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RETINAL TUMORIGENESIS AND NEURODEGENERATION: STRATEGIES TO PROMOTE TUMOR CELL DEATH AND SUPPORT RETINAL CELL SURVIVAL TO PRESERVE VISION

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Retinal Tumorigenesis and Neurodegeneration: Strategies to Promote Tumor Cell Death and Support Retinal Cell Survival to Preserve Vision

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To my family and friends

"The only thing worse than being blind is having sight but no vision"
- Helen Keller

ABSTRACT

Our vision is central to our quality of life enabling us to interact with our environment and lead productive lives. The ability to see begins in the retina, one of the paramount features of the eye, as it takes on the task of converting light signals to nerve impulses that are relayed to the visual centers of the brain. Proper functioning of the retina is essential for our visual experience. In this thesis, two different aspects of retinal dysfunction are highlighted together with potential therapeutic interventions. One of these is the childhood cancer retinoblastoma, which is characterized by uncontrolled cell proliferation after the loss of the *RB1* gene. And, the other is retinal neurodegeneration which is characterized by the dysfunction and death of the retinal cells. We targeted the pyrimidine ribonucleotide synthesis pathway as a possible strategy to prevent retinoblastoma cell growth and to promote cell death. In the case of retinal neurodegeneration, we explored the neuroprotective effect of human neural progenitor cell (hNPC) derived neurotrophic factors to support retinal cell survival.

The rationale to target pyrimidine ribonucleotide synthesis as a strategy to treat retinoblastoma cells was based on our findings in **paper I**. In this paper we discovered a novel dihydroorotate dehydrogenase (DHODH) inhibitor after screening a library of compounds for their ability to increase p53 transcription factor activity in two reporter cell lines, the ARN8 melanoma cell line and the T22 mouse fibroblasts. Amongst the hit compounds, we selected compound HZ00 because it was more active on the ARN8 cells than on the T22 fibroblasts and due to its favorable medicinal chemistry properties. Regarding the mechanism of action of HZ00 on the p53 pathway, we observed that it was able to induce p53 synthesis without affecting p53 mRNA levels. Moreover, HZ00 was able to kill cancer cells and also synergize with an inhibitor of p53 degradation both *in vitro* and *in vivo*. During our studies on the identification of the target for HZ00, we observed that short treatment times with HZ00 caused ARN8 cells to accumulate in S phase and long treatments led to an increase in the proportion of cells in SubG1. Moreover, the supplementation with an excess of uridine was able to prevent the cell death effect. Based on these and other facts, we eventually narrowed down the target for HZ00 to DHODH, and confirmed this finding with enzymatic assays using purified DHODH. A further search for more potent analogues of HZ00 led to the identification of HZ05. HZ05 was able to accumulate a number of different cancer cells in S phase, which was followed by increase in SubG1 levels. However, the U2OS cell line had a higher propensity to accumulate in S phase. Further analysis showed an increase in p53 expression in the S phase cells. When we pretreated the U2OS cells with HZ05 followed by nutlin-3a, cell death was observed. This indicated that DHODH inhibitors could sensitize cancer cells to p53 degradation inhibitors by accumulating them in S phase with high p53 levels.

Retinoblastoma is typically a *TP53* wild-type tumor characterized by inactivation of the *RB1* gene. This deficiency leads to the loss of the cell cycle's major G1-S checkpoint protein Rb. In **paper I**, we described that DHODH inhibitors can activate p53 and also cause cancer cells to accumulate in S phase, and that this is eventually followed by death. Since retinoblastoma

cells already have a dysfunctional G1-S checkpoint, the use of DHODH inhibitors seemed like a rational approach to promote retinoblastoma cell death. In **paper II**, we investigated the potential of the DHODH inhibitor brequinar to reduce retinoblastoma cell growth and promote cell death, both as a single agent and in combination with a nucleoside transport inhibitor, dipyridamole. Similar to the effects seen in paper I, we saw that brequinar as a single agent was able to accumulate retinoblastoma cells in S phase and to an extent, also cause an increase in cell death. However, the response was slow and required a treatment time of 6 days. When we treated the retinoblastoma cells with a combination of brequinar and dipyridamole, the cells responded with an S phase accumulation as early as 24 hours after treatment, with most of them driven towards cell death with increasing time. This synergistic effect was also seen with other DHODH and nucleoside transport inhibitors. Moreover, the combination treatment was effective in the presence of uridine at physiologic plasma concentrations. Further investigation showed activation of caspases 3 and 7 as well as an increased expression of cleaved PARP-1, indicating the onset of apoptosis. Additionally, the treatment of a p53 mutant retinoblastoma cell line also responded to brequinar and the combination treatment, suggesting that targeting pyrimidine ribonucleotide synthesis could be an attractive strategy to eliminate both p53 wild-type and p53 mutant retinoblastoma cells.

In **paper III** and **IV**, we investigated the neurodegenerative events in an *in vivo* and *in vitro* model of retinal neurodegeneration with an emphasis on photoreceptor degeneration, second order neuron remodeling and glia reactivity. Furthermore, in **paper IV** we assessed the neuroprotective potential of hNPC derived neurotrophic factors in porcine retinal explant cultures. In **paper III**, the *in vivo* pdgf-b$^{ret/ret}$ mouse model showed severe vascular defects due to the detachment of pericytes from the vascular endothelium. Degenerative events were followed on postnatal day (P) 7, 10, 15 and 28. These events were quite evident at P15 and worsened by P28, and included photoreceptor cell death, shortening of the cone outer segments and synaptic disassembly in the outer plexiform layer (OPL). Rod bipolar cells underwent remodeling and the Müller cells showed increased expression of GFAP (glial fibrillary acidic protein). The microglia also changed to their reactive amoeboid-like phenotype. For the *in vitro* porcine retinal explant model in **paper IV**, photoreceptor death increased significantly by 3 days *in vitro*. This was associated to the loss of the cone outer segments, mislocalization of opsin and synaptic disassembly in the OPL. Furthermore, we observed a loss and remodeling of horizontal cells, as well as severe gliosis of the Müller cells. The hNPC cocultured explants were observed to maintain photoreceptor survival through preservation of the cone outer segments, better opsin trafficking and retaining synaptic integrity. However, Müller cell gliosis was only alleviated by a decreased density of GFAP immunoreactive Müller cells. Both the *in vivo* and *in vitro* model of neurodegeneration demonstrated the vulnerability of photoreceptors to different mechanisms of retinal injury. The hNPC derived neurotrophic factors had the potential to preserve photoreceptors in the porcine retinal explants, but were not able to completely eliminate Müller cell gliosis.

PREFACE

The retina is an amazing tissue that gives us the ability to see the world around us. It consists of one of the most complex yet extremely organized network of neurons and glia that allow us to see in light, dark, depth and motion throughout our lifetime. Looking into the proliferative side and the degenerative side of retinal dysfunction has made me highly appreciate the sensitive balance between the life and death of these neurons and the great impact they place in our ability to see. It is reassuring to know that there are many dedicated researchers who are always on an endless mission to find novel and effective therapies to treat such maladies. Having been part of a drug discovery project, I can understand all the hard work and painstaking efforts it takes for a team of researchers to identify a potential molecule or form of therapy that could one day eventually be used to improve the lives of patients.

Having said this, I would like to highlight that thesis is a continuation of the licentiate thesis which was completed in 2016, but was not published. In the Swedish academic system, a licentiate thesis can be done half-way through a doctoral program and is similar in structure to the doctoral thesis. Major parts of the licentiate thesis have been included in the current thesis.

LIST OF SCIENTIFIC PAPERS

I. Marcus J. G. W. Ladds*, Ingeborg M. M. van Leeuwen*, Catherine J. Drummond*, Su Chu, Alan R. Healy, Gergana Popova, Andrés Pastor Fernández, **Tanzina Mollick**, Suhas Darekar, Saikiran K. Sedimbi, Marta Nekulova, Marijke C. C. Sachweh, Johanna Campbell, Maureen Higgins, Chloe Tuck, Mihaela Popa, Mireia Mayoral Safont, Pascal Gelebart, Zinayida Fandalyuk, Alastair M. Thompson, Richard Svensson, Anna-Lena Gustavsson, Lars Johansson, Katarina Färnegårdh, Ulrika Yngve, Aljona Saleh, Martin Haraldsson, Agathe C. A. D'Hollander, Marcela Franco, Yan Zhao, Maria Håkansson, Björn Walse, Karin Larsson, Emma M. Peat, Vicent Pelechano, John Lunec, Borivoj Vojtesek, Mar Carmena, William C. Earnshaw, Anna R. McCarthy, Nicholas J. Westwood, Marie Arsenian-Henriksson, David P. Lane, Ravi Bhatia, Emmet McCormack, Sonia Laín. A DHODH inhibitor increases p53 synthesis and enhances tumor cell killing by p53 degradation blockage. Nature Communications 9, 1107 (2018).
   * Authors contributed equally

II. **Tanzina Mollick**, Saikiran Sedimbi, David Lane, Sonia Laín. Targeting pyrimidine ribonucleotide synthesis in retinoblastoma inhibits cancer cell growth and promotes cell death. Manuscript.

III. Guillem Genové, **Tanzina Mollick**, Kjell Johansson. Photoreceptor degeneration, structural remodeling and glial activation: a morphological study on a genetic mouse model for pericyte deficiency. Neuroscience 279, 269-284 (2014).

IV. **Tanzina Mollick***, Camilla Mohlin*, Kjell Johansson. Human neural progenitor cells decrease photoreceptor degeneration, normalize opsin distribution and support synapse structure in cultured porcine retina. Brain Research 1646, 522-534 (2016).
   * Authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

α-sma	alpha smooth muscle actin
ALL1	leukemia, acute lymphocytic, susceptibility to, 1
APC	adenomatosis polyposis coli
ATM	ataxia telangiectasia mutant
ATR	ataxia telangiectasia and rad3-related protein
BAX	bcl-2-associated X protein
BCL-2	b-cell lymphoma 2
Bcr-Abl	breakpoint cluster region protein – Abelson murine leukemia viral oncogene homologue 1
bFGF	basic fibroblast growth factor
BH3	bcl-2 homology region 3
BRCA1	breast Cancer Type 1 Susceptibility Protein
BRCA2	breast Cancer Type 2 Susceptibility Protein
B-Raf	v-raf murine sarcoma viral oncogene homolog B
BrdU	bromodeoxyuridine
CAD	carbamoylphosphate synthetase II, aspartate transcarbamylase and dihydroorotase
CAR-T	chimeric antigen receptors
cc-3	cleaved caspase-3
Cdc6	cell division cycle 6
CDH11	cadherin-11
CDK	cyclin-dependent kinase
CDK1	cyclin-dependent kinase 1
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
C/EBP	CCAAT enhancer binding protein alpha
CETSA	cellular thermal shift assay
cGMP	cyclic guanosine monophosphate
Chk1	checkpoint kinase 1

Cho	choroid
CNT1	concentrative nucleoside transporter 1
CNT2	concentrative nucleoside transporter 2
CNT3	concentrative nucleoside transporter 3
DEK	DNA-binding proto-oncogene
DHODH	dihydroorotate dehydrogenase
E2F	E2 factor
E2F3	E2F transcription factor 3
EBRT	external beam radiotherapy
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENT1	equilibrative nucleoside transporter 1
ENT2	equilibrative nucleoside transporter 2
ENT3	equilibrative nucleoside transporter 3
ENT4	equilibrative nucleoside transporter 4
FMN	flavin mononucleotide
ER	endoplasmic reticulum
FGF	fibroblast growth factor
FGF-2	basic fibroblast growth factor
GADD45	growth arrest and DNA damage inducible protein 45
GCL	ganglion cell layer
G-CSF	granulocyte colony-stimulating factor
GFAP	glial fibrillary acidic protein
HDAC	histone deacetylase
HDM2	human double minute 2
HDMX	human double minute 4
hNPC	human neural progenitor cell
HPV	human papillomavirus
Iba1	ionized calcium binding adapter molecule 1
IB4	isolectin B4
ID2	inhibitor of DNA binding protein 2

IGF-I	insulin-like growth factor I
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
INL	inner nuclear layer
IPL	inner plexiform layer
KIF14	kinesin family member 14
MCP-1	monocyte chemotactic protein 1
MDM2	murine double minute 2
MDM4	murine double minute 4
MDMX	murine double minute 4
Myc	v-myc avian myelocytomatosis viral oncogene homolog
NFL	nerve fiber layer
NG2	neuron-glial antigen 2
NMYC	neuroblastoma-derived v-myc avian myelocytomatosis viral oncogene homolog
NOD	non-obese diabetic mice
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
Nr	nervous
ON	optic nerve
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segment
P	postnatal day
$P14^{ARF}$	ARF tumor suppressor
P16	cyclin dependent kinase inhibitor 2A
$p21^{Waf1/Cif1}$	cyclin dependent kinase inhibitor 1
p27	cyclin dependent kinase inhibitor 1B
p107	retinoblastoma-like protein 1
p130	retinoblastoma-like protein 2

PCNA	proliferating cell nuclear antigen
PAIN	pan-assay interference compound
PALA	phosphonacetyl-l-aspartate
PARP-1	poly (ADP-ribose) polymerase-1
PDGF	platelet-derived growth factor
PDGF-B	platelet-derived growth factor B
PDGFR-β	platelet-derived growth factor receptor beta
pdgf-b$^{ret/ret}$	platelet-derived growth factor b retention motif knockout
PEDF	pigment-epithelium derived factor
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKCα	protein kinase C alpha
PNA	peanut agglutinin
PRPP	5-phosphoribosyl-1-pyrophosphate
PSD-95	postsynaptic density protein 95
PUMA	p53 upregulated modulator of apoptosis
RAS	rat sarcoma viral oncogene homolog
Rb	retinoblastoma protein
RB1	RB transcriptional corepressor 1
Rb2	retinoblastoma-like protein 2
RBL2	RB transcriptional corepressor like 2
Rd	retinal degeneration
Rho	rhodopsin
RPE	retinal pigment epithelium
SCID	severe combined immunodeficiency
SP1	specificity protein 1
SRB	sulforhodamine B
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
TIMP-1	matrix metalloproteinase-1
TNF-α	tumor necrosis factor alpha

TP53	tumor protein p53
TSPO	translocator protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UMP	uridine monophosphate
UMPS	uridine monophosphate synthetase
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 THE RETINA

Sight is one of our most precious senses that helps us to perceive and interact with the environment around us. Our ability to see begins in our eyes where incoming light is focused on the retina, a thin neurosensory tissue that lines the back of the eye (1-3) (Fig 1A). The retina is part of the central nervous system and is responsible for the visual processing of photons to nerve impulses that are ultimately sent to the vision centers of the brain for interpretation. The retina achieves this task through the collective effort of its multiple neurons and glial cells that are morphologically arranged in a highly organized fashion. The remarkable complexity and efficiency of this neuronal circuitry together with its integration to the brain gives us our visual experience in the form of color, shade, motion and depth (2-4).

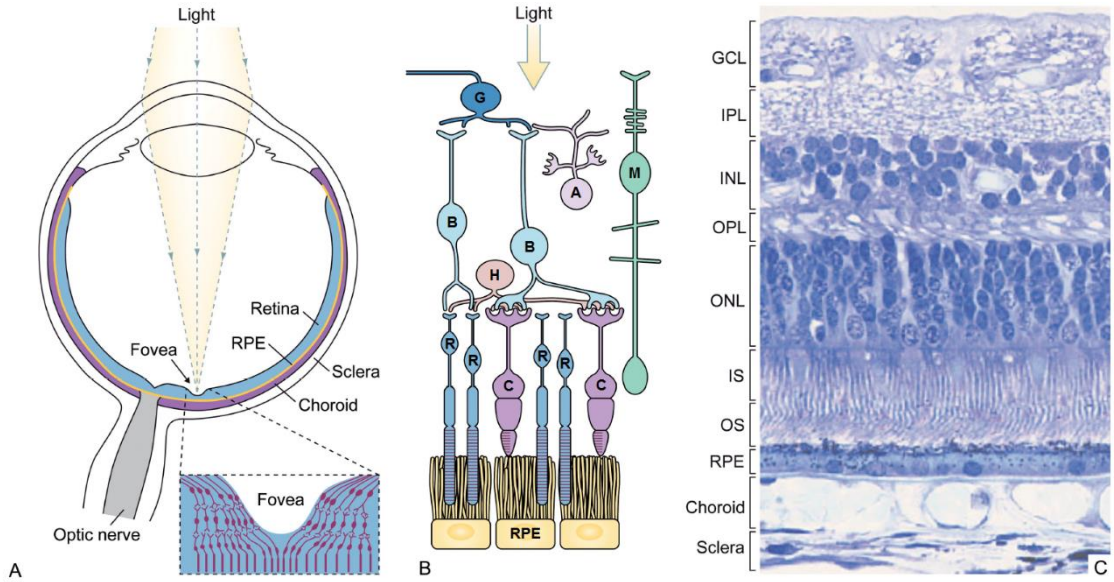

Figure 1. The human visual sense organ. (A) Diagram of the eye; an enlarged diagram of the fovea is shown in the box. The retina forms the inner lining of the most posterior part of the eye. The RPE is sandwiched between the retina and choroid, a vascularized and pigmented connective tissue. (B) Diagram of the organization of retinal cells. R, rod; C, cone; B, bipolar cell; H, horizontal cell; A, amacrine cell; G, ganglion cells; M, Müller cell; RPE, retinal pigment epithelium (C) A Richardson's methylene blue/azure II stained transverse section of human retina. The retina has laminated layers which include the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL), which are separated by the outer plexiform layer (OPL) and inner plexiform layer (IPL). Adjacent to the RPE lie the photoreceptor outer segments (OS), while the photoreceptor inner segments (IS) lie close to the ONL (4). Reproduced with permission from Rockefeller University Press all copyright retained (2010).

