Role of Wnt and Notch signalling pathways on the neural differentiation of human Müller stem cells and their modulation by growth factors

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This thesis is submitted to University College London

for the degree of Doctor of Philosophy

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June 2016

Declaration

I, Angshumonik Angbohang, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

June, 2016

Acknowledgements

I would like to take this opportunity to thank my supervisors Professors Astrid Limb and Karl Matter, without whom this thesis may not be possible. In particular, I am enormously grateful to Astrid for not only designing this wonderful project and providing me with an opportunity to obtain my PhD, but also giving me every opportunity to develop towards becoming a modern scientific researcher by encouraging me to attend various scientific conferences to present my research work, network with my scientific peers and develop potential future collaborations. In addition, I appreciated her positive way of thinking towards difficult situations and her critical analysis to solve scientific problems. It was an honour and a pleasure working for Astrid.

“The Müllers” had been incredible in making my last 3.5 years of PhD training enjoyable and rewarding. Without their care and support, PhD experience would not have been fulfilling. Therefore, I am very grateful to have met you guys and also become part of the Müller family. In no particular order, I would like to say thank you to some of the most awesome people, Dr Hari Jayaram, Dr Megan Jones, Dr Hussam Muntasser, Dr Karen Eastlake, Dr Na Wu, Dr Silke Becker, Dr Phillippa Cottrill, Erika Aquino, Thalis Charalambous and Stephanie Fletcher.

Lastly, I would like to say massive thanks to my family and relatives. In particular, to my wonderful parents without whom I may not have been possible, biologically speaking. In addition, for their wisdom and support throughout my life so far that has been important towards shaping me into the person I am today. I am most grateful

for their ability to install this idea in me that anything is possible in this world, if you are willing to believe and work for it. Thank you.

Abstract

Müller glia mediates retina regeneration in zebrafish. Despite exhibiting Müller glial with stem cell (hMSC) characteristics *in vitro* by the human retina, retinal regeneration mediated by Müller glia following disease or injury has not been demonstrated. Notch, Wnt, TGFβ and HBEGF signalling is well known to regulate retinal neurogenesis and inflammation, but the roles of these molecules in the neural differentiation of hMSC are not known. This thesis aimed to establish whether there is an interaction between these signalling pathways and the role that these factors play during retinal ganglion cell (RGC) and photoreceptor differentiation of hMSC.

The research showed that inhibition of Notch signalling caused downregulation of components of the canonical Wnt signalling pathway in these cells, as demonstrated by a decrease in mRNA expression of the Wnt ligand WNT2B and its target genes WISP-1 and AXIN2. Addition of TGFβ1 did not significantly change the expression of the Notch signalling target HES1 or the RGC marker BRN3A/B. Culture of hMSC with a combination of factors that induce their photoreceptor differentiation (FGF2, taurine, retinoic acid and Insulin growth factor; collectively called FTRI), markedly upregulated the expression of components of the canonical Wnt signalling pathway, including WNT2B, DKK1 and active β-catenin. Although FTRI did not modify mRNA expression of WNT5B, a component of the non-canonical/planar cell polarity Wnt pathway, it upregulated its secretion. Furthermore, TGFβ1 not only decreased WNT2B expression, but inhibited FTRI-induced photoreceptor differentiation of hMSC, as determined by expression of the photoreceptor markers NR2E3, rhodopsin and recoverin. Inhibition of TGFβ1 signalling by an ALK5 inhibitor prevented TGFβ1 induced changes in the expression of the two Wnt ligands

examined. More importantly, inhibition of the canonical Wnt signalling by XAV-939 prevented FTRI-induced photoreceptor differentiation. Similarly, HBEGF, a factor shown to be upregulated by FTRI also decreased Wnt signalling components such as WNT2B, WISP-1, DKK1 and AXIN2. Inhibition of HBEGF by its specific inhibitor CRM197 prevented photoreceptor differentiation. These observations suggest that both Notch and Wnt signalling pathways can regulate the neurogenicity of hMSC *in vitro* and that TGFβ as well as HBEGF play important roles in mediating key pathways leading to either RGC or photoreceptor differentiation of hMSC. Targeting components of both Notch and Wnt pathways may constitute targets for potential induction of endogenous regeneration of the human retina and this merits further studies.

Table of Contents:

List of Figures

Chapter 1:

Chapter 2:

List of Abbreviations

Atoh7	Atonal basic helix-loop-helix Transcription Factor 7
RPC	Retinal Progenitor cell
RGC	Retinal ganglion cell
MG	Müller glia
Pou4f2	POU Class 4 Homeobox 2
HES1	Hairy and enhancer of split-1
HES5	Hairy and enhancer of split-5
HESR2	Hes-Related Family BHLH Transcription Factor with YRPW Motif 2
RAX	Retina And Anterior Neural Fold Homeobox
ILM	Internal limiting membrane
GCL	Ganglion cell layer
IPL	Inner plexiform layer
INL	Inner nuclear layer
OPL	Outer plexiform layer
ONL	Outer nuclear layer
OLM	Outer limiting membrane
bHLH	Basic helix-loop-helix
CMZ	Ciliary Marginal Zone
RPE	Retinal pigment epithelium
BrdU	Bromodeoxyuridine
IdU	Iododeoxyuridine
Pax6	Paired box 6
HUD	ELAV-like protein 4
GFAP	Glial fibrillary acidic protein

PCNA	Proliferating cell nuclear antigen
NMDA	N-methyl-D-aspartate
EGF	Epidermal Growth Factor
Ascl1	Achaete-scute homolog 1
Stat3	Signal transducer and activator of transcription 3
BLBP	Brain lipid-binding protein
Rx1	retinal homeobox gene 1
Six3	SIX Homeobox 3
Olig2	Oligodendrocyte transcription factor 2
TGFa	transforming growth factor beta
bFGF	Basic fibroblast growth factor
GSK3β	Glycogen synthase kinase 3β
APC	Adenomatous polyposis coli
LRP	Low density lipoprotein receptor-related protein
ECD	extracellular domain
ICD	Intracellular domain
TCF	T-cell Factor
LEF	Lymphoid enhancer factor
PCP	Planar Cell Polarity signalling
CAMKII	Calmodulin-dependent kinase II
NFAT	Nuclear factor associated with T cells
NICD	Notch cleaved intracellular domain
CSL	CBF1/RBP-Jk, Suppressor of hairless and Lag-1
NSCLC	Non-small cell lung carcinoma cells
IL-6/-8	Interleukin -6/-8
Mash1	Mammalian achaete scute homolog-1
Ngn2	Neurogenin 2

NMDA	N-methyl-D-aspartate receptor
Shh	Sonic hedgehog
HBEGF	Heparin-Binding EGF-Like Growth Factor
RT-PCR	Reverse transcription Polymerase chain reaction
RGC	Retinal ganglion cells
MSC	Müller stem cells
BMP	Bone morphogenic protein
HSC	Hematopoietic stem cells
PVR	Proliferative vitreoretinopathy
TGIF	TGFB-Induced Factor Homeobox 1
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
DKK1	Dickkopf-1
NOD-SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
GSK3	Glycogen synthase kinase 3
Fz3	Frizzled-3
RPE	Retinal pigment epithelium
ALK5	Activin A Receptor Type II-Like Kinase
JNK	c-Jun N-terminal kinases
FTRI	FGF2, taurine, retinoic acid and Insulin-like growth factor 1
GNAT1	Guanine Nucleotide Binding Protein (G Protein), Alpha Transducing Activity Polypeptide 1
BDNF	Brain-derived neurotrophic factor
RA	Retinoic acid
NR2E3	Nuclear Receptor Subfamily 2, Group E, Member 3
SVZ	Sub ventricular zone
PLZF	Promyelocytic leukaemia zinc finger

Work contributed by lab members:

Dr. Karen Eastlake	Figure 2.6
Dr. Na Wu	Fig 2.7A, Fig 3.3B, Fig 3.5, Fig 3.9, Fig 3.11 and Fig 3.12
Stephanie Fletcher	Fig 3.6B (ELISA)

Chapter 1: General Introduction and Objectives

1.1 Retinal neurogenesis

The eye is a specialised organ responsible for the visual perception. It is made up of three different layers (Fig 1.1). The outer layer is formed by the cornea and the sclera. The cornea functions by directing the light through the lens into the retina, the neural part of the eye, whilst the sclera maintains the eye shape by forming a connective tissue coat around the eye. The middle layer is formed by the iris, ciliary body and choroid. By controlling pupil size, the iris regulates the amount of light entering the eye, whilst the ciliary body controls the shape of the lens and the choroid provides nutrients to the retina. The inner layer is formed by the neural retina, which is considered the most essential part of the eye as it is responsible for detecting light. It is also the part of the eye that is connected to the central nervous system through the optic nerve (Willoughby et al., 2010, McCaa, 1982). The retina is formed of neurons and glial cells which originate from retinal progenitor cells (RPCs) during development (Harris, 1997, Cepko, 1999). This process, known as retinal histogenesis has been shown to be evolutionary conserved across species including lower vertebrates and mammals. The retinal neurons and Müller glia created during this process are formed during a specific order of events in a specific arrangement: 1) retinal ganglion cells (RGC), 2) cone photoreceptor, 3) horizontal cell, 4) amacrine cell 5) rods, 6) Müller glia (MG) and and lastly 7) bipolar cell (Fig. 2A) (Young, 1985, Kahn, 1974, Prada et al., 1991, Cepko et al., 1996).

Using birth dating and lineage analysis experiments, various studies have established that RGCs are the earliest neural cells to develop, whilst MG are formed towards the end of retinal histogenesis (Altshuler et al., 1991, Turner and Cepko, 1987). This has been illustrated by generating an approximate timeline of these cells

in various species including mammals (Fig 1.2) (Rapaport, 2006). In mice and rat, the RGCs are observed at E11.5 and E13 (embryonic days) respectively, whilst MG are observed at P4 and P5 respectively (postnatal days) (Fig. 2C) (Martins and Pearson, 2008). In comparison, the human RGCs develop from 6.5 weeks onwards while MG is generated from 16.5 week onwards (Fig. 2C) (Rapaport et al., 2004, Martins and Pearson, 2008, Donovan and Dyer, 2005). During RGC formation in mice, the expression of Atoh7, a basic Helix loop Helix gene (Brown et al., 2001, Wang et al., 2001) and Pou4f2, a homeobox gene, are essential in mice (Yang et al., 2003). By contrast, inhibition of either of these genes causes a loss in the generation of RGCs (Wang et al., 2001, Yang et al., 2003), whilst MG are formed through the expression of transcription factors Hes1, Hes5, Hesr2 and Rax (Furukawa et al., 2000a, Satow et al., 2001, Hojo et al., 2000) by the undifferentiated RPCs following retinal neuron differentiation (Ohsawa and Kageyama, 2008). Interestingly, the studies performed by Turner and Cepko highlighted the fact that both retinal neurons as well as MG were formed by the same multipotent RPCs during retinal histogenesis (Turner and Cepko, 1987). This was demonstrated by retroviral labeling of progenitor cells in the postnatal rat retina that facilitated tracing the newly formed neurons at different stages. They observed that these multipotent RPCs always differentiated into clones of MG and neurons (e.g. rod photoreceptors), whilst they did not form only single colonies of either retinal neurons or MG (Turner and Cepko, 1987). This phenomenon may be unique to retinal histogenesis in the eye as glial specific progenitors producing only glial cell clones are found in other areas of the central nervous system such as the cerebral cortex (Cai et al., 2000, Levison et al., 1993).

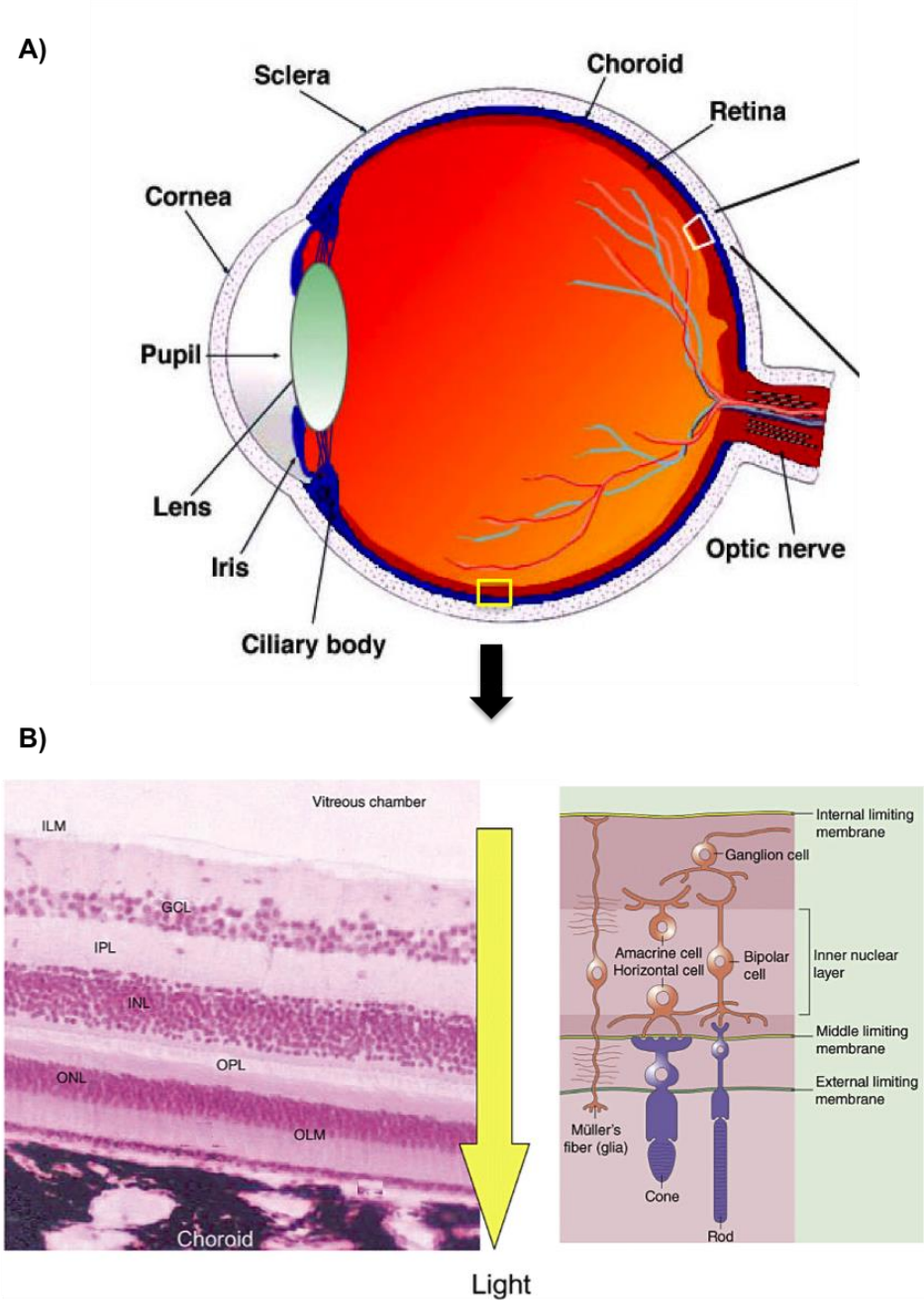

Figure 1.1 Anatomy of a human eye and retina A) Diagrammatic representation of various structures of the human eye. Adapted from (Kolb, 2003) **B)** Histological cross section and representation of different layers of the retina as well as the location of retinal neurons and Müller glia within the retina. From (Willoughby et al., 2010). ILM (internal limiting membrane); GCL (ganglion cell layer); IPL (inner plexiform layer); INL (inner nuclear layer); OPL (outer plexiform layer); ONL (outer nuclear layer); OLM (outer limiting membrane)

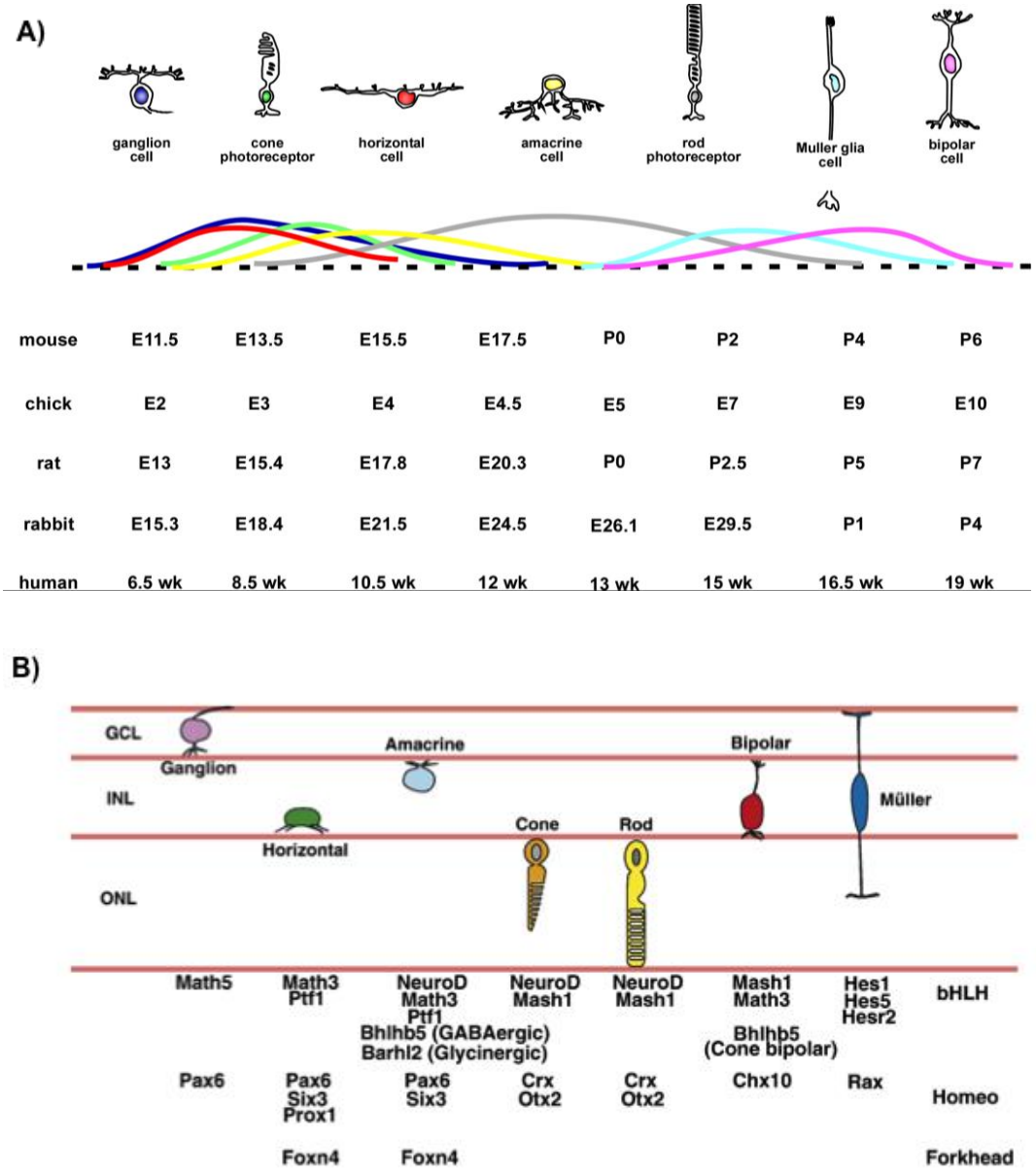

Figure 1.2 Retinal histogenesis. A) A timeline of retinal neurons and Müller glia generated during retinal histogenesis in various species. From the figure, retinal ganglion cells are formed earliest whilst Müller glia cells are formed late during this process. From (Martins and Pearson, 2008). B) List of known transcription factors involved in the formation of different retinal neurons and Müller glia during retinal histogenesis. From (Ohsawa and Kageyama, 2008, Kageyama et al., 2005). bHLH (basic helix-loop-helix); Homeo (Homeobox).

1.1.1 Retinal regeneration by Müller Glia across species

The lower vertebrates such as urodeles, frogs and fish are able to regenerate retina during adult life following retinal damage. For example, in frogs, new retinal neurons are continually added during development as well as after retinal injury from a specialised region containing retinal progenitor cells called the ciliary marginal zone (CMZ) (Perron and Harris, 2000). Other sources for endogenous retinal regeneration have been identified as the retinal pigment epithelium (RPE) and Müller glia (Perron and Harris, 2000).

MG are primarily responsible for retinal regeneration following retinal damage in fish. Early evidence of the MG stem cell properties came from various studies investigating rod photoreceptor generation in fish (Ahlbert, 1976, Johns, 1982, Julian et al., 1998, Otteson et al., 2001). However, at these early stages of MG research these cells were not confirmed as MG. In 1976, Ahlbert et al, observed the presence of mitotic cells in the ONL of larval salmon and suggested that these cells may be responsible for forming rod photoreceptor cells (Ahlbert, 1976). Later, these mitotic cells were shown to incorporate ^{3}H-thymidine labeling and undergo rod photoreceptor differentiation in various fish including larval goldfish (Johns, 1981). Furthermore, in a larval goldfish study it was observed that the RPCs in the INL, which labeled with ^{3}H-thymidine, migrated apically towards ONL by forming chains of elongated cells (Johns, 1982, Raymond and Rivlin, 1987). This finding was further supported by a similar migratory pattern of these thymidine labeled cells in the INL from electron microscope autoradiography and serial reconstruction observations (Raymond and Rivlin, 1987). In addition, by using sequential labeling of thymidine analogs bromodeoxyuridine (BrdU) and iododeoxyuridine (IdU) for several days,

Julian et al., demonstrated that these INL RPCs migrated to the ONL and are responsible for generating rod precursor cells (Julian et al., 1998) .This was confirmed by the observation of IdU labeled cells in the ONL of the rainbow trout. Following 43 hours of incubation in IdU containing water, when the fish were switched to BrdU containing water, cells labeled with IdU only were observed in the ONL. In addition, these IdU only labeled cells increased transiently in the ONL even after 24 hours of BrdU incubation. This suggested that RPCs were able to migrate from the INL to ONL , as any new cells created in the ONL after BrdU switch would have contained BrdU labeling (Julian et al., 1998). Similarly, another study by Otteson et al., which also employed a long term use of BrdU to label these cells in the INL, reported that the number of BrdU labeled cells in INL did not change upto 30 days, whilst the number of BrdU labeled rod precursors increased in the ONL (Otteson et al., 2001). Moreover, they observed a subset of these cells in the INL expressed Pax6, which suggested that these stem cells are responsible for rod photoreceptor generation (Otteson et al., 2001). Interestingly, it was seen that in goldfish retina, MG proliferated and migrated of into empty spaces following photoreceptor damage by laser treatment (Braisted et al., 1994). The most convincing evidence that these INL mitotic cells are MG and are responsible for rod photoreceptor generation in fish, as well as for retinal progenitors after injury, came from the studies conducted in the 2000s by Fausett and Goldman, 2006, Bernardos and Raymond, 2006, Bernardos et al., 2007 and Fimbel et al., 2007. Using transgenic zebrafish with alpha 1 tubulin GFP-tag, a neuron specific protein previously shown to mediate RGC differentiation and proliferating MG after retinal injury, Fausett and Goldman demonstrated that following retinal damage, the MG cells showed GFP expression as well as BrdU labeling. In addition, these cells also expressed the RPC marker Pax6 and after 7 days they expressed HuC/D, RGC and amacrine cell markers (Fausett and Goldman, 2006). Furthermore, Bernardos and

Raymond also conducted similar studies in transgenic zebrafish by labeling cytoplasmic and nuclear Glial fibrillary acidic protein (GFAP) , a protein exclusively expressed by MG in the retina (Bernardos and Raymond, 2006, Bernardos et al., 2007). Using these species, they found that after retinal injury, GFAP labeled MG cells proliferated by expressing markers such as BrdU and PCNA within 1-2 days post injury. At days 3-4, Pax6 expressing RPCs also expressed GFAP labeling and were found in the INL. Furthermore, at days 4-5, the early photoreceptor marker Crx expressing cells in the ONL also were labeled with GFAP and co-expressed zpr1, a cone specific antibody (Bernardos et al., 2007). These results were further supported by a separate regeneration study conducted by Fimbel et al., 2007 where the GCL and INL were damaged through intraocular injection of ouabain in three different transgenic lines of zebrafish labeling for GFP in MG, RPCs and differentiating RGCs through expression of gfap, olig2 and atoh7 respectively. It was shown that within 1 day, there was increased expression of the proliferation marker PCNA in both these layers. MG cells primarily expressed PCNA by day 3. However, by day 5 the RPC expressing olig2 expressed this marker more than MG. After 7 days, these RPCs differentiated towards RGCs by expressing the transgenes atoh7 and zn5 antigens (Fimbel et al., 2007).

In chick, after NMDA induced neurotoxic damage, MG re-entered cell cycle and initially, the dividing cells expressed retinal progenitor markers such as Pax6, followed by the co-expression of retinal neural markers after 7 days (Fischer and Reh, 2001). However, the MG response and its ability to regenerate new retinal neurons after injury are limited in the chick as compared to the fish. This is because only a sub-population of MG cells is able to differentiate in chick as they can only undergo one round of cell cycle (Fischer and Reh, 2002, Hayes et al., 2007). By

contrast, most MG cells in the fish are capable of repairing retinal damage by undergoing multiple rounds of cell cycles (Raymond et al., 2006).

The ability of MG to generate new retinal neurons following retinal injury is also conserved in small mammals such as rodents. However, the regenerating ability is even more limited than in the chick. Like any other injury induced glial response in the mammalian CNS, retinal injury also causes up-regulation of the intermediate filament protein GFAP (Glial fibrillary acidic protein), cellular hypertrophy, changes in ion transport properties and proliferation of MG in a process called reactive gliosis (Bringmann et al., 2009). Reactive gliosis eventually inhibits retinal regeneration as a result of glial scar formation, but during the early phase of reactive gliosis it demonstrates neuroprotective functions by the release of neurotrophic factors such as VEGF (vascular endothelial growth factor) which support survival of retinal neurons (Yamada et al., 1999), secretion of free radical scavengers such as lysozyme, heme oxygenase (Hollborn et al., 2008) and glutamate uptake to prevent neuronal toxicity (Kawasaki et al., 2000). In addition, during the early phase of reactive gliosis a subpopulation of Müller glia re-enters cell cycle (Sahel et al., 1991). Using BrdU labelling on NMDA induced retinal damage of an adult rat , Ooto et al., demonstrated that a subpopulation of proliferating Müller glia had differentiated into other retinal neurons as these subsets of BrdU labelled cells were found co-expressing markers of rod photoreceptors, bipolar cells, horizontal cells and amacrine cells (Ooto et al., 2004). In mice the proliferation of Müller glia appears to be more limited than in the rat as Oubain induced retinal damaged followed by BrdU labelling only demonstrated occasional proliferation (Dyer and Cepko, 2000). Using growth factors such as EGF, Müller glia proliferation was shown to increase in rats (Karl et al., 2008).

In summary, based on these studies, adult MG have the ability to regenerate new retinal neurons across species including lower vertebrates, chick and mammals. Despite the regenerative ability of the MG becoming more limited in higher mammals, by using various growth factors, limited MG proliferation can be promoted in small mammals.

1.1.2 Comparison of Müller glia functions between zebrafish and mammals

Whilst the zebrafish retina can endogenously repair after retinal damage (Raymond et al., 2006), mammalian retina cannot self-repair. In a normal retina, Müller glia provide support to neighbouring retinal neurons by maintaining retinal homeostasis through exchange of ions, water and neurotransmitters, as well as visual function (Reichenbach and Bringmann, 2010). The stem cell-like characteristics of MG in zebrafish is proposed to come from the retinal progenitors which give rise to these cells during development (Jadhav et al., 2009). Studies of retinal histogenesis in small rodents show that, MG acquire glial characteristics whilst still maintaining the stem cell properties of RPCs. This theory is supported by evidences from various genomic array studies that have highlighted similarities in the genetic profile between mammalian Müller glia and late stage retinal progenitors (Blackshaw et al., 2004, Roesch et al., 2008, Jadhav et al., 2009).

In the zebrafish, MG also respond to retinal damage by first inducing reactive gliosis in a similar fashion as that observed in the mammalian retina, followed by initiation of the regeneration cascade (Thomas et al., 2016) (Fig 1.3). Genes such as Ascl1,

Lin-28 and Stat3 are observed to be upregulated during retinal regeneration in the zebrafish (Fausett et al., 2008, Ramachandran et al., 2010). In addition, epigenetic studies by Powell et al showed a decrease in the DNA methylation in MG during this regenerative period. The findings that methylation of pluripotency gene promoters, which changed during iPSC (induced pluripotent stem cell) generation, remain unchanged and stayed in a hypomethylated form (Powell et al., 2013), suggest that hypomethylation of pluripotent gene promoters may induce MG derived retinal progenitor formation after retinal injury. This observations provide support for the hypothesis that MG have conserved RPC properties (Powell et al., 2013). Furthermore, the nuclei of zebrafish MG undergo apical migration for mitosis, similar to the interkinetic nuclear movement made by RPCs during development (Lahne and Hyde, 2016) (Fig 1.3). Undergoing a single asymmetric cell division, MG self-renew and also generate a multipotent RPC (Lenkowski and Raymond, 2014). Furthermore, clusters of RPCs are promptly generated by repeat divisions of this multipotent RPC. These cells in the cluster migrate to the areas of damage cells where they differentiate and repair the damage neurons using the radial processes of the dedifferentiated MG (Fausett and Goldman, 2006, Bernardos et al., 2007, Nagashima et al., 2013). Throughout regeneration, zebrafish MG maintain their radial shape to preserve the retinal structure (Lenkowski and Raymond, 2014).

Although endogenous neurogenesis has not been demonstrated in adult mammalian MG after injury, these cells however undergo reactive gliosis. Reactive gliosis has similar features associated with early stages of the zebrafish regenerative process such as MG proliferation, morphological changes, upregulation of regeneration markers, de-differentiation and apical nuclear migration (Dyer and Cepko, 2000, Bringmann et al., 2009). More recently, Löffler et al demonstrated in *ex vivo* mouse retinal explants, where there is continuous loss of neurons, that MGs

display these different phases: a quiescent state, a dedifferentiation state, a proliferation state and neurogenesis of amacrine cells as widely described in the zebrafish (Löffler et al., 2015). The regenerative process was aided by the stimulation with EGF growth factor (Löffler et al., 2015).

