (100% contrast) was projected as a virtual cylinder in three-dimensional space on computer monitors arranged in a quadrangle around a testing arena (OptoMotry; CerebralMechanics, Lethbridge, Alberta, Canada). Unrestrained mice were placed on the elevated platform at the center of the equipment. An observer used a video monitor to ensure that the virtual cylinder was kept centered on the animal's head and recorded head movements in response to cylinder rotation. The highest spatial frequency that resulted in a response was documented and group means were calculated and compared at each time points. Sample size (n) were 6 for all the cohorts for both single and multiple injection studies.

5.2.9 Histology

Animals were euthanized by CO₂ asphyxiation. Eyes were enucleated and marked at the 12 o'clock position, by leaving a piece of white conjunctiva muscle for orientation. Eyes were immersed in half strength Karnovsky's fixative for 1 h, and the anterior segment removed by excision at the ora serata. Other posterior segments were embedded in paraffin, cut sagittally to a thickness of 5 µm, and counterstained with hematoxylin and eosin. Schisis formations within the inner nuclear layer were analysed. To standardize schisis area measurement from different eyes, only sagittal sections that included the optic nerve were employed. Images were overlaid with a 200 µm-length rectangular template using Adobe Photoshop CS2. This template was positioned at four predetermined coordinates to give counts from two central (behind the equator) and two

peripheral (in front of the equator) coordinates [10]. These four images for each eye were then imported into ImageJ (v 1.37), converted to greyscale, inverted and schisis areas were measured by saturating the black areas. For the multiple injection study, the same four 200 μm area were used to count the outer nuclear layer cell number as well. Sample size (n) were 6 for all the cohorts for both single and multiple injection studies.

5.2.10 ELISA

Retinal lysates were analyzed through ELISA to detect RS1 expression following the previously described protocol [11]. Briefly, isolated neurosensory retinas from mouse eyes were collected and homogenized with a 25G needle using 200 μL cold PBS. Homogenates were then centrifuged for 10 min at 13,000 \times g and the protein concentration was then determined in the cleared supernatants. RS1 concentrations were measured using a sandwich ELISA (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's instruction [12]. Visualization of the chromogenic substrates was measured at 450nm using a POLARstar Omega plate reader (BMG LABTECH, GmbH, Germany). All samples were analyzed in duplicates and results were calculated as pg/ml. For all the data n=3.

5.2.11 Immunohistochemistry

For immunofluorescence studies, eyes enucleated from three different cohorts (cMSC, iMSC and untreated) and for both single (all three time points) and multiple injections eyes were paraformaldehyde-fixed (overnight) and subsequently incubated in 25% (W/V) sucrose for 24 hrs. Eyes were embedded in Tissue-Tek Cryomolds (Sakura Finetek USA Inc., CA USA) with Polyfreeze Tissue Freezing Medium (Polysciences, Inc., PA USA). 10 micron thick sections were cut using a Microm HM525 (Germany) microtome. Immunostaining was performed following established protocol [44]. Briefly, cryosections were blocked with PBS containing 0.2% Triton X-100 (PBS-T) and 10% (vol/vol) goat serum for 20 min and labeled overnight with the primary antibody (3R10, generous gift from Dr. Molday's Lab, UBC). The samples were then rinsed in PBS and labeled for 1 h with the secondary antibody conjugated to Alexa 594 (red) (Life Technologies).

5.2.12 Statistical analysis

The significance of differences between the groups was assessed by performing ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant. Data are presented as the mean \pm SEM.

5.3 Results

5.3.1 Characterization of transgene expression by MSCs

Transfection efficiency and Rs1 expression of the pIRES2-DsRed2-RS1 plasmid in cMSCs was characterized by rtPCR, western blot and immunolabeling (Figure 5.1). The transient transfection efficiency was assessed by observing the expression of the reporter gene, DsRed, under the confocal microscope 48 hours after electroporation. At this stage the transfected cells morphologically looked the same as unmodified MSCs when examined under light microscope. These cells were selected in presence to G418 for two weeks to get stably transfected cMSCs. RS1 expression of these cMSCs was assayed for at cellular level (Figure 5.1E), transcription level (Figure 5.1A) and protein level (Figure 5.1C). Similarly the cMSCs displayed morphology identical to control cells.

For the inducible system, the iMSCs were also characterized by rtPCR, western blot and immunolabeling of RS1. After the first round of electroporation with the pCMV-Tet3G vector, the transfection efficiency was assayed by observing the expression of the Tet3G protein via western blot. Cells with confirmed Tet3G expression were then transfected with pTRE3G-BI-mCherry-RS1 plasmid and transient transfection efficiency was assessed by observing the expression of the reporter gene, mCherry. Once stably transfected cells (iMSC) were obtained (through selection with both G418 and Hygromycin), RS1 expression was assayed for Dox induction with 0 ng/ml and 100ng/ml Dox concentrations *in vitro*. Both at transcript level (Figure 5.1B) and protein level (Figure 5.1D) RS1 expression was induced by the presence of Dox, whereas RS1 was not expressed without Dox. RS1 expression was also analyzed at the cellular level (Figure 5.1E) by immunolabeling the cells after inducing with Dox.