1.1.1 Structural organization of the retina

The vertebrate retina consists of a distinct laminated cytoarchitecture that is divided into neuronal and plexiform layers that contain the neuron cell bodies and synapses respectively (2-4) (Fig 1B and C). The neurons present in these different layers will be discussed in more detail in the next section. The first set of neurons in the outermost region of the retina are the light-sensitive photoreceptor cells. These include the rods, which mediate vision in dim light,

and the cones that allow us to see color in bright light. The cell bodies of the photoreceptors form the outer nuclear layer (ONL) of the retina, and their axon terminals form synapses with the bipolar cell dendrites and the horizontal cell processes. This synaptic layer is referred to as the outer plexiform layer (OPL). The OPL is followed by the inner nuclear layer (INL) that consists of the cell bodies of the second order neurons which include the bipolar, horizontal and amacrine cells. Signals are transmitted from the bipolar and amacrine cells through synapses with ganglion cell dendrites present in the inner plexiform layer (IPL). The ganglion cell bodies form the ganglion cell layer (GCL) and their axons collectively form the nerve fiber layer (NFL) which converge and exit the eye as the optic nerve (2-4).

Considering retinal topography, the retina can be broadly divided into a central and peripheral region (1-4). The central part of the retina comprises of a circular region known as the macula lutea which is located about 3.5 mm temporal to the optic disc and is approximately 6 mm in diameter (2, 5). The macula lutea (Latin for 'yellow spot') is so called because of its yellowish hue which occurs due to the presence of the xanthophyll pigments lutein and zeaxanthin (1, 2, 5). These pigments are known to filter light of short wavelength (3, 5-7) and also serve as antioxidants (2, 3, 5, 7). Such properties help to decrease light scatter and prevent retinal damage due to photo-oxidation (2, 5-7). The centermost region of the macula contains an important anatomical feature called the fovea that is demarcated by a gentle depression in the retina and is 700 µm in diameter (1, 2, 4) (Fig 1A). Structural modifications in the fovea make it specially designed for high visual acuity. The cone photoreceptors are present in this region at one of the highest densities, reaching about 199,000 to 300,000/mm^2 (1, 2, 8). The depression in the structure is attributed to the lateral shifting of the inner regions of the retina such that light can directly reach the cones without interference from the other retinal layers. The absence of retinal vasculature in this region also directly exposes the cones to incident light. Additionally, the ratio of cones to ganglions cells in the fovea reaches 1:1, allowing us to see color images in great detail (1, 2, 4, 5).

The peripheral retina starts at the boundary of the central retina and stretches to the ora serrata which marks the beginning of the ciliary body (1, 2, 5). This area of the retina is mainly responsible for our peripheral vision, which is more sensitive to movement detection rather than image resolution. This means that images appear less clear but the slightest change in motion is easily detected and usually attracts our eyes or head towards the motion. The cone density decreases the farther away we deviate from the fovea and the photoreceptor population becomes more rod dominant in the peripheral retina where rods outnumber cones 20:1. The convergence from photoreceptors to ganglion cells is much higher in this region, all of which leads to greater spatial summation rather than spatial resolution (2, 5).

It should be noted that the structure of the vertebrate retina can vary significantly between different species, and emphasis has been placed on the human retina in this thesis.

1.1.2 Cellular components of the retina and the visual pathway

The major cellular components of the retina include the neurons, glial cells and retinal pigment epithelium (RPE). Among the neurons, the photoreceptors, bipolar cells and ganglion cells are responsible for the phototransduction of light while the horizontal and amacrine cells help in the modulation of neural signals. The glial cells provide support for the efficient functioning of these neurons and include the Müller cells, microglia and astrocytes. The RPE is a dark pigmented layer of epithelial cells that lies adjacent to the photoreceptors. It is known to perform a number of functions, some of which include the absorption of scattered light and the maintenance of photoreceptor health (1, 2). The morphology and function of these cellular components together with their association in the visual pathway are given in more detail below.

1.1.2.1 First order neuron: Photoreceptors

Photoreceptors are highly specialized neurons that are responsible for initiating the phototransduction cascade, which can be described as the process by which photons are converted to neuronal signals (1-4). As mentioned previously, photoreceptors are of two types, the rods and the cones. The human retina consists of rods and three different kinds of cones which can be distinguished by their sensitivities to different wavelengths of light. These include the long wavelength (L) cones that detect red, mid wavelength (M) cones that detect green and the short wavelength (S) cones that detect blue; thus, the human retina can be referred to as having trichromatic vision (1, 3, 9). Both rods and cones share similar structural features which comprise of an outer segment (OS), cilium, inner segment (IS), cell body, axon (also called inner fiber) and synaptic terminal (Fig 2). The OS is the light sensitive unit that is densely packed with membrane discs that enclose the photopigments rhodopsin and opsin, in rods and cones respectively. In rods, these membrane discs appear to float in the OS cytoplasm, whereas in cones they are continuous with the OS plasma membrane and form stacked invaginations. Upon continuous light irradiation in a high oxygen environment, the photoreceptors produce free radicals that damage the disc membranes (1, 4). This is compensated for by the regular renewal of the discs, where newly formed discs at the inner end of the OS gradually displace older ones outwards until they are sloughed off and phagocytosed by the RPE. The OS is connected to the IS of the photoreceptor by means of a physical bridge called the cilium. The IS contains numerous mitochondria in the ellipsoid region to meet the high metabolic demands of the photoreceptor, as well as necessary organelles in the myoid region for protein synthesis. Moving towards the more inner regions, the cell body contains the nucleus and this is followed by the axon that transmits the signals received from the outer parts of the cell to the synaptic terminal (1-4).

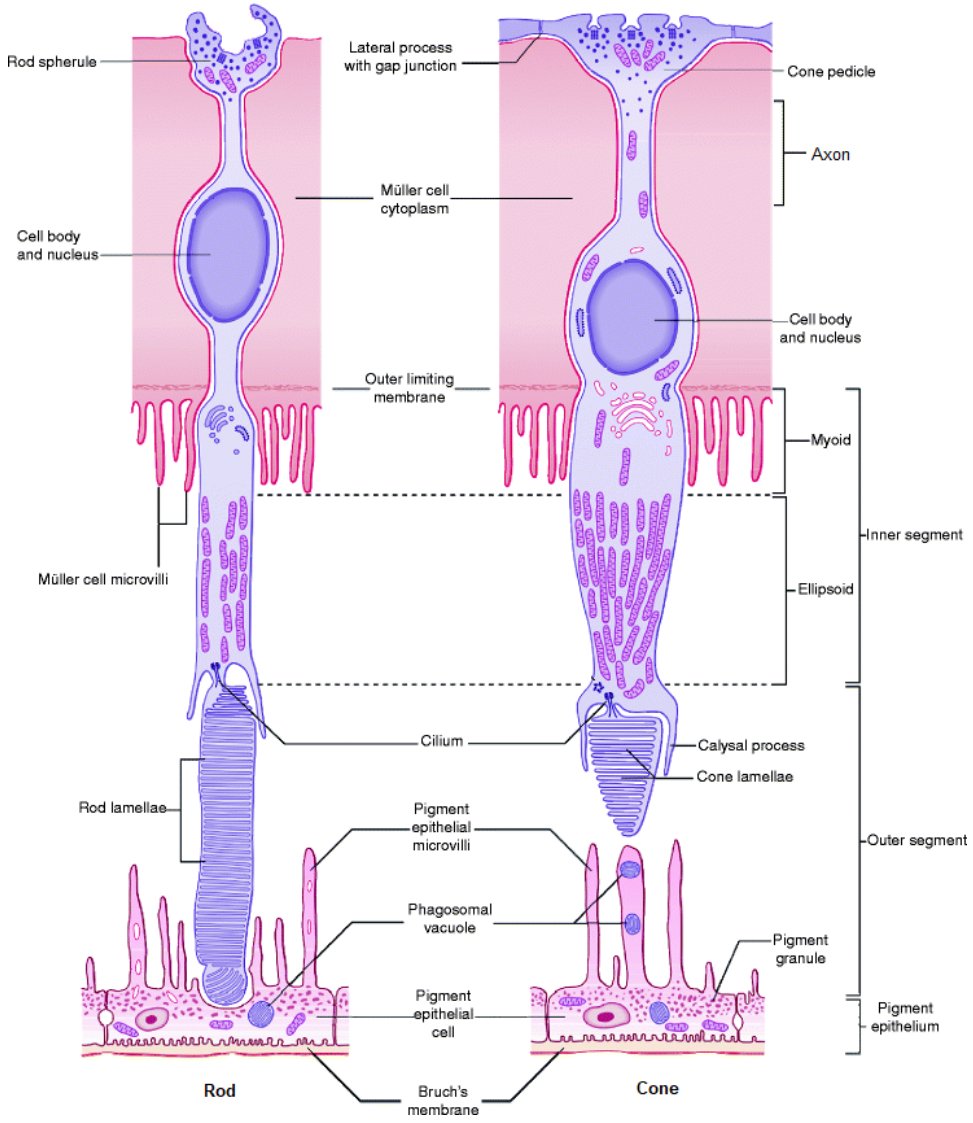

Figure 2. Structure of the rod and cone photoreceptors (1). Reproduced with permission from Springer Nature all copyright retained (2011).

The synaptic terminals of the rods are called spherules and they have a small rounded pair-like shape (Fig 2). Spherules have a deep invagination in their internal surface where usually two presynaptic ribbon units each form a synaptic complex with a pair of horizontal cell processes and one or more bipolar cell dendrites (2, 3, 10). Ribbons are special structures that are located perpendicular to the active zone of the synaptic membrane where neurotransmitter containing vesicles undergo exocytosis. The ribbon tethers a large number of these vesicles providing a reservoir of release-ready neurotransmitters which allow for high speed graded neurotransmission (11, 12). The presence of two ribbon synaptic complexes in the spherule and the deep invagination that houses postsynaptic processes close to the release site is thought to prevent rod signal spillover and the detection of single photons of light with high efficiency (10, 13). The cone synaptic terminals are called pedicles, and these are relatively larger than the spherules with broad flattened structures that have several superficial invaginations (Fig 2). Each of these invaginations contain a presynaptic ribbon that forms a synaptic complex in a similar fashion as the spherules, i.e. a pair of horizontal cell processes

and one or more bipolar cell dendrites (10, 14, 15). Cone pedicles also form basal junctions with bipolar cell dendrites at their flat bases. Additionally, they can also form gap junctions, which are reflective of electrical synapse transmission, with neighboring spherules and pedicles through projections that extend from their sides and bases, allowing crosstalk between the photoreceptors (15-18). Coupling between the cones is thought to reinforce signals and improve signal-to-noise ratio (13, 19). Whereas, coupling between cones and rods is thought to help detect intermediate light intensities by circumventing saturation at the rod synapses and providing an alternate route for the rod driven pathways (13, 20).

The photopigments of the photoreceptor OSs are the first components to detect photons from incident light and initiate the phototransduction process. The photopigments are composed of a protein unit, i.e. the rod and cone opsins, which is covalently linked to a chromaphore 11-cis-retinal (a derivative of vitamin A) (1-4, 21). Whereas the opsins can have different molecular structures (3, 22, 23), the chromaphore is the same in all mammalian photoreceptors (2). The spectral characteristics of the different opsins is based on their interactions with 11-cis-retinal making them photosensitive to specific wavelengths of light (21, 23). When photons reach the photopigments, it leads to the isomerization of 11-cis-retinal to all-trans-retinal; the only light dependent step in the visual cascade. This event leads to a conformational change in the opsins, leading to the formation of a light activated opsin. This reacts with a G protein called transducin that contains GDP bound to its α-subunit. The interaction catalyzes the exchange of GDP for GTP, consequentially activating the α-subunit. This dissociates from transducin and activates phosphodiesterase by removing its two regulatory γ-subunits. In turn, phosphodiesterase hydrolyses cyclic GMP (cGMP) to 5'-GMP. This decreases the levels of cGMP and results in the closing of cGMP-gated cation channels. Inhibition of the influx of cations across the plasma membrane causes the photoreceptors to hyperpolarize, i.e. the intracellular voltage becomes more negative, and inhibit neurotransmitter release from the synaptic terminals (1-4).

In order for the next phototransduction cascade to take place, the photoreceptors must undergo a recovery phase where the activated photopigment is deactivated. This is accomplished by (a) phosphorylation of the activated photopigment followed by binding to arrestin, (b) inactivation of transducin and phosphodiesterase, and (c) restoration of cGMP by the action of guanylate cyclase. Once the activated photopigment is deactivated, all-trans-retinal is released from the opsin and then reduced to all-trans-retinol in the OS cytoplasm. This is then transported to the RPE to be isomerized to 11-cis-retinal. The 11-cis-retinal is transported back to the OS where it can reconjugate with the opsins to form photopigment. This process is known as the classical visual cycle, and usually refers to the rods (1, 3, 4, 23). In case of cones, the recycling of the chromophore can additionally occur in the Müller cells in order to rapidly supply chromaphore during sustained daylight vision; this is known as the cone visual cycle (2, 3, 23, 24).

1.1.2.2 Second order neurons: Bipolar, horizontal and amacrine cells

The second order neurons include the bipolar, horizontal and amacrine cells. The bipolar cells provide a direct link for the photoreceptor signals to be transmitted to the ganglion cells through a forward or vertical pathway of neural transmission (1-4, 25). The horizontal and amacrine cells, mainly allow for the lateral communication and integration of signals in the OPL and IPL respectively. Bipolar cell dendrites branch in the OPL and form synapses with photoreceptors and horizontal cells, whereas their axons form synaptic bulbs in the IPL and synapse with amacrine and ganglion cells. They are known to exclusively contact either rods or cones and thus can be broadly classified as rod bipolar cells and cone bipolar cells. The rod bipolar cells can only transmit information obtained from the rods to the amacrine cells and rarely synapse with ganglion cells (1-3, 25). The amacrine cells then send these signals either directly to the ganglion cells or to the cone bipolar cells which in turn communicate with ganglion cells (1, 3). Such a synaptic arrangement helps to converge the rod and cone signaling pathways. In case of the cone pathway, the cone bipolar cells can directly transmit nerve signals from the cones to the ganglion cells. The bipolar cells can be divided further to ON or OFF subtypes depending on their electrophysiological characteristics. The ON bipolar cells are known to depolarize upon light stimulation whereas the OFF bipolar cells hyperpolarize. The rod bipolar cells are inherently of the ON type, while the cone bipolar cells can be ON or OFF (1-3, 25).

The horizontal cells lie near the outer aspect of the INL with their axons and dendritic tufts lying parallel to the retinal surface and terminating in the OPL. These cells form synapses with photoreceptors and bipolar cells as well as gap junctions with other horizontal cells enabling lateral communication and integration of signals. They provide inhibitory feedback to photoreceptors and inhibitory feedforward to the bipolar cells (1-3, 26). These signaling patterns are thought to contribute to contrast enhancement and color opponency (1, 27, 28). In the human retina, the horizontal cells can be divided into three types: HI, HII and HIII (1, 2, 29). The HI horizontal cell dendrites contact cones while its axon terminals contact rods. All the HII processes are only specific for cones possibly of the blue type, whereas the HIII contacts red/green cones and possibly rods (2, 29).

Amacrine cells usually reside in the inner aspect of the INL with their processes, which have both dendritic and axon like characteristics, in the IPL (1-3, 30). In some cases, amacrine cells have also been found to be in the GCL and the IPL. Similar to the horizontal cells, the amacrine cells also enable lateral communication by forming synapses with bipolar cells and other amacrine cells. However, they can also have direct synaptic links with the ganglion cells. Due to the wide coverage of their processes they have an important role in modulating the signals transmitted to the GCL. There are about 30 to 40 different types of amacrine cells which can be classified into four groups according to the extent of coverage of their branching processes; these include narrow field, small field, medium field, and large field. Amongst the numerous types of amacrine cells, the narrow field A2 type has been well characterized. Its function has been related to the transferring of signals from the rod bipolar cells to the ganglion cells, or channeling the rod bipolar signals to ON cone bipolar cells by

means of gap junctions, or OFF cone bipolar cells through chemical synapses (2, 3, 30). Another wide field A17 cell is thought to modulate the rod bipolar signals before they are received by the A2 cells (2, 30).

1.1.2.3 Third order neuron: Ganglion cell

The ganglion cells are the third order neurons which are last to receive the nerve impulses. Their cell bodies lie in the GCL and their dendrites are localized to the IPL where they make contacts with the amacrine and bipolar cells. The axons bundle together to form the nerve fiber layer that ultimately exits the eye as the optic nerve (1-3, 13). About 90% of the ganglion cells send their transmissions to the lateral geniculate nucleus of the brain which processes visual information, and 10% to the supraoptic and subthalamic regions which are responsible for pupillary reflexes and circadian rhythm regulation (3). The presence of nearly 20 different types of ganglion cells have been described in the human retina (1-3, 13). Interestingly, a sub-population of ganglion cells have been reported to contain the photopigment melanopsin, although it was generally considered that photopigments are only restricted to the photoreceptors. These cells are thought to participate in nonvisual processing tasks (3, 13, 31, 32).