Interestingly, in this study following retinal damage, the regeneration-mediating gene Ascl1 was found to be upregulated in a sub population of MG in young mice, whilst their levels were decreased in older mice (Löffler et al., 2015). Overexpression of Ascl1 has been shown to induce mouse MG mediated differentiation *in vitro* (Pollak et al., 2013). Although this gene was overexpressed in MG of young mice *in vivo*, no changes were observed in uninjured retina. It however induced cell cycle entry as well as formation of amacrine, bipolar and photoreceptors in injured retina (Ueki et al., 2015). These studies suggest that a low level of Ascl1 expression found in mammalian MG when compared to the zebrafish is potentially one of the factors responsible for the limited regeneration observed in mammalian retina. Furthermore, the promoters of mice Ascl1 were observed to be hypomethylated in the quiescent MG as well as in the zebrafish (Powell et al., 2013). This further provides support that mammalian MG have the potential for regeneration similar to that seen in the zebrafish.

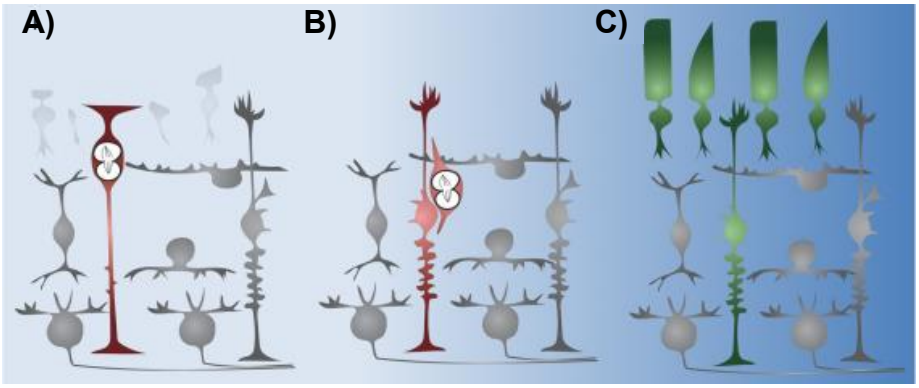

Figure 1.3 A model of retinal regeneration in the zebrafish. A) Following retinal damage, the Müller glia nuclei undergo apical migration within 1 day (Lahne and Hyde, 2016). **B)** Through asymmetric cell division, MG self-renew and generate multipotent RPC within 3-5 days (Lenkowski and Raymond, 2014). **C)** Using radial processes of the MG, these RPCs migrate to neural damage sites where they undergo neural differentiation after 14 days. From (Hamon et al., 2016)

1.2 Müller glia express retinal progenitor cell markers

Following retinal injury in the zebrafish, various studies have shown that activated Müller glia start expressing factors observed in RPCs during development. The expression of RPC markers such as BLBP, Rx1, Pax6, Vsx2 (orthologs of Chx10), Six3 and Olig2 have been reported to be upregulated within the first 24 hours post injury by genetic and protein analysis (Nagashima et al., 2013, Raymond et al., 2006, Hsieh, 2012, Lenkowski et al., 2013, Hafler et al., 2012). One of the RPC markers, Pax-6 is a member of the paired-box (Pax) family characterised by the presence of two DNA binding domains (Paired-domain and Homeodomain). It is conserved across species including lower vertebrates and mammals (Bopp et al., 1986, Bopp et al., 1989). During development, Pax-6 is found in all RPCs and its functions include regulating cell cycle and differentiation in the retina (Oron-Karni et al., 2008). In adult zebrafish, Pax6 is expressed at low levels. During retinal regeneration in the zebrafish, it was shown that Pax6, which exists in two isoforms, Pax6a and Pax6b, is required for differentiation of MG into cone photoreceptors, but not rod photoreceptors (Thummel et al., 2010). Also in mammalian MG, Pax6 was also observed at low expression in cell microarray study in mice (Roesch et al., 2008). Furthermore, despite the increased expression of Pax6 in mice MG, no proliferation of these cell was observed (Joly et al., 2011).

Six3 is an important transcription factor for eye development that directly interacts with Gemini, a cell-cycle inhibitor to regulate cell proliferation and differentiation (Del Bene et al., 2004). In zebrafish, Six3b (one of the three Six paralogs) was shown to be upregulated following retinal damage (Qin et al., 2009). Furthermore, morpholino induced knockdown of Six3, also shown to inhibit TGFβ signalling during zebrafish

brain development (Inbal et al., 2007), mediated a decrease in the formation of RPC under the same conditions (Lenkowski et al., 2013).

As indicated above, an important transcription factor known to promote regeneration is Ascl1a, a basic helix loop helix factor reported to be critical for MG derived regeneration in zebrafish (Fausett et al., 2008), as well as for MG differentiation in mammals (Pollak et al., 2013, Ueki et al., 2015) . Following 6 hours after retinal stab injury in the zebrafish this factor has been shown to be highly upregulated in MG (Fausett et al., 2008). In addition, following acute light damage, Ascl1a expression has been shown to increase by 36 hpi (Qin et al., 2009) and was maintained for 7 days after the injury (Cameron et al., 2005). Whilst downregulation of Ascl1a by morpholino knock down has been shown to reduce MG mediated RPC proliferation (Fausett et al., 2008), other reports suggest that TGFβ signalling may also need to be downregulated for the inhibition of MG mediated regeneration in the zebrafish (Lenkowski et al., 2013).

DNA microarrays comparing genes found in RPCs, MG and neuronal cells have highlighted a similar distribution of many genes across retinal progenitors and MG. Livesey et al., reported that RPCs have more genes in common with MG (43%) than with neuronal cells (Livesey et al., 2004). In addition, from 63 transcripts identified in MG alone by a SAGE study with subsequent in situ hybridization, 61 of these transcripts were also found in RPCs (Blackshaw et al., 2004). In addition, microarray studies conducted in single cells from developing and mature mice retinae (Roesch et al., 2008), where the examined sample cells were categorized into early and late progenitors, showed that the late progenitors contained genes which were highly present in MG, including Sox9 and retinaldehyde binding protein 1 (Roesch et al., 2008).

1.3 Factors involved in Müller glia differentiation

Müller glia has been shown to release various factors that are known to be involved in retinal development and regenerative processes across species. These include HBEGF (Heparin Binding EGF like Growth Factor) and TGFβ (Transforming Growth Factor β) (Wan et al., 2012, Lenkowski et al., 2013). Studies in the zebrafish have shown that factors such as HBEGF promote MG derived retinal regeneration in the injured retina (Wan et al., 2012, Zhao et al., 2014), whilst TGFβ has also been shown to play an important role during this process (Lenkowski et al., 2013). Other factors such as EGF, FGF or FGF1 and insulin have also been shown to induce proliferation of MG cells and appeared to be responsible for the induction of expression of RPC markers such as Pax6 following NMDA induced retinal damage in mice (Karl et al., 2008). It was found that a small population of these cells differentiated into amacrine cells as judged by the expression of markers such as Calretinin, NeuN and Prox1 (Karl et al., 2008).

According to the study by Wan et al. in a stab model of retina injury in the zebrafish, it was found that the MG cells express HBEGF. Within an hour after the injury had been induced, Hbegfa expression increased in the retina and led to proliferation of MG cells followed by their dedifferentiation (Wan et al., 2012, Zhao et al., 2014). In contrast, inhibition of Hbegfa expression during this period using either morpholino constructs targeting HBEGF or EGF receptor inhibitors decreased proliferation of these cells (Wan et al., 2012). However, in a model of zebrafish retinal injury caused by light damage in the zebrafish, it was shown that morpholino mediated downregulation of HBEGF did not alter the proliferation of RPCs (Nelson et al.,

2013). HBEGF expression has been shown to be controlled by the transcription factor Insm1a, as determined by a chromatin immunoprecipitation assay, which indicated that Insm1a binds directly to the hbegf promoter in the injured retina (Ramachandran et al., 2012).

In contrast, TGFβ, an anti-proliferative factor, has been shown to prevent the MG derived regenerative process by inhibiting MG proliferation (Close et al., 2005, Lenkowski et al., 2013). From the zebrafish study, it was shown that following retinal injury there is an initial increase in TGFβ signalling, followed by a decrease in the components of this signalling pathway (Lenkowski et al., 2013). This was demonstrated by increase in TGFβ ligand activin (by 6 hpi) (Craig 2008), and also the TGFβ target tgfβi (transforming growth factor beta induced) (8 hpi) (Qin et al., 2009). These TGFβ factors decreased by 16 hours post injury, whilst TGFβ transcriptional repressors such as tgif and six3b remained increased (Qin et al., 2009). Furthermore, inhibition of either of these TGFβ transcriptional repressors with a zebrafish mutant expressing a truncated form of tgif or morpholino, induced downregulation of six3b with consequent decrease in the proliferation of MG derived RPCs. Furthermore, under these conditions a decrease in the generation of cone photoreceptors in the injured zebrafish was also observed (Lenkowski et al., 2013).

1.4 Retinal gliosis and Müller Glia

Müller glia are the main glial cells present in the retina. During retinal injury, these cells undergo morphological, biochemical and physiological changes in order to protect the undamaged cells and promote repair. This process is known as reactive gliosis. Reactive gliosis is characterized by both unspecific and specific gliotic responses. Unspecific MG responses include cellular hypertrophy, proliferation and

upregulation of intermediate filaments (Bringmann et al., 2009). The increase in GFAP (non-specific marker) is considered as universal cellular marker for retinal injury/MG activation (retinal stress indicator) (Bignami and Dahl, 1979, Bringmann and Reichenbach, 2001, Lewis and Fisher, 2003). Similarly, activation of ERK1/2 (extracellular signal-regulated kinases 1 and 2) is an example of another non-specific marker for retinal damage and it is observed in various models of retinal injury (Geller et al., 2001, Tezel et al., 2003). In contrast, glutamine synthetase (GS), an enzyme responsible for neurotransmitter recycling and ammonia detoxification, is thought to constitute a specific gliotic response and is often downregulated in MG when glumate producing neural cells such as the photoreceptors are damaged (Grosche et al., 1995, Landiev et al., 2006). These changes were not observed in MG during diabetic induced retinopathy and optic nerve crush studies (Mizutani et al., 1998, Chen and Weber, 2002).

Gliosis is categorised into Conservative/non-proliferative and Massive/proliferative gliosis. Conservative gliosis is described as an upregulation of GFAP, cellular hypertrophy, a moderate/or no decrease in potassium currents followed by a slight membrane depolarization (by 10 mV), a moderate transient or no proliferation of MG, and a decrease in the expression of GS, CRALBP and carbonic anhydrase. These features have been observed in various animal models of retinal degeneration such as inherited photoreceptor dystrophy in rds mice and RCS rats (Landiev et al., 2006, Felmy et al., 2001). In massive gliosis, there is no expression of proteins that promote retinal neuron support. In addition, MG cell membrane potassium conductance decreases, resulting in uncontrolled proliferation of these cells and formation of cell masses known as the gliotic scar in the sub-retinal or /epi-retinal surfaces (Bringmann et al., 1999, Bringmann et al., 2009). It has been shown that upregulation of intermediate filaments such as GFAP and vimentin are essential

for glial scarring. By using mice lacking these intermediate filaments, it was shown that there was reduced glial scarring in the central nervous system (Pekny et al., 1999, Kinouchi et al., 2003) as well as in the subretinal space of experimental eyes (Nakazawa et al., 2007, Verardo et al., 2008)

Reactive MG can form a fibrotic layer within the subretinal space and also occupy the empty spaces left by dead retinal neural cells, RPE and blood vessels (Burke and Smith, 1981, Fisher and Lewis, 2003). These gliotic scar has been thought to prevent retinal regeneration in mammals as compared to zebrafish, where scarring do not occur (Lenkowski and Raymond, 2014, Bringmann et al., 2009). This fibrotic layer often contains abnormal deposition of extracellular matrix proteins including chondroitin sulfate proteoglycans such as neurocan and cell adhesion molecules such as hyaluronan-binding glycoprotein CD44 (Chaitin et al., 1996, Krishnamoorthy et al., 2000, Zhang et al., 2003), which inhibit axon growth and regeneration of retinal neurons (Bringmann and Wiedemann, 2012, Ponta et al., 2003).

During reactive gliosis MG cells have also been reported to have neuroprotective effect. By secreting neurotrophic factors and growth factors such as bFGF which act directly on retinal neurons, MG can protect retinal neural cell death (Wen et al., 1995, Garcia and Vecino, 2003). In addition, by producing antioxidants such as glutathione and pyruvate (Hollborn et al., 2008, Frenzel et al., 2005), they provide neuroprotection (Francke et al., 2001)

1.5 Canonical and non-canonical Wnt Signalling

The Wnt signalling pathway is an important signalling cascade that contributes to both development and adult life in mammals. During development, it functions in cell proliferation, differentiation, body axis formation and tissue/ organ development. In adult, its role includes tissue homeostasis, cell renewal and regeneration (Valenta et al., 2012). The Wnt signalling cascade is initiated by Wnt ligands which are evolutionary conserved secreted lipoproteins rich in cysteine (Kikuchi et al., 2011). There are 19 human genes encoding WNTs, which bind to different receptors and activate the Wnt signalling cascade. In general, Wnt signalling can be categorised into 2 types: Canonical and Non-Canonical signalling (Kim et al., 2013b).

The canonical Wnt signalling cascade occurs via β-catenin, which translocate into the nucleus for transcription of Wnt-target genes (Fig 1.4). When Wnt signalling is not activated, cytosolic β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) for ubiquitin-dependent degradation (Fig 1.4A). The β-catenin phosphorylation takes place within a destruction complex consisting of Axin, Adenomatous polyposis coli (APC) and GSK3β. During activation of the canonical Wnt signalling, Wnt ligand proteins such as Wnt2b, bind to Wnt Frizzled receptors and co-receptors LRP5/LRP6 (Fig 1.4B). The co-receptor LRP6 is a large single transmembrane protein that interacts with Wnt ligand proteins and Frizzled receptors via its extracellular domain (ECD). This leads to the formation of a complex between Wnt, Frizzled and LRP6, resulting in phosphorylation of the intracellular domain (ICD) containing Pro-Pro-Pro-Ser/Thr-Pro repeats (PPPSP) present in LRP5/LRP6 (Zeng et al., 2005). The PPPSP site in the LRP5/LRP6 undergoes dual phosphorylation. First GSK3β phosphorylates the PPPSP site, which is then

followed by another phosphorylation at adjacent serine residues (PPPSPXS) by Casein Kinase Iγ (Davidson et al., 2005). Consequently, the fully phosphorylated Wnt co-receptor LRP6 acts as a docking site for Axin, which then binds to the destruction complex. The phosphorylated LRP6 inhibits GSK3β mediated β-catenin phosphorylation by immediately binding to GSK3β and sequestering them into multivesicular bodies (Piao et al., 2008). Thus, there is accumulation of β-catenin in the cytoplasm which enters the nucleus to bind T-cell factor (TCF)/ Lymphoid enhancer factor (LEF) and knocks off transcriptional co-repressor Groucho for Wnt-target gene transcription (Fig 1.4B) (Kim et al., 2013b)

The Non-canonical Wnt signalling (Planar Cell Polarity signalling (PCP) and Wnt-Calcium) cascade occurs independently of β-catenin activation (Fig 1.5). PCP uses co-receptors ROR and PTK7 for its activation and downstream signalling occurs via activation of small G-proteins such as Rho/Rac, which results in actin polymerisation and microtubule stabilisation (Sato et al., 2010) (Fig 1.5A). Thus, it leads to changes in cell polarity, motility and migration, as observed during cell movement occurring in gastrulation and neural tube closure (Clark et al., 2012). Wnt-Calcium signalling which has been shown to be important in cancer and inflammation; it acts via Ca^{2+}/ Calmodulin-dependent kinase II (CAMKII), Protein kinase C and Calcineurin. Furthermore, Calcineurin activates the transcriptional regulator nuclear factor associated with T cells (NFAT), leading to transcription of genes associated with cell fate and migration (De, 2011) (Fig 1.5B).

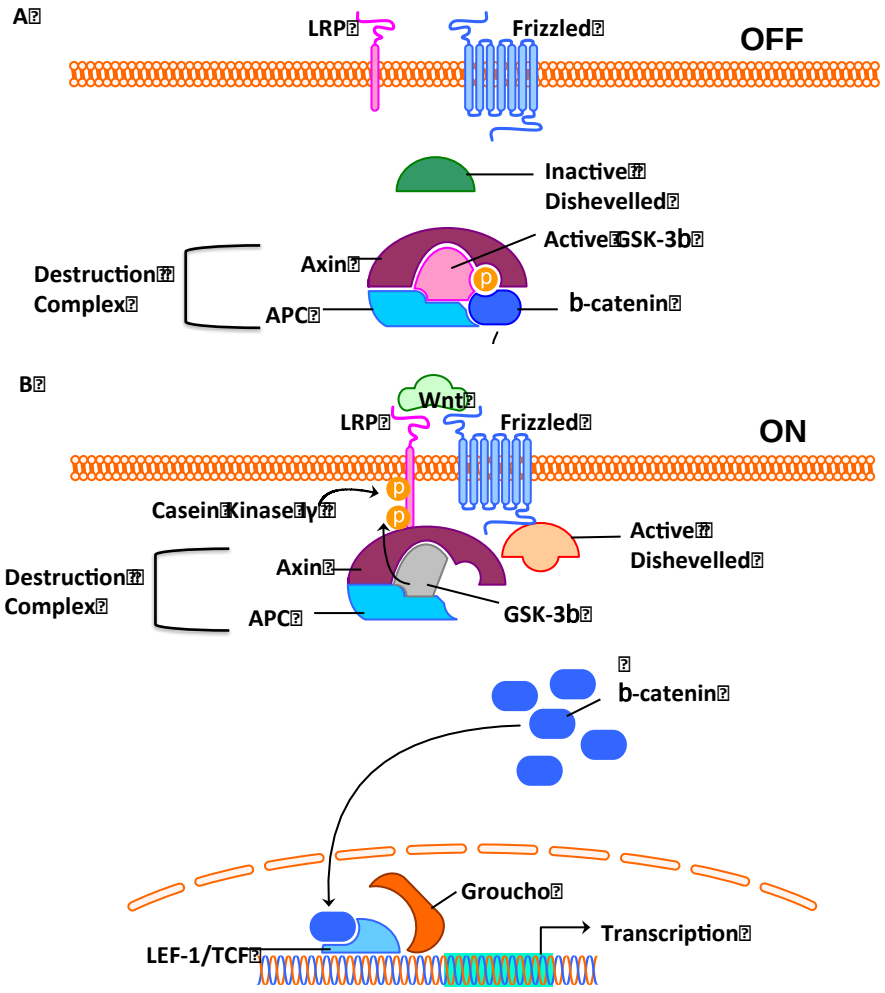

Figure 1.4 Canonical Wnt Signalling pathway. A) In the absence of Wnt ligand, β-catenin in the cytosol is phosphorylated by GSK3β (glycogen synthase kinase 3β) for ubiquitin-dependent degradation of β-catenin. **B)** In the presence of Wnt signalling and scaffold protein dishevelled, these phosphorylation sites on Wnt co-receptor LRP are dual-phosphorylated by GSK3β followed by by Casein Kinase Iγ (Davidson et al., 2005). The fully phosphorylated LRP6 acts as a docking site for Axin, which subsequently binds the destruction complex. Phosphorylated LRP6 inhibits phosphorylation of β-catenin by GSK3β via immediately binding to GSK3β and sequestering them into multivesicular bodies (Piao et al., 2008). Thus, there is accumulation of β-catenin in the cytoplasm which enters the nucleus to bind TCF (T-cell factor)/ LEF (Lymphoid enhancer factor) for Wnt-target gene transcription.

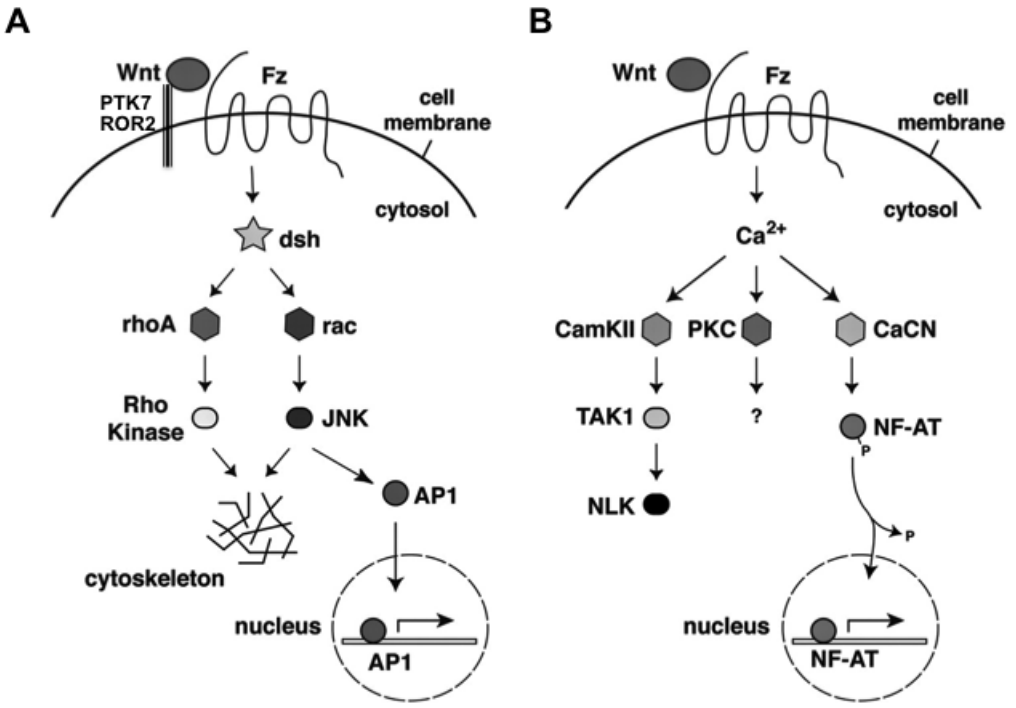

Figure 1.5 Non-canonical Wnt Signalling pathways. A) The Non-canonical Planar Cell Polarity signalling PCP act via co-receptors ROR and PTK7 for its activation. Downstream signalling occurs via activation of small G-proteins Rho/Rac, which results in actin polymerisation and microtubule stabilization leading to changes in cell polarity, motility and migration (Sato et al., 2010, Clark et al., 2012). **B)** Wnt-Calcium signalling act via Ca^{2+}/ Calmodulin-dependent kinase II (CAMKII), Protein kinase C and Calcineurin. Calcineurin activates NFAT to induce cell fate and migration associated genes transcription (De, 2011). Furthermore, activation of both NLK and NFAT has been reported to inhibit β-catenin mediated transcription (Ishitani et al., 2003, Saneyoshi et al., 2002). Adapted from (Gessert and Kuhl, 2010)

1.6 Notch Signalling Pathway

The Notch signalling pathway is an evolutionary conserved system that has many important functions during development such as maintaining undifferentiated progenitors during neurogenesis and promoting Müller glia cell fate during retinal development (Ghai et al., 2010). There are four Notch receptors (Notch1-4) and five Notch ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1 and Jagged 2) currently identified in mammals.

Notch receptors are heterodimers made up of an extracellular domain and a transmembrane domain that undergo S1 cleavage for the release of a precursor protein (Logeat et al., 1998) (Fig 1.6). Notch ligands expressed on one cell bind to Notch receptor on neighbouring cells via EGF-like repeats or DSL binding sites (Delta-Serrate-Lag2 domain) that results in S2 cleavage and removal of the extracellular domain (Koch et al., 2001). Subsequently, S3 cleavage occurs within the transmembrane domain by γ-secretases, leading to the release of Notch cleaved intracellular domain (NICD). NICD translocates to the nucleus and associates with the constitutive DNA binding protein CSL (CBF1/RBP-Jk, Suppressor of hairless and Lag-1) (Lai, 2002). In addition, mastermind adaptor protein also binds to the CSL complex and results in the transcription of Notch target genes such as HES family genes (Wu et al., 2000, Kageyama et al., 2005) (Fig 1.6).

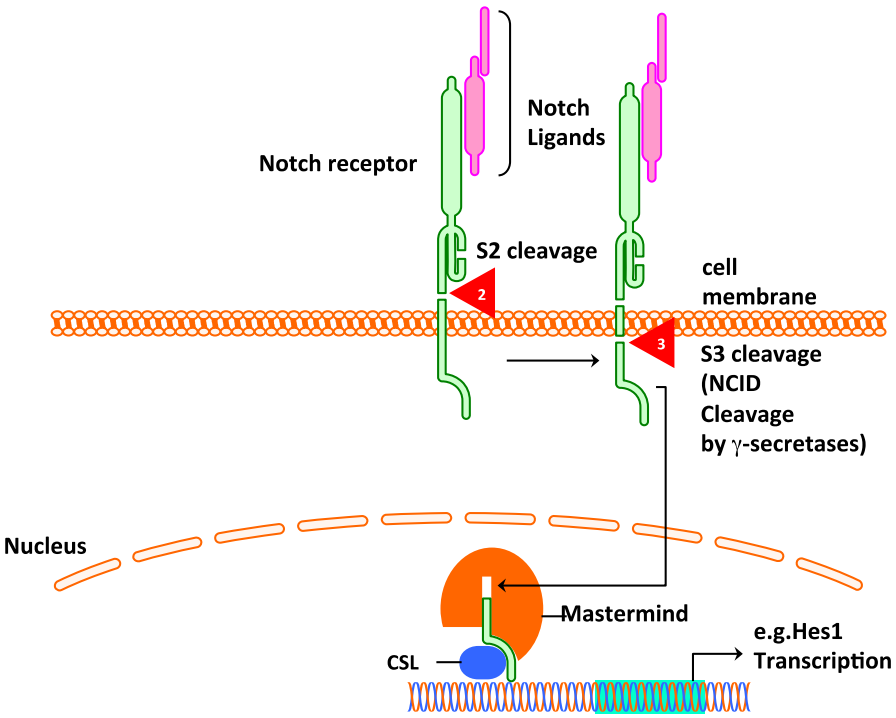

Figure 1.6 The Notch Signalling pathway. Notch ligands expressed on one cell bind to Notch receptor on neighbouring cells via EGF-like repeats or DSL binding sites (Delta-Serrate-Lag2 domain) that results in S2 cleavage and removal of the extracellular domain (Koch et al., 2001). Subsequently, S3 cleavage occurs by γ-secretases releasing Notch cleaved intracellular domain (NICD). NICD translocates to the nucleus and associates with the constitutive DNA binding protein CSL (CBF1/RBP-Jk, Suppressor of hairless, Lag-1) and mastermind adaptor protein. This results in transcription of Notch target genes such as HES family genes. Adapted from (Wu et al., 2000, Kageyama et al., 2005).

1.6.1 Notch inhibition *in vivo and in vitro*

Notch receptor is cleaved by γ-secretases to release the NICD for Notch target gene transcription (Fig. 2). γ-secretase is a protease complex consisting of 4 different proteins (presenilin 1 and 2, nicastrin, Pen2 and Aph1). Presenilin contains the catalytic site and nicastrin is thought to contain the substrate-binding site (De Strooper et al., 1998, Shah et al., 2005). The γ-secretase complex cleaves substrates such as amyloid precursor protein (APP), neuregulin and Notch receptors (Zhao et al., 2010). γ-secretase can be pharmaceutically inhibited by γ-secretase inhibitors such as DAPT and RO4929097. RO4929097 is a γ-secretase inhibitor that blocks Notch signalling by preventing cleavage of the Notch transmembrane at the S3 cleavage site (Fig. 2). Thus, the NICD cannot be released and translocate into the nucleus to promote the activity of Notch target genes. It was initially created to prevent APP cleavage via γ-secretase inhibition for Alzheimer's Disease research. However, now RO4929097 has been specifically used in cancer research to down-regulate Notch signalling. This has been demonstrated *in vitro* by the decrease in Notch target genes such as Hes1 in various human cancer lines (e.g. human non-small cell lung carcinoma cells and human primary melanoma cell lines). Furthermore, addition of RO4929097 to cells in culture resulted in less transformed, flattened and slower growing phenotype in non-small cell lung carcinoma cells (NSCLC). Furthermore, *in vivo* NSCLC mouse models demonstrated prevention in tumour growth by RO4929097 even after completion of the treatment (Luistro et al., 2009). Similarly, RO4929097 prevented cell proliferation and colony formation in human primary melanoma cell lines in culture. In vivo, RO4929097 decreased tumour volume and prevented growth of metastatic melanoma cell lines (Huynh et al., 2011). Therefore, R04929097 is currently used in phase I or phase II clinical

trials (alone or in combination with other pharmaceutical drugs) for the treatment of various types of tumours such as advanced solid tumours, Glioblastoma and Metastatic Colorectal Cancer (http://www.cancer.gov/drugdictionary/?CdrID=662240.) However, RO4929097 seems to be less effective in tumour cells with high levels of IL-6 and IL-8 (factors known to promote tumour angiogenesis). Overexpression of IL-6 and IL-8 induce RO4929097 sensitive cells to become resistant to this inhibitor (Nilsson et al., 2005, Luistro et al., 2009).