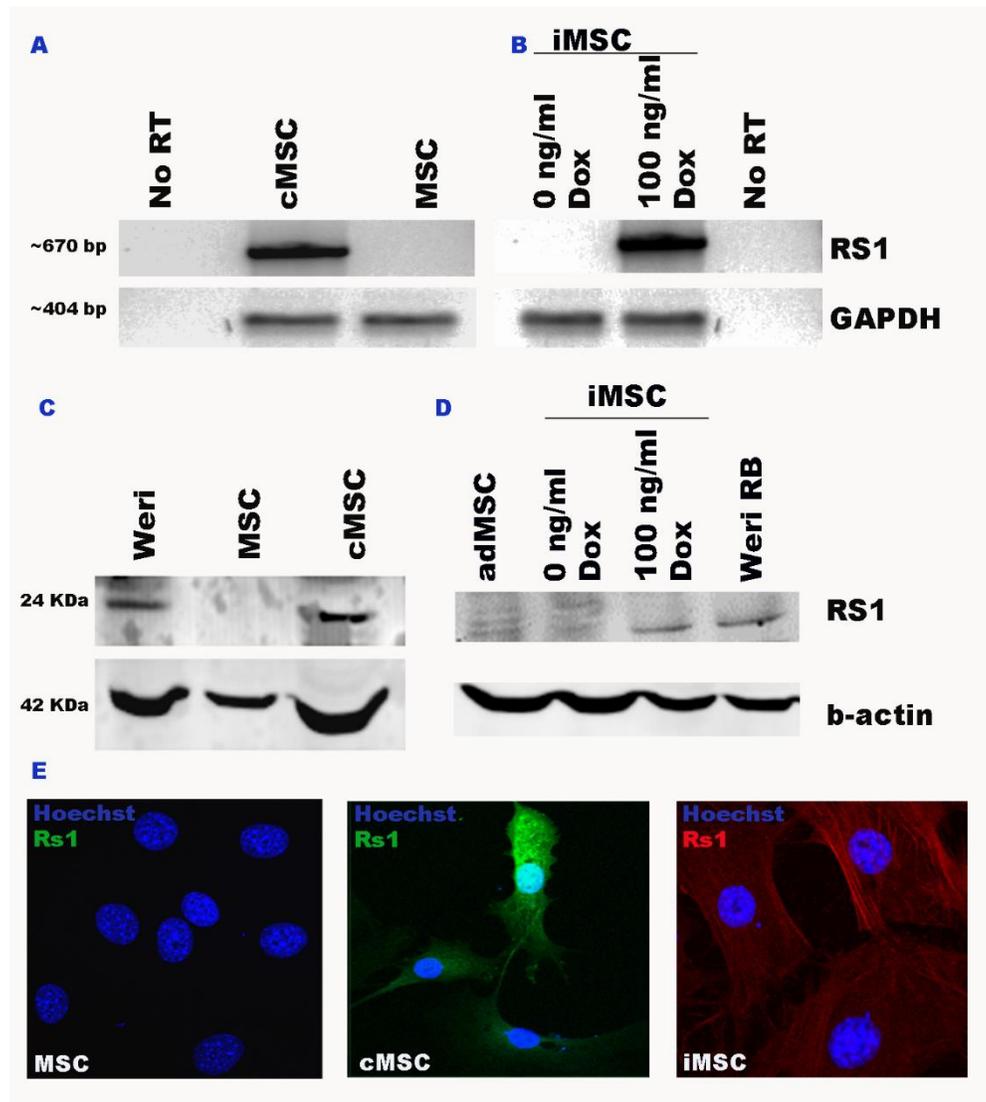


Figure 5.1: **Expression and characterization of the genetically modified MSCs.** Stably transfected MSCs with constitutive vector (cMSC) showed RS1 mRNA expression (A) and protein expression (C). MSCs transfected with the inducible system (iMSC) showed mRNA (B) and protein (D) expression in presence of Dox, whereas no expression was detected without Dox. Immunocytochemistry with a RS1 specific Ab (3R10) showed expression of RS1 from stably transfected cMSCs and iMSCs (E).

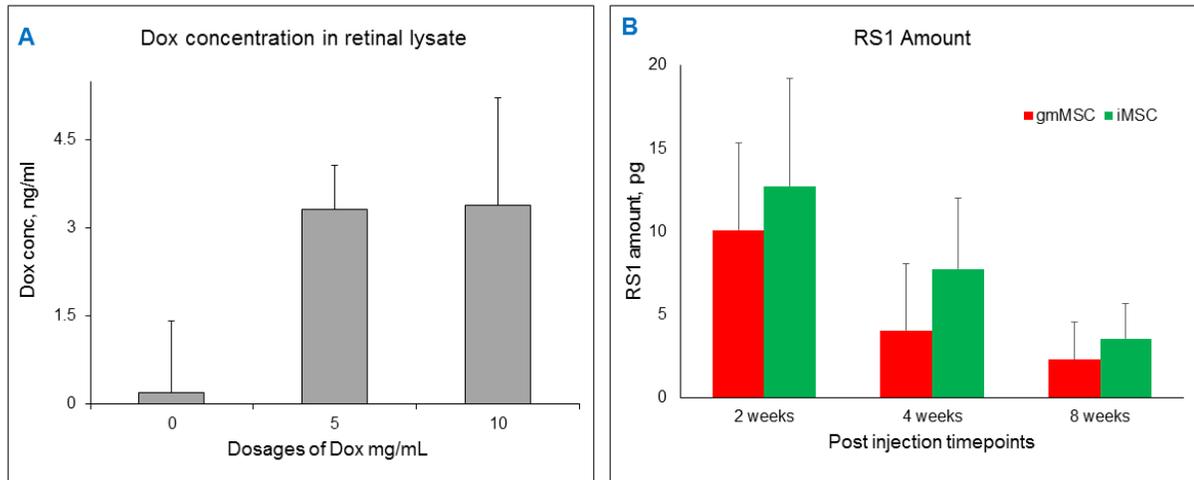


Figure 5.2: Dox and RS1 concentration in the retina. (A) Two different concentrations, 5mg/ml and 10mg/ml of Dox were topically applied via droppers to RS1KO mouse to assay their penetration capability. ELISA on retinal lysates showed almost similar amount of Dox were accumulated for both the dosages. (B) Quantification of the amount of RS1 in the retina by cMSC and iMSC at all three post injection time points. For all samples, n=6. Data are presented as mean \pm standard error of the mean.

5.3.2 Penetration of Dox in the posterior site of the eye

To determine the optimal concentration of topical Dox for penetration into the vitreous cavity and retina, three different concentrations of topical Dox were used and resultant Dox concentrations in the retina were assessed using ELISA (Figure 5.2A). Optimal concentrations was determined to be 5 mg/mL.

5.3.3 Single injection

5.3.3.1 Quantification of RS1 Expression in the retina

The amount of RS1 protein in the RS1KO mouse retina after intravitreal injection of cMSCs and iMSCs was quantified by ELISA (Figure 5.2B). Higher amounts of RS1 were detected in eyes treated with iMSCs at all timepoints suggesting that the inducible system was more efficient than the constitutively active system. No expression was detected in untreated eyes. A gradual decline in RS1 expression was documented over time in both treatment groups (Figure 5.2B) which might correlate with previous observations that MSCs do not survive in the retinal microenvironment for more than 6-8 weeks [11].