1.1.2.4 The retinal pigment epithelium

The RPE is a single layer of postmitotic cuboidal epithelial cells that is located at the outermost part of the retina (1, 33) (Fig 1 and 2). Its basal aspect is adjacent to the Bruch's membrane of the choroid whilst the apical aspect is oriented towards the photoreceptor OSs. The presence of the pigment melanin in these cells gives the RPE its name. Apart from absorbing light and preventing excess light scattering, the RPE plays a supportive role in maintaining the survival and functioning of the photoreceptors (1, 2, 33). As previously mentioned, it can phagocytose the shedding discs of the photoreceptor OSs, and with the help of its numerous lysosomes it can ingest almost 2000 discs on a daily basis (2). In conjunction to this, it plays an important role in the visual cycle by metabolizing, storing and transporting the chromophore required for the photosensitivity of the opsins. Its production of antioxidants helps to prevent cellular damage from free radicals that are generated due to its constant exposure to light, high oxygen environment as well as high metabolic activity. The RPE also has the ability to secrete a number of different growth factors like vascular endothelial growth factor (VEGF), pigment-epithelium derived factor (PEDF), platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF) as well as many others that are thought to help maintain the photoreceptors and the choriocapillaris (1, 2, 33). Probably one of the most essential roles played by the RPE is its contribution to the formation of the blood-retinal barrier. In addition, the RPE also controls the selective movement of nutrients and metabolites from the choriocapillaris to the retina and the removal of waste products from the retina to the choriocapillaris. This is achieved by the presence of tight intracellular junctional complexes and facilitated transport mechanisms for ions and molecules (1, 2, 33-35).

1.1.2.5 Glial cells: Müller cells, astrocytes and microglia

The human retina consists of three types of glial cells which include the Müller cells, astrocytes and microglia. The Müller cells are the principle glia of the retina. They radially span the entire expanse of the neural retina with their cell bodies residing in the INL and processes ensheathing retinal neurons, blood vessels and the ganglion cell axons. Their basal extensions flatten and expand to form the endfeet that contribute to the formation of the inner limiting membrane. Their apical processes terminate near the photoreceptor ISs where they form a series of junctional complexes that appear to make a membrane which is referred to as the outer limiting membrane (1-3). Besides giving structure, the Müller cells are known to provide a number of beneficial functions that help maintain the health, homeostasis and functionality of the retina. Amongst these include maintaining ion balance; maintaining the pH of the extracellular space; recycling neurotransmitters; recycling chromophores for the cone opsins in the cone visual pathway; producing antioxidants, cytokines and growth factors; providing a source of glucose by storing glycogen and helping to maintain the blood-retinal barrier (1, 2, 36, 37). They are also referred to as living optical fibers because they can guide light through the inner retinal layers and help reduce light scatter (36, 37).

The astrocytes are star shaped cells that are found surrounding the NFL, GCL and superficial retinal vasculature. They are thought to migrate from the optic nerve and enter the retina to help in the development of the retinal vasculature. By ensheathing the glial cell axons they help to form axon bundles of the nerve fiber layer. Amongst their other supportive roles include neurotrophic support, supplying metabolites like glucose, taking up K^+ and neurotransmitters from the extracellular space and maintenance of the blood-retinal barrier (2, 37).

The microglia are important for the retina's defense mechanism in case of pathological events. They are phagocytic cells that are usually located in the inner layers of the retina. They have two morphological phenotypes that determine their immune status. In case of a ramified morphology they are in a resting state and perform surveillance of the surrounding tissue with their processes. When they change to an amoeboid morphology they are said to be in an activated state ready to perform their phagocytic function. Apart from this, the microglia may also secrete cytokines and neurotrophic factors in response to certain stimuli, help to remodel neuronal circuits as well as remove cell debris and damaged synapses (37-39).

1.1.3 Development of the retina

The nervous system originates from the ectodermal neural plate. During embryogenesis, this plate is rolled up in the midline of the embryo to form the neural tube. The retina originates from the neuroepithelium of the anterior neural tube that evaginates to form the optic vesicles. During this process, the optical vesicles remain in contact with the neural tube by means of the optic stalk. At the next stage, the optic vesicle invaginates to form a double layered structure called the optic cup. The inner layer of the optic cup transforms into the

neural retina and the outer layer forms the RPE. While the optic cup forms, the optic stalk is reduced to a narrow bridge of cells that provides the scaffold to form the optic nerve (3, 40).

During the morphological transformation of the optic vesicle to the optic cup, the neuroblasts that make up the inner layer already start to proliferate. They have the potential to differentiate into all types of cells of the neural retina as well as the Müller glia. During proliferation, their nuclei repeatedly migrate from the outer to the inner regions of the retina to form a pseudostratified epithelium. Once the cells cease to divide, they differentiate and migrate to their final destinations. The first neuron born is the ganglion cell, followed by the cone, amacrine and horizontal cells, and then the rod and bipolar cells. The Müller cell is the last to differentiate. At around the same time, the cells of the outer wall of the optic cup proliferate in a single plane to give rise to the RPE (3, 40). The determination of cell fate during retinal development is dependent on a number of extrinsic and intrinsic factors as well as the response of the multipotent progenitors to spatial and temporal cues. These could involve transcription factors and growth factors that have regulatory roles in cell cycle or cell migration (3, 41).

Another important aspect during retinal development involves programmed cell death or apoptosis as a physiological process during development. In the retina, it has been shown that apoptosis can take place at an early phase during neurogenesis, cell migration and cell differentiation, as well as at a later phase when connections and synapses are already established, allowing for the selective elimination of aberrant neuronal wiring. These events help to fine tune the retina such that it can provide optimal performance (42). Some of the factors that may regulate apoptosis include the availability of neurotrophic factors, the interaction of neuron neurites with glia, and microRNAs (42, 43).

1.1.4 Vascular system of the retina

The mature retina is a highly metabolically active tissue that has one of the highest oxygen demands in the body (44). This demand is met by the formation of two independent vascular networks, which includes the choroid and the retinal vasculature. During the embryonic stages of eye development, the oxygen requirements are fulfilled by the choroidal vessels as well as a transient vascular system referred to as the hyaloid system. The hyaloid system forms a dense intraocular circulatory system that in later stages of development regresses as the retina matures. While the hyaloid vessels regress, a vascular plexus emerges from the optic nerve head, which later matures to form the retinal vasculature that supports the inner retinal layers (1, 44, 45). The transition from the hyaloid system to the retinal vasculature is thought to occur around mid-gestation for humans (45). The vascular plexus, which originates from the central retinal artery, expands towards the peripheral regions of the retina through biochemical cues received from the astrocytes. The astrocytes emerge in the retina before the formation of the retinal vasculature and are thought to form an astrocyte cellular network in the inner retina that act as a template for the development of these vessels. Just

before vascular development the astrocytes express VEGF, which is a primary growth factor for angiogenesis and endothelial cell survival. The differentiating retina and its continuous increase in metabolic activities creates a hypoxic environment, which triggers the expression of VEGF from the astrocytes. Sprouting endothelial tip cells of the vascular plexus respond to VEGF and undergo an angiogenic response. Once the primary vascular plexus reaches the margins of the retina travelling along the nerve fiber layer, angiogenic sprouts from the veins, venules and capillaries start to innervate the inner layers of the retina perpendicular to the plexus along the processes of the Müller cells in response to VEGF secreted by the Müller cells and somatas in the INL. Once the sprouts reach the inner and outer borders of the INL they change their direction sideways to form two additional vascular networks that are oriented parallel to the primary vascular plexus. At this stage, the vessels in the nerve fiber layer are referred to as the superficial capillary network and those reaching the INL and its borders the deep capillary network (1, 2, 44-46).

Once the inner retinal layers are vascularized, the vessels undergo further remodeling and maturation. Endothelial cells may be relocalized and strengthened whilst others may be removed by apoptosis in a process known as vessel pruning. During the sprouting and remodeling of the vessels, endothelial cells recruit pericytes by secreting PDGF-B which in turn interact with PDGF receptor β on the pericytes. Pericytes are known to promote vessel survival, maturation and stabilization by means of paracrine signaling and physical contact with the endothelial cells. Pericytes can signal endothelial cells by their secretion of angiopoietin 1 which binds to the Tie2 receptors on endothelial cells. Tight junctions like the zonula occludens hold the endothelial cells tightly together making the vessels unfenestrated. The involvement of tight junctions, pericytes and the glial cells help form the blood-retinal barrier in the retinal vasculature. The outer region of the retina remains avascular and is supported by the choroidal blood supply (2, 44, 45, 47).

In humans, the development of the choroid completes by 22 weeks of gestation. The choroid provides oxygen and nutrition to the early retina by means of diffusion. It is known to branch into a dense network of capillaries, called the choriocapillaris, separated from the RPE by Bruch's membrane (1, 2, 46). The generation of the choroid and its choriocapillaris is thought to be closely linked to the RPE. During choriocapillaris development the RPE highly expresses VEGF. It is thought that the secretion of angiogenic factors from the RPE promote choroidal development and differentiation (44). Unlike the retinal vasculature, the choriocapillaris is fenestrated and permeable allowing large molecules to easily escape and pass Bruch's membrane. However, the blood-retinal barrier formed by the tight junctions in the RPE prevent entry of unwanted molecules to the retina (1-3).

1.2 RETINAL TUMORIGENESIS

The development of the retina is a highly controlled process, however, certain genetic anomalies can lead to uncontrolled cell division causing retinal tumorigenesis and cancer, as is seen in the case of the childhood cancer retinoblastoma. Before going into more depth about retinoblastoma, it is important to have an understanding of cancer and its underlying mechanisms.

1.2.1 Tumorigenesis and cancer

Tumorigenesis can be defined as a multistep process that drives the transformation of normal cells to cancerous derivatives. This transformation is linked to the occurrence of a succession of genetic alterations that confer growth advantage to affected cells (48, 49). This means that the cell will lose its ability to regulate the cell cycle and start to divide uncontrollably. The underlying characteristics that permit this state of immortality can be understood by the well summarized work of Hanahan and Weinberg in the year 2000 as the 6 hallmarks of cancer (50), which they later expanded to 10 hallmarks in 2011 based on emerging research in the field (49). These include: genomic instability and mutation, inducing angiogenesis, activating invasion and metastasis, enabling replicative immortality, evading growth suppressors, sustaining proliferative signaling, evading cell death, deregulating cellular energetics, avoiding immune destruction and tumor-promoting inflammation (49, 50) (Fig 3).

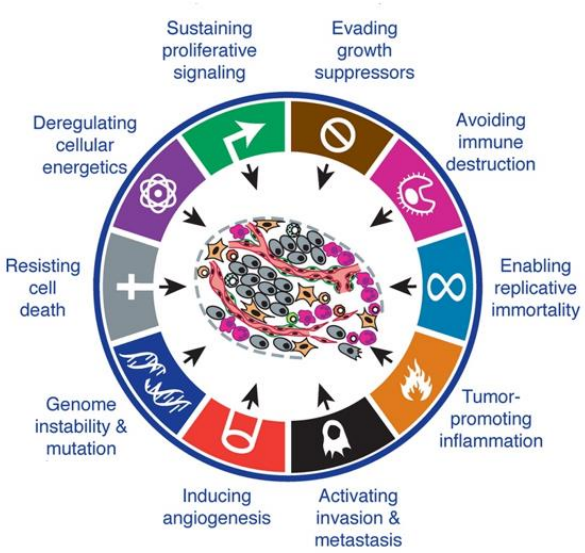

Figure 3. The hallmarks of cancer (49). Reproduced with permission from Elsevier all copyright retained (2011).

1.2.1.1 Causes of cancer

According to the WHO, cancer is the second leading cause of death with approximately 9.6 million deaths in 2018; the most frequently occurring types include lung, prostate, colorectal, stomach, liver, breast, cervical and thyroid cancers. The cause of cancer can be related to a large number of factors, which could be genetic or environmental in nature. Cancers that

develop due to germline mutations usually occur early in life; some of these include Li-fraumeni syndrome (*TP53*) (51), retinoblastoma (*RB1*) (52) and hereditary breast cancer (*BRCA1* and *BRCA2*) (53). Environmental factors that are associated to cancer can be biological, physical or chemical in nature. Biological factors may be related to viruses like human papilloma virus (HPV) or hepatitis C and B, which has been linked to cervical cancer (54) and liver cancer respectively (55). Physical factors like UV radiation has been shown to increase risk of skin cancer (56), and asbestos exposure is directly linked to development of mesothelioma (57). Both natural and synthetic chemicals can also increase cancer risk (58). As for example, aflatoxin produced by certain *Aspergillus* molds can cause liver cancer when ingested or lung cancer when inhaled (58, 59).

1.2.1.2 Current treatment strategies for cancer

The current treatment strategies for cancer include conventional chemotherapy, radiotherapy and surgery. Conventional chemotherapeutic agents cause cell death by interfering with DNA or interacting with key proteins that participate in cell division. These drugs can be classified as DNA alkylating agents (e.g. cisplatin, carboplatin), DNA intercalating agents (e.g. doxorubicin), topoisomerase inhibitors (e.g. etoposide, topotecan), tubulin binding drugs (e.g. vincristine, paclitaxel) and anti-metabolites (e.g. gemcitabine, methotrexate). These agents are not specific and can target both cancer and normally dividing cells. As a consequence, there are significant toxicities associated, which include thrombocytopenia, myelosuppression, anemia, mucous membrane ulceration and alopecia. Moreover, these agents may also have genotoxic effects and cause secondary tumors after therapy; and the possibility of introducing further mutations is quite high, leading to more resistant or aggressive cancers (60). For example, leukemia was reported to develop in patients with colon adenocarcinoma (61), osteosarcoma (62) and bladder cancer (63) as a second malignancy after chemotherapy. Radiotherapy is the use of ionizing radiation to kill cancer cells. Similar to chemotherapy, it is not specific and does not discriminate between normal and cancerous cells. Even though it has a more localized treatment approach where only tumor sites are targeted, there are still reports of second malignancies among patients (64, 65). Surgery is usually a better option, considering the side effects of these two forms of therapy, however, only tumors localized near non-vital organs can be removed.

Targeted therapy is another option that involves a direct approach of killing specific types of cancers with small molecule drugs or antibodies that target one or more key cellular mechanisms. This approach is based on a thorough understanding of cancer pathways. Examples include erlotinib, that targets epidermal growth factor receptor in non-small cell lung cancer, and vemurafenib that inhibits the B-Raf serine/threonine protein kinase for melanoma (66). More recent therapeutic approaches involve immunotherapy, which is an indirect approach where the patient's immune cells can be activated to kill the cancer, by the use of cytokines, antibodies, cancer vaccines or the more novel modified T cell therapies (e.g. CAR-T) (67). Although these therapies are promising, the problem of developing resistance and possible cancer relapse, as well as acquired toxicity in case of immunotherapy, are still

concerns. One way to overcome this is by employing combination therapies that target two different mechanisms to obtain synergistic or additive outcomes which can kill cancer cells with reduced risk of developing resistance (68, 69).

1.2.2 Tumor suppressors and oncogenes

As mentioned previously, cancer involves genetic alterations that lead to uncontrollable cell proliferation. These alterations mainly occur in genes that encode for proteins that help regulate cell growth and division, and include both tumor suppressor genes and oncogenes (70, 71).

1.2.2.1 Oncogenes

Proto-oncogenes encode for proteins that stimulate cell cycle progression and cell growth under normal physiological conditions. When proto-oncogenes are activated by mutations, amplifications or chromosomal rearrangements, they are referred to as oncogenes (72). The products of oncogenes can be divided into six groups: (a) transcription factors (e.g. MYC), (b) chromatin remodelers (e.g. ALL1), (c) growth factors (e.g. PDGF), (d) growth factor receptors (e.g. EGFR), (e) signal transducers (e.g. RAS, PI3K, AKT), and (f) anti-apoptotic proteins (e.g. BCL-2). Contrary to tumor suppressor genes, oncogenes undergo sustained activation which leads to uncontrolled hyperproliferation of cells (71, 72).

1.2.2.2 Tumor suppressor genes

As its name implies, tumor suppressor genes help to inhibit the formation of tumors. They encode for proteins that can be functionally categorized into five broad classes: (a) proteins that control cell cycle progression (e.g. Rb, p16) (b) receptors or signal transducers that prevent cell proliferation (e.g. TGF-β, APC), (c) checkpoint control proteins that trigger cell cycle arrest in response to chromosomal defects (e.g. p53, BRCA1, p16, $p14^{ARF}$), (d) proteins that induce apoptosis (e.g. p53), and (e) proteins involved in DNA repair (e.g. p53, BRCA1) (73-75). In order for cancer to develop, tumor suppressor genes must undergo what is known as Knudson's 'two-hits', where not one but both alleles need to be inactivated. In case one allele remains wild-type, it can still encode for the protein to carry out its functions (70, 73). However, there are exceptions to this rule; as for example, in case of p27 it has been seen that the remaining wild-type allele is insufficient to perform tumor suppression activity independently (76). Apart from these, cancer cells can also inactivate tumor suppressor genes through epigenetic silencing, and their encoded proteins by ubiquitin-proteasome degradation, overexpression of E3 ligases as well as protein mislocalization (73).