1.6.2 Role of Notch in neural progenitor proliferation and differentiation

Notch signalling has been demonstrated during retinal development to promote Müller glial cell-fate. For example, *in vivo* Notch1 gene expression by retrovirus in retinal progenitor cells of rat retina led to the expression of Müller glia markers such as cyclin D3 and CRALBP. In addition, in other stem cells such as neural crest stem cells, the activation of Notch was shown to promote glial differentiation while inhibit neuronal differentiation *in vivo* (Morrison et al., 2000). Also in lower vertebrates such as zebrafish, Notch1a activation caused retinal progenitor cells to promote glia cell-fate and inhibit neuronal differentiation (Scheer et al., 2001). The primary Notch downstream factors include the HES family genes such as Hes1, Hes5 and Hey2. These negative bHLH (Basic helix-loop-helix) genes have shown to promote glial cell fate (Hojo et al., 2000, Satow et al., 2001). For example, Hes1 null mice resulted in lack of Müller glial cell formation (Tomita et al., 1996), whilst Hes5 null retinas had approximately 40% reduction in Müller glial cells (Hojo et al., 2000) Conversely,

mutations in Notch downstream proneural genes such as Mash1 and Ngn2 resulted in increased Müller glia production (Akagi et al., 2004, Brown et al., 2001). Furthermore, the loss of functional studies in mouse has demonstrated that lack of Notch signalling results in the retinal progenitor cells differentiating into neuronal photoreceptor cell-fate. For example, Notch1 gene inactivation using the Cre/loxP mechanism in peripheral retinal progenitor cells resulted in increased cone photoreceptor precursor production instead of early and later born retinal neurons (Yaron et al., 2006, Jadhav et al., 2006b). However, the overexpression of the Notch intracellular domain (NICD) did not result in the expected glial fate in early mouse retina as it did in the postnatal retina. Instead, early progenitor cells were generated identified by markers such as frizzled-related protein 2 (Jadhav et al., 2006a). Therefore, the evidence presented above suggest that the Müller glial cell fate is promoted by the Notch signalling pathway and its downstream bHLH genes such as HES family. However, it seems that Müller glial cell fate is not solely dependent on Notch signalling pathway and its downstream bHLH genes, and thus other factors may also be involved in promoting a Müller glia cell-fate.

Notch signalling is found in adult Müller glia across species such as zebrafish and rodent retina; this was shown by the expression of HES family genes (Ghai et al., 2010, Bernardos et al., 2005, Nelson et al., 2011). The up-regulation of Notch signalling after retinal injury has been demonstrated across species such as zebrafish, chick and rodents. For example, there is increase in Notch1 and Notch3 after retinal injury in zebrafish (Raymond et al., 2006). Similarly, increase in Notch1 and Hes5 in Müller glia has been demonstrated after NMDA induced retinal injury in the chick (Ghai et al., 2010). Furthermore, Notch signalling has been demonstrated to be important for the proliferation of Müller glia derived progenitors, a function seen during retinal development (Dorsky et al., 1995).

By contrast, the inhibition of Notch signalling results in differentiation of Müller glia derived progenitors into retinal neurons. For example, treatment with the γ-secretase inhibitor DAPT (which also blocks Notch signalling by preventing cleavage of NICD) after retinal injury in chick, blocks MSC derived progenitor proliferation. Instead, Notch signalling inhibition results in promotion of MSC derived progenitor differentiation towards retinal neurons (Hayes et al., 2007). Furthermore, Notch signalling inhibition by DAPT has been shown to induce differentiation of human Müller glial stem cells (hMSC) into retinal ganglion cells *in vitro* (Singhal et al., 2012), and use of DAPT combined with Sonic hedgehog (Shh) have been shown to induce MG derived progenitors to differentiate into photoreceptors (Del Debbio et al., 2010)

Although Notch signalling is activated after injury across species it seems to have opposite functions on MSC derived progenitors. For example, in chick and mammals, Notch signalling activation promotes MSC derived progenitor proliferation, while Notch inhibition decreases progenitor proliferation. In zebrafish, the up-regulation of Notch signalling induces a decrease in MG derived progenitors. Instead, the down-regulation of notch signalling promotes MSC derived progenitor proliferation (Wan et al., 2012).

1.7 Objectives of the Thesis

Based on the current knowledge that following retinal injury, Müller glial cells are capable of differentiating into retinal neurons in the zebrafish or in small mammals *in vivo* with the aid of exogenous growth factors, the initial aim of this thesis was to test the role of those signalling pathways in human Müller stem cells (hMSC) cultures during their proliferation and neural differentiation into RGC and photoreceptors. Furthermore, factors such as TGFβ1 and HBEGF, which are highly expressed during retinal damage and known to promote gliosis, were examined for their effect on these signalling pathways in hMSC cultures. Accordingly, the following objectives were formulated:

1. To examine expression of components of the Notch and Wnt signalling pathways and growth factors associated with retinogenesis in hMSC cultures.
2. To investigate the role of the Notch and Wnt signalling pathways during hMSC differentiation towards photoreceptors.
3. To investigate the interaction of the growth factors TGFβ and HBEGF with these signalling pathways and to understand their role on RGC and photoreceptor differentiation of hMSC.
4. To understand the specific role of TGFβ and HBEGF towards hMSC differentiation by inhibition of these factors with pharmacological inhibitors.

CHAPTER 2: Effect of Notch inhibition on the expression of components of the Notch and Wnt signalling pathways by Müller stem cell lines- Modulation of Notch targets by TGFβ1

2.1 Regulation of Müller glia progenicity by Notch signalling

The Notch signalling pathway plays an important role during retinal development in both zebrafish and mammals (Bernardos et al., 2005, Jadhav et al., 2006b, Maurer et al., 2014). Notch signalling mediates several functions in Müller glia, including maintenance of neuronal progenicity, promotion of cell cycle exit for differentiation and retention of glial properties. It exerts these effects on a time dependent manner during development (Chapouton et al., 2010, Furukawa et al., 2000b, Del Bene et al., 2008). Interestingly, growing evidence suggests that Notch signalling is also crucial for Müller glia derived retinal regeneration in adult zebrafish (Wan et al., 2012, Conner et al., 2014), as well as for mammalian regeneration in the mice *in vivo* (Del Debbio et al., 2010). In the zebrafish, following acute light injury, there is a decrease in Notch signalling genes during the 16 first hours after damage (Qin et al., 2009). This is a time point considered as the peak of cell death in this experimental model (Gorsuch and Hyde, 2014). After 16 hours post injury, there is an increase in a subset of Notch related genes including deltac, her9, her4.2 and jagged2 (Qin et al., 2009). It has been hypothesized that in zebrafish and chick the initial downregulation of Notch signalling permits Müller glia to dedifferentiate into neural progenitors and to express pro-neural genes such as ascl1a and pax6 (Lenkowski and Raymond, 2014). Upregulation of Notch signalling would then promote acquisition of glial cell properties and regulation of the cell cycle exit and cell fate (Lenkowski and Raymond, 2014). Inhibition of Notch signalling by DAPT has been shown to increase the expression of ascl1 by Müller glia derived progenitors in the zebrafish (Ramachandran et al., 2012, Wan et al., 2012) and the post-natal mouse (Nelson et al., 2011). Conversely, overexpression of the Notch

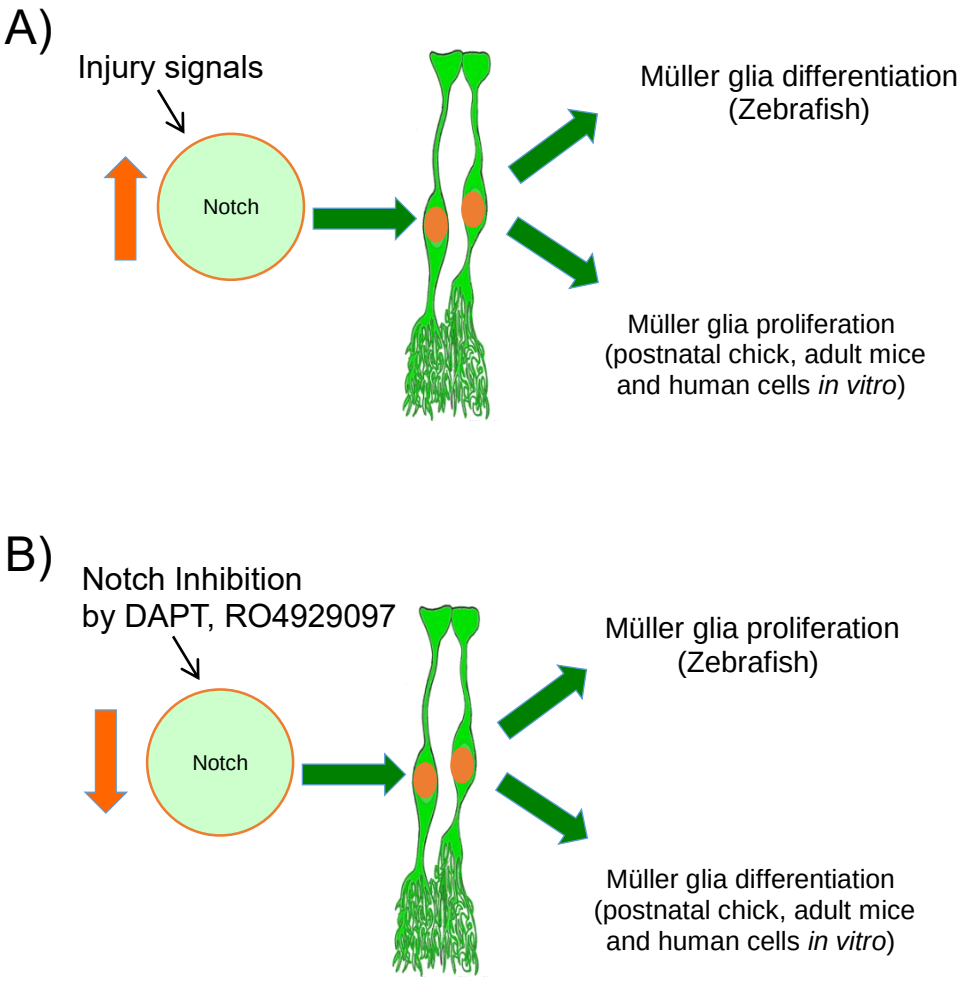

Figure 2.1 Effect of Notch signalling on Müller glia in zebrafish and mammals
(A) Upregulation of Notch signalling pathway by injury signals results in Müller glia differentiation in the zebrafish, whilst in vertebrates such as the postnatal chick, adult mice and human cells *in vitro*, upregulated Notch signalling causes Müller glia proliferation. (B) Conversely, downregulation of Notch signalling by the γ-secretase inhibitors DAPT or RO4929097 causes proliferation of Müller glia in the zebrafish, whilst inducing differentiation of these cells in mammals (Adapted from papers by Wan et al., 2012, Del Debbio et al., 2010, Singhal et al., 2012, Fischer and Bongini, 2010).

intracellular domain (NICD) decreased the expression of ascl1a (Wan et al., 2012). Interestingly, in the zebrafish Notch inhibition results in the proliferation of Müller glia derived progenitors in both damaged and undamaged retinae (Wan et al., 2012, Conner et al., 2014), whereas the proliferation of progenitors in the postnatal chick and adult mouse retina is promoted by the activation of Notch signalling (Das et al., 2006, Del Debbio et al., 2010, Ghai et al., 2010, Hayes et al., 2007).

2.1.1 Crosstalk between Notch and TGFβ signalling pathways

Both the Notch and TGFβ signalling pathways have been shown to play an important role during Müller glia derived progenitor proliferation and differentiation following retinal damage (Wan et al., 2012, Lenkowski et al., 2013). Various studies have demonstrated crosstalk between the components of these two pathways. The upregulation of HES1, primary target of Notch signalling, has been reported to be caused by activation of TGFβ signalling pathways and has been demonstrated in chick *in vitro* and *in vivo* (Blokzijl et al., 2003). This has been shown to be due to the direct interaction between NICD and Smad3, and the ability of Smad3 to bind to DNA binding sites in the presence of a complex between the DNA binding protein CSL (CBF1/RBP-Jk), suppressor of hairless (Lag-1) and NICD (Blokzijl et al., 2003) (Fig 2.2).

Further evidence of crosstalk has been demonstrated in the Epithelial to Mesenchymal cells transition (EMT) of cells from the mammary gland, kidney tubules and epidermis by upregulation of Notch target genes such as HES1, HEY1, HEY2 and HES5 by TGFβ signalling (Zavadil et al., 2001, Zavadil et al., 2004). In

contrast, downregulation of Notch signalling has been demonstrated to inhibit the TGFβ signalling (Zavadil et al., 2004). Furthermore, through inhibition of Notch3 expression, TGFβ signalling promotes smooth muscle cell differentiation (Kennard et al., 2008), whilst activation of Notch signalling is essential for BMP4 mediated inhibition of muscle stem cell differentiation (Dahlqvist et al., 2003) as well as inhibition of endothelial cell migration (Itoh et al., 2004).

2.1.2 Crosstalk between Notch and Wnt signalling pathways

The Notch and Wnt signalling pathways are both important for the development of retinal cells as well as for retinal regeneration following retinal damage in both zebrafish and mammals (Wan et al., 2012, Ramachandran et al., 2011, Del Debbio et al., 2010). According to Collu et al, the interaction between these two pathways can occur into three forms: i) co-operative regulation of transcriptional targets, ii) transcription-dependent interaction and iii) direct molecular crosstalk between signal transduction machinery (Collu et al., 2014) (Fig 2.3).

Co-operative regulation of transcription targets has been shown during drosophila wing development, where synergic activation of Notch and Wnt signalling pathways induces an enhancer element which regulates vestigial expression required for boundary formation between the dorsal and ventral surfaces (Klein and Arias, 1999) (Fig 2.3A). Similarly, the formation of a complex between RBP-Jk, NICD and β-catenin following co-activation of Notch and Wnt signalling pathways were required to form arterial endothelial cells, but not venous endothelial cells from embryonic stem cells

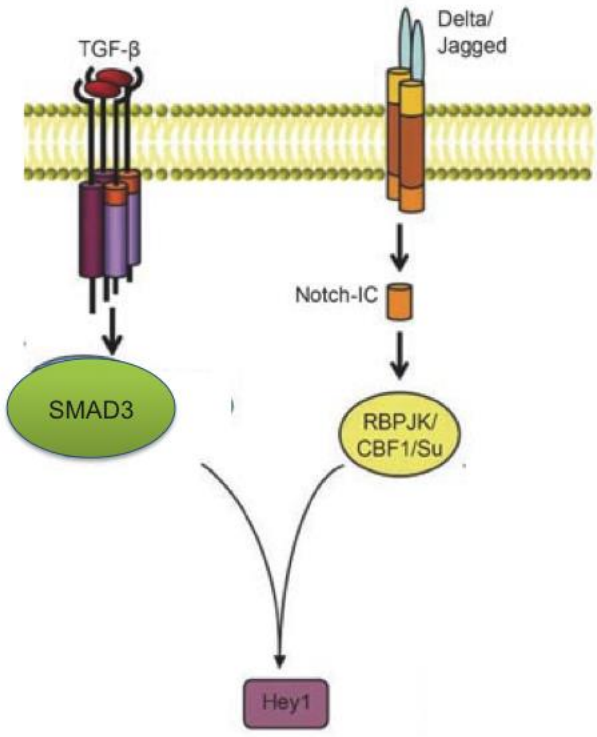

Figure 2.2 Crosstalk between Notch and TGFβ and Wnt signalling pathways. Interaction between Notch and TGFβ can occur through binding between intracellular cytoplasmic domain (NICD) and CSL (RBP-JK/CBF1/Su) complex with SMAD proteins in chick (Blokzijl et al., 2003). From (Xu et al., 2009)

and *in vivo* (Yamamizu et al., 2010). Transcription-dependent interaction occurs during development to generate temporal and spatial separation of Notch and Wnt activity, such as formation of dorsal and ventral surfaces in drosophila wing development (Klein and Arias, 1998). Notch signalling is initially activated at the dorsal-ventral boundary cells, inducing Wnt signalling to help growth and patterning of the wing. In turn, Wnt signalling controls the induction of Notch ligand expression to maintain Notch and Wnt signalling active (Micchelli et al., 1997, Micchelli and Blair, 1999) (Fig 2.3B).

Direct molecular crosstalk between Notch and Wnt signalling pathways has been demonstrated as follows: In the drosophila sensory organ precursor cells (SOP), the Wnt signalling component dishevelled has been shown to directly interact with Notch *in vivo*, resulting in inhibition of the Notch induced specification of SOP cells (Axelrod et al., 1996) (Fig 2.3C). Furthermore, during Xenopus cell fate specification, it has been shown that dishevelled can directly interact with RBPj and regulate Notch signalling (Collu et al., 2012). In addition, it has been demonstrated that membrane bound Notch can bind to active β-catenin, promoting its degradation in stem and colon cancer cells (Kwon et al., 2011). By contrast, NICD has also been shown to inhibit canonical Wnt signalling, possibly through expression of HES1 (Deregowski et al., 2006) or through modulation of the histone status of Wnt target genes (Kim et al., 2012). Additionally, it has been suggested that NICD can modulate Wnt signalling by forming a complex between NICD/RBP-Jk and β-catenin that prevents β-catenin binding to its target sites (Collu et al., 2014).

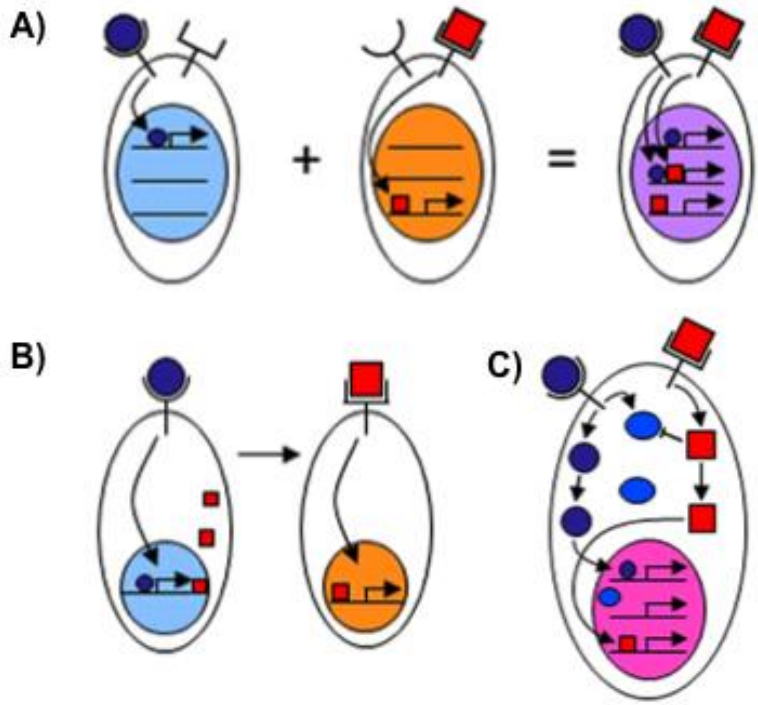

Figure 2.3 Crosstalk between Notch and Wnt signalling pathways. A) Co-operative regulation of transcriptional targets. When activation of both Notch (red Square) and Wnt (blue circle) signalling pathways lead to transcription of their target genes. **B)** Transcription-dependent interaction. Activation of one pathway (For e.g. Wnt) lead to sequential activation of the other pathway (e.g. Notch) by promoting their ligand expression. **C)** Direct molecular crosstalk. When a intracellular component of Wnt (e.g. dishevelled) (red Square) can directly interact with intracellular component of Notch pathway (e.g. RBPJK) (light blue oval) and inhibit gene transcription for the Notch induced specification SOP cells in drosophila (Axelrod et al., 1996) or regulate Notch signalling in *Xenopus* (Collu et al., 2012). From (Collu et al., 2014)

2.2 Function of the basic helix-loop-helix (bHLH) genes in Müller glia

The hairy and enhancer of split (HES) family is a type of basic helix-loop-helix (bHLH) genes, some of which are known Notch targets that regulate neural stem cells maintenance and promote gliogenesis (Kageyama et al., 2005). HES1 is one of the 7 members of the HES family, which is highly expressed by neural stem cells (Sasai et al., 1992) and is known to promote Müller glia formation. HES family proteins can bind to DNA templates via their conserved amino-terminal conserved bHLH domain. Through the bHLH domain, HES can repress transcription by binding to two transcription sequences, E box and N box. HES1 is known to have stronger affinity for N box, whereas most others including HES3 bind strongly to the E box (Sasai et al., 1992). This is due to the presence of a proline residue within the conserved bHLH domain (Sasai et al., 1992). Using the WRPW domain at the carboxy-terminal, HES can bind the co-repressors TLE/Groucho resulting in transcriptional repression of the HES family (Paroush et al., 1994, Grbavec and Stifani, 1996). Additionally, HES can be repressed at protein level by forming a non-functional heterodimer with activator-type bHLH factors such as Mash1, preventing Mash1 from dimerizing with another activator-type bHLH factor such as E-47, which would normally result in transcription (Sasai et al., 1992)

HES1 expression is controlled by Notch signalling (Jarriault et al., 1995), however, the expression of not all members of the HES family are directly regulated by Notch signalling such as HES3 (Nishimura et al., 1998). The absence of HES1 expression prevents Notch signalling from inhibiting neuronal differentiation, suggesting that HES1 is a key target for neural differentiation of Müller glia (Ohtsuka et al., 1999). Activation of Notch signalling results in the formation of a complex between the DNA

binding protein RBP-Jk and the Notch intracellular domain (NICD), which leads to transcription of HES1 (Honjo, 1996). In the absence of NICD, RBP-Jk represses transcription of HES1 by binding to their promoters (Selkoe and Kopan, 2003).

2.2.1 Role of BRN3 in retinal ganglion cell development

The BRN3 family of factors consists of three classes of IV POU domain transcription factors; BRN3A, BRN3B and BRN3C. It has been shown that BRN3B is expressed early during retinal ganglion cell differentiation (Gan et al., 1999), whilst BRN3A and BRN3C are expressed later during differentiation (Wang et al., 2002). Initially, it was assumed that BRN3B played the most important role amongst the BRN3 family of transcription factors as a single Knockout of each of the BRN3 members in mice showed that only BRN3B mutants had a significant deterioration on RGC development (Gan et al., 1996, Erkman et al., 1996, Wang et al., 2002, Wang et al., 2000). However, double knockout studies of BRN3B with BRN3C or BRN3B and BRN3A demonstrated that these mutants showed more severe defects as compared to the single BRN3B mutation(Wang et al., 2002), indicating the importance of both BRN3A and BRN3C in RGC development.

BRN3B has been shown to play an important role in the maturation of retinal ganglion cells. In the absence of BRN3B expression, RGC axons do not develop normally, often presenting with short, disorganized axons mimicking dendritic features. In addition, they display pathfinding defects and migratory problems within the ganglion layer (Erkman et al., 1996), and about 70% of these RGCs die during

early development, resulting in the formation of a thin optic nerve (Gan et al., 1999, Wang et al., 2000)

2.3 Objectives

The importance of the Notch signalling pathways during retinal development as well as in Müller glia mediated differentiation following injury has been demonstrated in species such as the zebrafish and small mammals. In addition, inhibition of the Notch signalling has been demonstrated to induce RGC formation in human Müller glia stem cells under culture conditions. However, we do not know the effect of Notch inhibition on components of the Wnt signalling, a pathway shown to be important during MG mediated retinal regeneration in the zebrafish. Also, the effect of factors released during retinal gliosis such as TGFβ are unknown during this process. Therefore, this study aimed to demonstrate whether Notch inhibition has any effect on the components of Wnt signalling pathway and whether modulation of Notch downstream targets by TGFβ1 may affect human Müller stem cell proliferation and differentiation.

On this basis, this chapter aims were as follows:

1. To investigate the effect of various concentration of γ-secretase inhibitor RO4929097 on the expression of downstream target HES1 in various hMSC lines.
2. To examine the effect of the various concentrations of the γ-secretase inhibitor RO4929097 on hMSC proliferation.
3. To study the effect of TGFβ on Notch targets during RGC differentiation.
4. To investigate the effect of Notch inhibition on components of the canonical and non-canonical Wnt signalling pathway.

To achieve these objectives, the following experiments were conducted:

1. Müller stem cells were cultured with various concentration of the γ-secretase inhibitor RO4929097 to identify the optimum concentrations that downregulate HES1 mRNA and protein expression and that could be used for assessing functional effects on human MSC.

2. Human Muller stem cells were cultured in the presence or absence of the Notch inhibitors RO4929097 or DAPT to examine gene and protein expression of components of the canonical and non-canonical Wnt signalling pathway. Selective gene components of canonical Wnt signalling, including WNT2B, DKK1, WISP-1 and AXIN2 were identified by RT-PCR of isolated mRNA, whilst intracellular protein was identified by western blotting analysis of isolated protein. In addition, protein expression was also identified by quantification of immunostained cells.

3. To investigate the effect of TGFβ1 on RGC differentiation of human MSC, cells were cultured with DAPT/FGF in the presence or absence of this factor. Gene expression of the RGC markers HES1 and BRN3A were examined by RT-PCR whilst protein expression was identified by immuno-cytochemical staining.

4. The effect of Notch inhibition on human MSC cell proliferation was analysed by immuno-cytochemical staining of Ki67 and the Hexosamindase assay.

2.4 Results

2.4.1 The γ-secretase inhibitor RO4929097 downregulates the expression of the Notch target gene HES1 in human Müller stem cell lines

Culture of MIO-M1 cells with the γ-secretase inhibitor RO4929097 at concentrations ranging from 0.0005 μM to 50 μM demonstrated that this inhibitor significantly decreased the mRNA expression of HES1 in a dose response manner when used at concentrations ranging from 0.5 μM to 50 μM ($p<0.05$ for 0.5 μM. and 5 μM; $p<0.01$ for 50 μM) as compared to control cells cultured with medium alone (Fig 2.4A). When RO4929097 was used at lower concentrations (0.0005 μM and 0.005 μM) it did not modify HES1 mRNA expression when compared to control cells (Fig 2.4A).

Corresponding to mRNA expression, Western blot analysis of whole cell lysates from MIO-M1 cells cultured with 0.5 μM RO4929097 also showed a significant decrease in the levels of intracellular HES1 protein as compared to cells cultured with medium alone ($p<0.05$) (Fig 2.4B).

Similarly, culture of three different human Müller stem cell lines with 0.5 μM RO4929097, showed that two of the cell preparations investigated (6426 and 6387) underwent a significant decrease in the mRNA expression of HES1 as compared to the control cells, ($p<0.01$) and ($p<0.05$) respectively (Fig 2.5). However, no significant downregulation of HES1 mRNA expression was observed in the third cell line investigated (6391) when compared to the control cells (Fig 2.5). As these cell

lines were generated from different patients, there is always a possibility that they may respond differently to this γ-secretase inhibitor. A study published by He et al., had previously reported that high levels of interleukin protein (IL-6 and IL-8) expression in tumour cells inhibit the activity of RO4929097 (He et al., 2011). The examination of IL-6 and IL-8 interleukin proteins of whole cell lysates from these hMSC lines demonstrated that hMSC line 6391 showed the highest protein expression for both interleukin proteins amongst all the cell lines when compared to control normal human retina (Fig 2.6). hMSC lines MIO-M1 and 6426 had similar level protein expression of these interleukins, whilst 6387 cell line had higher IL-6 expression than these two cell lines but similar IL-8 protein expression, when compared to control cells (Fig 2.6).

The findings from these experiments suggest that the γ-secretase inhibitor RO4929097 can significantly downregulate the Notch signalling target gene HES1 in various human Müller glia cell lines. The Notch inhibition activity of RO4929097 on these cells may be affected by IL-6 and IL-8 interleukin protein expression levels, as the cell line 6391 which displayed the highest expression of these proteins was not inhibited by this γ-secretase inhibitor.

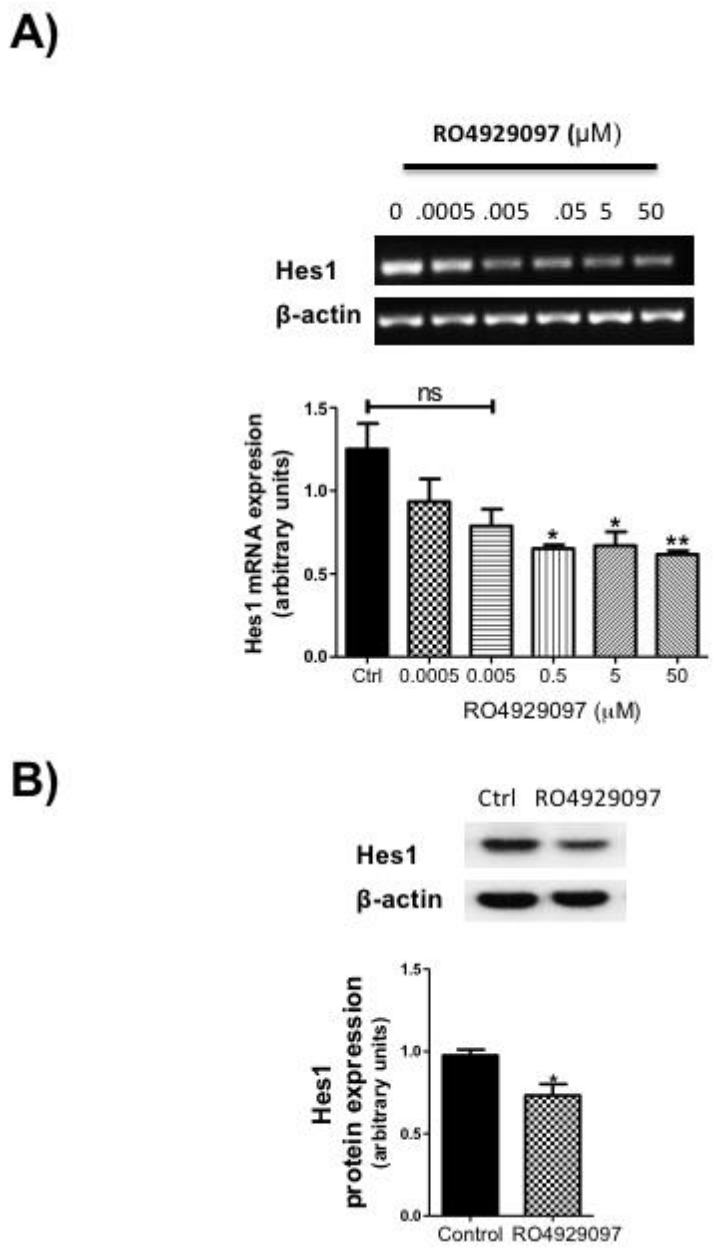

Figure 2.4 Inhibition of the Notch signalling target HES1 in MIO-M1 cells is dependent on the concentration of γ-secretase inhibitor (A) Culture of MIO-M1 cells with the γ-secretase inhibitor RO4929097 significantly downregulated mRNA expression of the Notch primary target HES1 at concentrations ranging between 0.5 μM to 50 μM; n=3. Anova test. *p<0.05 v. control (B) Western blot analysis of lysates from cells cultured with 0.5 μM RO4929097 also showed a significant decrease in the expression of HES1 protein; n=3. Student's t-test; *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above histograms.