5.3.3.2 Secretion of RS1 in the eye

RS1 expression and distribution patterns in the retina were analysed by immunolabeling the treated (both cMSC and iMSC) and untreated RS1KO eyes. A similar pattern of distribution and migration was observed for the both the cell types. 24 hours post injection, most of the cells were localized in the vitreous, with a few found on internal limiting membrane. At later time points (Figure 5.3A) cells were seen to have migrated into the neurosensory retina. At 2 weeks post injection, this migration was confined to the ganglion cell and inner plexiform layers. At later time points (4 and 8 weeks post injection), cells were also seen to have migrated as far as the inner nuclear layer, with occasional labelled MSCs seen in the outer nuclear layer. The distribution of the secreted protein was around the cell and also along the axonal length of the adjacent neuronal cells at all time points (Figure 5.3A).

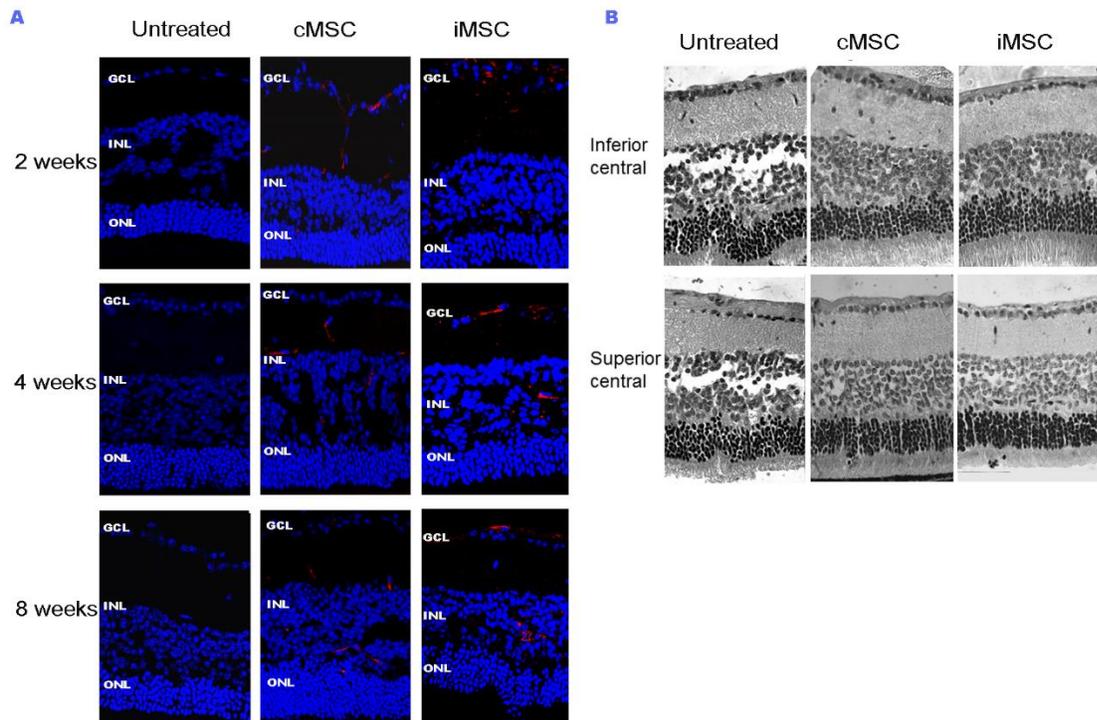


Figure 5.3: Expression and distribution of RS1 in the retina at different time points post intravitreal injection. (A) Immunohistochemistry identified the localization of the RS1 protein secreted by intravitreally injected cMSCs and iMSCs at 2 weeks, 4 weeks and 8 weeks post injection. GCL-ganglion cell layer, INL-inner nuclear layer, ONL-outer nuclear layer. (B) H & E staining of the histology sections from the superior and inferior central quadrant showing smaller schisis cavities in the iMSC and cMSC injected eyes compare to the untreated eye at the same time point.