Two important tumor suppressor proteins that have emphasis in this thesis are described below.

1.2.2.3 The tumor suppressor protein p53

The tumor suppressor gene *TP53* encodes for the protein p53. p53 mainly acts as a tightly regulated transcription factor which becomes activated in response to cellular stresses such as oncogene activation, nutrient deprivation, DNA damage and hypoxia. In response to these stimuli, it mediates a number of cellular responses including cycle arrest, DNA damage repair, apoptosis or senescence (77-79). Additionally, p53 can also cause apoptosis through its transcription factor-independent role as a pro-apoptotic BH3 (Bcl-2 homology domain 3) like factor (80). Hence, the function of p53 is paramount in ensuring the prevention of unregulated cell proliferation which could lead to tumorigenesis. Due to playing such a central role in cancer, p53 has been referred to as 'the guardian of the genome' (81). In agreement with its tumor suppressive role, *TP53* mutation is quite frequent in many cancers; and in cases where the gene is intact, usually the p53 signaling pathway is inactivated by alterations in upstream regulators (77-79).

In absence of stress signals, p53 is maintained at low basal levels by its negative regulator MDM2 (murine double minute 2, or HDM2 in case of humans), which is an E3 ubiquitin ligase. High cellular levels of MDM2 poly-ubiquitinates p53 for proteasomal degradation, whereas, low levels of MDM2 cause mono-ubiquitination of p53, and its nuclear export (77, 82). These two proteins follow a negative-feedback loop where p53 can also transactivate MDM2 expression and maintain the cellular levels of its negative regulator. MDM2 is a weak E3 ligase, and its dimerization with a structurally related protein, MDMX (also known as MDM4, or HDMX in case of humans), is thought to enhance its ubiquitination activity. A positive regulator of p53, $p14^{ARF}$, is also a tumor suppressor that interacts with MDM2 to prevent p53 degradation and stabilize it. In tumors where *TP53* is not mutated, usually the p53 pathway is inactivated through increased expression of MDM2 and MDMX and deletion or epigenetic inactivation of the positive regulator $p14^{ARF}$ (77-79).

As mentioned earlier, p53 can be activated in response to cellular stress. This leads to the transcription of a number of downstream genes. Some of these targeted genes, related to cell cycle arrest, DNA repair and apoptosis, are highlighted.

Cell cycle arrest: One of the primary aspects of p53 activation is the inhibition of cell division by causing cell cycle arrest. One of the most well studied way that this is achieved is through stimulating the expression of $p21^{Waf1/Cif1}$, which, among its many other roles, is a cyclin-dependent kinase (CDK) inhibitor. CDKs form complexes with cyclin proteins to allow transition through the cell cycle. p21 is known to inhibit CDK1 and CDK2, which in turn results in cell cycle arrest and/or senescence (77, 79, 83). Apart from this, p21 can also prevent cell cycle progression by binding to proliferating cell nuclear antigen (PCNA) which is known to participate in DNA repair and replication. This interaction prevents DNA synthesis and, thus, arrests the cell cycle (83). p53 has also been reported to prevent G2/M transition by mediating transcriptional repression of cyclin B1 through the Sp1 transcription factor (84).

Genomic stability and DNA repair: p53 activation can help to maintain genomic stability by aiding its repair in case of damage. It can do this by targeting the expression of growth arrest and DNA damage inducible protein 45 (GADD45). GADD45 plays an important role in global genomic repair, as well as in DNA excision repair through interaction with PCNA (77, 85).

Apoptosis: In some cases, p53 may need to mediate cell apoptosis when cell damage is beyond repair. As a result, it is known to initiate the transcription of several pro-apoptotic factors, some of which include Bcl-2-associated X protein (BAX), p53 upregulated modulator of apoptosis (PUMA) and NOXA (77).

It is comprehensible that the loss of p53 activity would prove detrimental to normal physiological functions. Since approximately 50% of cancers still harbor wild-type p53, pharmacological interventions have been used to reactivate the p53 pathway (86). Considering the diverse signaling cascades in the p53 network, many strategies can be employed to activate p53. Some of these involve inhibiting the interaction of MDM2/MDMX with p53; as for example, small molecules and stapled-peptides that competitively bind to MDM2 and prevent p53 degradation (e.g. nutlin-3, SAH-p53) (87, 88). Other molecules, like leptomycin B, can prevent the nuclear export of p53.

Although 50% of cancers retain wild-type p53, we cannot ignore the fact that the other 50% have p53 mutations. The most frequently occurring mutations are missense mutations that usually occur in the p53 DNA binding domain. Drugs targeting p53 mutant cancers usually aim to restore p53 function (89). One such molecule is PRIMA-1 which can covalently modify the thiol groups in the mutant p53 DNA binding domain and help restore the protein's conformation (89, 90). Another molecule, ZMC1 is believed to reactivate the R175H p53 mutant by chelating zinc. It has been reported that defective zinc binding can destabilize the structure of the p53 protein and affect its transcriptional activity. ZMC1 is able to provide zinc to the mutant protein and help regain its structural integrity (89, 91).

1.2.2.4 The tumor suppressor protein Rb

The tumor suppressor gene *RB1* encodes for the protein Rb. Rb was one of the first tumor suppressors to be identified. It is known to negatively control the cell cycle by regulating the G1/S checkpoint and blocking entry into S phase. Rb belongs to the family of pocket proteins which include p107 and Rb2/p130. All of these proteins have a binding region, termed as a 'pocket', for E2F transcription factors. E2F transcription factors activate genes that are responsible for cell cycle regulation, DNA replication, DNA repair, apoptosis, and other key regulatory functions (92). During the cell cycle, Rb undergoes phosphorylation by CDK 2, 4 and 6 which determines its physical interaction with E2F (93, 94). In absence of growth stimuli, Rb remains active in a hypo-phosphorylated state that is bound to E2F; this prevents E2F from activating genes necessary for S phase entry. However, when there is a growth signal CDKs phosphorylate Rb, which causes it to unbind from E2F, allowing E2F to perform its transcriptional activities so that the cell can progress through to the S phase (94).

The tumor suppressing activities of Rb, however, are not only due to its well-known regulation of the cell cycle. Rb is also known to promote cell differentiation by enforcing cell cycle exit. In case of neuronal differentiation, Rb sequesters a protein, ID2, which is known to inhibit basic helix-loop-helix transcription factors that are important for differentiation (95). Rb can also induce the transcriptional activities of C/EBP proteins, which participate in the terminal differentiation of a variety of tissues, like adipocytes (96) and leukocytes (97). Apart from this, Rb is also known to play important roles in the maintenance of chromosome stability, and the repression of genes through chromatin remodeling (93, 94).

The inactivating *RB1* mutation is usually seen in a few types of cancers, which include retinoblastoma (more details in next section) and small cell lung cancer. However, the Rb pathway is usually disrupted in most cancers, some of which could be due to hyperphosphorylation (98), viral oncoprotein binding (99) or methylation of the *RB1* gene that prevents its transcription (100). Some of the therapeutic interventions currently used and under investigation for retinoblastoma treatment are mentioned in the next section.

1.2.3 Retinoblastoma

Retinoblastoma is a pediatric intraocular tumor that originates during retinal development in young children, with an incidence of approximately 9000 new cases per year globally (101, 102). Although quite rare, it accounts for the most common form of eye cancer in children (102, 103). The disease burden is quite high in developing nations where the mortality rate is 40 -70%, compared to 3-5% for developed nations. Due to the young age of patients and modest symptoms, retinoblastoma cases are usually left undiagnosed until late stage of tumor development. The initial signs of retinoblastoma are leukocoria (a white reflex visible through the pupil) and strabismus (misaligned eyes). Lack of awareness of these signs, poor socioeconomic conditions and limited access to healthcare are thought to contribute to the higher mortality in developing nations (102, 104).

1.2.3.1 Genetic basis of retinoblastoma

Retinoblastoma was the first model used to establish Alfred Knudson's two-hit hypotheses for tumorigenesis, which states that both alleles of a tumor suppressor must be lost in order for cancer to develop (105). Retinoblastoma occurs due to the biallelic inactivation of the *RB1* tumor suppressor gene. In case of sporadic retinoblastoma, one retinal cell must undergo two mutations (hits), i.e. loss of both the wild-type alleles for *RB1* (Fig 4). In case of a germline mutation, a retinal cell would need just one more 'hit' to lose the wild type allele.

Usually patients with sporadic retinoblastoma have the disease in only one eye (unilateral), whereas patients with germline mutation tend to have the disease in both their eyes (bilateral). Numerous types of mutations have been reported for the *RB1* gene, which include missense, nonsense, splicing mutation, frameshifts, microdeletions, macrodeletions and promoter methylation (106). Intriguingly, some sporadic retinoblastomas arise in absence of *RB1* loss, and are instead driven by the amplification of the *NMYC* oncogene (107) (Fig 4). Although *RB1* loss poses a risk for malignancy, it only initiates tumor development with the formation of a retinoma, which is the benign form of retinoblastoma. Further genomic changes are required for the tumor to progress to the highly invasive form of the cancer. This is usually referred to as the additional hits required for retinoblastoma development from a retinoma (108) (Fig 4). These can be characterized as distinct patterns of genomic gains and losses, with gains at 1q, 2p and 6p, and loss at 16q chromosomal sites. The genes affected in these regions which are thought to participate in disease progression include *KIF14* (kinesin family member 14) and *MDMX* on 1q, *NMYC* on 2p as well as *DEK* (DNA-binding proto-oncogene) and *E2F3* transcription factor on 6p. Losses at 16q include *CDH11* (cadherin-11) and *RBL2* (also called p130). Among these genes, amplification of *MDMX* has been related to blocking the p53 pathway in retinoblastoma, which otherwise has an intact p53 gene (109, 110).

1.2.3.2 Current treatment strategies

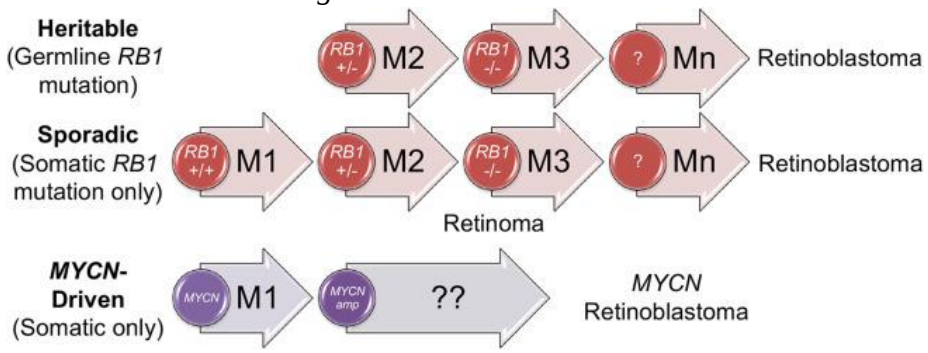

Figure 4. Retinoblastoma genetics. The cancer can initiate with a germline *RB1* mutation followed by a single somatic hit, or with two somatic mutations (M1 and M2). Loss of *RB1* leads to the benign retinoma; further mutations (M3 to Mn) are required for malignancy. Retinoblastoma can also initiate by amplification of *MYCN*, with other genomic changes as yet unknown (104). Reproduced with permission from John Wiley and Sons all copyright retained (2018).

Treatments that are currently used for retinoblastoma include focal therapy, chemotherapy, external beam radiotherapy (EBRT) and enucleation (surgical removal of the eye). Usually, one or a combination of these treatment strategies are employed based on tumor laterality and disease stage as shown in table 1 (110). Focal therapy is used to locally treat small tumors in cases where there is no vitreous seeding. These include photocoagulation, cryotherapy, thermotherapy and brachytherapy (110, 111). Photocoagulation employs the use of a diode laser, cryotherapy uses freeze-thaw cycles, thermotherapy uses infra-red, ultrasound or microwaves and brachytherapy uses radiation with custom designed plaques (111). Larger tumors are treated with chemotherapy to reduce tumor size usually using three classes of drugs: DNA alkylating agents (e.g. cisplatin, carboplatin), topoisomerase II inhibitors (e.g. etoposide, teniposide), and tubulin polymerization inhibitors (e.g. vincristine). The common

combination of selected agents used in the clinic include vincristine, etoposide, and carboplatin. Usually chemoreduction is used together with focal therapies. EBRT is no longer used due to the risk of developing secondary malignances, however, exceptions may be made when other treatment options do not yield satisfactory results (103, 110). Enucleation is based on whether a patient has unilateral or bilateral retinoblastoma. To avoid the risk of disease dissemination, patients with unilateral retinoblastoma usually undergo enucleation. However, in case of bilateral patients, the worst affected eye is enucleated and the remaining eye is salvaged so that the child can at least retain vision (103, 110, 111).

Table 1: The choice of chemotherapy based on tumor laterality and disease stage (110). Reproduced with permission from Bentham Science Publishers Ltd. all copyright retained (2016).

Group	Globe Salvage Likelihood	Quick Reference	Specific Clinical Characteristics	Treatment
A	**Very Favorable** (good visual and overall prognosis)	Small tumors away from fovea and disc	Tumors ≤ 3 mm in greatest dimension confined to the retina, and Located at least 3 mm from the foveola and 1.5 mm from the optic disc	Argon-YAG laser Diode laser-induced hyperthermia Cryotherapy Brachytherapy
B	**Favorable** (good visual prognosis)	Larger tumor	All remaining tumors confined to the retina not in Group A Subretinal fluid (without subretinal seeding) ≤ 3 mm from the base of the tumor	VCR + low dose CBP Focal therapy for 2-5 cycles
C	**Doubtful** (visual prognosis variable)	Localized seeding	Local subretinal fluid alone > 3 to ≤ 6 mm from the tumor Vitreous seeding or subretinal seeding ≤ 3 mm from the tumor	VCR + high dose CBP + ETO + G-CSF, up to 6 cycles Focal therapy Possible local carboplatin
D	**Unfavorable** (high morbidity from treatment, visual prognosis variable)	Diffuse seeding	Subretinal fluid alone > 6 mm from the tumor Vitreous seeding or subretinal seeding > 3 mm from the tumor	VCR + high dose CBP + ETO + G-CSF, up to 6 cycles EBRT Possible local carboplatin
E	**Very unfavorable** (high morbidity from treatment, no visual potential)	Extensive	Presence of one or more of the following poor prognosis features: - More than 2/3 globe filled with tumor - Tumor in anterior segment - Tumor in or on the ciliary body - Iris neovascularization - Neovascular glaucoma - Opaque media from hemorrhage - Tumor necrosis with aseptic orbital cellulitis - Phthisis bulbi	Enucleation Prophylactic 3-agent chemotherapy if high-risk features for disease dissemination observed on consensus pathologic evaluation

Abbreviations: CBP = carboplatin, EBRT = external beam radiation therapy, ETO = etoposide, G-CSF = granulocyte colony-stimulating factor, PD = progressive disease, VCR = vincristine

Although these current treatment regimens have been used successfully to treat many retinoblastoma patients, they are not devoid of side effects. Focal treatments may increase the chance of vitreous seeding, vascular occlusion, retinal fibrosis, traction, retinal tears and detachment, proliferative vitreoretinopathy, chorioretinal atrophy and cataracts (110, 111).

Brachytherapy can lead to radiation-induced retinopathy and optic neuropathy. Chemotherapy, comes with a myriad of adverse effects whether given systemically or intra-arterially. Systemic administration can lead to neurotoxicity, nephrotoxicity, hyponatremia, ototoxicity, nephrotoxicity and secondary leukemia; whereas intra-arterial administration leads to vitreous hemorrhage, myositis, eyelid edema, microemboli to the retina and choroid, orbital congestion, dysmotility, choroidal atrophy, ophthalmic artery stenosis, optic atrophy, and branch-retinal artery occlusion which could lead to blindness (111). Enucleation means the loss of an eye, which comes with cosmetic defects and partial vision loss (110, 112).

Due to the numerous side effects and possible complications of the current treatments, there is a need for safe and more effective alternatives. Efforts are being made at investigating new treatment modalities for retinoblastoma. *RB1* gene replacement therapies are under investigation, however, there are still challenges considering the unpredictable effects of gene dosage and gene delivery strategies (94, 113). Other interventions involve the use of small molecule drugs. Some of these include reactivation of the p53 pathway by inhibiting MDMX (109), and targeting E2F1 transcription factor activity by blocking histone deacetylases (HDAC) (114).

1.3 PYRIMIDINE RIBONUCLEOTIDES

Pyrimidine ribonucleotides are primarily known as the building blocks for RNA and as the precursors for the pyrimidine deoxynucleotides for the synthesis of DNA. The pyrimidine bases uracil, cytosine and thymine are composed of heterocyclic, six-membered, nitrogen-containing carbon ring structures. These are bound to ribose to form nucleosides uridine, thymidine and cytidine, respectively. Phosphorylation of these nucleosides gives rise to monophosphate, diphosphate and triphosphate pyrimidine ribonucleotides. Apart from being the precursors of nucleic acids, pyrimidine ribonucleotides play essential roles in various biosynthetic processes involved in cell growth and metabolism. Among these are the synthesis of cell membrane phospholipids and polysaccharides, glycosylation of proteins and lipids, as well as glucuronidation in detoxification processes (115).