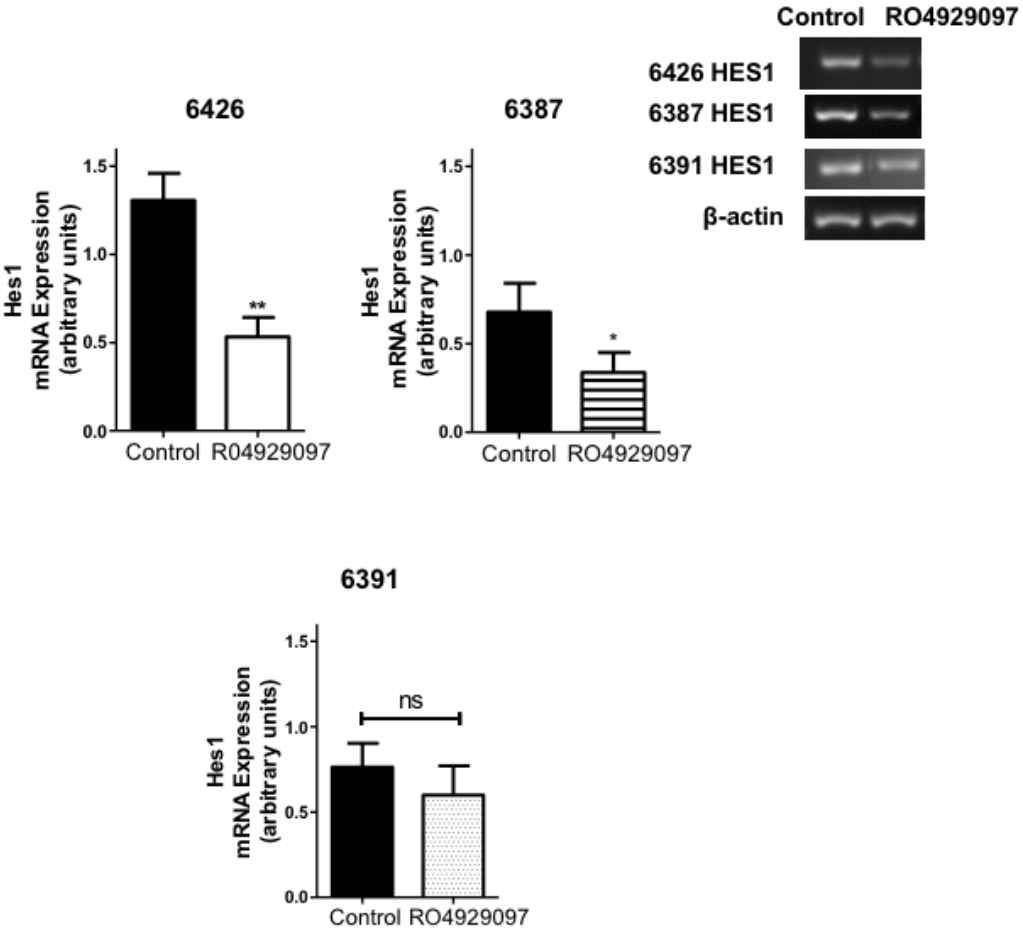

Figure 2.5 Inhibition of the Notch signalling target HES1 in three different human Müller stem cell lines: 6426, 6387 and 6291. Culture of the Müller stem cell lines 6426 and 6387 with 0.5 μM of RO4929097 resulted in a significant decrease in mRNA expression of the Notch primary target HES1. No changes were observed in the 6391 cell line; n=3-4.Student's t-test; *p<0.05 v. control, **p<0.01 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms

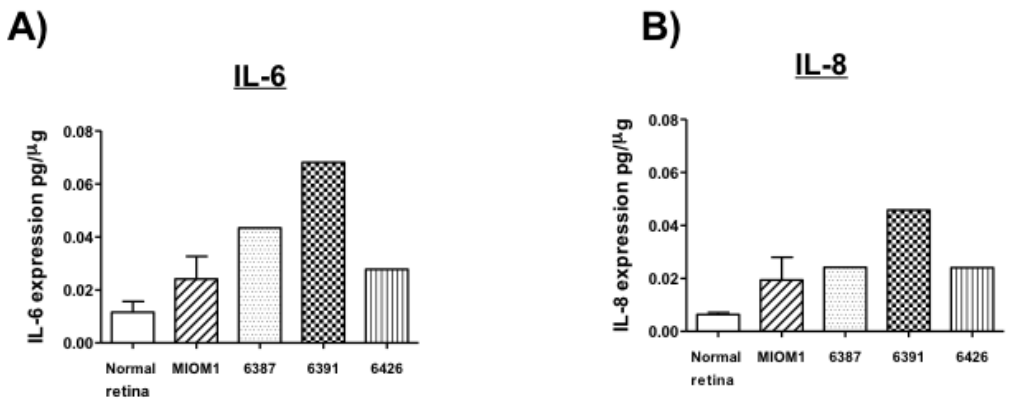

Figure 2.6 Expression of IL-6 and IL-8 proteins in Normal retina and Müller cell lines. **A)** IL-6 protein expression varies in different human Müller cell lines. Amongst all the cell lines examined, 6391 showed the highest protein expression as compared to control normal retina. **B)** Similarly, in these same cell lines, human Müller cell line 6391 also demonstrated the highest protein expression of IL-8 as compared to control normal retina.

2.4.2 Examination of the effect of TGFβ1 on the Notch signalling target HES1 and the retinal ganglion cell markers BRN3A and BRN3B

Both Notch and TGFβ signalling pathways have been shown to regulate Müller glia proliferation and differentiation. Evidence of the interaction between the components of these two signalling pathways has been demonstrated by the upregulation of HES1 by TGFβ signalling (Zavadil et al., 2004). In addition, it has been reported that Notch inhibition leads to the differentiation of human Müller stem cells towards a retinal ganglion cell phenotype *in vitro* (Singhal et al., 2012, Becker et al., 2013), and that TGFβ, which is capable of inhibiting cell proliferation, is highly expressed during retinal gliosis (Hoerster et al., 2014). Therefore, we examined the effect of TGFβ1 alone or in the presence of γ-secretase inhibitor DAPT on the mRNA expression of the Notch primary target HES1 and the RGC marker BRN3B in hMSC.

Culture of hMSC line MIO-M1 with 50 ng/ml of TGFβ1 for 7 days did not modify the mRNA expression of HES1 as compared to control cells cultured with medium alone (Fig 2.7A). By contrast, HES1 mRNA expression was significantly downregulated by DAPT in the presence of FGF2 as compared to control cells ($p<0.001$). Addition of 50 ng/ml TGFβ1 to cells undergoing Notch inhibition by DAPT in the presence of FGF2 did not modify HES1 mRNA expression in these cells as compared to cells cultured with DAPT and FGF2 (Fig 2.7A).

Similarly, 50 ng/ml TGFβ1 did not induce any significant changes in the mRNA expression of the RGC marker BRN3B when compared to control cells (Fig 2.7B). In contrast, and as previously reported by (Singhal et al., 2012, Becker et al., 2013), hMSC line MIO-M1 cultured with DAPT in the presence of FGF2 showed a significant upregulation in their mRNA expression of BRN3B ($p<0.05$) as compared to control cells (Fig 2.7B). However, addition of 50 ng/ml TGFβ1 to these cells undergoing RGC differentiation did not modify the mRNA expression of BRN3B when compared to cells undergoing RGC differentiation (Fig 2.7B). Furthermore, immuno-cytochemical examination of nuclear BRN3A protein showed similar pattern as the BRN3A mRNA expression. There were an increased percentage of cells with nuclear BRN3A immunostaining in the hMSC cultured with the Notch inhibitor when compared to control cells cultured with medium alone (Fig 2.8). However, addition of TGFβ1 to hMSC undergoing RGC differentiation did not modify the percentage of cells staining for this marker (Fig 2.8).

Taken together, the results from these experiments suggest that the TGFβ1 did not modify the expressions of the Notch signalling downstream targets HES1, BRN3A and BRN3B in hMSC line MIO-M1.

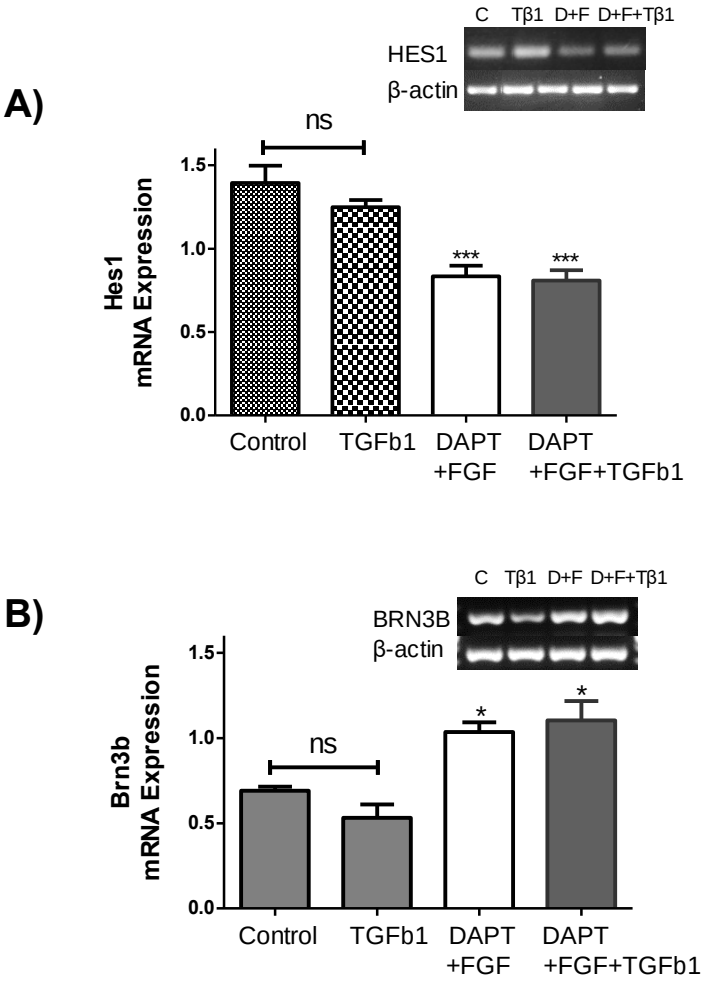

Figure 2.7 Effect of TGFβ1 on mRNA expression of the Notch primary target HES1 and the retinal ganglion cell marker BRN3B in MIO-M1 cells. (A) Culture of MIO-M1 cells with 50 ng/ml TGFβ1 for 7 days did not cause changes in the expression of HES1 mRNA as compared to control cells. In contrast, downregulation of HES1 mRNA was observed in cells cultured with the 50 μM Notch inhibitor DAPT in the presence of FGF2. Addition of TGFβ1 to cells undergoing Notch downregulation did not modify the downregulation of HES1 by this inhibitor; n=4. Student's t-test; ***p<0.001 v. control. **(B)** Similarly, culture of MIO-M1 cells with 50 ng/ml TGFβ1 for 7 days did not modify the mRNA expression of BRN3B as compared to control cells. In addition, the upregulated BRN3B mRNA expression caused by cells cultured with 50 μM DAPT in the presence of FGF2 was also not changed by the addition of TGFβ1. Conversely, TGFβ1 neither caused changes in BRN3B mRNA expression compared to control nor inhibited the increase in the BRN3B mRNA expression caused by the Notch inhibitor; n=4. Student's t-test; *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin.Representative bands are shown above histograms.

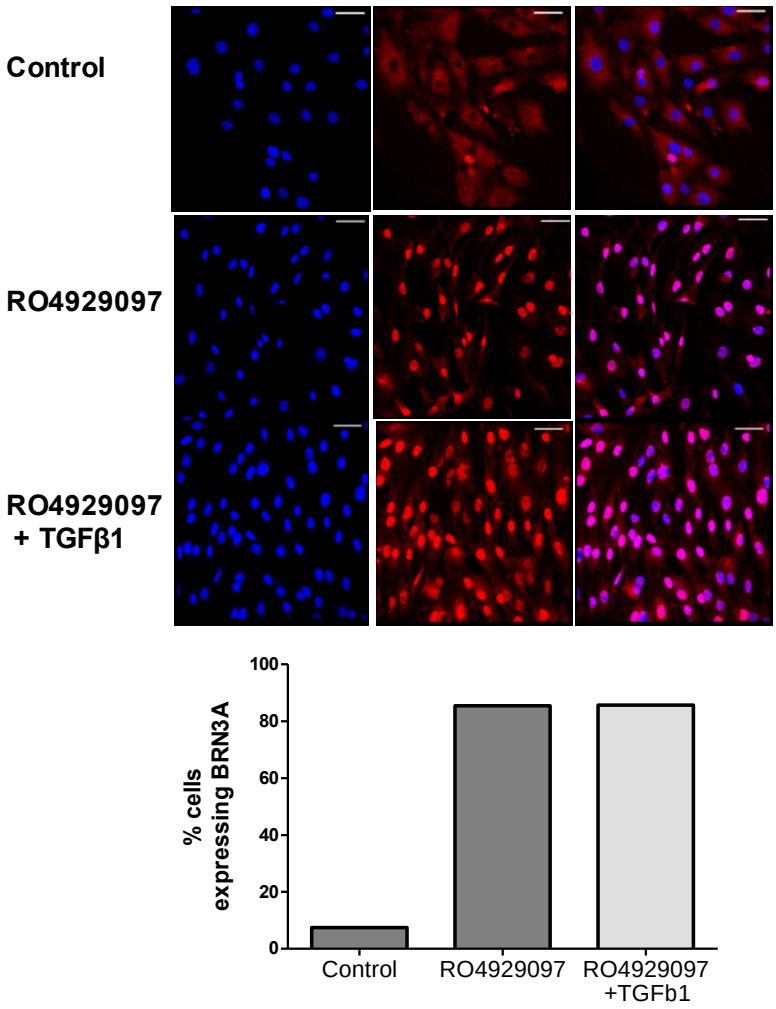

Figure 2.8 Effect of RO4929097 and TGFβ1 on nuclear BRN3A protein expression in MIO-M1 cells. Notch inhibition by 0.5 μM RO4929097 caused a marked increase in the nuclear BRN3A protein expression (Alexa 555, red) as compared to control cells. Addition of 50 ng/ml TGFβ1 to these cells with Notch inhibition did not modify BRN3A expression when compared to RO4929097 alone. Cell nuclei counterstained with DAPI (blue). Scale bars 50 μm.

2.4.3 Effect of Notch inhibition on the regulation of components of the canonical and non-canonical Wnt signalling pathways in hMSC

The Notch and Wnt signalling pathways have been shown to be involved in Müller glia proliferation and differentiation across various species, including zebrafish and small mammals (Wan et al., 2012, Ramachandran et al., 2011, Del Debbio et al., 2010). In addition, it has been demonstrated that activation of the Notch intracellular domain can inhibit the canonical Wnt signalling pathway possibly through expression of HES1 (Deregowski et al., 2006). Thus, we investigated the effect of Notch inhibition on the canonical and non-canonical Wnt signalling components in various hMSC lines.

Culture of MIO-M1 cells with 0.5 μM RO4929097 resulted in a significant decrease in WNT2B mRNA expression ($p<0.01$) as compared to cells cultured with medium alone (Fig 2.9A). Additionally, western blot analysis of whole cell lysates of MIO-M1 cells cultured with RO4929097 demonstrated a decrease in WNT2B protein expression ($p<0.05$) upon Notch inhibition as compared to control cells (Fig 2.9B). When three different cell lines (6426, 6387 and 6391) were cultured with the Notch inhibitor RO4929097, a significant decrease in WNT2B mRNA expression was observed in the cell line 6387 ($p<0.05$) when compared to the control cells (Fig 2.9). However, RO4929097 did not cause any changes in the mRNA expression of this gene in the other cell lines 6426 and 6391 as compared to control cells (Fig 2.9).

Corresponding to the decrease observed in WNT2B mRNA and protein expression by 0.5 μM RO4929097 in MIO-M1 (Fig 2.9) and mRNA expression in 6387 hMSC cell line (Fig 2.10) respectively, Notch inhibition also caused a significant decrease

in the mRNA expression of the canonical Wnt signalling targets WISP-1 ($p<0.01$) and AXIN2 ($p<0.05$) in MIO-M1 cells, whilst DKK1 mRNA expression was increased in this hMSC line as compared to control cells (2.11A). In 6387 cell line, a significant decrease in DKK1 ($p<0.05$) and WISP-1 ($p<0.05$) mRNA expressions were caused by RO4929097 when compared to control cells (Fig 2.11B). However, no significant change was observed in mRNA coding for AXIN2 by RO4929097 in this cell line (Fig 2.11B). When three different hMSC lines MIO-M1, 6426 and 6387 were cultured with 0.5 μM RO4929097 for the examination of non-canonical Wnt signalling ligand WNT5B, there was a significant decrease in WNT5B mRNA expression ($p<0.001$) in MIO-M1 cells as compared to the control cells (Fig 2.12). However, 0.5 μM RO4929097 did not modify the WNT5B mRNA expression in hMSC lines 6426 and 6387 as compared to controls (Fig 2.12).

The results suggest that Notch inhibition by RO4929097 inhibits the components of the canonical Wnt signalling including WNT2B, AXIN2 and WISP-1 in hMSC. Additionally, non-canonical Wnt signalling ligand WNT5B is also downregulated by inhibition of the Notch signalling in hMSC. However, similar to the variation seen in different hMSC lines in the downregulation of HES1 by this γ-secretase inhibitor, differences in the downregulation of the WNT components were also observed in the different examined hMSC lines.

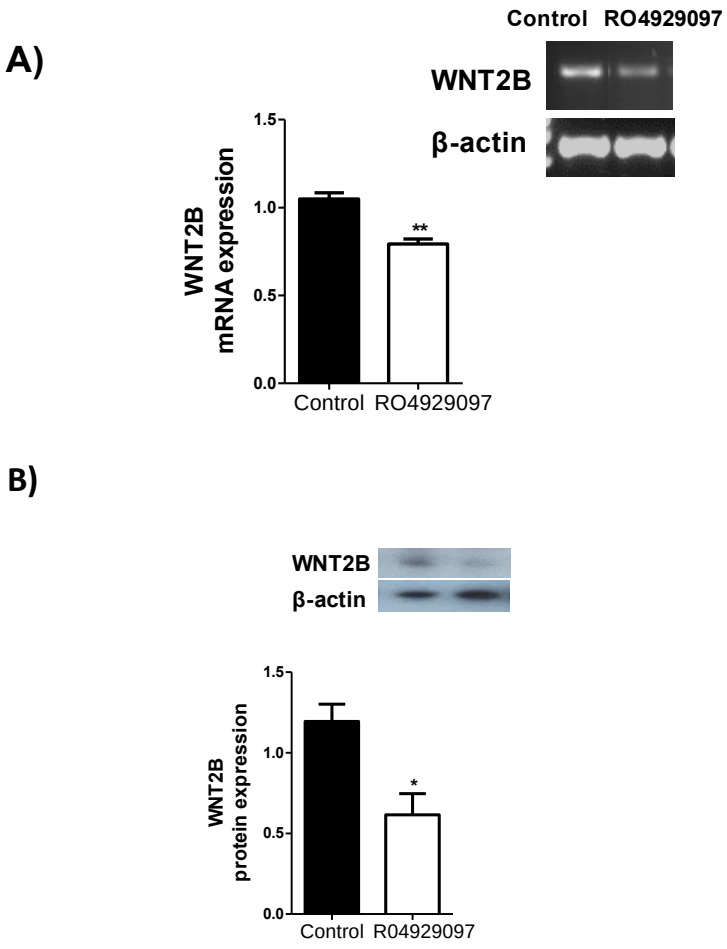

Figure 2.9 Effect of Notch inhibition on the canonical Wnt signalling ligand WNT2B in MIO-M1 cells. **(A)** Notch inhibition in MIO-M1 cells by RO4929097 induced downregulation of WNT2B mRNA expression in these cells; n=3. Student's t-test; **p<0.01 v. control. **(B)** Western blot analysis of cell lysates from MIO-M1 cells showed a mark decrease of WNT2B intracellular protein in cells treated with RO4929097 at a concentration of 0.5 μM as compared to control cells; n=3. Student's t-test, *p<0.05 v. control. . Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above histograms.

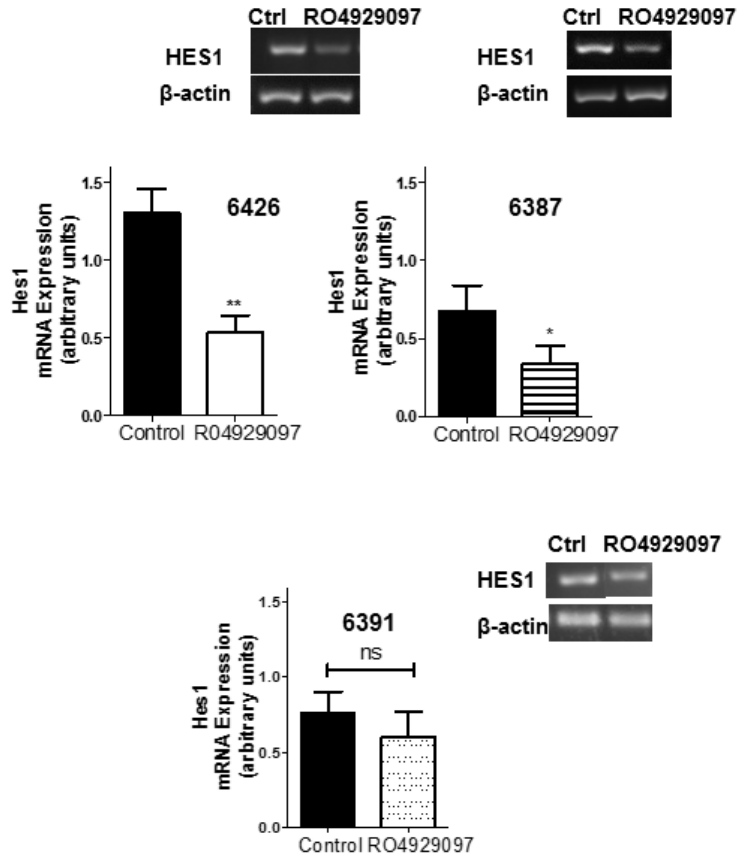

Figure 2.10 Effect of Notch inhibition on the canonical Wnt signalling ligand WNT2B in various Müller stem cell lines (6426, 6387 and 6391). Notch inhibition by 0.5 μM RO4929097 induced downregulation in the expression of WNT2B mRNA in the 6387 cell-line, whilst there were no changes were observed in the cell lines 6426 and 639; n=3-4. Student's t-test; *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms.

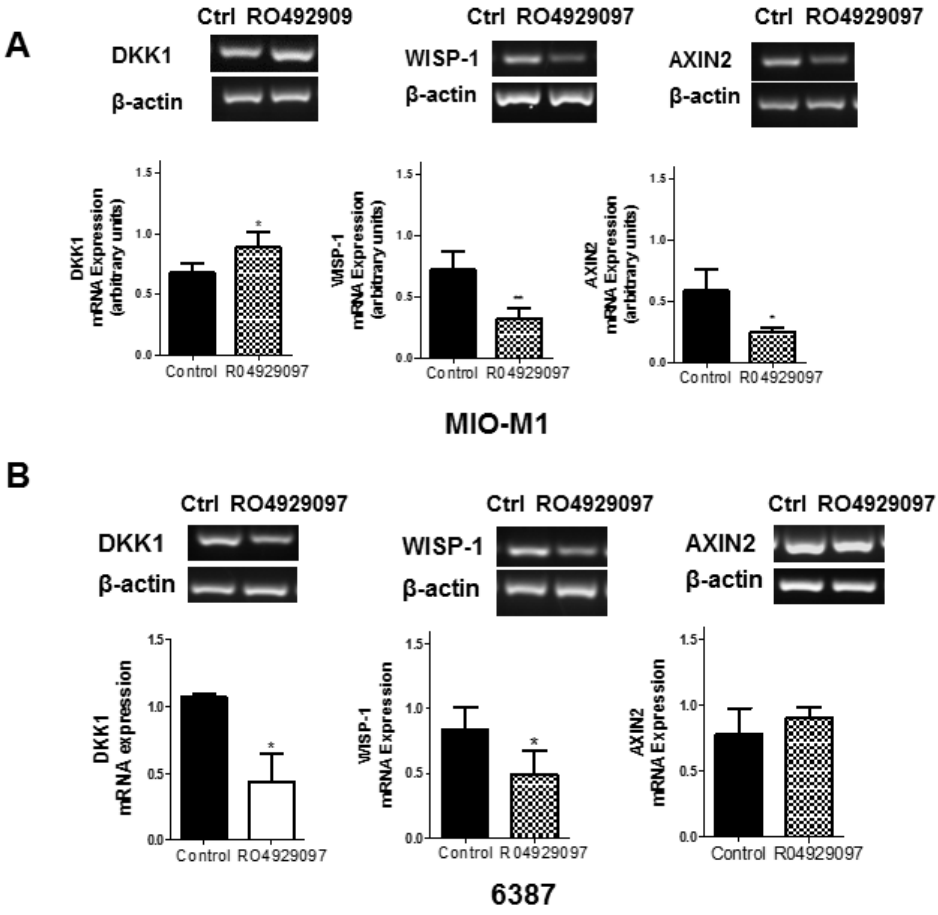

Figure 2.11 Effect of Notch inhibition on the canonical Wnt signalling component β-catenin and Wnt target genes DKK1, WISP-1 and AXIN2 in in Müller stem cell lines (MIO-M1, 6387 and 6391). A) In MIO-M1 cell line, Notch inhibition by 0.5 μM RO4929097 caused upregulation in mRNA expression of DKK1. In contrast, RO4929097 used at the same concentration caused downregulation in mRNA expression of WISP-1 and AXIN2 in these cells; n=3-4. Student's t-test; *p<0.05 v. control. B) Culture of 6387 cells with RO4929097 at the same concentration caused downregulation in mRNA expressions of DKK1 and WISP-1 in these cells but did not alter AXIN2 mRNA expression; n=3-4. Student's t-test; *p<0.05 v. control.

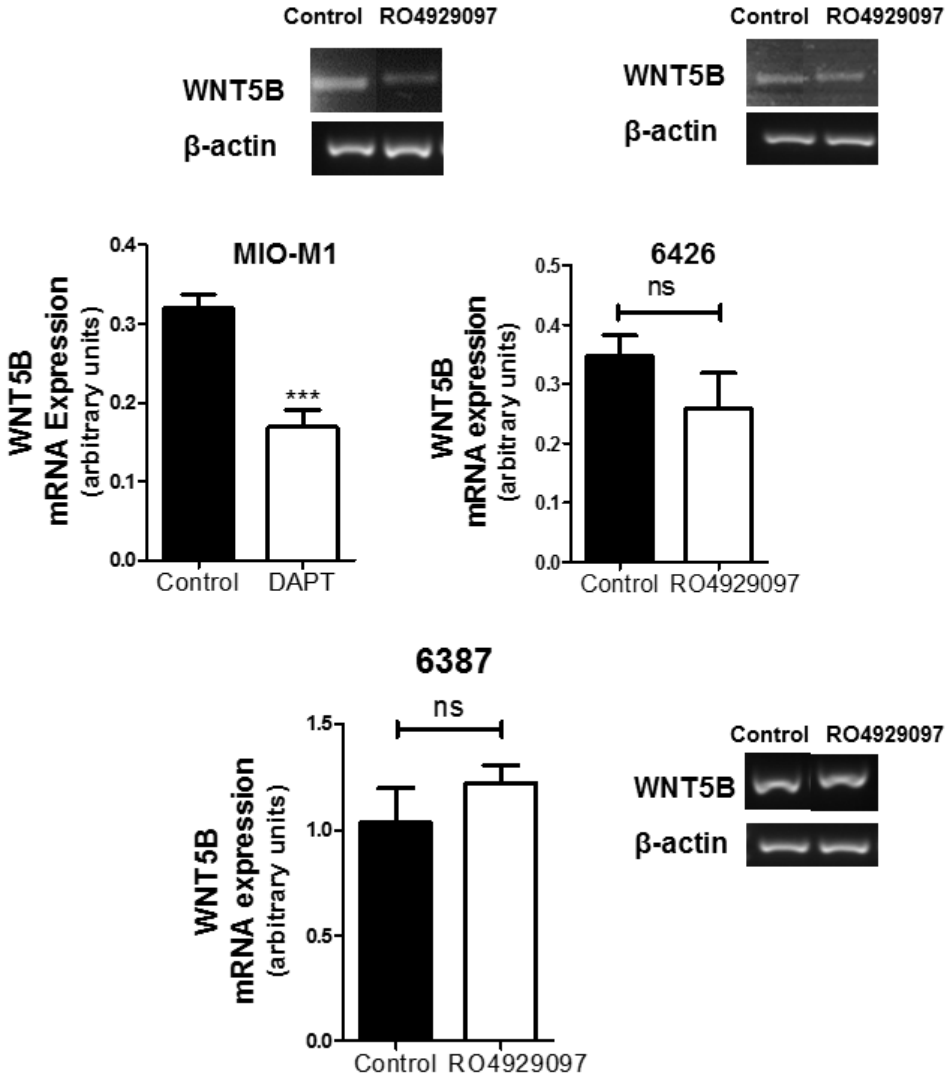

Figure 2.12 Effect of Notch inhibition on the non-canonical Wnt signalling ligand WNT5B in the Müller glia stem cell lines MIO-M1, 6426 and 6387. Notch inhibition by 0.5 μM RO4929097 caused a decrease in mRNA expression of WNT5B in the MIO-M1 cell line, whilst it did not induce changes in the 6426 and 6387 cells; n=3. Student's t-test; *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms.

2.4.4 Effect of Notch inhibition on cell proliferation of MIO-M1 cells

In mammals, the Notch signalling pathway is known to promote cell proliferation in different cell types including Müller glia (Del Debbio et al., 2010). Thus, the effect of RO4929097 mediated Notch inhibition on hMSC line MIO-M1 cell proliferation was examined by culturing these cells with different concentrations of RO4929097 (0.005 µM to 50 µM) for 7 days. A dose response decrease in number of MIO-M1 cells was observed with increasing $\log_{10}$ concentrations of RO4929097 ($p<0.001$) when compared to control cells cultured with medium alone (Fig 2.13). In addition, immuno-cytochemical staining of these hMSC cultured with the various concentrations of γ-secretase inhibitor RO4929097 (0.005 µM, 0.5 µM and 50 µM showed a similar dose dependent decrease in the percentage of cells labelling for cell proliferation marker Ki67 ($p<0.05$ for 0.005 µM; $p<0.001$ for 0.5 µM and $p<0.001$ for 50 µM) as compared to control cells (Fig 2.14). Therefore, the results suggest that the inhibition of Notch signalling by RO4929097 cause downregulation of the hMSC cell proliferation and may possibly promote differentiation.