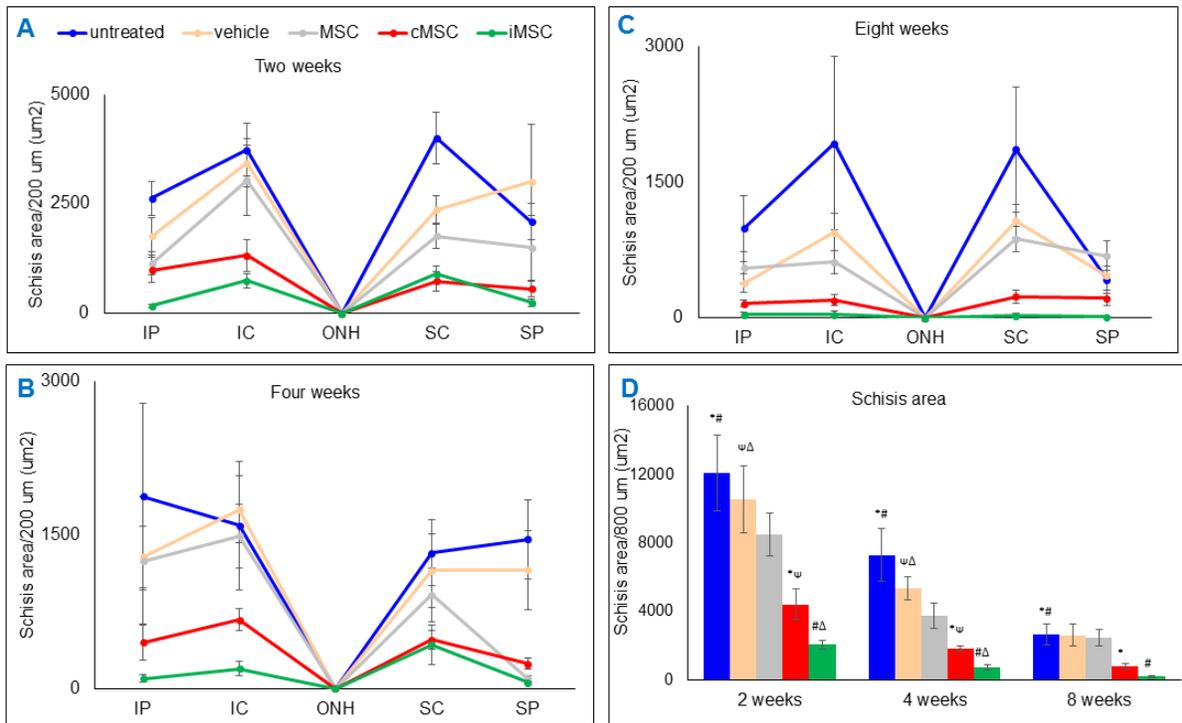


Figure 5.4: **Structural restoration from the injected cMSC and iMSC.** (A-C) Spidergrams of three different post injection time points comparing the schisis area in the four quadrants of the retina of the five different groups. (D) Total schisis area from all four quadrants were calculated and compared among groups at all three time points. Data are presented as mean \pm standard error of the mean. IP-inferior peripheral, IC-inferior central, SC-superior central, SP-superior peripheral. *, #, Δ and ψ imply statistically significant where $p > 0.05$. For all samples, $n = 6$.

5.3.3.3 Structural restoration

A representative images demonstrating the difference of the structural restoration between the treated (cMSC and iMSC) and untreated groups of 4 weeks post injection time point are shown in Figure 3B. To quantify retinal damage, the schisis area of a 200um span in the inner nuclear layer from four different sites (superior peripheral, superior central, inferior central and inferior peripheral) was measured and compared between 5 groups at 3 time points (Figure 5.4A-C). The largest sized cavities were observed at the 2 week post-injection time point in all four quadrants. Treated animals exhibited smaller schisis cavities in all four quadrants compared to control groups. This was particularly evident in animals treated with iMSCs (Figure 5.4A). The size of the cavities declined with time for all five groups (Figure 5.4B and 4C). To assess the statistical significance of differences in schisis cavity seen in different groups, the summation of the schisis area from all four quadrants were measured (total 800 μm) at each timepoint (Figure 5.4D). At 2 weeks post injection, animals treated with either cMSCs or iMSCs showed significantly smaller schisis area compare to the untreated (p value .0001 for both) and vehicle (p value .001 and .003 respectively) groups (Figure 5.4D). Similarly at 4 weeks post injection, cMSC and iMSC treated groups had significantly smaller schisis area than untreated (p value .0001 and .003 respectively) and vehicle (p value .004 and .038 respectively). At 8 week time points, only the untreated group had significantly larger schisis area than iMSC (p value .005) and gmMSC (p value .016) treated groups. Among the treated groups, iMSC has showed better preservation than the gmMSC at all time points (although not significant).

5.3.3.4 Functional rescue

To assess the functional benefit of the therapy the scotopic b/a wave ratio was measured for all groups at all time points. As scotopic b-wave is drastically reduced in XLRS while the a-wave is relatively preserved in early stages of XLRS, a higher ratio indicates better functional response. The b/a wave ratios of scotopic ERG of the treated eyes (cMSC and iMSC) were compared at each time points with the untreated and control groups. At all time points the treated groups showed higher ratio than the other groups (Figure 5.5A). iMSC treated groups showed significantly higher response at 4 weeks compare to the untreated (p value .049) and vehicle (p value .012) groups. At 8 week time point iMSC ratio was significantly higher than all the four

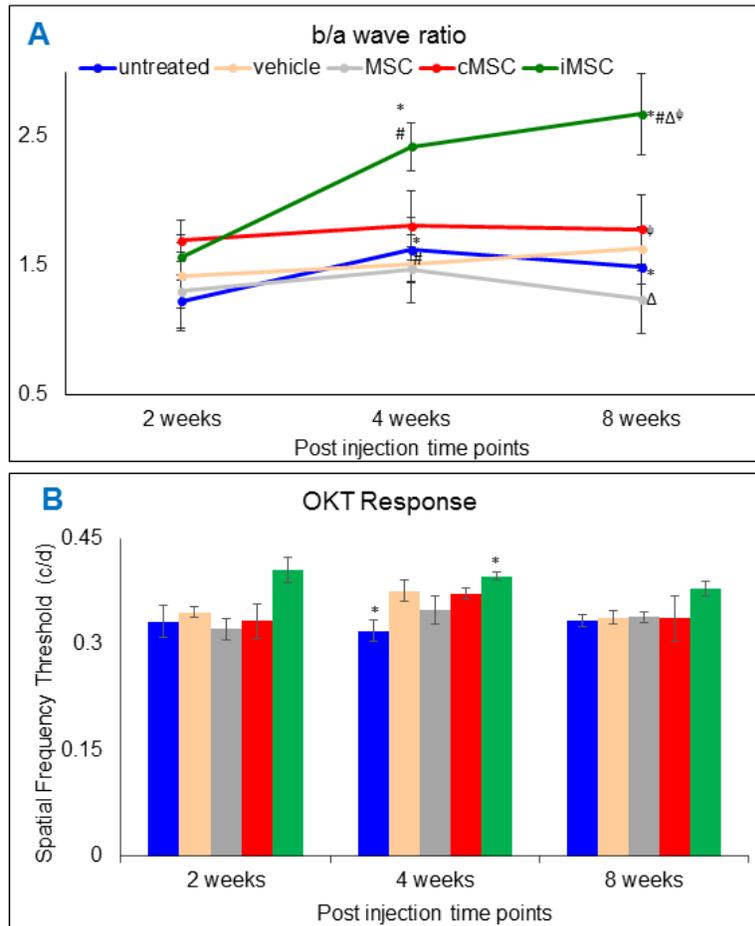


Figure 5.5: **Functional and behavioural benefits of the therapy.** (A) The scotopic ERG was recorded for all groups at 2, 4, and 8 weeks post injection time points. The mean b/a-wave ratio were compared among groups at each time points. (B) Spatial frequency threshold were measured and compared among groups at all time points. The mean of each group are shown. Data are presented as mean \pm standard error of the mean. Data are presented as mean \pm standard error of the mean. *, #, Δ and ψ imply statistically significant where $p > 0.05$. For all samples, $n=6$.