1.3.1 Pyrimidine ribonucleotide synthesis pathways

Pyrimidine ribonucleotides can be synthesized through the *de novo* or the salvage pathways. In *de novo* synthesis, the pyrimidine ring is formed from the starting molecules glutamine, aspartate and 5-phosphoribosyl-1-pyrophosphate (PRPP). In the salvage pathway, the breakdown products of nucleic acids are recycled and/or nucleosides are imported from extracellular sources through membrane transporters (115, 116). The relative contribution of these two pathways depends on the active needs of the cell. Highly proliferating cells, like cancer cells or activated immune cells, are more dependent on the *de novo* pathway due to their greater need for nucleic acids and cellular components such as cell membrane precursors and second messengers (117, 118). Non-dividing or resting cells, on the contrary, have relatively lower demands and can satisfy their requirements by the less energy dependent

salvage pathway (117). The molecule that is central to both these pathways is uridine monophosphate (UMP), which is the precursor for all pyrimidine ribonucleotides.

1.3.1.1 De novo pyrimidine ribonucleotide synthesis and DHODH

In the *de novo* synthesis pathway, UMP is synthesized from glutamine, aspartate and PRPP by the catalytic activity of three different enzymes. First, the precursors are catalyzed in three steps by the multifunctional enzyme CAD (carbamoylphosphate synthetase II, aspartate transcarbamylase and dihydroorotase) to form dihydroorotate. Next, this is oxidized to orotate by the action of dihydroorotate dehydrogenase (DHODH), which is the only enzyme in the pathway located in the mitochondria. The last enzyme of the pathway, the bifunctional uridine monophosphate synthetase (UMPS), catalyzes the formation of UMP in two steps through the action of its orotate phosphoribosyltransferase and orotidine monophosphate decarboxylase activities (115, 116) (Fig 5).

As mentioned above, DHODH catalyzes the fourth step in the *de novo* pyrimidine ribonucleotide synthesis pathway. In mammals, it is located in the inner mitochondrial membrane where it is known to catalyze the oxidation of dihydroorotate to orotate in the presence of the cofactor flavin mononucleotide (FMN) by the transfer of electrons to ubiquinone (CoQ) to form ubiquinol ($CoQH_2$). Ubiquinol is used by complex III of the electron transport chain (117, 119). DHODH has two domains; a large catalytic domain (CAT) which contains the active site, and a small domain referred to as the quinone tunnel (QT) where ubiquinone binds (117). The QT is considered the most important site for enzyme inhibition, based on the binding characteristics of the enzyme's two established inhibitors brequinar and teriflunomide (120, 121).

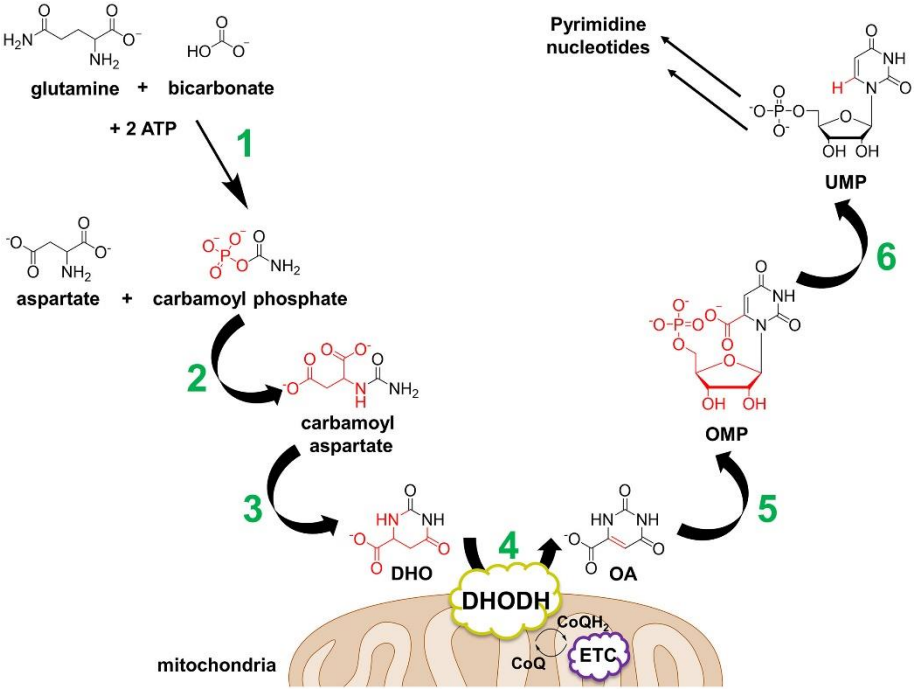

Figure 5. *De novo* pyrimidine nucleotide synthesis pathway (129). Reproduced with permission from Elsevier all copyright retained (2019).

1.3.1.2 The salvage pathway of pyrimidine ribonucleotide synthesis

The precursors to the salvage pathway can be obtained from endogenous sources, like the breakdown products of nucleic acids, as well from the extracellular environment. In this case, precursors must be imported through membrane transporters. The salvaged products can then be processed by the salvage pathway enzymes to synthesize UMP. These enzymes include uridine phosphorylase, that converts uracil to uridine, and uridine kinase, which converts uridine to UMP (115, 122). Another important enzyme, cytidine deaminase, is responsible for converting cytidine to uridine (123).

The import of nucleosides requires the use of specific nucleoside transporters due to the hydrophilic property of these molecules. Nucleoside transporters can be divided into two classes: equilibrative and concentrative. The human equilibrative nucleoside transporters (ENT1-4, also known as SLC29A1-4) are bidirectional sodium independent transporters. ENT1 and 2 transport purine and pyrimidine nucleosides, whereas ENT2 and 3 additionally transport nucleobases (124, 125). ENT4, however, is only selective for adenosine and organic cations (126). Considering cellular localization, ENT1 and ENT2 can be found both on cytoplasmic and intracellular membranes, whereas ENT3 is primarily found on intracellular membranes and ENT4 on cytoplasmic membranes (see table 2 for tissue specific information).

Table 2: The SLC28 and SLC29 transporter families (125). Reproduced with permission from Elsevier all copyright retained (2013).

Human gene name and locus	Protein name and UniProt accession	Transport type/cation coupling stoichiometry	Predominant tissue and subcellular distributions
SLC28A1 15q25.3	CNT1 O00337	Concentrative 1:1 sodium:nucleoside	Kidney, liver, small intestine. Primarily plasma membrane-located.
SLC28A2 15q15	CNT2 (alias SPNT) O43868	Concentrative 1:1 sodium:nucleoside	Heart, skeletal muscle, liver, kidney, intestine, pancreas, placenta and brain. Primarily plasma membrane-located.
SLC28A3 9q22.2	CNT3 Q9HAS3	Concentrative 2:1 sodium: nucleoside 1:1 proton: nucleoside probably 1:1:1 sodium:proton:nucleoside	Widely expressed, most abundant in mammary gland, pancreas, bone marrow, trachea and intestine. Primarily in plasma membrane, but intracellular in some cell types and splice variants.
SLC29A1 6p21.1	ENT1 Q99808	Facilitated diffusion	Widely expressed, primarily in the plasma membrane but also in nuclear and mitochondrial membranes.
SLC29A2 11q13	ENT2 Q14542	Facilitated diffusion	Particularly abundant in skeletal muscle but widely expressed, primarily plasma membrane-located but has also been detected in nuclear membranes.
SLC29A3 10q22.1	ENT3 Q9BZD2	Unclear, possibly proton-linked.	Widely expressed, intracellular (late endosomal/lysosomal and mitochondrial membranes).
SLC29A4 7p22.1	ENT4 (alias PMAT) Q7RTT9	Unclear, possibly proton-linked.	Heart, brain, and skeletal muscle. Primarily plasma membrane-located.

The human concentrative nucleoside transporters (CNT1-3, also known as SLC28A1-3) are unidirectional sodium dependent active transporters. CNT1 preferentially transports pyrimidine nucleosides, CNT2 purine nucleosides and CNT3 both pyrimidine and purine nucleosides (124, 125). CNT1 and 2 are primarily located in the cytoplasmic membrane, but CNT3 can be found both on intracellular and cytoplasmic membranes (see table 2) (125). All the CNTs can transport uridine, and have a higher affinity for uridine than the ENTs (124, 125) but a lower turnover rate of transport (125, 127).

1.3.2 DHODH inhibitors with relevance to cancer

The relevance of DHODH in cancer was first reported in 1960 when its high expression was seen in leukemia cells (128). Afterwards, several investigations that had encouraging results in preclinical studies led to the discovery of the potent DHODH inhibitor, brequinar (129).

Initial studies in the 1980's with brequinar on tumor xenograft models revealed promising anticancer effects on breast, colon, lung and stomach tumors (130). Later, the mechanism of action of brequinar was linked to its ability to inhibit DHODH (131). Further studies on cancer cell lines showed that continuous brequinar treatment was necessary to prevent cell proliferation, and that rapidly dividing cells were more sensitive relative to slower ones (130, 132). With the success in preclinical results, brequinar was tested on patients. However, the drug demonstrated unsatisfactory responses in a number of different cancers, including breast (133), melanoma (134), colon (135), lung (136) and gastrointestinal (137). Therapeutic responses were not detected even at higher doses of the compound. To help understand the failure of these patients to respond to brequinar treatment, one needs to look at the drugging schedule and plasma uridine levels. Most patients in these trials were administered brequinar at high dose once every week. Furthermore, plasma uridine levels were reported to decrease by 40 to 80% after 6 hours to 4 days of drug administration. However, approximately a week after the treatment, plasma uridine levels were reported to spike to above 150%. As mentioned earlier, a continuous treatment with brequinar is necessary to show anti-proliferative effects. It is likely that the dosing schedule of the trials did not lead to continuous suppression of DHODH and that plasma uridine levels weakened the response to the compound. In light of the poor results in the clinical trials, no further clinical testing was continued with brequinar (129).

It was not until 2016 that brequinar once again resurfaced in the cancer field as a strong inducer of differentiation in acute myeloid leukemia cells (138). Since then, many potent DHODH inhibitors have been synthesized and are being tested as anticancer agents. Bayer has started clinical trials with a very potent DHODH inhibitor, BAY 2402234, for acute myeloid leukemia. Teriflunomide, the active metabolite of the prodrug leflunomide, is a DHODH inhibitor that is clinically approved for multiple sclerosis (139). Leflunomide is also clinically approved for rheumatoid and psoriatic arthritis (140). Despite being marketed as an anti-inflammatory drug, leflunomide also demonstrates anticancer activity in multiple cancer

cell lines such as multiple myeloma (141), neuroblastoma (142), non-small cell lung carcinoma (143) and medullary thyroid cancer (144). It is also undergoing clinical trials for multiple myeloma (NCT02509052). However, a point worth noting is that even though teriflunomide and leflunomide are well established DHODH inhibitors, they are not specific and have multiple targets, some of which include activation of aryl hydrocarbon receptor, inhibition of tyrosine kinases, as well as activation of PDGF and EGF (129, 144). Hence, the DHODH inhibitory activity of these drugs may not be the sole contributor for anticancer effects. Moreover, the half-life of teriflunomide is around 2 weeks, and it can still be detected in plasma for up to two years after end of treatment (145).

1.3.3 Combination therapy with DHODH inhibitors

Uridine is present in our plasma (varies between 3.1 to 4.8 μM in concentration) (146), and this may influence the response towards DHODH inhibitors. This is also highlighted in the past clinical trials where high uridine in the plasma most likely prevented an effective therapeutic response in patients treated with brequinar. It should be noted that even when the *de novo* synthesis of pyrimidine ribonucleotides is blocked, the salvage pathway is still available to compensate for decreasing concentrations of UMP. In such cases, combining a DHODH inhibitor with a nucleoside transport inhibitor may prove beneficial so that both pathways are blocked.

There are many known nucleoside transport inhibitors (124, 147), and one of particular interest to this thesis is dipyridamole. Dipyridamole is a phosphodiesterase inhibitor that is normally used in the clinic as an anti-thrombotic drug (148); additionally, it is also a potent inhibitor of ENT1, as well as ENT2-4 with varying sensitivities (124). Furthermore, this drug has previously been investigated in different ocular disorders, using different routes of administration, such as intravitreal and oral, with no noted side effects (149). Interestingly, dipyridamole has been previously shown to reduce plasma uridine levels alone and in combination with PALA, an inhibitor of the aspartate transcarbamoylase activity of CAD, in cancer patients. However, the treatment did not prove to be as effective, possibly due to the inefficient inhibition of *de novo* pyrimidine synthesis by PALA (150, 151). Another potent ENT1 inhibitor is nilotinib, which is well-known as a Bcr-Abl tyrosine kinase inhibitor for treating chronic myeloid leukemia (147). CNTs can also be blocked to inhibit the salvage pathway. CNTS have a higher affinity to uridine but a slower turnover rate (ENT1: 200 uridine molecules/s; CNT1: 10 uridine molecules/s) (124, 125). Thienopyrimidine 2′-deoxynucleoside has been recently reported to be a potent CNT1 inhibitor (152). And among the tyrosine kinase inhibitors, imatinib has also shown inhibitory activity toward CNT2 (147).

ENT and CNT expression can vary among different tumor tissues which have been reported to show higher or lower mRNA levels for the transporters relative to their normal counterparts. Higher mRNA levels for ENT1 have been reported in lung, breast, stomach and rectal cancers, whereas for ENT2 it was highly seen in breast, kidney, colon, stomach and

prostate cancers. CNT1 mRNA levels were high in ovarian cancers, and CNT2 in a majority of lung, prostate, breast, uterine and ovarian cancers (153). However, it should be noted that higher mRNA levels may not necessarily correlate to higher expression of these transporters.

DHODH inhibitors may also be combined with drugs that can target downstream responses to DHODH inhibitors. As for example, the depletion of pyrimidine ribonucleotides is known to activate p53, and combination with an inhibitor of p53 degradation (e.g. MDM2 inhibitor) can potentiate the effect of the DHODH inhibitor (paper I). DHODH inhibitors can also sensitize cancer cells to chemotherapy and overcome resistance. In this regard, treatment of triple negative breast cancer with doxorubicin increases nucleotide synthesis to aid DNA repair. Pretreatment with leflunomide is able to deplete pyrimidine ribonucleotide synthesis and overcome doxorubicin resistance (154). Combination treatment of brequinar with cyclosporine was also reported to show synergistic effects in *in vitro* and *in vivo* models of melanoma (155).

1.4 RETINAL NEURODEGENERATION

Retinal neurodegeneration involves a group of heterogeneous disorders that progressively lead to vision impairment. The retina's function may be compromised as a result of inherited mutations leading to protein dysfunction, such as retinitis pigmentosa (156); vascular defects in the retinal vasculature and choriocapillaris, including diabetic retinopathy and retinopathy of prematurity (46, 47); aging, e.g. age-related macular degeneration (44); and environmental insults, such as light-induced damage (157). Retinopathy has also been observed in patients treated with radiation (158) or chemotherapeutic drugs like cisplatin, etoposide and tamoxifen (159-161). Although the underlying mechanisms of these causes differ, the deconstruction and loss of photoreceptors, the remodeling and loss of inner retinal neurons as well as glia cell activation usually characterize the neurodegenerative events. The progressive neuronal cell death and limited synaptic connectivity in the retina lead to sensory deafferentation and loss of structural integrity (157, 162, 163).

Retinal remodeling is a phenomenon that is evident in most retinal neurodegenerative disorders; especially those that involve photoreceptor degeneration. It occurs in three phases of structural and functional changes. The first phase involves photoreceptor stress and in some cases RPE cell stress (162-165). Usually, this begins with the rod photoreceptors followed by the cones if it is a rod-initiated degeneration or vice versa for a cone-initiated degeneration (163). Based on histological observations, the first sign of photoreceptor cell stress involves the mislocalization of opsins in the rod and cone photoreceptors from the OSs to other parts of the cell (162). The OSs of the photoreceptors also deconstruct and become shorter. The rods may start to develop neurites that either extend into the INL or retract further down into the ONL (163). Additionally, changes also start to occur in bipolar cell dendrite glutamate receptors. Phase two is accompanied by photoreceptor cell death and Müller cell gliosis where the Müller cells undergo hypertrophy and increased expression of

glial fibrillary acidic protein (GFAP). This results in the formation of the glial seal that isolates the retina from the RPE and choroidal blood supply (162-165). Microglia are activated and may cause further cell death (163, 165). At this phase, the bipolar cells also become completely deafferented by the loss of their glutamate receptors in the OPL and retraction of the dendrites into the INL. Sprouting of processes of bipolar, horizontal and amacrine cells may also occur in cone-sparing forms of retinal degenerations. Finally, in phase three the retina undergoes further remodeling as a result of bidirectional migration of surviving neurons along the vertical axis of the retinal tissue. This may involve the migration of surviving bipolar or amacrine cells to the GCL or the migration of ganglion cells to the INL, leading to complete disorganization of the retinal morphology. The processes of these neurons may also continue to sprout and form neurites that could tangle up and also cover great distances in the neural retina (162-165). In cases where some cone photoreceptors continue to survive, rod bipolar cells tend to rewire with the remaining cone pedicles. Such events lead to corruptive signaling circuits (162, 164, 165).