A)

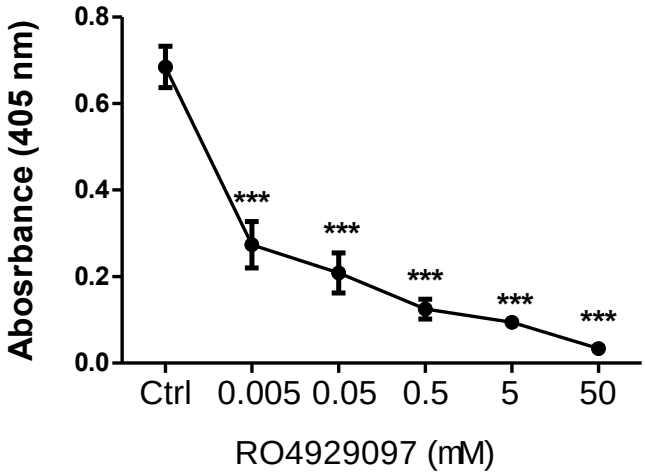

Figure 2.13 Effect of Notch inhibition on the proliferation of the MIO-M1 stem cell line. Culture of MIO-M1 cells in the presence of RO4929097 at range of concentrations from 0.005 to 50 μM for 7 days caused a decrease in cell proliferation in a dose response manner, as assessed by the hexosaminidase assay. The change in cell number/cell growth is expressed as the absorbance (Wendeler and Sandhoff, 2009)

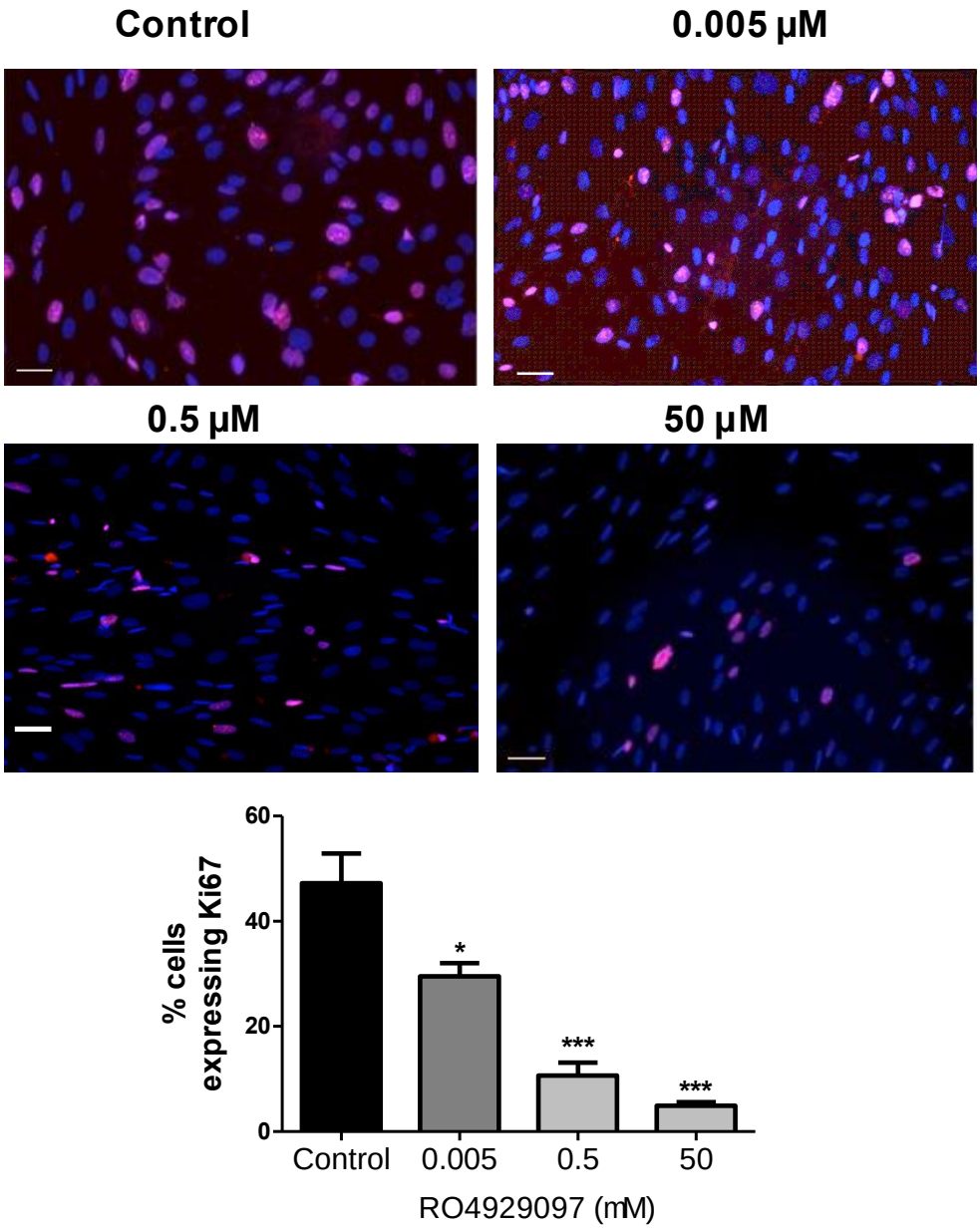

Figure 2.14 Effect of Notch inhibition on the proliferation of MIO-M1 cells.
Confocal images confirmed that culture of MIO-M1 cells with various concentrations of RO4929097 caused a decreased expression of the cell proliferation marker Ki67 (Alexa 555, red). Cell nuclei counterstained with DAPI (blue). Scale bars 50µm. x20 Magnification. Histogram represents the percentage of cells stained with Ki67 following 7 days culture with different RO4929097 concentrations; n = 3. ANOVA test; *p<0.05 v. control; ***p<0.001 v. control.

2.5 Discussion

The Müller glial cells constitute the primary sources of cells responsible for repairing damaged neurons in adult zebrafish following injury *in vivo* (Wan et al., 2012, Lenkowski et al., 2013, Lenkowski and Raymond, 2014). These cells have also shown their ability to differentiate *in vivo* into retinal neurons in small mammals such as mice and rats upon intraocular injection of growth factors (Del Debbio et al., 2010, Osakada et al., 2007). Müller glia with stem cell characteristics have been also isolated from the adult human eye and can be induced to differentiate into retinal neurons *in vitro* by growth and differentiation factors (Singhal et al., 2012, Jayaram et al., 2014).

Activation of the Notch signalling pathway occurs upon cleavage of the Notch NICD fragment, leading to activation of signalling cascades that promote proliferation and neural differentiation of Müller glial cells (Wan et al., 2012, Del Debbio et al., 2010). Here it was demonstrated that the Notch signalling pathway can be inhibited in hMSC using γ-secretase inhibitors RO4929097 or DAPT, which block the cleavage of NICD and subsequent Notch signalling mediated gene transcription. It was observed that various concentration of RO4929097 downregulation hMSC cell proliferation (Fig 2.13 and Fig 2.14). In this chapter, the Notch signalling mediated gene transcription of HES1 was inhibited in three different human Müller stem cell lines (MIO-M1, 6426 and 6387) (Fig 2.4 and Fig 2.5), but no effect was seen in the fourth cell line investigated (6391) (Fig 2.5). One of the factors for this variability observed in different hMSC lines may be ascribed to the genetic differences between different donors, resulting in variability in the Müller stem cells isolated from these individual human cadaveric eyes as judged by the differences in the time taken by different hMSC lines to become fully confluent. A study examining 60 cell lines

created from various tumour tissues showed that variation in gene expression pattern was linked to the differences in the tissue used to derive those cell lines (Ross et al., 2000). It is also possible that the different levels of interleukin proteins IL-6 and IL-8 found in various hMSC lines affected the inhibitory activity of RO4929097. It has been demonstrated in the literature that cell cultures showing high overexpression of these two interleukins prevented this γ-secretase inhibitor from inhibiting the Notch signalling pathway (He et al., 2011). Agreeing with this study, the results showed that the hMSC line 6391 where HES1 mRNA expression was not modified by RO492097, expressed the highest protein levels of both IL-6 and IL-8 amongst the other cell lines examined (Fig 2.6). In contrast, MIO-M1 cells where HES1 was downregulated by this γ-secretase inhibitor, levels of IL6 and IL8 interleukin proteins were at similar level to that of normal retina (Fig 2.6).

Examination of the effect of TGFβ1 on the Notch signalling pathway showed that this factor did not affect the expression of Notch signalling target gene HES1 in MIO-M1 cells cultured in the presence of Notch inhibitor DAPT (Fig 2.7). In addition, TGFβ1 did not alter the mRNA expression of the RGC markers BRN3A or BRN3B in these hMSC cultured in the presence of the same Notch inhibitor, which is known to induce human Müller stem cell differentiation into the RGC fate (Singhal et al., 2012) (Fig 2.7B and Fig 2.8). These present findings contrast with previously reported results in the chick *in vitro* and *in vivo* where an interaction between TGFβ signalling intracellular component SMAD3 and Notch signalling complex CSL or NICD leading to upregulation of HES1 mRNA expression was observed (Blokzijl et al., 2003). Similarly, cross-talk between the Notch and TGF-beta signaling pathways has been demonstrated in Epithelial to Mesenchymal transition of cells from mammary gland and kidney tubules (Zavadil et al., 2001, Zavadil et al., 2004). Therefore, although TGFβ1 did not have any effect on Notch signalling targets HES1

and BRN3A/B in hMSC MIO-M1, it is possible that TGFβ1 may induce an effect on these Notch targets on other hMSC cell lines. This is supported by studies that showed that the TGFβ signalling pathway is important for regulation of zebrafish Müller glia derived differentiation (Lenkowski and Raymond, 2014) and similarly activation of Notch signalling is essential during this process (Wan et al., 2012).

The effect of Notch inhibition on the components of the Wnt signalling pathway was next analysed in this chapter. Inhibition of Notch signalling by RO4929097 caused a downregulation of the Wnt signalling ligand WNT2B in two of the hMSC lines examined (MIO-M1 and 6387) (Fig 2.9 and Fig 2.10). However, RO4929097 did not induce any changes in other two cell lines (6426 and 6391) (Fig 2.10). Furthermore, Notch inhibition resulted in downregulation of the canonical Wnt signalling target genes such as WISP-1 and AXIN2 or DKK1 mRNA in the two examined hMSC lines. Therefore, these results suggest that there is an interaction between the Notch and Wnt signalling pathways in hMSC, and that Notch inhibition in these cells results in downregulation of the components of canonical Wnt signalling pathway. The downregulation of canonical Wnt signalling has been previously shown in adult zebrafish Müller glia through the regulation of Insm1a, a downstream gene of Notch signalling. Activation of Notch signalling in this species results in upregulation of Insm1a, which promotes canonical Wnt signalling activation by inhibition of DKK1 expression (Ramachandran et al., 2012). Conversely, inhibition of Notch signalling results in downregulation of this transcription factor resulting in canonical Wnt signalling inhibition (Ramachandran et al., 2012). Agreeing to this study, a significant downregulation of DKK1 mRNA expression was observed in the hMSC line 6387 (Fig 2.11B), where HES1 was also found to be downregulated by RO4929097 induced Notch inhibition (Fig 2.5). However, in the literature, there are also studies that suggest activation of Notch signalling inhibit canonical Wnt signalling. In stem

and colon cancer cells, it has been shown that Notch is able to downregulate Wnt signalling by binding to β-catenin and targeting it for degradation (Kwon et al., 2011). Furthermore, Notch signalling can also regulate Wnt signalling through modulation of the Wnt target genes histone status (Kim et al., 2012) and by forming a complex consisting of NICD/RBP-Jk and ng a complex consisting of u-catenin mediated transcription of Wnt target genes (Collu et al., 2014). Similarly, culture of hMSC with RO4929097 also caused a downregulation in the mRNA expression of non-canonical Wnt signalling ligand WNT5B in MIO-M1 cells. However, no such change was observed in the cell line 6387 (Fig 2.12). Although there was a decreased WNT5B expression by RO492907 in MIO-M1 cells, previous studies in the literature suggest that non-canonical signalling preferentially act in opposite to that of canonical Wnt signalling and inhibit canonical Wnt signalling by acting on β-catenin (Schubert, 2003). Taken together, based on the present results of this chapter the Notch inhibition can inhibit components of both canonical and non-canonical Wnt signalling pathways. This suggests that both these Wnt signalling pathways may potentially be important for hMSC RGC differentiation induced by Notch inhibition.

In summary, the results presented in this chapter suggest that the downregulation of the Notch signalling pathway by γ-secretase inhibitor RO4929097 occurred in most human Müller stem cell lines. The variation observed between the different hMSC lines may possibly due to the identity of the donor tissue used for cell isolation (Ross et al., 2000) and may also be a result of different levels of the interleukin protein expressions of IL-6 or IL-8 in different hMSC lines, which are known to affect the activity of RO4929097 (He et al., 2011). In addition, TGFβ1 did not modify the Notch signalling targets HES1 and BRN3A/B during RGC differentiation in the examined hMSC line MIO-M1, but given the importance of both the Notch and TGFβ signalling pathways in zebrafish Müller glia mediated retinal regeneration, an

interaction between these pathways may be observed in other hMSC lines. Furthermore, the canonical Wnt signalling pathway ligand WNT2B and target gene WISP-1 were downregulated by Notch inhibition in hMSC cell lines (MIO-M1 and 6387), and non-canonical Wnt ligand WNT5B downregulated in MIO-M1 cells.

Chapter 3: Effect of TGFβ on the expression of Wnt signalling components by human Müller stem cells

3.1 The TGFβ signalling pathway

The TGFβ superfamily consist of large number of secreted growth factor proteins. Members of this family include TGFβ molecules (three isoforms: TGFβ1, TGFβ2 and TGFβ3), bone morphogenic proteins (BMPs), activin and inhibin, growth and differentiation factors (GDFs), Nodals, leftys and anti-Muellerian Hormone (AMH) (Massague, 1998, Derynck and Miyazono, 2008). All TGFβ molecules are secreted as a complex consisting of pro-domain linked to active TGFβ form. The pro-domain is further bound to latent The TGFβ binding protein, which is attached to extracellular matrix (Massague, 1998, Annes et al., 2003). Thus, cleavage by serine proteases in the extracellular matrix releases the active TGFβ ligand and combines with another active TGFβ ligand to form homodimer and become functional (Boettner et al., 2000).

The TGFβ signalling is mediated through three different types of receptors: type I (TGFβRI or Activin-like kinases (ALK)), type II (TGFβRII) and type III (TGFβRIII). There are seven different isoforms of type I receptor (ALK1-7) and four different for type II receptor (TGFβRII, BMPRII, ActRIIA/B) (Nickel et al., 2009). Whilst type I and type II receptors are involved in TGFβ signalling, type III helps binding of TGFβ2 ligand to TGFβRII (Bierie and Moses, 2006). The TGFβ signalling via type I receptor is activated when TGFβ ligands bind to type II receptor kinase homodimer. This results in formation of a complex consisting of homodimers of type II and type I receptors along with the TGFβ ligand (Yamashita et al., 1994). During this process, GS-domain (rich in glycine and serine conserved domain) of type I receptor is trans-phospholyrated by type II receptor kinase and activating the type I receptor kinase

(Huse et al., 2001, Dennler et al., 2002), which in turn activates the intracellular mediator proteins such as Smads in the cytoplasm (Fig 3.1).

There are three types of Smad proteins: 1) receptor regulated or R-Smads; 2) Complex or Co-Smads and 3) Inhibitory or I-Smads (Dennler et al., 2002). R-Smads include Smad1, Smad2, Smad3, Smad5 and Smad8, and are activated by trans-phosphorylated by type I receptor kinase (Souchelnytskyi et al., 1997). Of these, Smad 2 and 3 are by commonly activated by TGFβ, whilst the others are activated by BMP ligands (Feng and Derynck, 2005). This is followed by the binding of R-Smads with Co-Smads 4 and formation of a complex which is translocated into the nucleus to transcription of TGFβ mediated target genes (Dennler et al., 2002). The I-smads (Smad6 and Smad7) regulate Smad function by inhibiting the function of both R-Smads and Co-Smads (Imamura et al., 1997, Nakao et al., 1997). In addition, TGFβ signalling is also mediated independent of Smad proteins. Some of the pathways activated are Ras/MAPK (Yu et al., 2002), JNK (Engel et al., 1999), Rho/ROCK (Bhowmick et al., 2001) and PI3K-Akt pathway (Yi et al., 2005) (Fig 3.2).

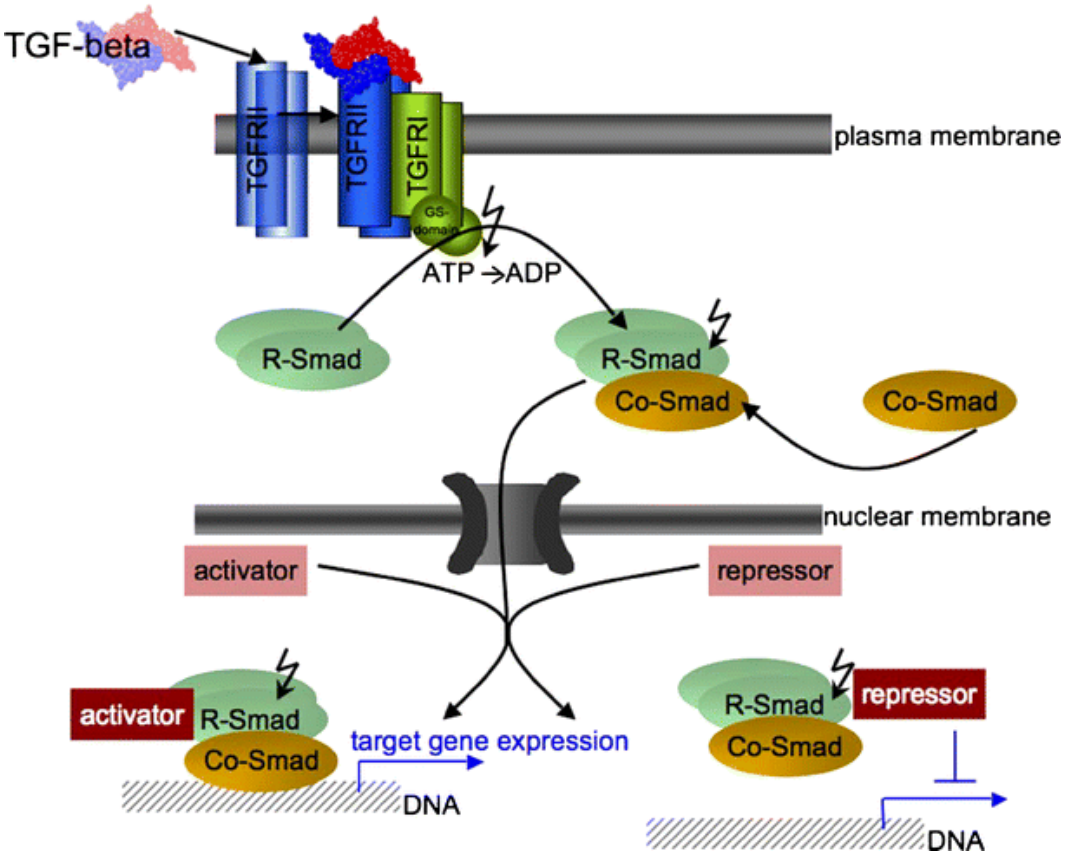

Figure 3.1 Smad dependent TGFβ signalling pathway. The homodimer of TGFβ ligands binds type II receptor, followed by formation of complex with type I receptor. The type II receptor kinase trans-phosphorylates the GS-domain (conserved Glycine and serine sequence) in type I, and activating it in the process. Likewise, in the cytoplasm the type I receptor kinase activates R-Smads (e.g. Smad2 and Smad3). The activated R-Smads bind with Co-Smads and translocate into nucleus for transcription of TGFβ mediated genes. R-Smads and Co-Smads complex formation can be inhibited by I-Smads (Massague, 2000) From (Aigner and Bogdahn, 2008).

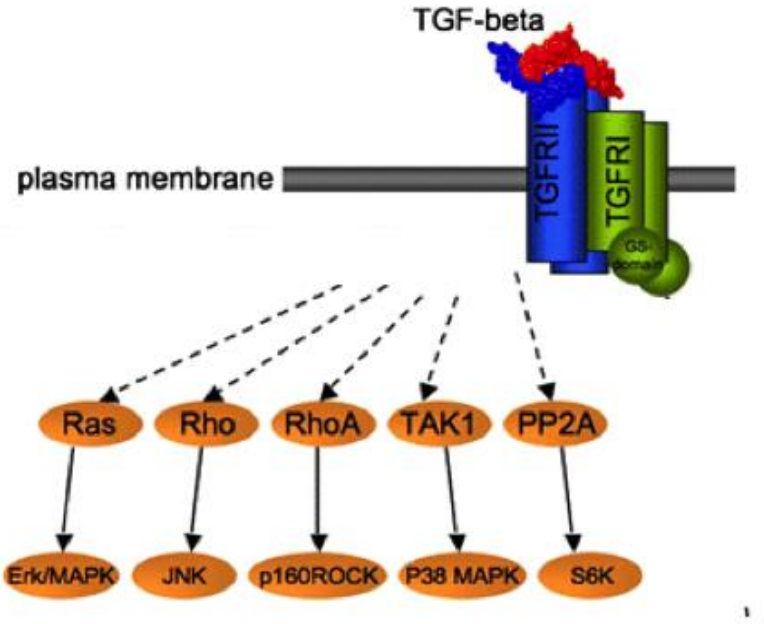

Figure 3.2 Smad independent TGFβ signalling pathway. Ras/MAPK (Yu et al., 2002), JNK (Engel et al., 1999), Rho/ROCK (Bhowmick et al., 2001) and PI3K-Akt pathway (Yi et al., 2005) are activated by TGFβ signalling in a Smad independent way. From (Aigner and Bogdahn, 2008)

3.2 Role of TGFβ signalling in stem cells

The TGFβ signalling pathway is one of the most important regulators of cellular functions such as cell proliferation, differentiation, apoptosis, adhesion and interactions with the cellular microenvironments in various cell types including epithelial, endothelial and hematopoietic cells (Blobe et al., 2000). In addition, TGFβ has also been shown to be important for maintaining pluripotency of both human and mouse embryonic stem cells as TGFβ signalling has been shown to regulate the expression of Nanog, one of the master pluripotency controlling transcription factor. This regulation is mediated by directly binding of the TGFβ signalling transducing factors Smad2 or Smad3 proteins to the Nanog promoter region (Xu et al., 2008, Vallier et al., 2009), resulting in the transcription of Nanog. It has been recently shown that TGFβ signalling occurs in both neural stem cells and progenitors found in the subgranular zone of the hippocampus, where adult neurogenesis occurs. When bone morphogenic protein (BMP), a protein of the TGFβ superfamily of ligands, was inhibited in the brain, neural stem cells initially proliferated but later this effect diminished (Mira et al., 2010). In addition, inhibition of TGFβ type II receptor gene has been shown to increase self-renewal of neural stem cells, resulting in an enlarged midbrain (Falk et al., 2008). In Hematopoietic stem cells (HSC) which give rise to all blood cell types found in the bone borrow, TGFβ signalling has been suggested to maintain HSC quiescence by limiting the activation of TGFβ signalling (Yamazaki et al., 2009). Furthermore, it has been hypothesized that activation of TGFβ signalling is triggered by contact of quiescent HSC with non-myelinating Schwann cells, resulting in the activation of these quiescent cells when required (Yamazaki et al., 2011).

Proliferation of intestinal stem cells is negatively regulated by BMP signalling in the crypts. It has been suggested that by acting in a paracrine manner, BMP signalling acts on the non-proliferating epithelial cells and stem cells. This is supported by findings that the intravillus mesenchyme highly expresses BMP-4 protein, whilst the BMP activated Smads are found in differentiated intestinal stem cells (He et al., 2004). Similarly, BMP signalling is involved in the maintenance of the quiescent state of the hair follicle stem cells. This was demonstrated by observations that removal of the Bmpr1a gene from the postnatal skin epithelium, resulted in the proliferation of the quiescent hair follicle stem cells and lead to the production of tumor like cells which failed to generate hair. This contrasted with the findings that activation of Bmpr1a resulted in early hair follicle differentiation. (Kobielak et al., 2007). These reports suggested that BMP signalling controls hair follicle stem cells and that a balance in BMP expression is required to maintain its quiescent and activated states.

Although TGFβ has been shown to promote and maintain various stem cells, during injury this factor is highly released (Hoerster et al., 2014). Moreover, TGFβ is known to promote gliosis and inhibit proliferation of different cell types in the central nervous system in vertebrates (Bringmann et al., 2009, Robel et al., 2011) including Müller glia in the retina (Close et al., 2005, Lenkowski et al., 2013). That TGFβ plays a role in retinal gliosis has been further supported by findings that administration of the TGFβ signalling inhibitor decorin prior to surgery in an experimental PVR rabbit model decreased the glial scarring (Nassar et al., 2011).

In the adult zebrafish, TGFβ signaling mediated through Smad 2/3, which is controlled by transcriptional co-repressors such as *tgif1* and *six3b*, has also been demonstrated to regulate Müller glia derived photoreceptor regeneration in the adult

zebrafish (Lenkowski et al., 2013). Microarray data has also shown that TGFβ signalling is initially upregulated after acute light lesions, which is followed by its downregulation in the zebrafish (Qin et al., 2009). In the same study it was observed that the co-repressors tgif1 and six3b stayed at high levels whilst tgfβi and its receptor were decreased (Qin et al., 2009). These observations further show that TGFβ signalling also plays a critical role in Müller glial cell functions during retinal damage and subsequent retinal regeneration.

3.2 Role of Wnt signalling in neural progenitor proliferation and differentiation

The Wnt signalling (primarily canonical) has been demonstrated to be important for the regulation of various tissue stem cells. These include stem cells found in the intestine (crypt-villus), epidermis (hair follicle) and haematopoietic systems (HSC) (Reya and Clevers, 2005). For example, stem cells (Lgr5+) are found in the crypts of the intestine. As the intestine undergoes mechanical and chemical stress, these stem cells enables the intestine to maintain their cellular homeostasis by self renewing every 4-5 days (Gerbe et al., 2011). In this process, stem cells differentiate and replace the cells that have been lost at the tip of the villus by migrating along crypt-villus axis (Roth et al., 1991). The cell fate of these intestinal stem cells has been shown to be regulated by canonical Wnt signalling as nuclear β-catenin is observed throughout the crypts. This is supported by studies in the neonatal mice with mutation in T-cell factor, a downstream effector of the canonical Wnt signalling which forms a nuclear complex with β-catenin for transcription of Wnt target genes, resulting in the absence of crypt progenitor compartment. This demonstrated the importance of Wnt signalling for the maintenance of crypt progenitor cells (Korinek

et al., 1998). Furthermore, inhibition of canonical Wnt signalling by using Dickkopf-1 (DKK1), a naturally occurring canonical Wnt signalling inhibitor, resulted in loss of crypts in adult mice (Kuhnert et al., 2004).

Another example of the role of Wnt signalling regulating stem cells is that observed with multipotent epidermal stem cells found in the bulge region of hair follicles that gives rise to hair. In these cells, β-catenin expression promotes de novo hair growth (Gat et al., 1998, Vangenderen et al., 1994) and mutations in Lef1, a nuclear protein that is required for Wnt transcription, result in loss of hair formation (Vangenderen et al., 1994). Interestingly, conditional β-catenin deletion leads to complete hair loss after hair follicle formation (Huelsken et al., 2001). In addition, Wnt signalling maintains expression of the nuclear protein TCF3, which is required for Wnt mediated transcription and is specifically found in bulge region stem cells This promotes the bulge stem cells characteristics and prevents differentiation towards keratinocytes (DasGupta and Fuchs, 1999).

Several studies suggest a role for Wnt signalling in the maintenance and self-renewal of HSCs which are present in adult bone marrow. In vitro murine haematopoietic progenitors, Wnt proteins have been shown to promote proliferation while inhibiting their differentiation (Austin et al., 1997). Furthermore, *in vitro* expression of β-catenin and Wnt3A, which are components of the canonical Wnt signalling pathway, has been demonstrated to rescue the haematopoietic system in lethally irradiated mice *in vivo* (Willert et al., 2003). Human haematopoietic progenitor proliferation is promoted by treatment with Wnt5A, a component of the non-canonical Wnt signalling pathway, in the presence of stromal cell contact *in vitro* (Van den Berg et al., 1998). In addition, Wnt5A treatment in Non-obese

Diabetic/Severe Combined Immunodeficiency (NOD/SCID) model results in human HSC re-population in mice (Murdoch et al., 2003).

In neural stem and progenitor cells of mice, conditional deletion of β-catenin results in the decreased expansion of the brain and spinal cord cells. Conversely, conditional activation of β-catenin in mice results in increased expansion of the cells from the same areas, suggesting that Wnt signalling is important for controlling neural stem and progenitor growth and differentiation (Zechner et al., 2003). Furthermore, treatment of mouse embryonic stem cells with the Glycogen synthase kinase 3 (GSK3) inhibitor promotes in the maintenance of pluripotency *in vitro* (Sato et al., 2004), and Adenomatous polyposis coli (APC) mutations in mouse embryonic stem cells results in increased inhibition of differentiation *in vivo*/vitro (Kielman et al., 2003).