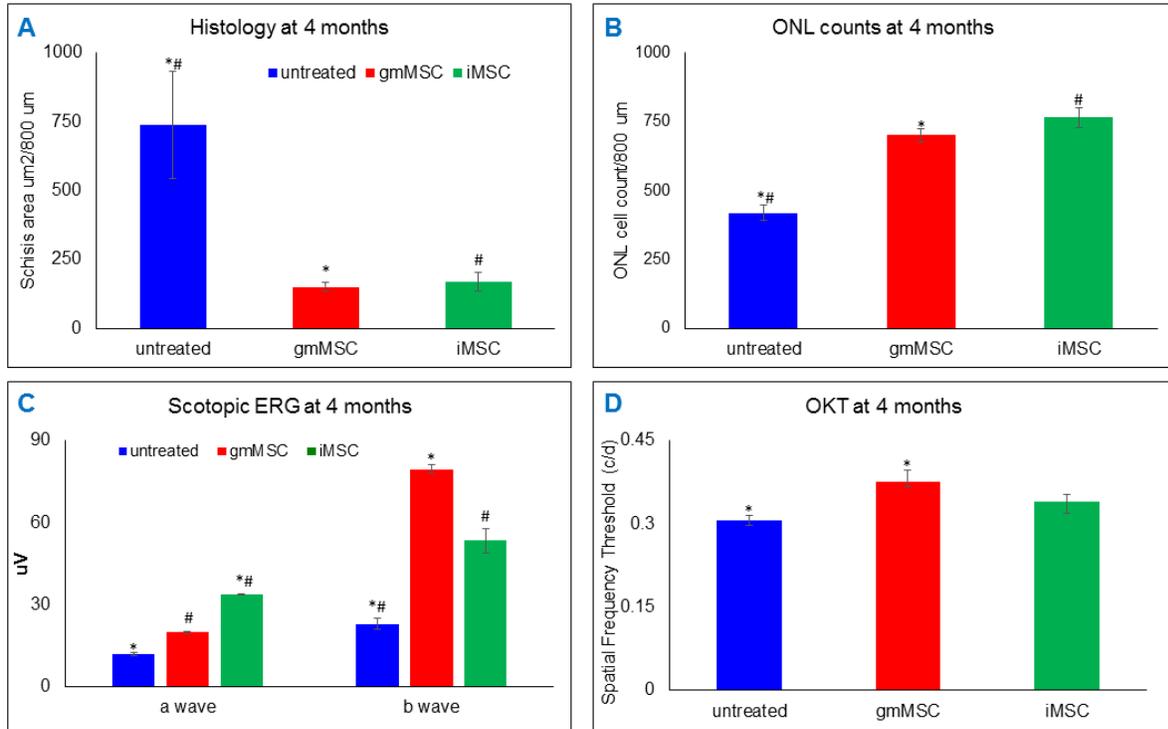


Figure 5.6: **Multiple injections study.** (A) Total schisis area from four quadrants were calculated and compared among three groups. (B) Number of photoreceptor cell (ONL) were counted and compared among groups. (C) a- and b-wave from scotopic ERG were compared separately among groups. (D) Visual acuity of the animals were observed by measuring the spatial frequency threshold. The mean of each group are shown. Data are presented as mean \pm standard error of the mean. * and # imply statistically significant where $p > 0.05$. For all samples, $n=6$.

groups (p value .006, .002, .002 and .014 respectively). Among the treated groups, iMSC showed higher ratio at 4 and 8 week time points over cMSC.

Visual behavioral response of the animals to treatment was also studied by assessing optokinetic tracking response (OKT) (Figure 5.5B). At all time points iMSC treated groups showed the best preserved spatial frequency thresholds (with significantly higher response at 4 weeks compare to the untreated, $p=0.001$). The highest response by iMSC group was recorded at 2 weeks post injection and from there it declined slightly with time. cMSC treated groups showed better response than other control groups only at the 4 week time point (Figure 5.5B).

5.3.4 Multiple injection

5.3.4.1 Structural benefits

The summation of the schisis cavities from all four quadrants (total 800um) were measured and compared among the three cohorts. Both the treated groups, cMSC ($p=.009$) and iMSC ($p=.015$), had significantly smaller schisis area at p120. However, there was no significant difference in schisis area between the two treated groups, although cMSC showed slightly smaller (not significant) cavities (Figure 5.6A). The number of outer nuclear layer (ONL) cells was also measured at those four quadrants and compared among cohorts. The treated cohorts have significantly ($p=.0001$ for both cMSC and iMSC) higher numbers of cells in the ONL compare to the untreated cohort (Figure 5.6B). Unlike schisis area, iMSC had higher number of ONL cells compare to cMSC group.

5.3.4.2 Functional response

The outer nuclear layer of the retina in RS1KO mouse model starts to get affected by the disease after p90 [44]. The a-wave values from ERG also start to change at this point as they are generated from the photoreceptors. As a result both a- and b- waves were measured and compared separately for multiple injection cohorts. Both cMSC ($p=.0001$) and iMSC ($p=.013$) showed higher b-wave response than the untreated cohorts. Between the groups, the cMSC group had a better response than iMSC, which is consistent with the finding from schisis area