1.5 MODELS OF RETINAL NEURODEGENERATION AND ADVANTAGES OF ORGANOTYPIC RETINAL EXPLANT CULTURE

The biology and disease etiologies of the retina have been extensively studied on a number of different models in order to improve our knowledge of its functional mechanisms and for the development of therapeutic approaches. The models studied include *in vivo*, *in vitro* and organotypic culture systems.

Mouse models have proven to be powerful tools in retinal research. Considering their similarity to the human genome and physiology, fast reproduction rates and low cost they are suitable for experimental research. However, what makes them ideal is that they can easily be modified by genetic interventions in order to recapitulate disease phenotypes. This has led to a wide variety of transgenic mice that carry mutations which alter developmental pathways or cellular mechanisms to help investigate relative human disorders and therapeutic strategies (166-168). Moreover, these mice also provide reproducible experimental systems since the genetic backgrounds can be controlled and the mutant and control mice only differ by the mutated gene being studied. This makes it possible to study the implications of a mutated gene in the same sex with same age littermates. Another advantage of using this model is their accelerated life span that can allow researchers to follow the progression of retinal disorders in a relatively short time period (166, 168).

Although the mouse model has proved to be invaluable in retinal research, it is important to consider the fact that mouse retinas are not exactly the same as humans. The clearest distinguishing feature is the size of the eye which for mice has a diameter of ~ 4 mm and for humans ~25 mm (169). They are nocturnal animals and have a rod to cone ratio of 35:1 (167) whereas in humans it is 20:1 (170). The mouse retina is also dichromatic and does not have a macula (169). Pigs being diurnal animals have retinas similar in size and physiology to

humans, and, although they do not have a fovea, they have an area centralis consisting of a high density of cones (171, 172). Due to such features, the porcine model is also becoming quite popular in retinal research and transgenic pig models have also been generated (172).

Although *in vivo* studies on animals are quite important for retinal research, another model that uses an *in vitro* culturing system for retinal tissue has received much attention. Organotypic retinal tissue culture systems provide an essential approach for direct experimental manipulation of therapeutic agents in a controlled environment (173). This model is superior to single cell culture systems in that it conserves the tissue morphology and most importantly the cell-to-cell contact and biochemical features for several days (174, 175). Intraocular injections into the eyes of mice may prove to be quite difficult since they are small and have a vitreous volume of ~7 µL leaving little space for drug intervention (176); however, in the *in vitro* organotypic cultures this restriction is eliminated. The disadvantage of this model is their short life spans in culture outside the *in vivo* environment; a number of modified culture systems are still under research to improve tissue survival (173, 174, 177). However, this feature of organotypic culture models is beneficial for investigating retinal degeneration and potential neuroprotective strategies (173). Since organotypic cultures share the benefits of *in vivo* and *in vitro* systems, they minimize the animals required for experimentation and help decrease ethical burden (174, 175).

1.6 HUMAN NEURAL PROGENITOR CELL (HNPC) DERIVED NEUROTROPHIC SUPPORT AS A TREATMENT STRATEGY

The regenerative capacity of the human retina is limited, so the loss of photoreceptors cannot be replaced. Currently there is no treatment for retinitis pigmentosa, which is a genetic disorder characterized by the loss of rods (178). Only supportive care is provided, as for example, by the use of nutritional supplements like vitamin A (179). The treatments for diabetic retinopathy target vascular defects and inflammation. However, the loss of photoreceptors is not accounted for (180, 181). The same holds true for other retinal neurodegenerative disorders.

Gene therapy and stem cell therapy are under investigation to help prevent and replace photoreceptor loss. Although these therapies are very promising, there are still underlying problems. Gene therapy still comes with the challenges of gene delivery strategies, unpredictable dosage and different transfection rates in the retina and RPE (182). Moreover, gene therapy is mutation specific and may be difficult, although not impossible, to employ in retinitis pigmentosa (179). Stem cell therapy is aimed at replacing lost retinal cells by transplanting newly differentiated ones irrelevant of the disease mechanism. Successful transplantation of RPE cells have been reported, but photoreceptor cell transplantation may be challenging due to synaptic integration and the complex circuitry involved, although some preclinical studies have shown successful results (182-184).

Neuroprotective therapies aim to provide an environment to prolong retinal cell survival through altering their biochemical pathways independent of the underlying etiology of degeneration. A number of neurotrophic factors have been shown to have a neuroprotective effect on photoreceptor survival in retinal degeneration. Basic fibroblast growth factor (bFGF) was demonstrated to have an anti-apoptotic response in degenerating photoreceptors of the Royal College of Surgeons (RCS) rats that have inherited retinal dystrophy (185). In another study, it was shown that administration of ciliary neurotrophic factor protected photoreceptors in three different mutant mouse models; rd/rd (retinal degeneration), nr/nr (nervous) and Q344ter (truncated carboxyl terminus where last five amino acids of rhodopsin are removed); as well as in presence of light-induced damage (186). The beneficial effects of neurotrophic factors in combination, like VEGF and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), or brain derived nerve factor and ciliary neurotrophic factor was also reported and explained to interact synergistically for photoreceptor survival (187, 188).

The concept of using neurotrophic factors in combination can be further refined by the use of neural stem/progenitor cells. Neural progenitor cells are multipotent, have self-renewal capacity and can be conveniently expanded in culture (189). Unlike neural stem cells, they are more specific and this reduces their tumorigenic potential. Interestingly, human neural progenitor cells (hNPCs) have been used as a source of neurotrophic factors in retinal degeneration studies. Gamm and colleagues (2007) have shown hNPCs to survive for prolonged periods (up to 130 days post transplantation) and provide neurotrophic support to rescue photoreceptors in the RCS rat. The transplanted hNPCs were found to migrate and integrate into the retina forming a pigmented sub-retinal cell layer between the RPE and photoreceptors as well as distribute as individual cells in the inner retinal layers (190). In other studies, hNPCs used as feeder layers in mouse and rat retinal explant cultures were seen to provide neuroprotection solely by their ability to secrete a combination of diffusible factors through the culture medium (191, 192).

Although hNPCs have their relevance in retinal neuroprotective therapy, they do not have the ability to regenerate photoreceptors once cell death has taken place. The use of hNPCs to treat retinal degenerative disorders may prove to be beneficial only when a significant number of photoreceptors still remain unaffected. If this window of opportunity is met, hNPCs can help prevent or slow the progression of further damage. Hence, it may be advantageous to combine hNPC neuroprotection with additional strategies like cell replacement therapy to help restore and maintain vision.

2 AIMS OF THE THESIS

The overall aim of the thesis was to investigate ways to treat two different aspects of retinal dysfunction: (1) retinoblastoma, which is characterized by uncontrolled cell proliferation; and (2) retinal neurodegeneration, which is related to loss of retinal cells and functionality. Depletion of pyrimidine ribonucleotides through targeting the *de novo* and salvage pathways of synthesis was employed as a possible strategy to inhibit retinoblastoma cell growth and promote cell death; whereas, hNPCs were selected as a means to preserve retinal cells and their functionality in case of neurodegenerative events.

The specific aims of the papers were:

Paper I: To characterize a new class of molecules that activate p53, elucidate their mechanism of action and identify their target. Once the target was identified as DHODH, we wanted to further assess therapeutic implications.

Paper II: To investigate if DHODH inhibitors as a single agent or in combination with nucleoside transport inhibitors can inhibit retinoblastoma cell growth and promote cell death.

Paper III and IV: To characterize retinal neurodegenerative events in the *in vivo* pdgf-b$^{ret/ret}$ retina and *in vitro* cultured porcine retinal explant with emphasis on photoreceptor degeneration, second order neuron remodeling and glia reactivity.

Paper IV: To investigate whether treatment of porcine retinal explant cultures with hNPCs can preserve photoreceptors and minimize further neurodegenerative events.

3 RESULTS AND DISCUSSION

3.1 DISCOVERY OF A NOVEL DHODH INHIBITOR (PAPER I)

3.1.1 Screening for novel p53 activating compounds

In paper I, we set out to find novel small molecules that could activate p53 in tumors retaining wild-type *TP53*. To do this, we performed a phenotypic screen of 10,000 molecules from the DIVERSet and 10,000 additional molecules from the CombiSet libraries obtained from ChemBridge on two different p53 reporter cell lines. These p53 reporter cell lines had been previously obtained from T22 murine fibroblasts and the A375 melanoma cell line, by stably transfecting a pRGC-ΔFosLacZ construct expressing the bacterial lacZ-gene, encoding for β-galactosidase, under a p53-dependent promoter. After transfection, these cell lines were designated as the T22 pRGC-ΔFosLacZ and the ARN8 melanoma cell line respectively. Among these 20,000 molecules, 20 showed p53 transcriptional activity >1.5 fold in the ARN8 cells and <1.5 fold or no activation in the T22 cells. One of these molecules showed favorable medicinal chemistry characteristics which included the presence of a chiral center and absence of PAIN (pan-assay interference compound) (193) like features. This molecule, which was named HZ00 based on its tetrahydroindazole structure, was selected for further investigation.

3.1.2 Characterization of HZ00

To determine the mechanism of action of HZ00, we first did a large kinase panel assay with the compound to see if it had kinase inhibiting properties. The assay revealed no such activity for HZ00 and also confirmed a non-promiscuous targeting profile for the compound. To see if p53 activation was elicited as a result of DNA damage, the expression of proteins involved in the DNA damage pathway were investigated, which included phosphorylated (p)-ATM, p-ATR, p-chk1 and p-serine-15 p53. Our results did not show any increase in expression for these proteins and also highlighted that HZ00 is not a genotoxic agent. The next reasonable option was that HZ00 may be an HDM2 inhibitor like the well-established molecule nutlin-3, which is known to stabilize p53 by blocking its degradation. Before engaging in this line of thought, we first went ahead to see the effect of HZ00 on the viability of ARN8 cells and normal HNDFs (human normal dermal fibroblasts) as well as determine its selectivity in comparison to nutlin-3 at similar concentrations. Compared to nutlin-3, HZ00 was more selective for ARN8 cells and portrayed a potentially better therapeutic window. Afterwards, we did cellular thermal shift assays (CETSA) with nutlin-3 and HZ00 to assess whether HZ00 is able to bind to HDM2 or HDMX. The CETSA assay shows the thermostability of a molecule upon binding to a ligand; the bound molecule is relatively more thermostable than the unbound molecule (194). Our results indicated that HZ00 was not able to bind to either protein whereas nutlin-3 was able to bind HDM2 but not HDMX. These results suggested that HZ00 was possibly able to stabilize p53 through some other mechanism. We performed a cycloheximide chase study (195) to see whether HZ00 was able to stabilize p53 and prevent its degradation by the protein synthesis inhibitor. However, there was no indication that the

main mechanism of action of the molecule was to stabilize p53. The remaining explanation was that HZ00 may increase p53 synthesis. To investigate this, we did a pulse-chase experiment using ^{35}S labelled methionine and cysteine (196) to see the incorporation of these amino acids into newly synthesized protein in the presence of HZ00 or nutlin-3. The results showed that, indeed, HZ00 was able to promote the synthesis of p53 protein whereas nutlin-3 did not have any significant effect at the studied time point. Further qRT-PCR experiments demonstrated no noticeable changes to p53 mRNA levels, which suggested that the p53 synthesis induced by HZ00 may be related to increased translation rather than the increase in transcription of p53 mRNA.

Since HZ00 was able to induce p53 synthesis and nutlin-3 can prevent p53 degradation, we thought that combining the drugs would illustrate a greater p53 effect in cancer cells by enhancing cell death. We did western blots to see p53 protein expression with both the agents as single treatments and in combination. And indeed, we saw that the drugs in combination showed much higher p53 expression compared to the single agents. To assess whether the combination would lead to increased tumor cell death, we did propidium iodide (PI) flow cytometry and analyzed the SubG1 percentage of cells. Compared to the single agents, the drug combination showed higher SubG1 percentage in ARN8 cell cultures. Additionally, SubG1 levels in the HNDFs were negligible in all cases. By this time, we had also tested that the *R* enantiomer of HZ00 was the active enantiomer responsible for activating p53's transcriptional activity. Since the *in vitro* studies showed promising results, we performed xenograft tumor studies on NOD/SCID mice using *R*-HZ00 and nutlin-3. Compared to control, the increase in tumor volume was delayed by both the single agents and the combination, with the combination being more effective. Taking these results together, we continued our search for the drug target.

3.1.3 *R*-HZ00 is a DHODH inhibitor

To begin the process of target elucidation, we first did a time course analysis using BrdU/PI bidimensional flow cytometry with HZ00. The results showed that short treatment times caused ARN8 cells to accumulate in S phase and long treatments lead to increase in SubG1 levels. Furthermore, HZ00 decreased cdc6 (cell division cycle 6) which plays an important role in licensing replication origins. A reduced expression of cdc6 may be associated to decreasing nucleotide levels (197). Based on these observations, we considered the possibility of a depletion of nucleotides to be related to the activity of HZ00. In support of this theory, we saw that HZ00 was also able to lower total RNA levels. So as a next step, we started to add nucleosides to see whether this would alter the response to HZ00 treatment. Among the nucleosides, only uridine was able to prevent *R*-HZ00 activity on ARN8 cell growth and p53 transcriptional activity. This indicated that the drug was targeting pyrimidine ribonucleotide synthesis. To figure out which enzyme of the pathway was being targeted, we started to supplement ARN8 cells treated with *R*-HZ00 with the substrates and products of the enzymes in the *de novo* pyrimidine ribonucleotide synthesis pathway. From this, we concluded that the possible target for *R*-HZ00 was DHODH. In particular, we observed that supplementation

with the product of DHODH, orotate, rescued the cells from the treatment whereas supplementation with the DHODH substrate, dihydroorotate, had no effect. We confirmed this finding by treating the cells with two established DHODH inhibitors, brequinar and teriflunomide. Both these compounds were able to decrease ARN8 cell viability and increase p53 transcriptional activity, and both these effects were prevented by uridine supplementation. Thus, we could conclude ribonucleotide depletion, as a result of DHODH inhibition, was causing p53 activation. After these phenotypic studies, we tested *R*-HZ00 and *S*-HZ00 in an enzymatic assay with purified human DHODH enzyme. In agreement with the cell-based studies, *R*-HZ00 was able to potently inhibit DHODH while the *S* enantiomer had negligible effects.

3.1.4 HZ05 is a more potent HZ00 analogue

Since the therapeutic effects of HZ00 on cells was observed at relatively high concentrations, we wanted to find a more potent molecule with similar medicinal properties and selectivity. After testing a number of analogues with the DHODH enzymatic assay, we found a molecule that fit our criteria and was able to inhibit DHODH at nanomolar concentration. This compound was named HZ05. HZ05 was able to inhibit ARN8 cell viability and increase p53 transcriptional activity similar to HZ00, but at much lower concentrations. Furthermore, uridine supplementation was able to prevent these effects. Experiments in combination with nutlin-3a, the active enantiomer of nutlin-3, showed enhanced tumor cell killing both *in vitro* and *in vivo*. The similarity of its mechanism of action with HZ00 was also highlighted by an RNA sequencing experiment that was done with the two compounds. To investigate the binding mode of HZ05, we did crystallographic studies with racemic HZ05 and human DHODH. The crystal structure showed that the *R* enantiomer binds to the quinone tunnel of DHODH. This highlighted the specificity of the highly potent *R*-HZ05 compared to *S*-HZ05.

During this time, we also decided to rescreen the 20,000 molecules that we started with as well as another set of 30,000 molecules that were previously screened in a paper published in 2008 (198) to assess DHODH inhibition with the enzymatic assay. p53 transcriptional activity was also reassessed in the 30,000 molecules using the ARN8 cell line. Our findings showed that DHODH is a frequent target for a number of p53 activators. Moreover, we also discovered 12 different chemotypes that targeted DHODH.

3.1.5 HZ compounds accumulate tumor cells in S phase with high p53 levels

In our previous findings we observed that both HZ00 and HZ05 were able to accumulate ARN8 cells in S phase, and also enhance ARN8 cell death when combined with nutlin-3 or nutlin-3a. Next, we tried to see the effect of HZ00 and HZ05 in other cancer cell lines to ensure the effect is just not restricted to the ARN8s. Like the ARN8s, other cancer cell lines tested (SigM5, MV411 and U2OS) also responded with an initial accumulation of cells in S phase followed by increases in SubG1 levels. However, in the U2OS cell line, cells demonstrated a propensity to be in S phase. Further investigation showed that the cells at S phase had high levels of p53. This observation led us to think whether adding a p53

degradation inhibitor, when cells accumulate at S phase with high p53 levels, would enhance tumor cell death. To investigate this, we either co-treated U2OS cells with HZ05 and nutlin-3a, or we pretreated cells for 72 h with HZ05 and followed the treatment with the addition of nutlin-3a. Interestingly, co-treatment of the drugs resulted in an arrested phenotype, whereas the pretreatment condition with HZ05 followed by nutlin-3a lead to cancer cell killing. These observations indicated that DHODH inhibitors could sensitize cancer cells to p53 degradation inhibitors by accumulating them in S phase with high p53 levels.