In the retina, Wnt signalling is very important for various developmental processes such as establishing the eye formation from the anterior neural plate, promoting retinal stem cell progenitor maintenance and neuronal differentiation, as well as retinal vasculogenesis and development of lens and cornea (Lad et al., 2009). During the first stages of eye development in Xenopus, expression of the Wnt receptor Frizzled-3 (Fz3) changes from ubiquitous to restricted at the anterior neural plate to give rise to the early eye field (Shi et al., 1998) . Interestingly, Fz3 overexpression results in ectopic eye expression whereas, inhibition of Fz3 results in loss of eye development (Rasmussen et al., 2001). Whilst Fz4 and Fz5 expression is found in the chick distal optic vesicles (stage 10) and gives rise to the neural retina in this species (Fuhrmann et al., 2003), Wnt2b/ Wnt 13 are found in the ciliary marginal zone (CMZ) and the retinal pigment epithelium (RPE) of mice (Liu et al., 2003).

Wnt signalling molecules have been observed in the peripheral part of the retina in species such as zebrafish (Yamaguchi et al., 2005) and chicken embryo (Cho and Cepko, 2006). Wnt2b is expressed in the anterior rim of optic vesicles and its overexpression by *ovo* electroporation has demonstrated to inhibit differentiation of retinal progenitor cells in chick *in vivo*. By contrast, Wnt2b inhibition in the same species using dominant-negative form of LEF1, a nuclear protein required for Wnt mediated transcription, caused premature differentiation of peripheral retina into retinal ganglion cells *in vivo* (Kubo et al., 2003). In addition, Wnt2b maintains CMZ retinal stem cells in proliferative and undifferentiated state *in vitro*; expression of Wnt2b also keeps central retinal progenitor cells, which normally differentiates, in proliferative state (Kubo et al., 2005).

3.2.1 Effect of Wnt signalling on Müller glia and progenicity

Wnt signalling, in particular canonical Wnt signalling has been demonstrated to promote Müller glia derived progenitors in zebrafish. β-catenin accumulation has been shown to increase following retinal injury compared to non-injured retina in this species, suggesting that the canonical Wnt signalling is important for Müller derived progenitor proliferation (Ramachandran et al., 2011). Recent studies from the same group have shown that retinal injury in the zebrafish induces HBEGF expression which in turn increases Ascl1a, and activates Wnt signalling, resulting in Müller derived progenitor proliferation (Wan et al., 2012). Wnt signalling has also been demonstrated to have a similar role in the mammalian retina. Following activation of the canonical Wnt signalling by addition of Wnt3a or GSK-3β inhibitors, it has been observed that Wnt signalling promotes Müller derived progenitor proliferation

in rodents *in vivo*. On the contrary, it has been shown that blocking Wnt signalling by DKK-1 treatment, prevents Müller derived progenitor proliferation (Osakada et al., 2007). In vitro, the addition of Wnt2b results in promotion of neurosphere formation from Müller glial cells isolated from mice. By contrast, culture of the Müller glial cells in the presence of the Wnt antagonist, Fzd-CRD prevented neurosphere formation in these cells (Das et al., 2006). In the zebrafish, Wnt signalling is important for successful retinal regeneration by Müller glial cells in both larval (Meyers et al., 2012) and adult stages (Ramachandran et al., 2011). Furthermore, increased Wnt signalling in transgenic mice following laser injury of the retina, leads to Müller glia proliferation and differentiation into rhodopsin positive neural cells (Liu et al., 2013), whilst activation of Wnt and Notch signalling also causes differentiation of mice Müller cells into retinal cells expressing opsin (Del Debbio et al., 2010)

3.2.2 Crosstalk between Wnt and TGFβ signalling pathways

The Wnt and TGFβ signalling pathways are important regulatory elements known to control proliferation and differentiation of progenitor cells in different species including the zebrafish. Both pathways have been shown to interact throughout life. Furthermore, TGFβ and Wnt can reciprocally regulate their ligand production to establish extracellular gradient of these molecules. This is illustrated by observations that in chick embryos, in order to create left-right body axis, Wnt-8c induces the expression of Nodal in a β-catenin dependent manner (Rodriguez-Esteban et al., 2001). Similarly, in *Xenopus*, the patterning of the mesoderm occurs as a result of the control of Wnt-8 expression by BMP-2/-4 and the co-operation of both these ligands. In the colon crypts, where intestinal stem cells reside, it has been shown that Wnt activation can inhibit BMP signalling by promoting the expression of a

number of BMP antagonists (Kosinski et al., 2007). Furthermore, expression of the connective tissue growth factor (CTGF) is regulated by both TGFβ and Wnt signalling. However, CTGF can directly interact with ligands such as BMP-4 and TGFβ1 to prevent the binding of BMP-4, whilst promoting binding of TGFβ1 to its receptor (Abreu et al., 2002). Furthermore, production of CTGF also inhibits the BMP mediated osteoplastic differentiation of mesenchymal stem cells (Luo et al., 2004).

Crosstalk between Wnt and TGFβ signaling pathways also occurs in the nucleus, where the Smad/ β-catenin/ Lef protein complex can regulate shared target genes. In the Xenopus, during the regulation of the Spemann's organizer, an embryonic signalling center that controls the movement and fate of neighbouring cells (Niehrs, 2004), the organizer gene Xtwn is regulated synergistically through the formation of a complex between these three proteins (Labbe et al., 2000). In the absence of activation of any of these two pathways, reduced expression of Xtwn is observed (Nishita et al., 2000b). Furthermore, genes such as Emx2 and Msx2 which are important for neural development during embryonesis in mice, are shown to be upregulated co-operatively by Wnt and BMP signalling (Theil et al., 2002, Hussein et al., 2003). The interaction between these two pathways has been shown to occur in the cytoplasm. This is supported by findings that BMP inhibits Wnt induced β-actin translocation and cell proliferation in mouse mesenchymal cells as a result of Smad1 interaction with Dvl-1 in the cytosol (Liu et al., 2006). In addition, Axin also interacts with Smad proteins in the cytosol and can assist TGFβ signalling by presenting Smad3 to type I TGFβ receptor in COS7 cells (Furuhashi et al., 2002)

3.3 Objectives

The effects of TGFβ on Wnt signalling have been shown in different types of cells, including Müller glia, in species such as zebrafish and mice. However, there have not been reports in the literature on the effect of TGFβ on the expression of Wnt signalling components in human Müller glia cells. Given the significant roles that TGFβ and Wnt signalling play in retinal cell proliferation and differentiation in the zebrafish following retinal injuries, this study aimed to investigate whether there is any interaction between the components of these two important pathways during proliferation and neural differentiation of human Müller stem cells in vitro.

On this basis, the chapter aims were as follows:

1. To investigate the role of TGFβ1 on the components of the Wnt signalling pathway in human Müller stem cells.
2. To investigate the effect of exogenous TGFβ1 on proliferation and apoptosis of the human Müller stem cells.
3. To investigate the effect of TGFβ inhibitor on the expression of Wnt signalling components by human Müller stem cells.

To achieve these objectives, the following experiments were conducted:

1. Components of the Wnt signalling pathway were identified by performing RT-PCR on normal human Müller cells.

2. Human Müller stem cells were cultured with various concentration of TGFβ1 to identify the optimum concentration for further experiments.
3. After identifying the optimum concentration of TGFβ1 (50 ng/ml) the expression of Wnt signalling components were compared with methods such as RT-PCR, Western blot and Elisa methods.
4. The effect of TGFβ on cell proliferation was analysed by Hexosaminidase assay whilst the effect on cell apoptosis was analysed by Caspase-3/7 assay
5. The TGFβ inhibitors SB421542 and SP600125 which target ALK5 receptor and JNK respectively were used to study the effect of TGFβ inhibition on Wnt signalling components.

3.4 Results

3.4.1 Effect of TGFβ1 on the expression of components of the canonical and non-canonical Wnt signalling pathway by hMSC.

Examination of various Wnt pathway components in the human Müller glial cell line MIO-M1 using RT-PCR analysis showed that these cells express mRNA coding for WNT2B, WNT3A, WNT5B, WNT11, FZD1, FZD4, FZD7 and β-catenin. Transcripts for WNT8B and FZD5 were not detected despite the use of three different primers and variations in assay parameters in this cell line (Fig 3.3A). Three different hMSC cells lines named, MIO-M8, MIO-M7 and MIO-M1, when cultured with various concentrations of TGFβ1 for 7 days showed that mRNA expression coding for the WNT2B ligand consistently decreased in a dose response manner (Fig 3.3B). As compared with the controls, concentrations as low as 0.1 ng/ml reached significant differences in all the three cell lines. Increasing $\log_{10}$ concentrations between 1 and 100 ng/ml of TGFβ1 induced a further decrease ($p<0.001$ for all the cell lines examined) in the expression of this gene, without showing significant differences amongst them (Fig 3.3B). Corresponding to that seen with mRNA expression, western blot analysis of MIO-M1 cells cultured with 50 ng/ml of TGFβ1 showed a significant decrease ($p<0.05$) in intracellular WNT2B protein levels as compared with cells cultured in medium alone. Interestingly, the levels of WN2B ligand present in culture supernatants, as determined by ELISA methods, were minimally detected in both control and TGFβ1 treated cells (below 1pg/ml) and there were no differences between the two conditions (Fig 3.4). In addition, this could may be due to lower sensitivity of the ELISA kit for detecting this ligand at low starting material conditions as well as some technical difficulties while performing the experiment.

In contrast to the downregulation of WNT2B mRNA caused by TGFβ1 in hMSC, mRNA expression of the WNT5B ligand was consistently increased by TGFβ1 in a dose response manner (Fig 3.5). Although concentrations of 0.1 ng/ml of TGFβ1 did not cause significant changes in gene expression, increasing $\log_{10}$ concentrations ranging between 1 and 100 ng/ml of TGFβ1 induced a significant increase in the expression of WNT5B mRNA ($p<0.05$ for MIO-M8; $p<0.001$ for MIO-M7 and MIO-M1) (Fig 3.5). In agreement with the mRNA findings, culture of hMSC with TGFβ1 caused a significant increase ($p<0.05$) in the intracellular levels of WNT5B protein as compared with cells cultured in medium alone (Fig 3.6). Similarly to that seen with the levels of secreted WNT2B in the culture supernatant, secreted WNT5B protein was minimally detected by ELISA methods in both control and TGFβ1 treated cells and there were no difference between the two conditions (Fig. 3.6).

Examination of the ratio of phosphorylated β-catenin over β-catenin protein expression showed that the levels of phosphorylated β-catenin (which indicates that β-catenin is targeted for degradation) were increased by TGFβ1 in hMSC ($p<0.05$) (Fig 3.7A). Furthermore, TGFβ1 caused a significant decrease in the expression of mRNA coding for the canonical Wnt signalling target DKK1 ($p< 0.001$) and secreted DKK1 protein ($p<0.05$) compared to cells cultured with medium alone (Fig 3.7B). To test the effect of the WNT2B and WNT5B ligands on Wnt signalling in these cells, hMSC were cultured in the presence or absence of these two ligands. It was observed that recombinant WNT2B (100 ng/ml) significantly increased the levels of DKK1 mRNA ($p<0.05$) (Fig 3.8A), whilst recombinant WNT5B (500 ng/ml) markedly decreased the mRNA levels of this target gene in hMSC ($p<0.05$) (Fig 3.8B).

These findings indicate that different adult hMSC lines express various components of the Wnt signalling pathway and that TGFβ1 downregulates the canonical Wnt

signalling ligand WNT2B as well as the active form of β-catenin, which are important for canonical Wnt signalling. Furthermore, upregulation of the non-canonical Wnt ligand WNT5B by TGFβ1 may indicate the potential of this factor to inhibit the canonical Wnt signalling pathway.

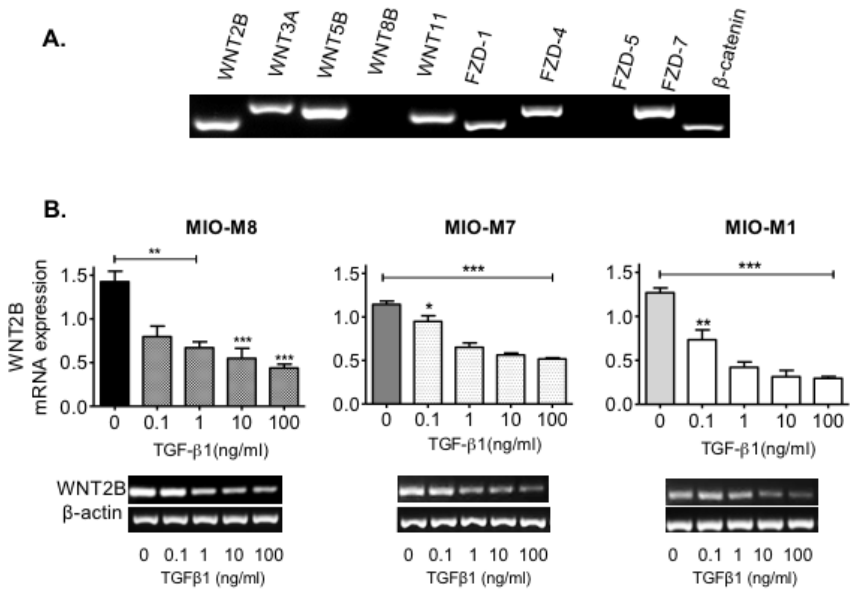

Figure 3.3 Expression of mRNA coding for molecules of the Wnt signalling pathway in hMSC and modulation of WNT2B expression by TGFβ1 (A) hMSC express mRNA coding for various components of the canonical and non-canonical Wnt signalling pathway. **(B)** TGFβ1 downregulation of the expression of mRNA coding for WNT2B occurred in a dose response manner in three different hMSC lines (MIO-M8, MIO-M7 and MIO-M1) after 7 days culture with concentrations of this factor ranging between 0.1 and 100 ng/ml. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown below histograms; n=3-4. Anova test, *p<0.05; **p<0.01; ***p<0.001.

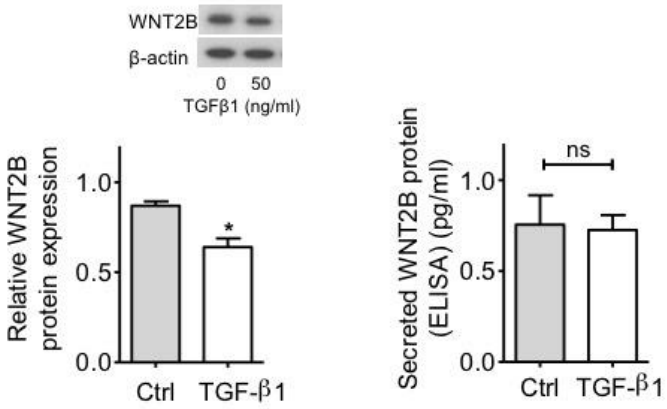

Figure 3.4 Expression of intracellular and secreted WNT2B protein expression by TGFβ1. A significant decrease in the expression of WNT2B protein was observed by western blot analysis of lysates from cells cultured with 50 ng/ml of TGFβ1. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms; n=3. Student's t-test; *p<0.05. Minimally detectable levels of secreted WNT2B examined by ELISA methods were observed in supernatants of cells cultured in the presence or absence of TGFβ1, and no differences between the two conditions were observed; n=3. Student's t-test, ns= No significant.

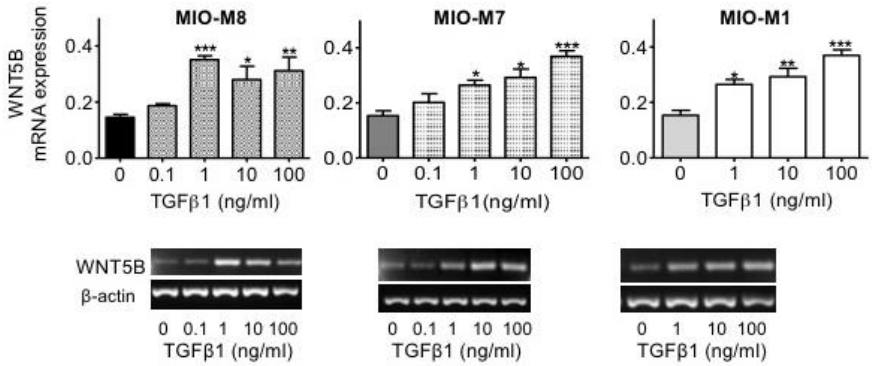

Figure 3.5 TGFβ1 modulation on the mRNA expressions of WNT5B in hMSC. TGFβ1 induced upregulation of the expression of mRNA coding for WNT5B occurred in a dose response manner in three different hMSC lines examined (MIO-M7, MIO-M8 and MIO-M1). Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown below histograms; n=4. Anova test, *p<0.05; **p<0.01; ***p<0.001.

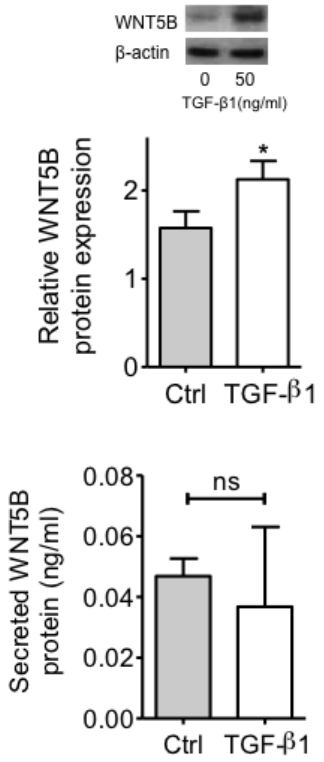

Figure 3.6 TGFβ1 modulation on the intracellular and secreted proteins of WNT5B in hMSC. TGFβ1 (50 ng/ml) induced upregulation of the expression of mRNA coding for WNT5B, whilst no changes was observed for the secreted protein. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown below histograms; n=4. Anova test, *p<0.05.

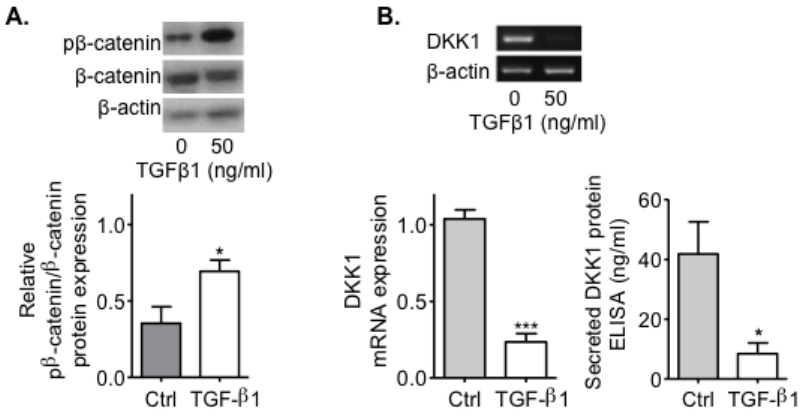

Figure 3.7 Modulation of pβ-catenin and DKK1 protein expressions by TGFβ1 (A). Western blot analysis revealed that culture of hMSC with 50 ng/ml of TGFβ1 induced a significant upregulation of the ratio of phospho-β-catenin/β-catenin. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms; n=5. Student's t-test, *p<0.05. pβ-catenin=phospho-β-catenin. **(B)** TGFβ1 caused a significant decrease in DKK1 mRNA expression in hMSC as revealed by RT-PCR analysis. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative bands are shown above histograms; n=8. Student's t-test; ***p<0.001. Secreted DKK1 protein levels as determined by ELISA methods were significantly decreased in culture supernatants of cells treated with 50 ng/ml of TGFβ1 as compared to controls; n=4. Student's t-test; *p<0.05.

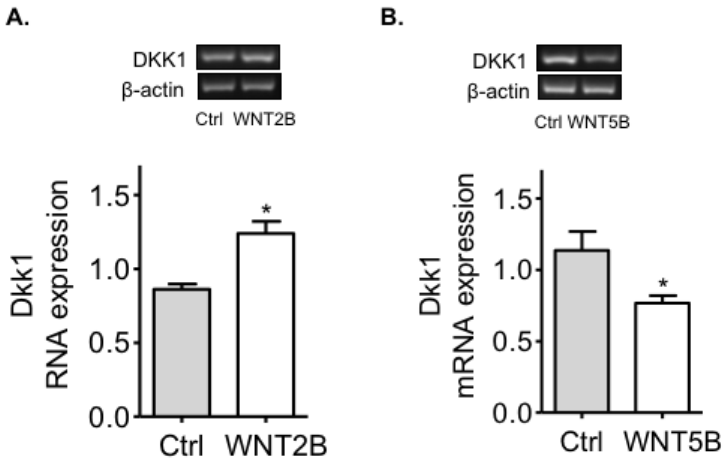

Figure 3.8 Effect of exogenous WNT2B and WNT5B ligands on DKK1 mRNA expression. A) Exogenous addition of recombinant WNT2B (100 ng/ml) into the culture medium induced a significant upregulation of DKK1 mRNA in hMSC; n=4. Student's t-test, *p<0.05. **B)** Addition of recombinant WNT5B (500 ng/ml) to cells in culture caused a significant down regulation of DKK1 mRNA expression; n=4. Student's t-test, *p<0.05.

3.4.2 The effect two different TGFβ inhibitors on the expression of Wnt ligands by hMSC

To assess whether inhibition of TGFβ signalling may modify the effect of this factor on expression of WNT2B and WNT5B, components of the TGFβ signalling pathway were inhibited using the type I receptor (ALK5) inhibitor SB431542 and the JNK inhibitor SP600125. As previously observed, Fig 2.6 shows that TGFβ1 alone caused a significant decrease in the expression of the WNT2B mRNA as compared with control cells ($p<0.01$). However, addition of SB431542 markedly inhibited this effect ($p<0.05$) (Fig 3.9). Unlike that seen with the ALK5 inhibitor, addition of the JNK inhibitor SP600125 did not cause any effect on the downregulation of WNT2B mRNA by TGFβ1 (Fig 3.9). This suggests that downregulation of the Wnt signalling ligand WNT2B by TGFβ1 is caused by activation of the SMAD2/3 signalling cascade. Similarly, the increase in WNT5B expression induced by TGFβ1 alone ($p<0.01$) was inhibited by addition of SB431542 ($p<0.01$) (Fig 3.10). This contrasts with the lack of inhibitory effect by JNK (SP600125) inhibitors (Fig 3.10). These observations suggest that the TGFβ1 induced upregulation of WNT5B expression in hMSC is also dependent of SMAD2/3 signalling but independent of JNK transcription signalling. Taken together, these results suggest that TGFβ1 may regulate hMSC photoreceptor differentiation by modifying the ligands WNT2B and WNT5B of the Wnt signalling pathway.

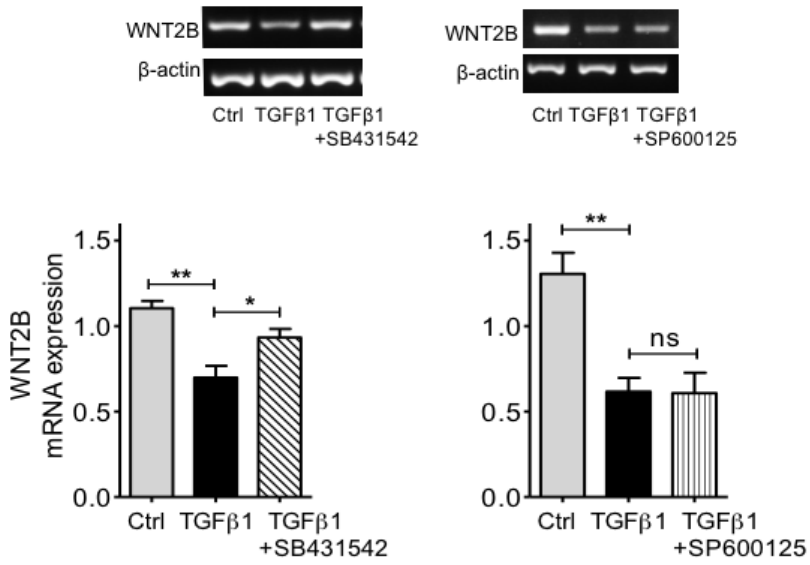

Figure 3.9 Effect of TGFβ1 inhibitors on the expression of the Wnt signalling ligands WNT2B and by hMSCs. Addition of the TGFβ type I receptor (ALK5) inhibitor SB431542 (10 µM) to cells cultured with TGFβ1 antagonized the inhibitory effects of this factor on WNT2B mRNA expression; n=5. ANOVA test; *p<0.05; **p<0.01. In contrast, addition of the JNK inhibitor SP600125 (20µM) to cells cultured in the presence of TGFβ1 did not modify the effect of this factor on WNT2B gene expression. Histograms represent the mean ± SEM from UV spectrophotometer readings of gel bands Representative bands are shown above histograms; n=3. ANOVA test, **p<0.01; ns = No significant.

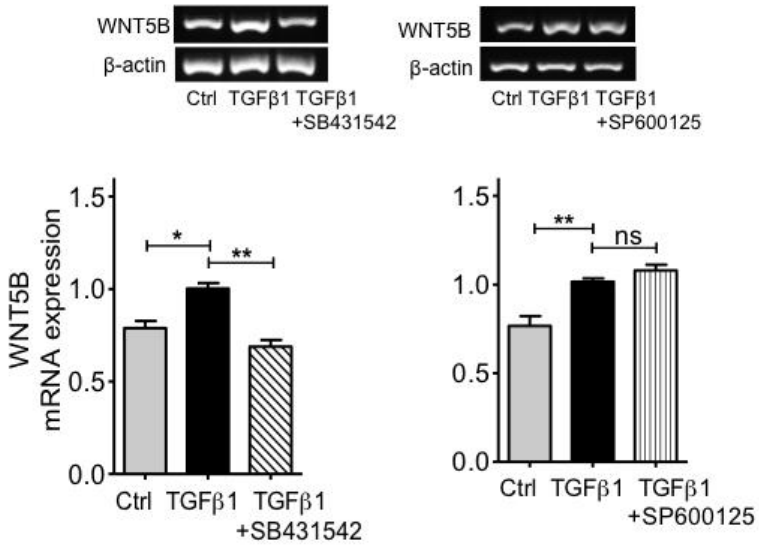

Figure 3.10 Effect of TGFβ1 inhibitors on the expression of the Wnt signalling ligand WNT5B by hMSC. ALK5 inhibitor SB431542 antagonized the upregulation of WNT5B mRNA by TGFβ1; n=3. ANOVA test; *p<0.05; **p<0.01, whilst the JNK inhibitor SP600125 did not modify the effects of this factor on the expression of this ligand gene; n=4. ANOVA test, **p<0.01; ns = No significant.

3.4.3 Effect of TGFβ1 on cell proliferation and apoptosis in MIO-M1 cells

Hexosaminidase examination of the effect of TGFβ1 on cell proliferation of MIO-M1 cells upon 7 days in culture showed a decrease in cell number with increasing concentrations of TGFβ1 used. Accordingly, there were no changes in cell number at concentrations 0.01 ng/ml and 0.1 ng/ml, but a dose response decrease in number of MIO-M1 cells was observed with increasing $\log_{10}$ concentrations of RO4929097 ($p<0.05$) from concentrations (1-100 ng/ml) as compared to control cells cultured with medium alone (Fig 3.11). The effect of TGFβ1 on cell apoptosis was determined using Caspase assay by comparing caspase 3/7 at different conditions. At the highest concentration of TGFβ (100 ng/ml), the proportion of cells showing apoptosis was similar to control cells cultured with medium alone. Thus, suggesting that TGFβ1 downregulate cell proliferation without promoting apoptosis (Fig 3.12).

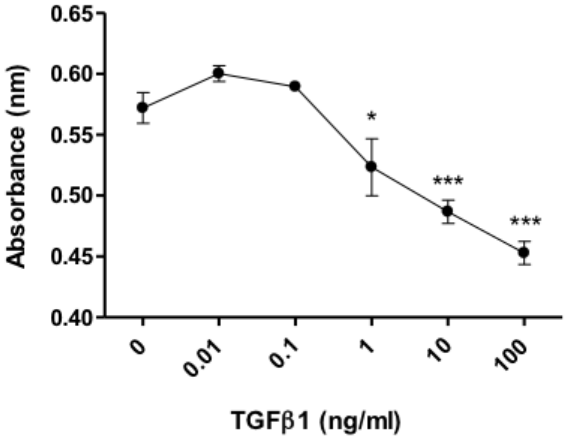

Figure 3.11 Inhibition of the proliferation of hMSC by TGFβ1. To examine whether TGFβ1 affects the cell growth and viability of hMSCs, we performed a hexosaminidiase assay in cells cultured with various concentrations of TGFβ1 for 7 days. Concentrations lower than 0.1 ng/ml of TGFβ1 did not modify the levels of intracellular hexosaminidase as compared with cells cultured in the absence of this factor. However, concentrations of TGFβ1 ranging from 1-100 ng/ml caused a dose dependent decrease in hexosaminidase levels, indicating a decrease in the number of cells.

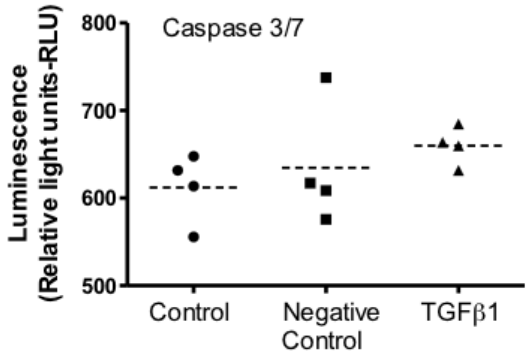

Figure 3.12 Effect of TGFβ1 on cell Apoptosis. Caspase-Glo® 3/7 assay performed after 7 days culture in the presence of 100 ng/ml of TGFβ1 showed that compared with the controls, TGFβ1 did not cause cell death. These observations suggest that TGFβ1 inhibits proliferation of hMSC but does not induce apoptosis at the highest concentration used (100ng/ml) to culture the cells; n=6.

3.5 Discussion

Spontaneous retina regeneration in the zebrafish is mediated by Müller glial cells (Lenkowski et al., 2013, Ramachandran et al., 2010). In small vertebrates, Müller glial cells have also demonstrated this regenerative ability in early postnatal life at a limited level (Osakada et al., 2007, Fischer and Bongini, 2010). Despite the isolation of a population of Müller glia from the adult human retina that show stem cell characteristics (hMSC) *in vitro* (Lawrence et al., 2007), no studies indicating the endogenous regenerative ability of these cells *in vivo* has been reported yet.