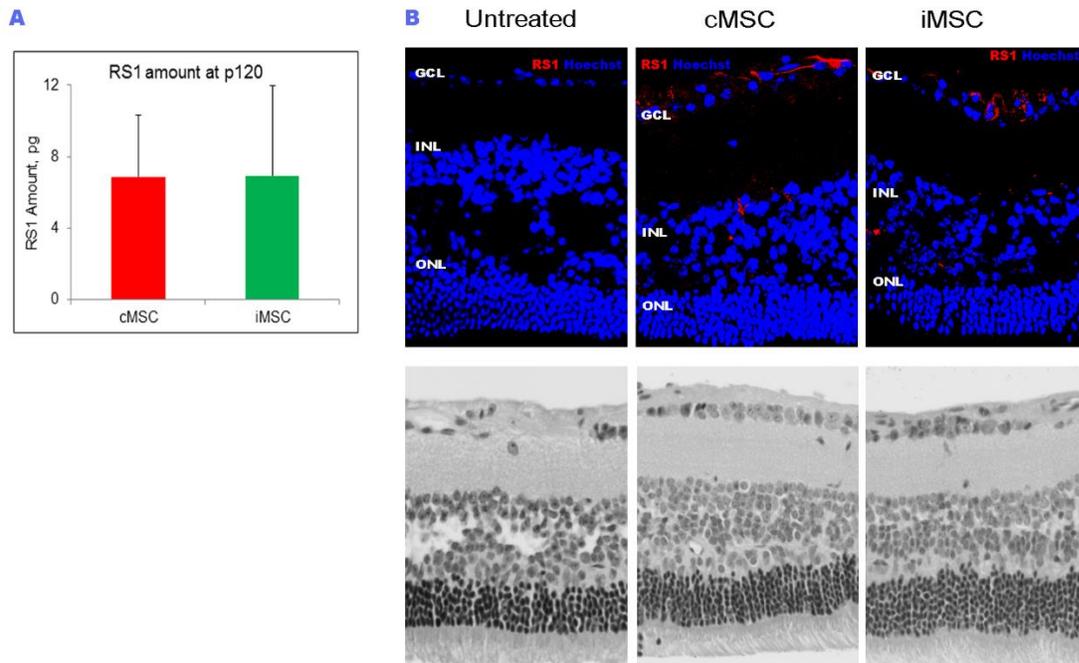


Figure 5.7: **RS1 amount and distribution.** (A) The amount of RS1 after multiple injection at p120 were measured and compared between the two treated groups. The mean of each group are shown with \pm standard error of the mean. (B) Migration of the injected cells and distribution of the RS1 were observed via immunohistochemistry. (C) Representative image of the inferior central quadrant of retina from each of three groups.

measurement. However, the iMSC group showed a significantly ($p=.02$) better scotopic a-wave response compared to the cMSC treated eyes (also compare to untreated, $p=.0001$) (Figure 5.6C). In terms of behavioral response, cMSC treated animals showed significantly ($p=.002$) better visual acuity compared to untreated ($p=.002$) and also the iMSC (not significant) treated ones (Figure 5.6D).

5.3.4.3 Cell migration and RS1 distribution

The migration and distribution of RS1 protein (by both cMSCs and iMSCs) was similar to that of the single injections. RS1 expression was observed through immunohistochemistry and ranged from ganglion cell layer to inner nuclear layer (Figure 5.7B). No RS1 expression was observed beyond outer plexiform layer.

5.4 Discussion:

XLRS is an inherited monogenic retinal degeneration with well characterized pathophysiology. These factors make it a promising candidate for *ex vivo* trials. Here we reported an adipose derived MSC based *ex vivo* gene therapy approach for the treatment of XLRS. Our approach showed that, in a knockout model of XLRS, a single intravitreal injection of genetically modified MSCs could achieve structural and functional benefits for up to 8 weeks post injection, whereas multiple monthly injections could retain that benefit for longer periods.

Mesenchymal stem cells (MSCs) were selected as a cell of choice source in this study because of their ability to provide paracrine benefits, as well as supplement the protein in question.

Mesenchymal stem cells have been extensively investigated and showed neuroprotective [340-342] and neurogenesis [343, 344] activity in a number of preclinical studies. In the study presented here, unmodified MSC treated groups did show some minor structural benefits over the vehicle and untreated groups, though not at a statistically significant level. There were, however, no significant functional or behavioral benefits. This might seem surprising since MSC therapy has been shown to be beneficial in other retinal dystrophies in the past [181, 304, 345]. This may demonstrate the special circumstances of the pathophysiology of XLRS where the

damaging structural effects of RS1 protein deficiency cannot be overcome by compensatory neuroprotective mechanisms [36]. We cannot therefore be certain that added paracrine effects would increase the benefits of supplying deficient RS1 with the cMSC and iMSC experiments, although other benefits of using MSCs over other cell types should be kept in mind. These include the ease with which they can be obtained, their low immunogenicity [345] and the fact that they are currently being used in some 389 clinical trials (which includes 4 studies in retinal degenerations) [<https://clinicaltrials.gov/>].

In any study where genetically modified, exogenous cells are injected into the eye, there is a risk of cells escaping the target site and expressing the therapeutic protein in other organs. It is also known that under certain conditions, MSCs can migrate and cross the blood-retina barrier [6]. Theoretically then, MSCs constitutively expressing RS1 could penetrate the blood vessels of the retina and lead to expression of RS1 in other organs with unknown consequences. In order to improve the safety of this *ex vivo* approach by anticipating such a scenario, we therefore also studied the effectiveness of an inducible iMSC cell line (in addition to the constitutively active cMSC cell line) that would only express RS1 in tissues exposed to doxycycline. Our results (showing that topical doxycycline can penetrate to the retina and trigger RS1 secretion) for the first time show that such an inducible *ex vivo* system is possible in the retina [181, 346, 347]. The topical application of the doxycycline to the eye significantly restricts doxycycline exposure in other organs and so prevent expression of RS1 from any iMSCs that might have made their way out of the eye. Interestingly, our results suggest that applying a higher concentration of topical doxycycline does not result in higher amounts of RS1 expression *in vivo* suggesting a plateau effect (Figure 5.2).

Delivery of cell-based therapy to the retina has focused on a subretinal injection technique in a number of previous works. This has been in response to other studies that show that most cell types, MSCs included, do not penetrate into the retina when delivered intravitreally [304] and also subretinal route results in migration of more cells in the photoreceptor layer [348]. In contrast, others have documented the capability of MSCs to penetrate the retina after intravitreal delivery [180, 349, 350]. Some studies have found that subretinal delivery can be associated with

significant local tissue damage [297]. In this study, it was observed that with intravitreal delivery, cells were able to reach different layers of retina, ranging from ganglion cell layer (GCL) to the outer nuclear layer, thus negating the need for subretinal delivery. Even though MSCs were not seen to penetrate the outer nuclear layer in large numbers, it could be assumed that since RS1 is secreted it would diffuse into deeper layers even if genetically modified MSC were mainly restricted to the inner retina [339, 351].

The migration of the two RS1 expressing cell lines in the retina resulted in structural restoration of the RS1KO mouse retina after single injection. One of the principal clinical pathologies of XLRS is the vitreo-retinal dystrophy characterized by formation of cystic cavities in the inner retinal layers [39]. The knock out mouse model also displays a similar phenotype as human patients [339]. However, unlike humans, the number and size of these cavities reduce and become absent as these animals age [45]. Similar observation was made in this study, where the size of the schisis cavities declined over time in all five groups. The treated groups had significantly smaller schisis cavities (overall) compared to other groups, with iMSC showing better results than cMSC, in all three post injection time points (Figure 5.3C). This group (iMSC) also has smallest schisis cavities when individual quadrants of retina were compared among the groups at different time points.