3.2 A DHODH INHIBITOR IN COMBINATION WITH A NUCLEOSIDE TRANSPORT INHIBITOR PROMOTES RETINOBLASTOMA CELL DEATH (PAPER II)

In paper I, we showed that DHODH inhibitors can activate p53 and lead to S phase accumulation followed by cancer cell death. Considering the molecular pathology of retinoblastoma, we thought that targeting DHODH may be a viable option. As previously mentioned in this thesis, retinoblastoma is typically a *TP53* wild type tumor, and inactivation of *RB1* leads to the loss of the cell cycle's major G1-S checkpoint Rb, which is known to protect cells from unscheduled S phase entry. Since DHODH inhibitors lead to cancer cell death by accumulating cells in S phase, the use of these agents seemed rational in a retinoblastoma setting.

3.2.1 DHODH inhibition affects retinoblastoma cell growth

At the beginning of this project, we tried to assess the effect of brequinar on retinoblastoma cell growth using a WST-1 metabolic assay for cell viability and PI cell cycle flow cytometry. We treated two commonly used cell lines in retinoblastoma research, the Weri-Rb1 and Y79, with brequinar at increasing concentrations (max 10 µM) for a period of 3 days. The metabolic activity of both cell lines were seen to decrease with increasing concentration until reaching a plateau after approximately 1 µM of brequinar. However, the cell cycle was unaffected by the treatment. These results were unexpected because we were anticipating a possible increase in S or SubG1 phase of the cell cycle based on our findings in paper I. Since the retinoblastoma cell lines proliferate slowly, we decided to prolong the treatment to 6 days. Indeed, this treatment time was seen to cause cells to accumulate in S phase at lower concentrations, whereas at higher concentrations there was a mixture of S phase and SubG1 cells. A point to be noted is that the WST-1 assay measures cell metabolic activity as a readout for viability and proliferation, and assessing a drug that targets the mitochondria may not give an accurate readout for proliferation. This is because a reduction in respiratory activity by the drug may not necessarily reflect a reduction in cell number (199). To account for this, in paper I an SRB (Sulforhodamine B) assay (200) was selected to determine cell growth and viability. This was not used in this project because the SRB assay is most suited for adherent cells. Therefore, we have to bear in mind that the readout from the WST-1 assay in these initial experiments, may actually reflect the metabolic activity of the cells and not the proliferation.

3.2.2 Combination of DHODH inhibitor with nucleoside transport inhibitor shows synergistic cancer cell killing effect

Combinations between therapeutics are becoming more frequently considered as a strategy in cancer because they enhance cancer cell death and, therefore, reduce the chance of metastasis. This may be highly applicable in retinoblastoma because of its invasive nature. Importantly, combination of therapeutics also diminish the risk of drug resistance, as well as enable the use of lower drug concentrations to obtain better therapeutic windows (68). To enhance the therapeutic effect of brequinar, we considered whether simultaneous blocking of the salvage synthesis pathway of pyrimidine ribonucleotides would give a synergistic effect. We selected a human ENT1 inhibitor, dipyridamole, based on its current use in the clinic, and also because it was previously investigated to mitigate several ocular disorders (149). Dipyridamole as a single agent did not show any decrease in retinoblastoma cell metabolic activity and did not affect cell cycle profiles. This may indicate that retinoblastoma cells are more dependent on the *de novo* pyrimidine ribonucleotide synthesis pathway than on the salvage pathway. Interestingly, the combination of brequinar and dipyridamole led to synergistic effects in retinoblastoma cell killing. Time course studies with the drug combination showed an accumulation of S phase cells by 24 hours of treatment with an increase in SubG1 cells over time. By six days of treatment, both cell lines showed considerable increases in cell death which nearly reached 100%, based on single cell population gating. Flow cytometry experiments with a live/dead marker, which is similar to a dye exclusion assay, also confirmed these results. Brequinar as a single agent at the concentration tested was also able to significantly cause cell death in the Weri-Rb1 cells when treated for 6 days. Since it is known that the presence of uridine can affect the activity of DHODH inhibitors, we supplemented 5 µM of uridine to the cell cultures when drugging. The concentration of uridine selected was based on reference values for human plasma uridine concentrations (146), as it was difficult to find literature on uridine levels in the human vitreous humor. The drug combination was still significantly effective ($p < 0.0001$) at causing retinoblastoma cell death, even in the presence of 5 µM uridine, however, brequinar as a single agent lost its ability to induce cell death.

To ensure that the combination therapy did not cause cell death in normal cells, we treated the retinal pigment epithelium ARPE-19 cells with the combination and did not see excessive cell death, however the proliferation of the ARPE-19 cells were slightly reduced. When considering retinal development, usually there is a sharp decline in retinal pigment epithelium cell proliferation after birth (201-203), which could mean that our current treatment strategy may not affect the retinal pigment epithelium. Retinal neuronal cells are usually postmitotic and are difficult to grow in culture unless they are immortalized. Hence, the effect of the treatment could not be assessed for normal retinal neurons. However, teriflunomide and leflunomide which are used in the clinic for multiple sclerosis and rheumatoid arthritis, respectively, as well as brequinar, have not been reported to cause adverse visual impairment to the best of our knowledge. On the other hand, as mentioned earlier in this thesis, current chemotherapeutic drugs can cause retinal damage as well as have other severe toxicities when

given systemically (158-160). The possible side effects of the combination of brequinar and dipyridamole on the retina, that might still be functional in retinoblastoma patients, need to be addressed through *in vivo* animal studies, *in vitro* organotypic studies or with retinal organoid systems.

3.2.3 Activation of caspase 3 and 7 precedes cell death

To try to determine the mechanism of cell death we did live cell imaging with a caspase 3/7 substrate and YOYO-3. The caspase 3/7 substrate is cleaved by activated caspases 3 and 7, after which it is capable of binding to DNA and emit a fluorescent signal, while YOYO-3 is a cell impermeable nuclear dye. Our results showed that the combination treatment activated caspase 3/7 prior to YOYO-3 death signals, indicating an apoptosis mediated cell death. The same also held true for brequinar as a single agent. To clarify further, we assessed the protein expression of cleaved poly (ADP-ribose) polymerase-1 (PARP-1) in the Weri-Rb1 cell line. The proteolytic cleavage of PARP-1 by activated caspases 3 and 7 is considered a characteristic event of apoptosis (204). The expression of cleaved PARP-1 was also increased in the combination after 48 h of treatment, which was in agreement with the previous findings where caspase 3/7 signal was seen to increase after 1.5 days. However, although these experiments are indicative of apoptosis, further experiments with the pan caspase inhibitor Z-VAD-FMK and the expression of other apoptosis markers (e.g. Bcl-2, Bax) need to be performed. Live cell imaging with the ARPE-19 cells using YOYO-3 did not show any death signal for the entire time span investigated.

3.2.4 DHODH and nucleoside transport inhibition promotes retinoblastoma cell death irrespective of p53 status

In paper I, we saw that DHODH inhibitors can activate p53. We wanted to see if the same was true in case of the retinoblastoma cells. We observed that p53 protein expression started to increase for the combination treatment after 48 hours. This reflected the fact that p53 activation may not have led to caspase activation, which was observed to start before 48 hours.

Another interesting finding was that both brequinar as a single agent and in combination with dipyridamole showed effect in a p53 mutant retinoblastoma cell line, which initially started with an S phase accumulation of cells, followed by increase in SubG1 levels. This p53 mutant cell line was derived from a metastatic site. Although p53 mutations in retinoblastoma are quite rare, metastatic tumors may tend to harbor p53 mutations (205). This highlighted the possibility for this treatment strategy to target both p53 wild-type and p53 mutant retinoblastoma cell backgrounds.

3.3 CHARACTERIZATION OF NEURODEGENERATIVE EVENTS IN AN *IN VIVO* AND *IN VITRO* MODEL OF RETINAL DEGENERATION

3.3.1 Pathways to photoreceptor degeneration (paper III and IV)

Photoreceptors are sensory neurons that have the ability to survive during the entire lifespan of an organism. However, they are susceptible to injury or death as a result of neurodegenerative pathologies. Since these neurons are postmitotic, their loss may have a profound impact on vision quality. If the underlying disease mechanisms are not properly addressed, the loss of the photoreceptors can eventually lead to blindness. In these papers, photoreceptor degeneration was assessed by studying cell death, cone outer segment shortening or loss, opsin mislocalization, and synaptic disassembly in the OPL.

3.3.1.1 Loss of the pdgf-b retention motif leads to photoreceptor cell death and cone outer segment shortening (paper III)

In case of the pdgf-b$^{ret/ret}$ mutant mouse retinas, vascular defects in the choriocapillaris and the intraretinal vessels were evident from postnatal day (P)10. In the older mutants, the defects became increasingly severe with total disorganization of the vascular bed including distorted and frequently hyperfused vessels. Aberrant NG2 profiles were also apparent, reflecting improper pericyte investment to the vessels. The abnormal tortuous phenotype of the vessels could be related to endothelial cell hyperproliferation due to defective pericyte recruitment which is known to negatively regulate endothelial cell proliferation (206, 207). Interestingly, cone outer segment shortening was observed to start from P10 and continued to shorten in the older mutants. Based on TUNEL assay and cc-3 immunostaining, there was a dramatic increase in photoreceptor death between P10 to P15. These observations could be explained by the insufficiency of the choroidal blood supply in the mutant mice to meet the high metabolic requirements of the photoreceptors, which increases when the mice open their eyes at around P14. The deficiency in proper nutrient and oxygen balance may start with shortening of outer segments and eventually lead to cell death when the actively functioning cones create an increasing burden on the already compromised vascular network. Another crucial factor leading to photoreceptor death and cone OS shortening could be retinal detachment from the RPE which was also observed at P15 and P28. Interestingly, the rod photoreceptors seemed to be more vulnerable to cell death compared to the cones. This was assessed by TUNEL and rhodopsin colabeled rod cells which were usually present towards the inner aspect of the ONL, and also by the fact that the cone photoreceptor density, quantified by the number of OSs/mm, was only significantly reduced at P28. Rod degeneration followed by cone degeneration has been shown in other models of retinal degeneration as well (164, 208, 209). Another interesting observation was the higher proportion of single TUNEL+ photoreceptor cells compared to cc-3+ cells. TUNEL labels fragmented DNA, which is known to occur in apoptotic and necrotic cells. Since a high number of cells were only TUNEL+ without cc-3 co-labeling, it could be that alternative death mechanisms are involved apart from apoptosis. In other studies, it has been demonstrated that photoreceptor death can also be linked to autophagy, necrosis and caspase-

independent calpain mediated pathways (210-212). However, another scenario may be that after cc-3 activates the endonuclease caspase activated DNAse to induce DNA fragmentation it is no longer detectable in the cell due to its rapid activity and transient nature.

3.3.1.2 Retinal explant culture leads to photoreceptor cell death, cone outer segment loss and opsin mislocalization (paper IV)

In the retinal explant cultures, photoreceptor cell death was assessed by TUNEL assay after 1, 2 and 3 days of culture. As expected, cell death increased with days *in vitro* (div) and a significant difference was seen at the third day of culture relative to uncultured controls. This fast rate of photoreceptor loss is thought to be related to the explantation procedure, which involves isolation of the retina from the systemic blood circulation and detachment from the RPE. The RPE and photoreceptor OSs together form a functional unit that allows for the proper health and maintenance of the photoreceptors (213). In fact, in previous studies with retinal explant cultures that included the RPE, there was a slower rate of photoreceptor degeneration compared to those that excluded the RPE (173, 214). When assessing cone OS shortening and conservation, it was observed that both cultured and hNPC cocultured explants underwent OS shortening; however, the only cultured explants had a significant reduction in OS conservation. The cone OS is known to contain opsin, which is the primary visual pigment in the cone phototransduction pathway. After opsin is synthesized in the endoplasmic reticulum it is transported to the OS. In a normal retina, opsin remains restricted to the OS unit, however, it has been reported that in neurodegenerative pathologies there may be opsin mislocalization to other parts of the cone cell, which may contribute to photoreceptor apoptosis (212, 215, 216). In this study it was demonstrated that after 3 div, the cultured explants had opsin mislocalized to the cell somata, axon and synaptic pedicle. Quantifying opsin labeled cone pedicles revealed a significantly higher number of cone photoreceptors to have mislocalized opsin than hNPC cocultures. The normal retinas had opsin restricted to the OS. Since the presence of the OS is crucial for the proper shuttling of the pigment, its loss may lead to the undesired accumulation of opsin in other components of the cell and increase cell burden. Consequentially, this may lead to an ER stress response causing cell apoptosis (212, 215-217). Interestingly, similar to paper III most of the TUNEL+ photoreceptors seemed to be labeled by the rod population, reflecting the vulnerability of these cells to the culture paradigm. Although rhodopsin, the visual pigment found in rods, was not investigated in this study it could be that rhodopsin accumulation may be a contributing factor to rod photoreceptor death. Studies on rat retinal explants with detachment from the RPE have previously connected rod photoreceptor death to rhodopsin accumulation (192).

3.3.1.3 Synaptic disassembly (paper III and IV)

The photoreceptor axon terminals are highly sophisticated functional structures that form synapses with the second order neurons in the OPL. Synaptic disassembly may lead to impaired transmission of phototransduction signals from the ONL to the INL and eventually compromised vision. Immunolabeling for synaptic proteins ribeye (paper III) and PSD-95

(paper IV) together with PNA staining for cone pedicles (paper III and IV) was used to assess synaptic integrity of the photoreceptor axon terminals. Ribeye is a major protein component of the unique photoreceptor ribbon synapses that allow for high rate neurotransmission. The ribbon, present at the active zone of the synaptic terminal, provides a reservoir of synaptic vesicles that are immediately available for exocytosis (11). In the immunofluorescence studies of paper III, ribeye immunostaining appeared as a uniform band of punctae in the OPL with a characteristic horseshoe shape in the P15 and P28 control mice. However, in the pdgf-b$^{ret/ret}$ P15 and P28 mutants the expression of ribeye was reduced and disorganized, especially in rosette dominant regions, and had altered morphology. In the P28 mutants, ribeye immunostaining was found to be scattered in the ONL. The reduction in ribeye expression could be due to disrupted transport of ribeye to the photoreceptor terminals or due to the loss of the photoreceptors. And the presence of the proteins in the ONL may be explained by photoreceptor axon retraction, which could be related to the rod population because PNA labeled cone pedicles mostly remained restricted to the OPL. The loss of the characteristic horseshoe shape may be because of the degradation of the ribbon complex. It has been reported that the stability of the ribbon complex can be modified by Ca^{++} homeostasis (218). In a mouse model of glaucoma, investigation of the ribbon synapses also showed altered ribeye protein shape and decreased expression, which were thought to be related to disrupted Ca^{++} homeostasis (219). In another study it was found that loss of the calcium binding protein, CaBP4, caused an imbalance in Ca^{++} homeostasis which eventually affected photoreceptor ribbon synapse integrity (220). In case of the cone pedicles, the older mutants had tendencies of losing their PNA staining possibly due to cone cell death. However, as mentioned earlier, the pedicles retained their position in the OPL.

In paper IV, synaptic integrity was investigated by PSD-95 expression in the photoreceptor synaptic terminals. PSD-95 is a scaffolding protein that aids neuronal signaling by means of interacting with different glutamatergic receptors, cell adhesion molecules, and cytoskeletal elements (221, 222). In normal retinas, PSD-95 appeared as a uniform and distinct band in the OPL. However, in the cultured and cocultured explants frequent gaps devoid of PSD-95 immunostaining was observed possibly due to the rapidly degenerating rod population. In a previously reported study on a transgenic porcine model of retinitis pigmentosa, it was shown that PSD-95 immunoreactivity was lost from rod axon terminals but persisted in cone pedicles as a result of early rod degeneration (223). In this study, photoreceptor terminals in both the experimental groups depicted three different features: (a) intact synaptic terminals with low intensity PSD-95 labeling (b) condensed terminals with high intensity PSD-95 labeling and (c) PSD-95 labeled terminals with high or low intensity L/M-opsin colabeling. In case of intact terminals, PSD-95 was seen to distinctly outline the plasma membrane of the cone pedicles and rod spherules at low intensity. Condensed synaptic terminals showed high intensity PSD-95 labeling and were considered to represent impaired synaptic integrity. The cultured explants were seen to have a significantly higher number of condensed PSD-95 labeled terminals compared to the hNPC cocultured explants. Moreover, these condensed PSD-95 labeled terminals would frequently colabel with high intensity L/M-opsin labeled

condensed cone terminals. Interestingly, in the same study by Blackmon and colleagues (223) it was also reported that PSD-95 labeled pedicles appeared condensed and small in the transgenic porcine model. The loss of the cone pedicle's triangular shape has also been observed in human retina explant cultures (224). Additionally, there was also a significant reduction in the number of PNA labeled cone pedicles in the cultured explants compared to the cocultures. Similar to paper III, the pedicles were restricted to the OPL.