In the literature, there are evidences on the importance of TGFβ and Wnt signalling in the regulation of zebrafish Müller glia mediated differentiation in the retina (Lenkowski et al., 2013, Meyers et al., 2012), as well as in the rodent retina (Close et al., 2005). In addition, these pathways are also involved in the eye patterning during embryonic development (Hagglund et al., 2013, Grocott et al., 2011). Nevertheless, the effect of TGFβ on the Wnt signalling pathway in human Müller glial cells has not been examined.

The present results from this chapter showed that TGFβ1 act as an anti-proliferative factor similar agreeing to reports in various studies (Massague, 2012) without inducing cell apoptosis in the human Müller stem cells (Fig 3.11 and 3.12). TGFβ1 also caused *in vitro* downregulation of the canonical Wnt signalling pathway in hMSC. This was demonstrated by a decrease in the expression of WNT2B, DKK1 and active β-catenin in cells cultured with this factor (Fig 3.3, Fig 3.4 and Fig 3.7). The crosstalk between the TGFβ and Wnt signalling pathway has been demonstrated to occur in various ways in different species. For example, in the

chick, it has been shown that they can regulate each other's ligand expression during embryonic development (Rodriguez-Esteban et al., 2001). Additionally, the interaction has been shown to occur through formation of a complex for transcription of target genes between TGFβ intracellular Smad proteins and Canonical Wnt component β-catenin/Lef in nucleus of *Xenopus* (Nishita et al., 2000) and also through interaction of their cytoplasmic components (Han et al., 2006). By interaction of Smad1 with Dishevelled-1 protein, the downregulation of canonical Wnt signalling has been reported in bone marrow stromal cells (Liu et al., 2006). Furthermore, the inhibitory Smad7 has been shown to downregulate canonical Wnt signalling by binding to β-catenin and targeting it for degradation in epidermal stem cells (Han et al., 2006).

In contrast, TGFβ1 upregulated the mRNA and intracellular protein expression of the non-canonical Wnt ligand WNT5B in hMSC cultures. In the literature, it has been documented that overexpression of Wnt5b in zebrafish causes downregulation of the canonical Wnt target Axin2, with consequent inhibition of tail fin regeneration (Stoick-Cooper et al., 2007). Agreeing to this study, it was found that culture of hMSCs with recombinant WNT5B downregulated the mRNA expression of canonical Wnt signalling target DKK1 (Fig 3.8B). This gene was upregulated in hMSC culture containing recombinant WNT2B, a canonical Wnt ligand (Schubert, 2003) (Fig 3.8A). Furthermore, loss of Wnt5a or TGFβ (which shares 80% protein sequence with human WNT5B (http://www.omim.org/entry/606361) in mouse mammary cells, results in increased Wnt/β-catenin activity (Roarty et al., 2009). Therefore, it is possible that TGFβ signalling may upregulate the non-canonical WNT5B ligand to suppress canonical Wnt signalling pathway as an alternative method.

Regulation of intracellular functions by TGFβ may upregulate the non-canonical WNT5B ligand to suppress canonical Wnt signalling pathway, as well as SMAD independent pathways such as those involving JNK and p38 activation (Derynck and Zhang, 2003). It was observed that SB431542, an inhibitor of type I receptor (ALK5) which selectively blocks the SMAD2/3 dependent pathway (Inman et al., 2002), antagonized the effect of this factor on the downregulation of WNT2B (Fig 3.9) in the examined hMSC cultures. Similarly, upregulation of WNT5B was antagonized by the ALK5 receptor inhibitor SB431542 (Fig 3.10). This contrasted with the lack of effect of the JNK inhibitor on the modulation of both ligands by TGFβ1. Taken together, these results suggest that modulation of the expression of WNT2B and WNT5B by TGFβ1 is dependent on Smad signalling.

In conclusion, the results presented in this chapter suggest that there is a crosstalk between TGFβ and Wnt signalling pathways in human Müller stem cells. TGFβ signalling induced by TGFβ1 in human Müller stem cells downregulated the components of canonical Wnt signalling pathway (WNT2B, DKK1 and active β-catenin, whilst it upregulated the non-canonical WNT ligand WNT5B in these cells. Furthermore, inhibition of TGFβ receptor type I by pharmacological inhibitor SB431412 prevented the effect on these Wnt ligands induced by TGFβ1. Therefore, TGFβ signalling mediated through Smad proteins may play an important role in regulating the Wnt signalling pathway in human Müller stem cells.

Chapter 4: The role of Wnt Signalling on the photoreceptor differentiation of human Müller glial stem cells

4.1 Differentiation of Müller stem cells into photoreceptors

Photoreceptors are specialized neurons in the retina that are tasked with detecting the light that enters the eye and converts these signals into electrical impulses that cascade into other neurons for transmission to the brain. They are located in the outmost layer of the retina and their structure is made up of an outer segment consisting of photoreceptor pigments such as opsins, a connecting cilium, nucleus, axon and synaptic terminal (Swaroop et al., 1999). There are two types of photoreceptors, known as rods and cones. Rod photoreceptors facilitate vision in low light conditions using the highly light-sensitive photopigment rhodopsin. In contrast, cone photoreceptors, which exists as various subtypes, each containing one or more opsins, assists in colour vision during normal light conditions (Brzezinski and Reh, 2015).

Müller glial stem cells are the primary sources of stem cells in lower vertebrates such as the zebrafish, that are responsible for the endogenous regeneration of damaged retinal cells after injury (Lenkowski and Raymond, 2014). In particular, it has been shown that during development Müller glial stem cells are responsible for the generation of rod progenitors *in vivo* (Raymond and Rivlin, 1987), and subsequent rod photoreceptors which accounts for almost all of the rod photoreceptors formation (Bernardos et al., 2007). In addition, using transgenic fish with complete or partially ablated rod photoreceptors, it was shown that Müller glia could endogenously repair the loss of rod photoreceptors. In this model it was found that Müller glial stem cells preferentially repair the retina when there is a complete loss of rod photoreceptors as compared to partial loss of rod photoreceptors (Montgomery et al., 2010).

In mammals such as rats, the differentiation of Müller glial stem cells into rod photoreceptors has been demonstrated in a neurotoxicity model induced by intravitreal injection of NMDA N-methyl-D-aspartate (NMDA). Under these conditions, it was found that Müller glial stem cells proliferated and expressed the rod photoreceptor markers rhodopsin and recoverin (Ooto et al., 2004). Furthermore, Del debbio et al., demonstrated in mice that by activating the Notch and Wnt signalling pathways, a subset of Müller cells undergoes proliferation and differentiation into rod photoreceptor lineages (Del Debbio et al., 2010). However, the percentage of rod photoreceptors generated in these mammalian species is very low compared to the number of cells generated in the zebrafish. This is potentially because Müller glial stem cells in the zebrafish are able to re-enter the cell cycle more readily than their mammalian counterparts, resulting in proliferating cell clusters (Raymond et al., 2006). In addition, in zebrafish, most Müller glia progeny are able to generate new retinal neurons (Hayes et al., 2007) as compared to only a sub population of mammalian Müller glia (Osakada et al., 2007).

Human Müller glia with stem cell characteristics have also been shown to differentiate into photoreceptors *in vitro* (Jayaram et al., 2014). Jayaram et al., demonstrated that the human Müller glial stem cell line MIO-M1 can be differentiated into photoreceptors in culture by the addition of FGF2, taurine, retinoic acid and Insulin-like growth factor 1 (FTRI). This was highlighted through the expression of photoreceptor markers such as NR2E3, recoverin and rhodopsin by these cells (Jayaram et al., 2014). In the same study, it was shown that upon transplantation into the P23H rat, a model of primary photoreceptor degeneration, these photoreceptor differentiated cells with neural-like projections migrated and integrated into the outer nuclear layer where normally the rod photoreceptors are located. Furthermore, photoreceptor functionality following transplantation was

investigated using electroretinography at 3 weeks. It was observed that the eye with transplanted photoreceptor cells showed an improvement in rod receptor function as compared to the unoperated control eye (Jayaram et al., 2014). Similarly, another research group, Giannelli et al., have also successfully differentiated human Müller glia stem cells into rod photoreceptors using PA6 feeder layers (consisting of mitomycin-inactivated PA6 mouse bone marrow stromal cells) and taurine. This was confirmed by the expression of the rod markers rhodopsin and Guanine Nucleotide Binding Protein (G Protein) and Alpha Transducing Activity Polypeptide 1 (GNAT1). In addition, these rod photoreceptors were functional as shown by patch clamp studies which suggested that their properties were similar to those of adult rod photoreceptors (Giannelli et al., 2011).

4.2 Role of Wnt signalling in photoreceptor survival and differentiation

The Wnt signalling pathway has many functions during development, including proliferation and differentiation of retinal progenitor cells across species such as zebrafish and mammals. In larval zebrafish, when light-lesioned larval zebrafish were incubated in the presence or absence of the Wnt signalling activator 1-azakenpaullone, a GSK3β inhibitor, it was observed that the continuous activation of Wnt signalling, promoted Müller glial stem cells derived progeny to migrate towards the ONL where only photoreceptors are found. In addition, these cells did not display Müller glia features such as radial process as observed in larval zebrafish incubated in medium alone (Meyers et al., 2012). However, this event did not occur in the uninjured larval zebrafish retina (Meyers et al., 2012, Lenkowski and Raymond, 2014).

In transgenic mice with increased Wnt signalling due to the loss of the Wnt regulator Axin2, it was shown that following laser induced retinal injury there was increased proliferation of a sub population of Müller glial stem cells responding to Wnt signals. In addition, these cells survived longer and also expressed the rod photoreceptor marker rhodopsin (Liu et al., 2013). Furthermore, Del Debbio et al. also reported that activation of Notch and Wnt signalling causes the differentiation of a sub population of mice Müller glial stem cells into photoreceptor cells (Del Debbio et al., 2010). The Wnt signalling pathway has also been shown to induce photoreceptor protection during inherited retinal degeneration, as activation of canonical Wnt signalling led to increased photoreceptor survival in rd6 mice. This Wnt mediated photoreceptor protection was effected by Müller glia (Patel et al., 2015). Furthermore, Müller glia cell have been shown to release neurotrophins such as BDNF to mediate photoreceptor protection (Harada et al., 2000).

4.2.1 Effect of FGF2, taurine, retinoic acid and IGF1 on Wnt signalling pathway

A combination of the factors FGF2, taurine, retinoic acid and IGF1 was shown to induce differentiation of human MIO-M1 cells into photoreceptors *in vitro* (Jayaram et al., 2014). FGF2 has been shown to affect Wnt signalling pathway in various studies. When FGF2 mutant mice was used in osteoblast differentiation and bone formation studies, there was a significant decrease in the mRNA and protein expressions of the Wnt genes Wnt10b and β-catenin (Fei et al., 2011). Similarly, in FGF2 induced lens fiber differentiation in rat lens explants, it was observed that there was an increase in the protein expression of the components of Wnt signalling such as Frizzled 3, Frizzled 6, Dishevelled 2 and Dishevelled 3 (Dawes et al., 2013).

Taurine (2-aminoethane sulfonic acid), found in most cells, has been shown to increase the proliferation of progenitors cells isolated from areas such as mice embryonic mesencephalon and hippocampus dentate gyrus (Hernandez-Benitez et al., 2010, Shivaraj et al., 2012). Through DNA microarray studies conducted in progenitor cell culture in the presence of taurine obtained from the adult mouse subventricular zone, it was recently shown that there was an increase in the transcripts of Wnt signalling pathway. In addition, quantitative real-time PCR demonstrated an increase in Wnt2 in the taurine treated samples as compared to control samples (Ramos-Mandujano et al., 2014).

Retinoic acid (RA) is the major form of bioactive retinoid, which is made up of vitamin A and its derivatives (Mark et al., 2006). This signalling molecule has been shown to be important during embryonic development and cellular differentiation (Gudas, 1994) including *in vitro* photoreceptor differentiation (Osakada et al., 2008, Eiraku et al., 2011, Zhong et al., 2014). During inhibition of adipogenesis of 3T3-L1 cells i *in vitro*, it was shown that addition of RA resulted in the increased expression of Wnt genes such as Wnt1, Wnt4 and β-catenin (Kim et al., 2013a). In contrast, addition of RA to cultures of mice ESC caused an increased in the expression of components of the non-canonical pathways such as Wnt5a, Wnt7a, Fzd2 and Fzd6, whilst reducing the expression of canonical Wnt signalling components such as phosphorylated β-catenin. Furthermore, in the same study they showed that addition of RA promotes Tcf3 association, thus promoting non-canonical signalling. In contrast, at the same time, addition of RA causes Tcf1 dissociation, with subsequently inhibition of the canonical Wnt signalling (Osei-Sarfo and Gudas, 2014).

IGF-1, which predominately act via the IGF1 receptor has been demonstrated to be important for the proliferation, survival and maturation of neural cells *in vitro* (Popken et al., 2005). It has been shown that in oligodendroglial cell cultures IGF-1 increases the protein expression of β-catenin (Ye et al., 2010). Furthermore, the same research group showed that IGF-1 signalling could interact with canonical Wnt signalling to promote neural proliferation *in vivo*. This was illustrated by the downregulation of canonical Wnt components β-catenin and phospho-GSKβ in the IGF-1 or IGFR mutant mice brain (Hu et al., 2012).

4.3 Objectives

The Wnt signalling pathway has been shown to be involved in the induction of Müller glial stem cell proliferation and differentiation in various species, including zebrafish and mice. In addition, various exogenous factors have been shown to promote photoreceptor differentiation of neural progenitors and stem cells. However, it is not known whether factors that induce photoreceptor differentiation of human Müller glia stem cell can modify the Wnt signalling pathway, or whether Wnt signalling may be involved in the photoreceptor differentiation of these cells *in vitro*.

On this basis, the aims of this chapter were as follows:

4. To investigate the effect of FTRI induced human MSC photoreceptor differentiation on the components of Wnt signalling pathway.
5. To investigate whether inhibition of β-catenin may affect the photoreceptor differentiation of human MSC.
6. To investigate the effect of TGFβ on the photoreceptor differentiation of human MSC.

To achieve these objectives, the following experiments were conducted:

5. Human Muller stem cells were cultured in the presence or absence of FTRI to examine gene and protein expression of components of the canonical and non-canonical Wnt signalling pathway. Selective gene components of canonical Wnt signalling studied, including WNT2B, β-catenin and DKK1. These factors were identified by RT-PCR of isolated mRNA, whilst intracellular protein was identified by western blotting analysis of isolated

protein. Wnt related proteins released into the supernatant were also examined by ELISA methods.

6. Müller stem cells were cultured with various concentration of the tankyrase inhibitor XAV-939 to identify the optimum concentrations that would inhibit the canonical Wnt signalling pathway by downregulating β-catenin protein expression and could be used for assessing its effects on MSC.
7. hMSC were cultured in the presence of FTRI alone or FTRI combined with tankyrase inhibitor XAV-939 to examine gene and protein expression of photoreceptor markers. These included NR2E3 and recoverin. Photoreceptor marker genes were identified by RT-PCR of isolated mRNA, whilst protein expression was identified by quantification of the immunostained cells.
8. Furthermore, to investigate the effect of TGFβ on the photoreceptor differentiation of human Müller stem cells, cells were cultured with FTRI in the presence or absence of this factor. Gene expression of the photoreceptor markers NR2E3, rhodopsin and recoverin were examined by RT-PCR whilst protein expression was identified by immuno-cytochemical staining.

4.4 Results

4.4.1 Upregulation of canonical Wnt signalling components by factors that induce photoreceptor differentiation of MIOM1 cell line

Previous studies have demonstrated that adult hMSC can be successfully differentiated into photoreceptors upon culture with FGF2, taurine, retinoic acid and Insulin-like growth factor 1 (FTRI) (Jayaram et al., 2014). Given the importance of Wnt signalling in neural stem cell proliferation and differentiation, we examined the effect of FTRI on the Wnt signalling components of hMSC. As previously reported, hMSC cultured with FTRI showed significant mRNA upregulation of the photoreceptor markers NR2E3 ($p<0.01$), recoverin ($p <0.01$), rhodopsin ($p<0.01$) and IRBP ($p<0.05$) compared to control cells (Fig 4.1). In addition, FTRI also caused a significant mRNA upregulation of Blimp1 ($p<0.01$) (Fig 4.1), a factor shown to inhibit expression of genes such as Chx10, which promote bipolar fate (Katoh et al., 2010). The present results also showed that conditions that induce photoreceptor differentiation of hMSC caused a significant increase in the expression of mRNA coding for the canonical Wnt signalling ligand WNT2B ($p<0.001$), but did not modify the mRNA expression of the non-canonical Wnt ligand WNT5B (Fig 4.2). Interestingly, a significant increase in the release of both WNT2B and WNT5B ligands into the culture supernatants ($p<0.05$ and $p<0.05$ respectively) was observed when compared to control undifferentiated cells (Fig 4.2). Western blot analysis of protein isolated from cells cultured with FTRI demonstrated that the ratio of phospho-β-catenin/β-catenin was significantly decreased in cells undergoing photoreceptor differentiation as compared to undifferentiated hMSC ($p<0.05$) (Fig 4.3). When culturing cells under photoreceptor differentiating conditions we also observed an

increase in mRNA expression of DKK1, a Wnt antagonist and a product of Wnt activation ($p=0.05$), as compared to cells cultured with medium alone (Fig 4.3).

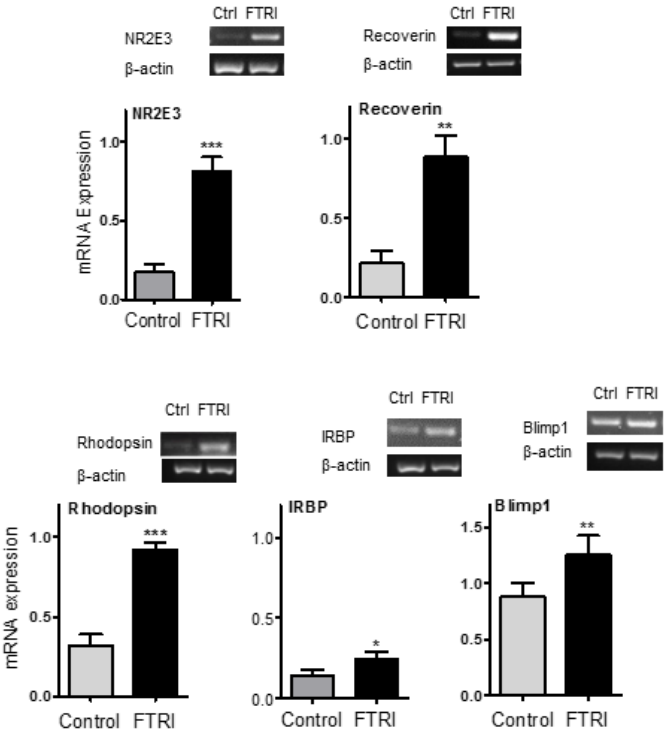

Figure 4.1 FTRI causes induction of photoreceptor differentiation markers in hMSCs. Culture of hMSC with FTRI caused an increase in mRNA expression coding for the photoreceptor markers NR2E3, rhodopsin, IRBP and Blimp1 as compared to control cells; n=6-8. Student's t- test. *p<0.05 v. control, **p = 0.01 v. control, ***p = 0.001 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

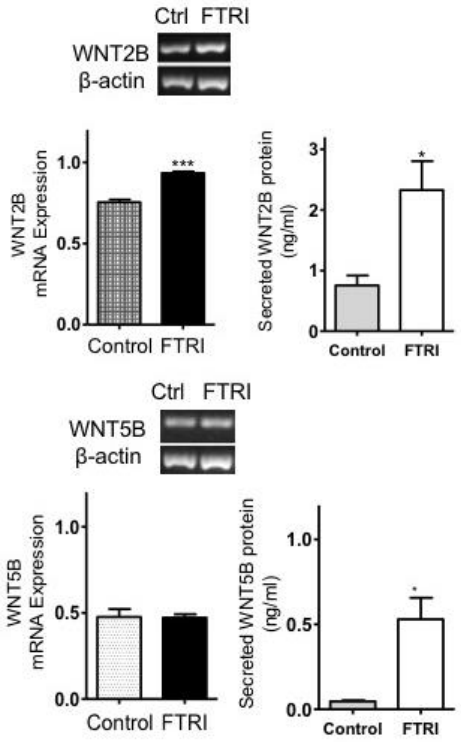

Figure 4.2 Induction of photoreceptor differentiation by FTRI causes changes in the expression of the Wnt signalling components WNT2B and WNT5B in hMSCs. Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of WNT2B mRNA, whilst no changes in the expression of WNT5B were observed in these cells. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above the histograms; n=5. Student's t-test, ***p<0.001; ns=No significant; FTRI = FGF2, taurine, retinoic acid and Insulin-like growth factor type1. (B) Quantification of the secreted ligands, as measured by ELISA, showed that both WNT2B and WNT5B were significantly increased in culture supernatants of hMSC cell treated with FTRI for 7 days; n=3. Student's t-test, *p<0.05.

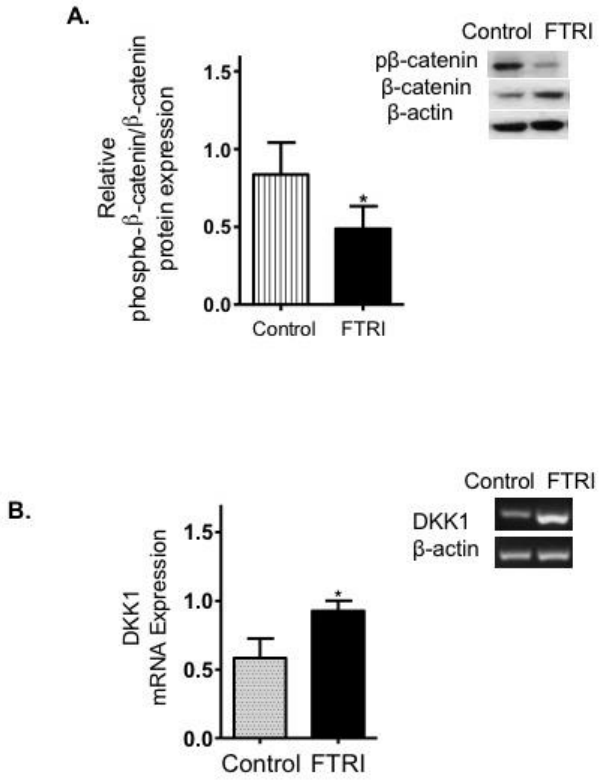

Figure 4.3 Induction of photoreceptor differentiation by FTRI causes changes in the expression of the Wnt signalling components β-catenin and DKK1 in hMSC. **(A)** Western blotting analysis showed that the ratio of phospho-β-catenin/β-catenin was decreased by FTRI treatment of hMSC; n=5. Student's t-test, *p<0.05. **(B)** A significant increase in the expressions of DKK1 mRNA was observed in hMSC cultured with FTRI for 7 days; n=3. Student's t-test, *p<0.05. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above the histograms.

4.4.2 The canonical Wnt signalling is required for photoreceptor differentiation of hMSC

Following observations that FTRI, which induces photoreceptor differentiation of hMSC, causes upregulation of canonical Wnt signalling components in these cells, we examined whether Wnt signalling is required for hMSC photoreceptor differentiation. We first tested the effect of the tankyrase inhibitor XAV-939, known to effectively block β-catenin by stabilizing axin (Huang et al., 2009), on undifferentiated cells. We observed that increasing $\log_{10}$ concentrations of this compound caused a gradual increase in the expression of phosphorylated β-catenin in hMSC, although a significant increase was only observed with concentrations above 10 nM ($p<0.05$) (Fig 4.4).

Addition of XAV-939 (10 nM) to hMSC cultured under photoreceptor differentiating conditions caused a significant inhibition of the effect of FTRI on the mRNA expression of the photoreceptor markers NR2E3 ($p<0.01$) and recoverin ($p<0.05$) (Fig 4.5). This inhibition was further confirmed by immuno-cytochemical analysis, which also demonstrated a significant decrease in the number of cells expressing NR2E3 ($p<0.01$) and recoverin ($p<0.05$) proteins in hMSC cultured with FTRI in the presence of XAV-939 ($p<0.01$) (Fig 4.6). Taken together, these results suggest that signalling through the canonical Wnt pathway precedes the activation of proneural factors involved in the differentiation of hMSC into photoreceptors.

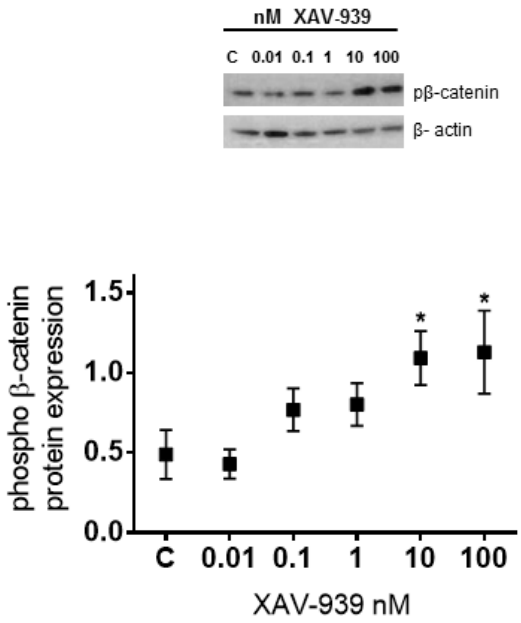

Figure 4.4 Effect of inhibition of β-catenin by tankyrase inhibitor XAV-939.
Dose response increase in phospho-β-catenin by tankyrase inhibitor XAV-939 concentrations of XAV-939 ranging between 0.01 and 100 nM. Western blot bands on the right show that XAV-939 significantly upregulated the expression of phospho-β-catenin protein at concentrations of 0.01 and 100 nM; n=4. Student's t-test, *p<0.05.

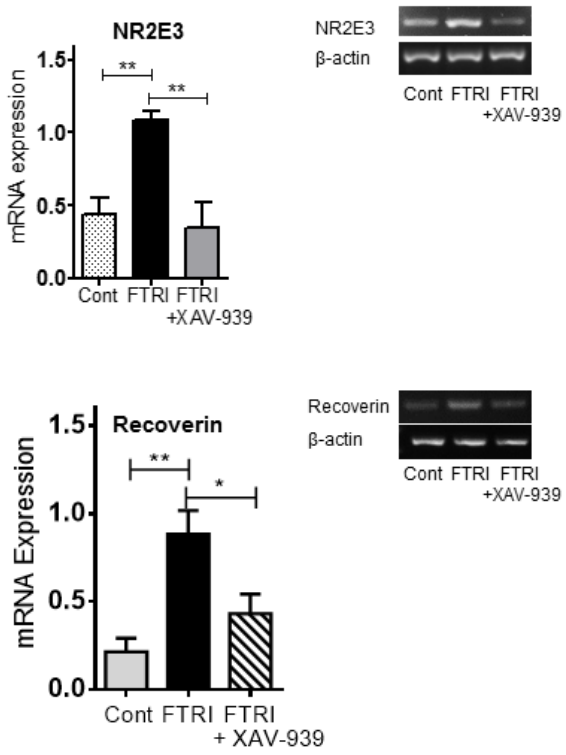

Figure 4.5 Effect of activation of β-catenin degradation on mRNA expression of the photoreceptor differentiation markers by hMSC. Addition of XAV-939 (10 nM) to hMSC cultured with FTRI inhibited the differentiation of these cells into photoreceptors, as judged by the expression of mRNA coding for NR2E3 and recoverin. Histograms on the left represent the mean ± SEM from UV spectrophotometer readings of gel bands. Representative bands are shown on right of the histograms; n=5-7. ANOVA test; *p<0.05; **p<0.01; FTRI= FGF2, taurine, retinoic acid and insulin-like growth factor type 1

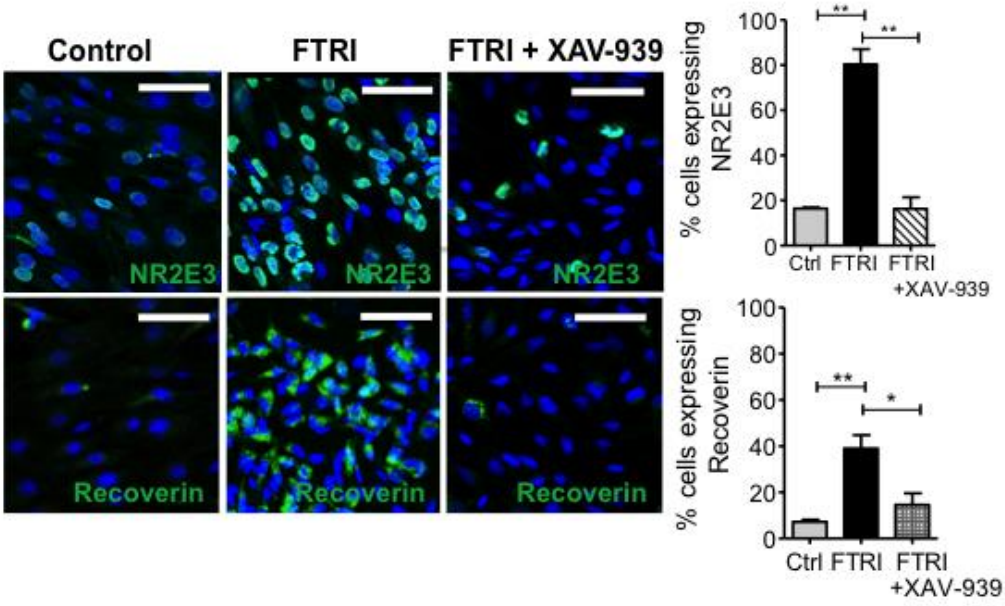

Figure 4.6 Effect of inhibition of β-catenin on the protein expression of photoreceptor differentiation markers by hMSC. Confocal images confirmed that addition of XAV-939 (10 nM) to hMSC cultured in the presence of FTRI caused a decrease in the expressions of NR2E3 and recoverin which is upregulated by FTRI alone (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm. Histogram represents the percentage of cells stained with NR2E3 following 7 days culture under the different conditions; n = 3. ANOVA test; *p<0.05; **p<0.01.

4.4.3 TGFβ1 inhibition of photoreceptor differentiation is associated with changes in the expression of Wnt ligands

Having shown that photoreceptor differentiation of hMSC promotes activation of the canonical Wnt signalling pathway (Fig 4.2 and Fig 4.3) and that TGFβ1 downregulates canonical Wnt signalling components whilst upregulating the non-canonical WNT5B ligand (Chapter 3, Fig 3.3 and 3.5), it was examined whether TGFβ1 can modulate photoreceptor differentiation of hMSC by modifying the Wnt signalling pathway in these cells.