Another hallmark diagnostic feature of human XLRS is an “electronegative ERG” which corresponds to a decreased b-wave response with a relatively preserved a-wave amplitude in a dark adapted electroretinogram (scotopic ERG). This is mainly caused by defects in the synaptic transmission due to disorganized photoreceptor-bipolar synapse. As mentioned earlier, the RS1KO mouse model shows similar pathological features as human XLRS patients and its ERG response is similar as well. To track functional rescue, scotopic b/a wave ratio was measured for the single injection study. The b/a wave ratio normalize the photoreceptor response to the second order neuronal response (bipolar cells, horizontal cells etc.). The b/a wave ratio of the iMSC injected group was significantly higher than all others at the 8 weeks time point; at 4 weeks, it was significantly greater than the untreated and vehicle groups. The response by this group was not just better than the others, it also improved over time. An analogous pattern of

response was observed in case of OKT, where the iMSC treated animals has increased visual acuity at all time points compare to other groups. The cMSC treated group showed better response than control groups in ERG (not significant) but did not produce similar result in OKT.

Both structural and functional data indicated a better response from treatment with iMSC than with cMSC. To better understand the reason behind this we measured the amount of RS1 produced by these two lines in the eye at all time points (Figure 5.2B). iMSC had higher expression at all times. This might be because of the CMV promoter driven expression of the protein in the constitutive system which could bottleneck the process. In case of inducible cassette, it is controlled by the activation of the Tet response element (TRE) which is dependent on the amount of Dox in the system. The expression profile of RS1 by the two cell lines also reveals a declining pattern over time. The reason for that might be the decrease in the amount of MSCs over time. It has been shown that MSCs survive in the eye for 6-8 weeks after intravitreal delivery [11]. Therefore, at 8 weeks time point there were fewer cells than at 2 weeks which resulted in lower amount of retinoschisin secretion.

A multiple injections study was performed to attain a more constant long-lasting supply of RS1 to the retina, where animals received 1 injection per month for 3 months, and the results were analysed at p120. In this study, only cMSC and iMSC groups were compared to an untreated group (excluding vehicle and adMSC). Treated groups had significantly smaller schisis cavities than the untreated. However, unlike in the single injection study, cMSC showed slightly smaller cavities than the iMSC group. At this stage (p90), the photoreceptor layer also starts to degenerate, hence the outer nuclear cell number (ONL) was counted and ERG a- and b- waves were analysed separately. The iMSC group has higher cell counts (Figure 5.6B) than cMSC which correlates with the higher ERG a-wave response (Figure 5.6C). On the other hand, higher b-wave response was correlated with smaller schisis cavities for the cMSC group. This group also showed a better response in OKT for the multiple injection study. When the RS1 amount was measured, a significant difference was not observed. The presence of Dox might contribute to the higher number of ONL cells, and better a-wave response. A recent study showed that oral Dox monohydrate can improve some functionality of the eye of patients with diabetic

retinopathy, when compared to placebo [352]. However, a detailed mechanism of how Dox can improve the pathophysiology of retinal degenerations has yet to be described.

Three different groups reported an *in vivo* gene therapy approach in the retinoschisin knock-out mouse model [20, 21, 23, 45, 339, 351, 353]. Three of them used viral vectors and a knockout mouse model, although viral serotype, mode of injection and promoter sequence were different. All three groups showed structural preservation and functional rescue (normal b-wave) in both the short and long term. As viral vectors were able to transfect a large number of cells from a single injection, RS1 expression was found from ganglion cells to the outer segment of the photoreceptors. However, one potential problem is that the disease models in these studies were not completely comparable to the human condition, as the mice were knockout models with no retinoschisin gene expression, whereas some human patients express mutant protein which may interfere with the endogenously expressed retinoschisin. Two studies showed that for some of the most commonly occurring disease mutations, exogenous wild type RS1 secretion was not affected by the ectopic expression of the mutant protein [354, 355]. However some of the mutated form co-assembled with the wild type polypeptide and failed to secrete from the cell. This type of retention and accumulation could lead to stress in the cell [355]. Highly metabolically active cells like photoreceptors might also behave in a different way in response to the accumulated protein than the cell lines that were used in these studies. Moreover, a recent study showed that neutralizing factor against AAV vector can be present in the vitreous which can limit the efficiency of *in vivo* gene therapy [356]. An *ex vivo* approach also has room for improvement. For instances, to maintain a steady and therapeutically relevant amount of the RS1 protein, repeated injections are required. Higher numbers of the intraocular injection could potentially increase the chance of collateral damage in the eye. A systemic delivery of the cells with a targeting mechanism to the eye can reduce that risk [11]. Another issue is the knockout mouse model, which does not represent the human disease physiology completely. But this is the only experimental model available to study the potential interventions.

The inducible *ex vivo* approach can also be applied to other similar disorders where deficiency of an extracellular protein functionality is the major cause of the disease. Sorsby's fundus dystrophy

is such a disease where a non-functional extracellular tissue inhibitor of metalloproteinases 3 (*TMP3*) could disrupt the controlled degradation of the ECM around the blood vessels in the retina [357]. This disruption leads to excessive degradation of the ECM and retinal neovascularization. A sub retinal delivery of the genetically modified MSCs expressing *Tmp3* protein has the potential to reverse the disease condition. Other multifactorial diseases can also be targeted with the inducible *ex vivo* approach. Guan Y et al showed that Dox inducible expression of erythropoietin from rat MSCs can protect and rescue RPE and retinal neurons after sodium iodide induced AMD-like (Age related macular degeneration) retinal degenerations [181].

In summary, this study indicates that a mesenchymal stem cell based *ex vivo* gene therapy approach for treatment of X-linked retinoschisis can prevent the disease conditions and restore the structure and function of the eye. The results collectively point to the potential that *ex vivo* gene therapy holds as a future therapy for XLRS without using any viral vector. Future investigation can focus on determining the appropriate transgene expression system that will provide lasting effect of the treatment with a minimally invasive procedure.