In both paper III and IV, it was difficult to confirm whether the loss of synaptic integrity occurred before or after photoreceptor degeneration because of the selected time points. In many studies it has been observed that synaptic integrity is compromised upon extensive photoreceptor loss (225-227). Due to the rapid death of photoreceptors in such short time frames; i.e. between P10 to P15 in the pdgf-b$^{ret/ret}$ mutant retinas and within only 3 days of culture for the retinal explants; it could be considered that photoreceptor cell death may be an upstream event for synapse disassembly.

3.3.2 Second order neuron responses to retinal neurodegeneration (paper III and IV)

It has been previously reported that a causative factor for second order neuron defects may be related to loss of synaptic connectivity with the photoreceptor terminals as a result of compromised photoreceptor terminal integrity (224, 228, 229). We hypothesized that extensive photoreceptor degeneration together with OPL disruptions would also translate to second order neuron impairment. Moreover, since the intra-retinal vasculature provide the necessary support to maintain the health and survival of the second order neurons, abnormalities of the vasculature in the pdgf-b$^{ret/ret}$ mutants as well as vascular regression and loss of vascular perfusion in the cultured explants could be additional contributing factors. As expected, and discussed below, rod bipolar cells and horizontal cells underwent remodeling in response to retinal degeneration.

3.3.2.1 Rod bipolar cell responses (paper III)

In paper III, rod bipolar cells were detected by PKC-α immunostaining. Compared to the controls, the pdgf-b$^{ret/ret}$ mutant P15 retinas rod bipolar cell defects were characterized by a reduction in their dendritic tufts, close proximity of the cell bodies to the ONL and the disorganized arrangement of the cells in the INL especially in rosette dominant regions. In case of the P28 mutants, an additional feature was the sprouting of rod bipolar cell dendrites to from single neurites that extended into the ONL. Bipolar cell sprouting and dendrite atrophy were also reported in the CaBP4 knockout mouse model (220), which highlights the fact that photoreceptor synaptic terminal defects may indeed be a consequential factor to bipolar cell remodeling in this study. An explanation for bipolar cell neurite sprouting to the ONL may be due to the retracting rod terminals, which is known to generate a tractional force that induces a growth response from postsynaptic neurons (228).

3.3.2.2 Horizontal cell responses (paper IV)

In paper IV, horizontal cell remodeling in the cultured retinal explants was detected by calbindin immunoreactivity. This included cellular defects related to dendrite atrophy, reduction in cell somata, mislocalization of the cells to the ONL and in rare occasions the formation of neurites extending to the ONL. In a study conducted on human retinal explant cultures (224), it was found that synaptic connections were lost in condensed presynaptic terminals due to alterations in synaptic invaginations required for horizontal dendrite contacts. This in turn led to dendrite atrophy. Other insults leading to neurite outgrowth may be related to defective calcium ion release machinery in photoreceptor synapses (230). It is possible that condensed PSD-95 immunoreactive synapses harbor defective PSD-95 proteins; since PSD-95 is known to regulate Ca^{++} pump localization in photoreceptor synapses (231) it is likely that these synapses may have defective synaptic activity which in turn leads to neurite formation from the second order neurons. These results indicate that the horizontal cell changes may be associated to photoreceptor degeneration and synaptic disassembly. Although morphological changes in horizontal cells were clearly defined, western blot and qRT-PCR results were somewhat inconsistent with the immunohistochemical findings. This may be due to the fact that calbindin also labels amacrine cells of the inner retina and it is likely that these may also have a contribution to the protein and gene expression profiles. It seems that in this case the immunohistochemical results provide a means of distinguishing multiple cells expressing calbindin. A similar discrepancy in visual and western blot results was also experienced by Nagar and colleagues in their studies on an inducible mouse model for retinal degeneration (229).

3.3.3 Glia responses (paper III and IV)

In response to retinal degenerative events, the glial cells are known to shift to a reactive gliosis phase where they show altered morphology and biochemistry. The hallmark features include increased expression of GFAP for the Müller cells and a change in morphological phenotype for the microglia. These characteristic events have been previously reported in pathologies related to macular degeneration (232), diabetic retinopathy (233) and retinal detachment (228). Although the reactivity of Müller cells may prove to be beneficial initially, continued gliosis may tend to be quite the contrary. Similarly, a continued microglial response may prove detrimental to the retinal tissue although it may serve to have a neuroprotective function initially. We observed gliotic responses in both the *in vivo* and *in vitro* models of retinal degeneration which are discussed below.

3.3.3.1 Müller cell gliosis (paper III and IV)

In paper III, Müller cell gliosis in the form of increased GFAP expression throughout the cell was seen in the P15 and P28 mutants. Usually in the P15 mutants, the gliotic response was more prominent in the rosette dominant regions. However, at P28 the Müller cells portrayed intense GFAP labeling and seemed to be hypertrophic. The P7 and P10 mutants, like all the control retinas, only showed GFAP labeled astrocytes near the vitreal aspect of the retina and the optic nerve. Müller cells are sensitive to vascular injury, (233) and interestingly the gliotic

response was seen at a time point between P10 and P15 during which time the hyaloids vessels are replaced by mature vessels (45). Since the mutants harbor a defect in the pdgf-b retention motif there is neovascularization failure and the vessels that form malfunction. Such an event is likely to trigger a gliotic response. During this response, cytokines are secreted that are neuroprotective but later are involved in severe gliosis (234-236) and possibly photoreceptor death. However, based on the time points studied it was difficult to say whether the gliotic response appeared before or after photoreceptor degeneration; the P13 time point may have been interesting for these investigations. Nonetheless, Müller cell gliosis intensified with photoreceptor death especially in rosette dominant regions of the P15 and P28 mutants. It could be that tractional pull from the gliotic Müller cells together with the distorted vasculature caused retinal detachment, which in turn induced photoreceptor death.

In paper IV, Müller cell gliosis is thought to increase as a result of retinal detachment from the RPE in the explants. Müller cell gliosis is a common feature of explants consisting of adult retinal tissue (237). In normal control porcine eyes, GFAP labeled thin strands of Müller cells were seen to span the retina from the inner to outer limiting membrane and is thought to be a common feature of porcine retinas (191, 237). In the 3 div cultures, the Müller cells were heavily gliotic and hypertrophic with GFAP labeling in their processes and cell bodies. Photoreceptor degeneration was also significantly high in the 3 div explants as assessed by cell death and synapse pathology which may have been factors either contributing to or influenced by the gliotic response; since GFAP immunolabeling was not investigated at earlier time points, it was difficult to determine. However, the immunohistochemistry results were not paralleled by the western blot findings, which indicated similar levels of GFAP expression in the normal and cultured explants. This may be because the 3 div cultures underwent a loss of neurons that resulted in thinner nuclear layers which reduced the entire height of the neuro retina. The Müller cells in the normal retinas, although not gliotic, may express the same level of GFAP as the gliotic 3 div cultures because the cells are longer, whereas in the 3 div cultures the cells are shorter but then again have thicker trunks due to hypertrophy. Another possibility may be due to different subsets of cells expressing GFAP, like the astrocytes. However, the qRT-PCR results did agree with the immunohistochemistry findings. A reasonable explanation for the discrepancy in results may be related to the animal set used for the different experiments. Immunohistochemical and qRT-PCR analysis were done on the same animal group whereas western blot data were generated on a different set of animals. This may reflect the importance of the set of animals used for such analysis, and also the presence of possible inter/intra species variation.

3.3.3.2 Microglia responses (paper III)

In the pdgf-b$^{ret/ret}$ mutant retinas, the majority of the microglia were found in their ramified state residing in the inner layers at P7 and P10, which were similar to the control retinas at all time points. In the P15 and P28 mutants a high population of the microglia were observed to morph into their reactive amoeboid like phenotype. The Iba1 or TSPO labeled reactive microglia were quantified at P10 and P15 and were found to be significantly higher in the

P15 mutants relative to control retinas; a time point characteristic of vascular defects, photoreceptor degeneration, retinal detachment and rod bipolar cell remodeling. In some cases, single reactive microglial cells were found in the ONL. As mentioned before, initially microglia reactivity is associated to neuronal protection, however a sustained reactive response contributes to further neuronal damage. Microglia reactivity is thought to be related to cytokine feedback mechanisms and a dysregulation of microglial cytokine production is associated to neurotoxicity (235, 238). There have been reports where cytokine release from reactive microglia and Müller cells has been correlated with the promotion of photoreceptor survival or induction of their apoptosis in retinal pathologies (156, 234, 238). In a study on retinoschisin-deficient mice it was found that microglia reactivity could trigger events that lead to photoreceptor apoptosis (239). Based on the time points selected and the results obtained from this study it is difficult to determine whether microglial reactivity precedes photoreceptor cell death. However, in the older mutants it can be speculated that continued microglial reactivity may be involved in further photoreceptor damage and loss.

3.4 HUMAN NEURAL PROGENITOR CELLS PROVIDE NEUROPROTECTION TO CULTURED RETINAL EXPLANTS (PAPER IV)

In paper IV, it was investigated whether hNPCs in coculture with porcine retinal explants could provide neuroprotection and prevent photoreceptor degeneration, second order neuron remodeling and Müller cell gliosis. Previous studies have reported that hNPCs secrete a combination of neurotrophic factors that may have a synergistic effect on the preservation of neurons in retinal pathologies (187, 190, 191). In this study, conditioned medium from hNPC cultures was found to consist of significant amounts of EGF, eotaxin, FGF-2, IL-2, IL-4, IL-6, IL-8, MCP-1, TGF-α and VEGF. A variety of different neurotrophic/growth factors are known to support photoreceptors in retinal degenerations. Initial studies on cultured rd1 mouse retinas showed that growth factors in combination produced synergistic effects that could significantly rescue photoreceptors (188). As hNPCs have the potential to secrete a multitude of these factors simultaneously, the cells have been reported to be subretinally injected (190) or used as feeder layers in explant cultures (191) to demonstrate their neuroprotective ability. Amongst these factors, FGF-2 and VEGF have been linked to photoreceptor survival (187, 190, 191, 240). EGF and TGF-α, both ligands of EGF-receptor, are known to regulate neuron development, survival and function; EGF has been shown to counteract toxic insults like excitotoxicity, oxidative stress, and ischemia (241-248). Amongst the immunomodulatory cytokines, the anti-inflammatory cytokine IL-4 has been found to inhibit microglia reactivity and also decrease their secretion of neurotoxic molecules. Moreover, IL-4 was found to enhance the survival of axotomized retinal ganglion cells either in culture or *in vivo* (249-251). MCP-1, IL-2, and IL-6 are known to have variable impacts on the central nervous system and their responses highly depend on the environmental milieu and simultaneous presence of other factors (251-255). For instance, in a study it was shown that MCP-1 had both neurodegenerative and regenerative roles after neurotoxin injection into

the intrahippocamal region of the brain. Shortly after injection, neuronal apoptosis was related to elevated MCP-1 levels, which later decreased when significant apoptosis had taken place. However, 3 weeks post-injection MCP-1 levels were observed to rise again during a phase of significant neuronal regeneration (252). In another study it was found that at specific concentrations, MCP-1 proved to be a neuroprotectant for retinal ganglion cell survival in experimental glaucoma (254). Additionally, it was found that transgenic mice lacking MCP-1 or its receptor showed age-related macular degeneration like characteristics (256). Both IL-2 and IL-6 have been implicated in neurodegenerative mechanisms; however, studies have also highlighted these cytokines' neuroprotective attributes in the central nervous system by supporting the survival and physiological functioning of various neurons (251). In fact, IL-2 and IL-6 have been shown to enhance the survival of axotomized retinal ganglion cells in culture (249, 257). Whereas, IL-6 has also been shown to preserve photoreceptors in an experimental model of retinal detachment (258).

It must be mentioned that the cytokines discussed here is only limited to the milliplex assay used to detect these factors. There may of course be various other cytokines at play to preserve the neurons from the degenerative cascade observed in the explant cultures. Moreover, it cannot be dismissed that the explants themselves also intrinsically produce factors in response to both the degenerative culture paradigm and the presence of hNPC-derived proteins, which means that the cocultures may have diverse combinations of factors at different time points based on necessary physiological stimuli. Since Müller cells are also known to secrete a variety of factors in response to neuronal damage, they may also have a major contribution. In this study, the hNPC feeder layers promoted photoreceptor survival, better synaptic integrity and horizontal cell survival. Müller cell gliosis was relieved in the form of reduced density of the cells in the cocultures, however, there was no change in GFAP protein expression relative to the only cultured explants. The results are discussed below.

3.4.1 Photoreceptor protection

In the cocultured retinal explants there was significantly decreased photoreceptor death, as indicated by TUNEL+ photoreceptors, relative to the cultured explants. As discussed in the earlier sections, photoreceptor death was thought to be related to the deconstruction of the OSs due to loss of the RPE. The arrangement of the cone inner and outer segments was better maintained in the hNPC cocultures, and although both the experimental groups underwent cone OS shortening, the cocultured explants had a significantly higher number of preserved OSs. This finding was also paralleled by a significantly lower number of L/M opsin labeled cone pedicles in the cocultures. Together, these observations highlight the neuroprotective effect of the hNPCs on photoreceptor survival. A means by which this is achieved may be related to the hNPCs secreting factors that are similar to those released by the RPE. The RPE is known to secrete various diffusible factors that are thought to regulate the transport of nutrients and waste products across the retina and also promote photoreceptor functionality and homeostasis; these include FGF, TGF-β, IGF-I, PDGF, VEGF, TNF-α (tumor necrosis factor – alpha) and members of the interleukin family (259). Since most of these factors were

also observed in the hNPC conditioned medium, it could be that the hNPCs share some of the functional properties of the RPE to help maintain photoreceptor survival. For instance, in detached retinas *in vivo* it was found that after intravitreal injections with brain-derived neurotrophic factor, rod OSs were better preserved and rod opsin redistribution to the plasma membrane in the ONL was also reduced (228, 260). A similar scenario may be applicable to the cocultures. Another possibility may be that the factors in the hNPC derived medium may collectively contribute to the suppression of apoptotic pathways and better regulation of intracellular degradation systems. In retinal detachment studies on C57BL mice, IL-$6^{-/-}$ mice and Brown Norway rats it was suggested that IL-6 plays an important role in preserving photoreceptors through the STAT3 pathway which is predominantly associated with cellular proliferation and is considered to be anti-apoptotic (258). In our previous studies, we showed that hNPC cocultured rodent explants could help prevent caspase 3, caspase 12 and calpain mediated death pathways which may also be applicable in this study (187, 191, 226).

3.4.2 Synaptic integrity

The beneficial effects of the hNPCs on photoreceptor survival were also hypothesized to result in better maintenance of presynaptic terminals. In the cocultured explants, synaptic integrity was demonstrated by a significantly higher number of PNA labeled cone pedicles and a significantly lower number of condensed terminals labeled by PSD-95, relative to the only cultured explants. Cone pedicles were seen to be subtly labeled by PSD-95 clearly showing their triangular morphology. However, although the immunohistochemical studies showed better synaptic maintenance, PSD-95 expression levels were similar at the protein and genetic levels for both experimental groups. The hNPC culture medium did not seem to have much influence on the reduced levels of PSD-95, but possibly helped maintain synaptic integrity through other mechanisms. The synaptic terminals are highly complex and consist of multiple functional units; further studies are needed to assess whether hNPC protective medium target other synaptic proteins. One possible generalized explanation may be that the presence of MCP-1 may help synaptic stability; in studies it was found that transgenic mice lacking MCP-1 and CX3CR1 developed age-related macular degeneration amongst which synaptic pathology was a characteristic phenomenon (256, 261).

3.4.3 Horizontal cell responses

Immunohistochemical quantification of calbindin labeled horizontal cells showed a significantly higher number of cells in the cocultured explants relative to the only cultured counterparts. Although dendritic atrophy was not entirely eliminated, it seemed that the dendrites were better maintained in the cocultures. It could be that hNPC culture medium may have indirect influence on horizontal cell maintenance through better photoreceptor and presynaptic terminal functionality in the 3 div cultures.

3.4.4 Müller cell responses

In the cocultured explants, Müller cell gliosis was attenuated in terms of a significantly reduced number of GFAP labeled cells compared to the only cultured explants. Although GFAP gene levels were consistent with the immunohistochemical findings, the GFAP protein levels were similar for the two experimental groups. These observations may be explained by the factors discussed in section 3.3.3.1. Although the results obtained for Müller cell responses are inconclusive in this study, it may still be suggested that hNPCs prevent an undesired excessive gliotic response. The neurotrophic factors derived from the hNPCs may have complex rescue mechanisms; it could be that they mimic the Müller cell/photoreceptor intercellular pathway to maintain photoreceptor survival in the cultured explants. It has been reported that Müller cells can protect photoreceptor viability through the secretion of a number of factors upon exogenous treatment with ciliary neurotrophic factor that binds to its cytokine receptor gp130 (262). The degenerating photoreceptors in the explant cultures may send rescue signals to the Müller cells to produce neuroprotective factors. As discussed before, these factors may initially support photoreceptor survival but with continued gliosis could lead to neuronal death. Alternatively, in the hNPC cocultured explants neurotrophic factors are secreted by the cells to preserve photoreceptors and this may minimize photoreceptor signaling to the Müller cells and thus reduce the gliotic response. Nonetheless, based on these findings it is difficult to give a conclusive interpretation of the complex interactions of the hNPC and Müller cell derived factors and their implication on photoreceptor survival.

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