Chapter 6. Conclusion

Blindness and vision incapacitation due to retinal degeneration affect millions of people worldwide. The relevance of vision loss both in a social and economic perspective is significant. An intervention, targeting at preventing vision loss as well as restoring vision, is therefore well sought after. Stem cell based approaches, which promise both prevention and regeneration are therefore attracting attention in the field of therapeutic research. The clinical trials for stem cell based cellular therapy for eye disorders have already begun, for instance, embryonic stem cells (ESCs) derived RPE has been used in the recent clinical trials for retinal degenerations. ESCs had been studied widely; however, ethical issues associated with them have limited their use. Somatic stem cells such as Mesenchymal stem cells, which are multipotent, of low immunogenicity and easily harvested from several sources, have multiple advantages over ESCs [345]. To date, MSCs have been extensively investigated and showed neuroprotective [340-342] and neurogenesis [343, 344] activity in a number of preclinical studies. Currently there are 389 clinical trials that have been launched with mesenchymal stem cells and of them 4 studies have used MSCs for the treatment of retinal degenerations. Here, MSCs have been used for providing neuroprotection in a model of retinitis pigmentosa through their paracrine activity and in a second model of X-linked retinoschisis as a vector for *ex vivo* gene therapy.

Based on the prior observation that, magnetically guided systemic delivery of mesenchymal stem cells resulted in higher concentration of neuroprotective factors in the targeted area of retina, I hypothesized that these MSCs will also provide structural and functional benefits in a rat model of retinitis pigmentosa. After performing experiment with proper controls, I found that magnetic nanoparticle labeled MSCs resulted in better preservation of photoreceptor cells when guided through magnetic targeting. The treated group also showed better response in electroretinography and optokinetic tracking response, when compared to vehicle treated group. This established the concept that magnetic targeting results in higher number of cells in the retina which provide better neuroprotection by secreting more neurotrophic factors.

The effect of magnetic nanoparticle (MNPs) on the cells and the targeted organ has been studied extensively. Here, I also investigated the effect of MNPs on MSC secretion profile. The secretome of MSCs is one of the most important features because it is this paracrine activity that was used in this study to provide neuroprotection. My results indicated some inhibitory effects on secretion of paracrine molecules followed by exposure to increasing concentrations of MNPs. However, recommending that lower dosages of MNPs be used in future studies may not always be necessary. For instance, the results demonstrated GDNF inhibition by increasing dosage, but no such effect on CNTF. Therefore, when the intended effect of MSC therapy would be through CNTF secretion then higher MNP dosage might have no deleterious effect.

My second hypothesis was that MSCs can be used as a cell source for an *ex vivo* gene therapy approach to provide neuroprotection in an experimental model of X-linked retinoschisis. The experimental results indicate that a single intravitreal injection of genetically modified MSCs achieved structural and functional benefits for up to 8 weeks post injection, whereas multiple monthly injections retained that benefit for longer periods. To restrict the expression of the therapeutic protein (retinoschisin, RS1), an inducible expression cassette was used which ensures the expression only when the induction (Doxycycline) is present. Whether the added step of inducing the expression of the RS1 impede the effectiveness of the treatment, the inducible MSCs were compared with a constitutive system for both single and multiple injection. The inducible system showed better results than the constitutive system with higher amount of RS1 expression. Both expression systems provided strong evidence that a mesenchymal stem cell based *ex vivo* gene therapy approach can prevent the disease conditions and restore the structure and function of the eye in a XLRS model. Although comparable viral vector based *in vivo* gene therapy studies showed better neuroprotection and structural preservation, further investigations need to be done to determine the safety profile of the two approaches.

X linked retinoschisis is not a very common inherited disorder. Spending vital resources on investigating rare disease might raise concerns when cures for common ones are also lacking. There are many reasons to justify that concern; first, XLRS patients are no less deserving of a cure than patients with common diseases. Second, research on rare disease can benefit not only

that specific disease or population, but also pave the way to therapies for other diseases. The success of gene therapy trial on Leber Congenital Amaurosis (LCA2) inspired similar work on choroideremia, Leber Hereditary Optic Neuropathy, and Hemophilia B. The *ex vivo* approach that we used here can also be applied for Sorsby's Fundus Dystrophy, which also caused by haplo-insufficiency of an extracellular protein. Then, research on rare diseases are often applicable to more prevalent disorders, as they provides fundamental mechanisms of human biology and pathological conditions. Lastly, this type of research can accumulate knowledge about the aetiology, pathophysiology and the natural history of the diseases. This would help genetic counsellor, healthcare practitioner and the patients to manage the disease better.

One major issue faced in both of the studies was to retain therapeutic cells in the retina for a longer time. Multiple delivery of intraocular or intravenous cells (with magnetic targeting) could resolve this issue. Both of delivery routes, however, require significant clinical set up and preparation. There are two potential solutions for this problem: one is to keep MSCs alive in the retinal microenvironment and, if possible, multiply. A second potential solution is to differentiate MSCs to retinal cell types. A number of studies showed that MSCs do not persist in the retina for a longer period of time (6-8 weeks). To analyze what makes MSCs survive longer in *in vivo* system, we need to know in which organs MSCs (exogenous or endogenous) survive longer, what are the cues in those organ/system that are keeping them viable, which signaling pathways they employ and lastly, how we can mimic the same environment or signaling in the retina. Concerning multiplication and differentiation, there are contradicting reports indicating both MSCs ability and inability to divide and differentiate in the retina. Successful reprogramming to retinal progenitor cells or photoreceptor like cells from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) in *in vitro* setup have been reported [358-360]. But no such studies have been reported for MSCs. Although there are some studies that reprogrammed MSCs into neuronal like cells, researchers have yet to reprogram MSCs to retinal progenitor cells. Even if MSCs can be reprogrammed to differentiate to retinal progenitor like cells, the integration process, after delivery, in the adult retinal circuit needs to be investigated.

Diseases of retina in general represent the leading cause of visual impairment and blindness in the western world. So far, the treatments for most of these disorders are limited. My work showed mesenchymal stem cells based two very potential therapeutic approaches that provided neuroprotection in two such disease models. Although these interventions may not cure the conditions, they can significantly delay the progression of the diseases. The greatest value of these approaches is their highly transferable physiological mechanisms and molecular techniques, which can be used to develop therapies for other diseases of similar mechanism in the eye as well as other organs with great potential.

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