Addition of TGFβ1 to hMSC cultured in the presence of FTRI caused a significant decrease ($p<0.001$) in WNT2B mRNA expression, as compared to hMSC cultured with FTRI alone (Fig 4.7A). Cells cultured under differentiating conditions in the presence of TGFβ1 also showed a significant increase in WNT5B mRNA expression ($p<0.01$) as compared to cells cultured in the presence of FTRI alone (Fig 4.7B). These results suggest that by modifying the expression of the Wnt ligands WNT2B and WNT5B, TGFβ1 inhibits the effect of FTRI on the expression of Wnt ligands. To assess whether this inhibitory effect was reflected on the ability of these cells to differentiate into photoreceptors, TGFβ1 was added to hMSC cultured with FTRI. Under photoreceptor differentiating conditions, TGFβ1 caused a significant downregulation of mRNA coding for the photoreceptor markers NR2E3 ($p<0.001$), recoverin ($p<0.05$) and rhodopsin ($p<0.001$) as compared to hMSC cultured with FTRI alone (Fig 4.8). This was also confirmed by a decrease in the number of cells expressing NR2E3 ($p<0.01$) and recoverin ($p<0.01$) when cells were cultured with FTRI in the presence of TGFβ1, as compared to cells cultured with FTRI alone (Fig

4.9). Taken together, these results suggest that TGFβ1 may regulate hMSC photoreceptor differentiation by modifying the ligands WNT2B and WNT5B of the Wnt signalling pathway.

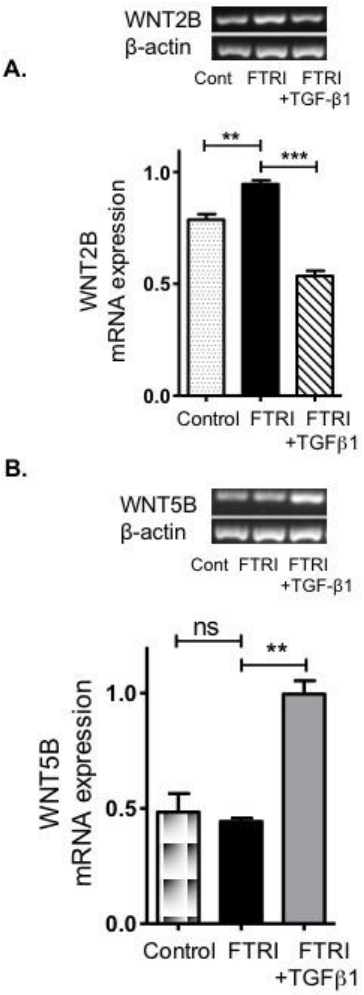

Figure 4.7 Effect of TGFβ1 on FTRI mediated changes in the mRNA expression of WNT2B and WNT5B in hMSC. (A) Culture of MIO-M1 cells with FTRI caused an increase in WNT2B mRNA expression, but addition of TGFβ1 to the differentiation medium inhibited this increase; n=4. ANOVA test, **p<0.01; ***p<0.001. **(B)** FTRI alone did not modify WNT5B mRNA expression, but addition of TGFβ1 to the differentiation cocktail increased WNT5B mRNA expression. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above the histograms.

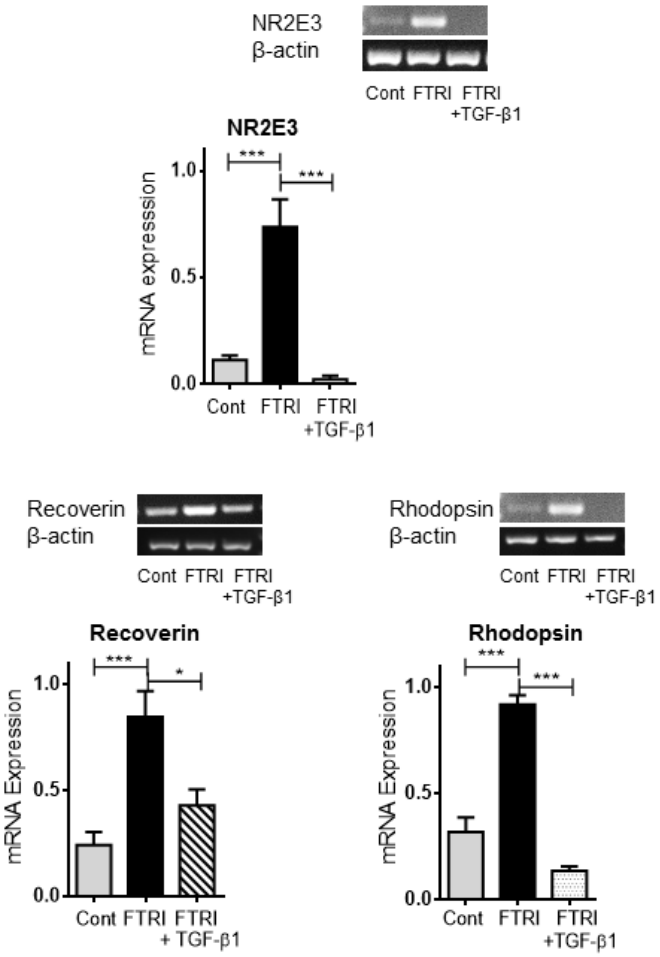

Figure 4.8 Inhibition of FTRI-induced photoreceptor differentiation of hMSC by TGFβ1. Addition of TGFβ1 to hMSC undergoing photoreceptor differentiation with FTRI inhibited the mRNA expression of NR2E3, recoverin and rhodopsin as compared with cells cultured with FTRI alone; n = 5-8. ANOVA test, *p<0.05; ***p<0.001. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown above the histograms.

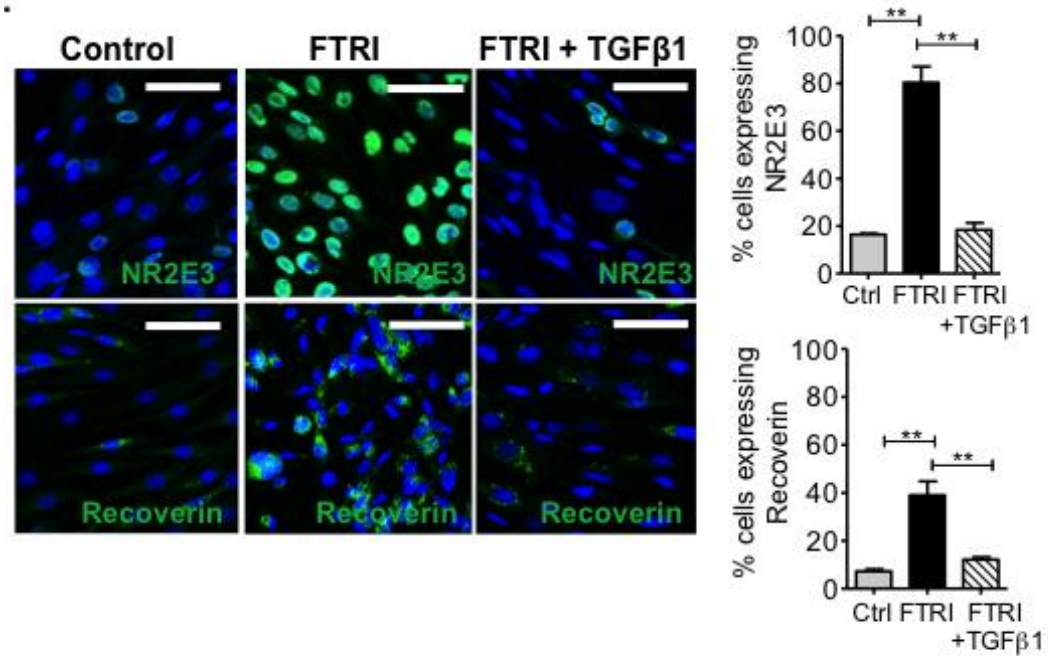

Figure 4.9 Inhibition of FTRI-induced photoreceptor differentiation of hMSC by TGFβ1. Immuno-staining for NR2E3 and recoverin confirmed that whilst FTRI alone caused a marked increase in the expression of this photoreceptor protein, addition of TGFβ1 to hMSC cultured with FTRI caused inhibition of photoreceptor differentiation (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm. Histograms on the right represent the proportion of cells immunostaining for each of the markers following 7 day culture under the different conditions; n=3. ANOVA test, **p<0.01.

4.5 Discussion

Müller glial cells have been shown to be important for the generation of photoreceptors during retinal development (Bernardos et al., 2007). In addition, Müller glia have also been demonstrated to give rise to photoreceptors following retinal injury in zebrafish (Montgomery et al., 2010) as well as in mammalian species (Del Debbio et al., 2010, Liu et al., 2013). Whilst Wnt signalling has been demonstrated to aid photoreceptor differentiation in both zebrafish and mice (Meyers et al., 2012, Del Debbio et al., 2010, Liu et al., 2013), the importance of Wnt signalling in the regulation of progenicity and neural differentiation of human Müller stem cells derived from the adult human eye has not been previously investigated.

The results from this chapter demonstrated that culture of hMSC with a combination of growth and differentiation factors (FTRI) induced expression of the photoreceptor markers NR2E3, recoverin, rhodopsin and IRBP in these cells *in vitro* as previously reported (Fig 4.1) (Jayaram et al., 2014). In addition, the differentiation of these cells into photoreceptors was further verified by their increased Blimp1 mRNA expression (Fig 4.1). In previous study conducted by Katoh et al, it was shown that ablation of Blimp1 resulted in a reduced number of photoreceptors, whilst increasing the number of bipolar cells by upregulating Chx10. In contrast, upregulation of Blimp1 resulted in increased number of photoreceptors following downregulation of Chx10 (Katoh et al., 2010). Addition of FGF-2, taurine or retinoic acid, either in single form or in combined together have been previously shown to induce neuronal differentiation of stem cells towards photoreceptor fate *in vitro* (Zhao et al., 2002, Eiraku et al., 2011), Furthermore, IGF-1 has also been reported to mediate production of rod photoreceptors in zebrafish (Zygar et al., 2005) and mouse retinae (Pinzon-Guzman et al., 2011) during retinal development . In these experiments

with hMSC, it was observed that FTRI upregulated the expression of genes and proteins associated with the canonical Wnt signalling pathway, including WNT2B, β–catenin and DKK1 (Fig 4.2 and Fig 4.3). The involvement of the canonical Wnt signalling pathway in the photoreceptor differentiation of hMSC *in vitro* is further supported by the observations that inhibition of the canonical Wnt signalling by inhibitor XAV-939 (which stabilizes axin and consequently targets β-catenin for degradation) in cells cultured with FTRI, prevented photoreceptor differentiation of these cells (Fig 4.6). Activation of canonical Wnt signalling is associated with the maintenance and proliferation of retinal progenitors in the embryonic chick and mouse retina (Cho and Cepko, 2006), whilst laser-injury in transgenic mice lacking the Wnt signalling regulator Axin2, induces amplification of Wnt signalling and generation of rhodopsin positive cells from Müller glia (Liu et al., 2013). Wnt signalling activation is also associated with Müller glia mediated regeneration in the zebrafish (Lenkowski and Raymond, 2014), and continuous activation of this pathway after acute injury in larval zebrafish also promotes the generation of neuronal progenitors from Müller glia (Meyers et al., 2012). Furthermore, activation of Wnt in association with Notch signalling has been shown to promote Müller glia mediated adult mammalian rod photoreceptor differentiation in mice (Del Debbio et al., 2010).

Individually, individual factors of FTRI used to induce photoreceptor differentiation of hMSC have been shown to modulate the expression of canonical Wnt signalling molecules. The loss of FGF-2 during bone formation studies in mice resulted in the downregulation of β–catenin under these conditions, suggesting that FGF-2 is able to promote the upregulation of canonical Wnt signalling pathway (Fei et al., 2011). Similarly, addition of taurine (known to be available in most mammalian cells) to cultures of adult mice subventricular zone cells also promoted upregulation of the

canonical Wnt signalling pathway ligands including Wnt2 (Ramos-Mandujano et al., 2014). In addition, Retinoic acid has also been shown to promote upregulation of canonical Wnt signalling component β–catenin during inhibition of adipogenisis *in vitro* (Kim et al., 2013a). Furthermore, the final factor of FTRI, IGF-1 also has been shown to upregulate the canonical Wnt signalling pathway by increasing the expression of β–catenin in oligodendroglial cell cultures (Ye et al., 2010). These findings suggest that as seen in the zebrafish and early postnatal life in small vertebrates, photoreceptor differentiation of hMSC requires activation of canonical Wnt signalling.

Addition of TGFβ1 to hMSC cultured with FTRI resulted in inhibition of photoreceptor differentiation (Fig 4.8 and Fig 4.9). The present findings that inhibition of photoreceptor differentiation by TGFβ1 in hMSC was accompanied by gene downregulation of WNT2B and upregulation of WNT5B, is in accordance with the reported functions of this ligand in promoting the activation of the canonical Wnt signalling pathway in other species. This suggests that by increasing WNT5B expression, TGFβ1 may potentiate its inhibitory effect that prevents photoreceptor differentiation of hMSC. The observation that FTRI upregulates the expression of WNT2B strongly suggests that the effect of these factors on the photoreceptor differentiation of hMSC may be effected through the activation of the canonical Wnt signalling pathway. Previous studies have shown that in the zebrafish regulation of Smad2/3 signalling in Müller glia is important for the proliferative and neurogenic response of these cells to retinal damage (Lenkowski et al., 2013). Hence, the present observations that known intracellular pathways of Müller cell differentiation observed in zebrafish are also active in human Müller glia *in vitro* suggests the existence of inhibitory mechanisms of these pathways in the adult human retina, which may prevent these cells from regenerating the retina *in vivo*. It also raises the

prospects that if we can control these mechanisms in hMSC *in vitro*, we could potentially induce Müller glia to regenerate the retina *in vivo*.

To summarize, this study has demonstrated that differentiation of hMSC into photoreceptors *in vitro* is dependent on the activation of the canonical Wnt signalling pathway and that TGFβ, which is highly upregulated during retinal gliosis (Hoerster et al., 2014), modifies the Wnt signalling mechanisms in hMSC (Fig 4.10) That hMSC express genes of the Wnt signalling pathway and that their activation regulates photoreceptor differentiation upon culture with differentiation factors may reflect their potential regenerative ability *in vivo*. Given that signalling cascades elicited by binding of TGFβ and Wnt ligands to their receptors involve cross talks of intracellular signalling pathways (Xu et al., 2009), it may be possible that regulation of TGFβ and Wnt signalling may have diversified during evolution to prevent uncontrolled growth and differentiation of human Müller glia in the adult retina. It may be also possible that factors released during inflammation and gliosis could inhibit the regenerative ability of these cells *in vivo*. On this basis, comparative investigations into mechanisms that control these pathways in zebrafish and human Müller glia may help to identify therapeutic targets that could be potentially used to promote endogenous regeneration of the human retina, and this merits further investigations.

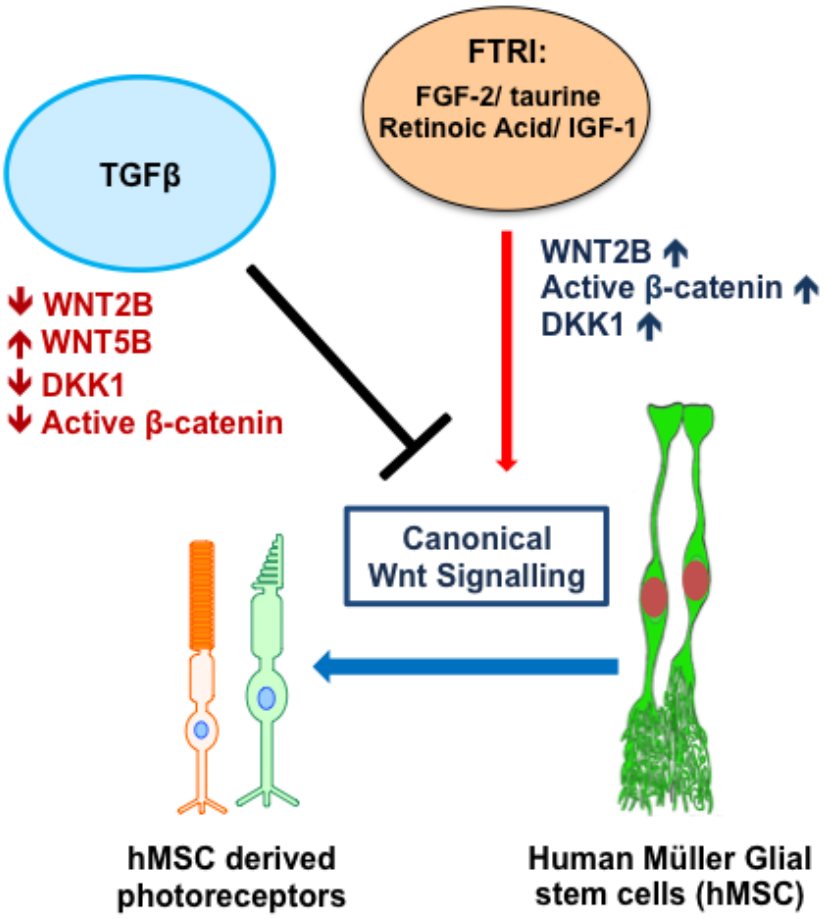

Figure 4.10 TGFβ1 inhibits the canonical Wnt signalling pathway necessary for the photoreceptor differentiation of hMSC *in vitro*. Schematic illustration summarizing the interactions of FTRI, WNT2B, WNT5B, TGFβ1 and DKK1 in hMSC. FTRI, which induces photoreceptor differentiation of hMSC, activated the canonical Wnt signalling pathway in these cells. Addition of *TGFβ1* to cells cultured with FTRI caused inhibition of the canonical Wnt signalling and consequently inhibited the photoreceptor differentiation of hMSC *in vitro*.

Chapter 5: Effect of HBEGF on the Notch and Wnt signalling pathways in human Müller glial stem cells

5.1 HBEGF

Heparin-binding EGF-like Growth Factor (HBEGF) is a member of the EGF family of growth factors. HBEGF was initially isolated as a mitogen for fibroblasts and smooth muscle cells from the conditioned medium of a human macrophage-like cell line (Higashiyama et al., 1991). HBEGF gene is expressed in various tissues such as the brain, lungs and heart. Within the CNS, HBEGF is highly expressed by neurons, astrocytes and oligodendrocytes (Nakagawa et al., 1998) .

HBEGF is synthesised as a transmembrane protein (ProHBEGF) which undergoes cleavage and results in the release of a 22- kDa O-glycosylated protein, mature soluble HBEGF (sHBEGF) (Fig. 4) (Higashiyama et al., 2008). Metalloproteinases such as A Disintegrin And Metalloprotease (ADAM), cleave ProHBEGF between Pro148 – Val149 and Glu151 – Asn152 to generate a sHBEGF in a process called ectodomain shedding. Ectodomain shedding is induced by stimuli such as phorbol esters, calcium ionophore and lysophosphatidic acid (LPA) (Hirata et al., 2001). Stimuli such as LPA act on G-protein coupled receptor (GPCR) which cause an activation of metalloproteinases such as ADAM (Prenzel et al., 1999). Ectodomain shedding is regulated by various signalling cascades such as MAP kinase and PKC pathways (Gechtman et al., 1999). The sHBEGF consists of EGF-like domain which has conserved six cysteine motif ($CX_7CX_{4-5}CX_{10-13}CXCX_8C$) present in all members of EGF family and heparin binding domain, an N-terminal extension to EGF-like domain, only present in HBEGF, and amphiregulin. HBEGF uses heparin binding domain to interact with the EGF receptor and may also regulate its activation with membrane bound heparin sulphate proteoglycans found on this receptor (Thompson et al., 1994). Through tyrosine phosphorylated activation of the EGF receptors (ErbB1 and ErbB4), sHBEGF induces MAPK activation which in turn activates cyclin

D-cyclin-dependent kinase (Cdk) complex resulting in cell cycle progression. Thus, HBEGF has mitogenic and chemoattractant effects that stimulate cell proliferation and migration in many cells, including epithelial cells and fibroblasts (Elenius et al., 1997). The ectodomain shedding also generates a carboxyl terminal fragment of HBEGF (HBEGF-CTF) that can translocate into the nucleus via endocytosis. Inside the nucleus, HBEGF-CTF can bind and cause nuclear export of a transcriptional repressor known as Promyelocytic leukaemia zinc finger (PLZF) (Fig. 5.1). This causes increase in expression of cyclin A and allows re-entry into the S-phase of the cell cycle, normally blocked by PLZF (Nanba et al., 2004). Therefore, it is evident that both sHBEGF and HBEGF-CTF regulate cell proliferation via different pathways.

5.1.1 HBEGF in retina regeneration

The expression of EGFRs by Müller glia have been demonstrated both *in vivo* and *in vitro* conditions (Milenkovic et al., 2003). Expression of EGFRs varies between the developmental and adult stages in the rat retina. EGFR expression is high in Müller glial cells and late retinal progenitor cells during the first two postnatal weeks compared to the early retinal progenitor cells. However, EGFR expression declines in Müller glial cells after two postnatal weeks (Close et al., 2006). Interestingly, it has been demonstrated that during injury induced by light in the adult rat retina, the EGFR expression increases in Müller glia, resembling that seen during early postnatal weeks. It has therefore been suggested that one of the reasons why EGFRs are down-regulated in the adult rat retina is possibly to maintain Müller glial cells in a mitotic quiescent state (Close et al., 2006).

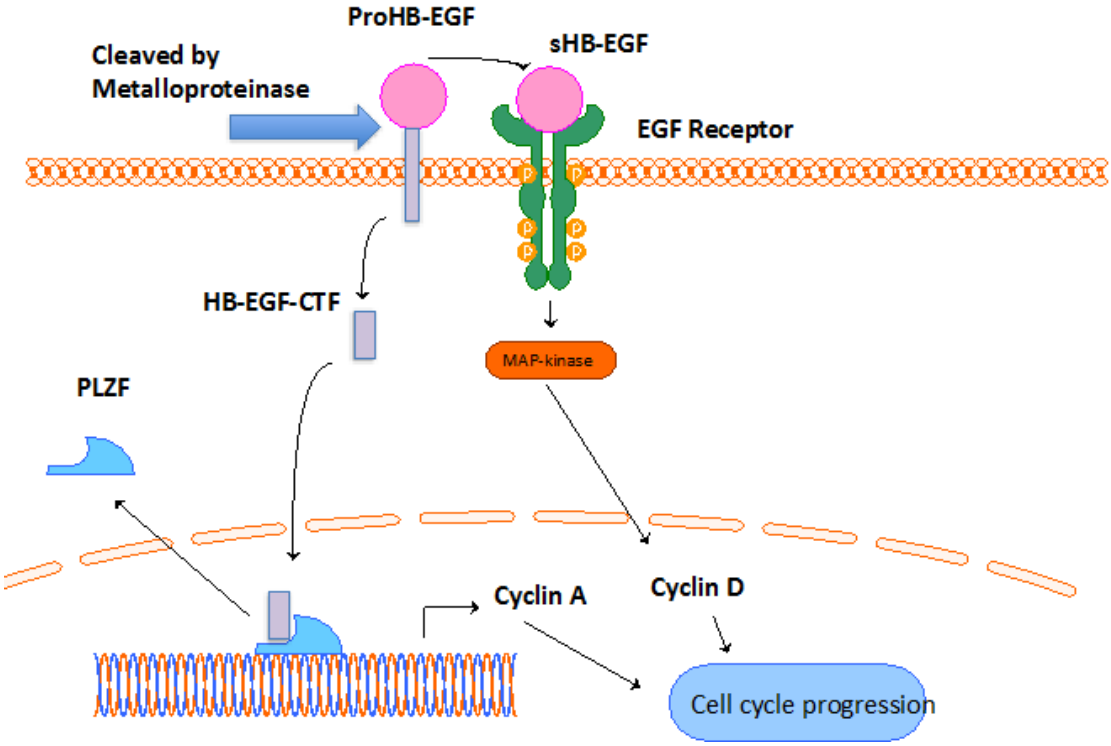

Figure 5.1 HBEGF signalling pathway. Metalloproteinases cleave ProHBEGF to generate a soluble HBEGF (sHBEGF) protein in a process called ectodomain shedding. sHBEGF binds to EGFR (Epidermal Growth Factor receptor) and induces in MAPK signaling activation and cyclin D-cyclin-dependent kinase (Cdk) complex formation, which leads to cell cycle progression. In addition, the carboxyl terminal fragment of HBEGF (HBEGF-CTF) can also translocate to the nucleus and export transcriptional repressor Promyelocytic leukaemia zinc finger (PLZF) to activate cyclin A expression, allowing re-entry into the S-phase of the cell cycle. From (Nishi and Klagsbrun, 2004)

In human proliferative vitroretinopathy (PVR), a dysregulated wound healing process due to overstimulation growth factors and factors, HBEGF has been shown to be upregulated compared to normal retinae. HBEGF, which is expressed during development was shown to exert mitogenic and chemotactic effects on adult human Müller glia *in vitro* (Hollborn et al., 2005). Recently, Wan et al., demonstrated that HBEGF can initiate Müller glia derived retinal regeneration with or without injury in the zebrafish (Wan et al., 2012). In this study, it was found that retinal injury induced rapid production and activation of HBEGF. Interestingly, it was shown that HBEGF activation via ectodomain shedding is required for Müller glia derived progenitor formation and subsequent retinal regeneration. This was confirmed by observations that the ectodomain shedding inhibition prevented Müller glia derived progenitor formation, as indicated by the absence of retinal progenitor genes such as Ascl1a and Pax6b. Furthermore, the study showed that the HBEGF/EGFR/MAPK pathway is involved in retinal regeneration, suggesting that the classical HBEGF pathway of EGF receptor activation is important for Müller glia derived progenitor formation and subsequent retinal regeneration in the zebrafish (Wan et al., 2012). In contrast with that seen in the zebrafish retina, EGF treatment in rodents only induced Müller glia proliferation in the injured retinae but not in uninjured retina, with strict formation of amacrine cells (Karl et al., 2008). Therefore, from the studies in injured mammalian retinae, it appears that EGFR activation does not fully activate Müller glia to form progenitors that can regenerate all types of retinal cells as demonstrated in the zebrafish (Wan et al., 2012). Nevertheless, these studies have indicated that through activation of HBEGF and EGFR, it may be possible to stimulate Müller glial cells for retinal regeneration across species such as zebrafish and mammals.

5.1.3 Interactions between HBEGF and the Wnt signalling pathways

Wan et al demonstrated that following injury of the zebrafish retina, HBEGF upregulate Müller glia derived progenitor proliferation. This factor also increases the expression of genes required for retinal regeneration such as Ascl1a (Wan et al., 2012) (Fig 5.2). The same research group had previously shown that activation of canonical Wnt signalling is required for the generation of a retinal progenitor population in the injured zebrafish retina (Ramachandran et al., 2011). They also observed that HBEGF is found upstream of the Wnt signalling pathway and when HBEGF is overexpressed, it activates the canonical WNT signalling pathway (Wan et al., 2012) (Fig 5.2). They found that HBEGF increased the nuclear β-catenin levels in Müller glia derived progenitors and that HBEGF enhanced mRNA expression of β-catenin in zebrafish expressing a Wnt signalling reporter (Wan et al., 2012). Intestinal ischemia studies in the adult rat have shown that PI3K/AKT is stimulated by HBEGF (Fig 5.3) (El-Assal and Besner, 2005). Furthermore, in rat intestinal epithelial IEC18 cells, it was demonstrated that PI3K/AKT pathway mediates inhibition of GSK3β by phosphorylation and thus upregulation of canonical Wnt signalling (Karrasch et al., 2011) (Fig 5.3). By contrast, when inhibition of the canonical Wnt signalling was examined under the same conditions, using the pharmacological inhibitor pyrvinium (Thorne et al., 2010), they found that HBEGF induced progenitor proliferation and regeneration and that the gene and protein expressions of Ascl1a were decreased.

In a mice model of induced intestinal ischemia/reperfusion injury (IR) by segmental mesenteric artery occlusion to the terminal ilium, it has been shown that there is decreased expression of β-catenin in the cell membranes and nuclei of intestinal

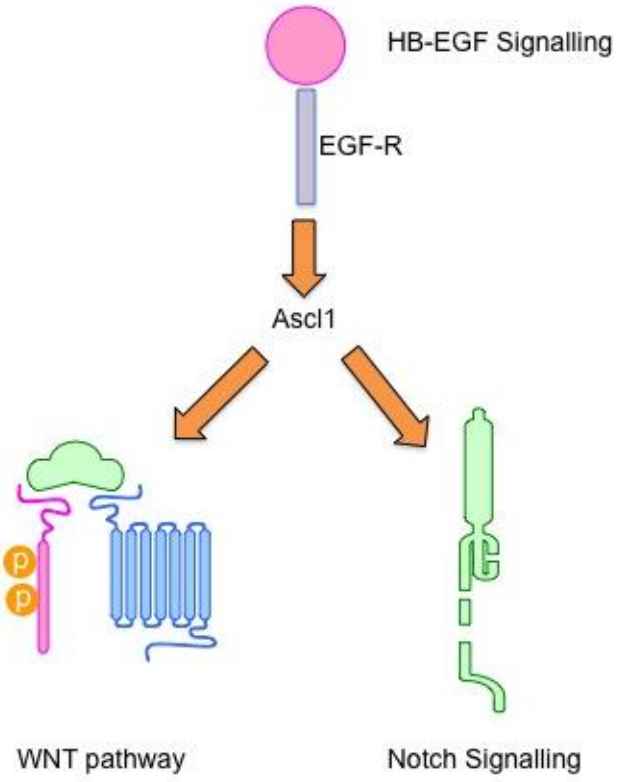

Figure 5.2 Activation of the Notch and Wnt signalling pathways by HBEGF in Müller glial stem cells. In zebrafish and mice, it has been shown that HBEGF signalling through the EGF receptor can activate the WNT and the Notch signalling pathways in Müller glial stem cells. Ascl1 signalling pathways are found downstream of the HBEGF signalling pathway. Adapted from (Wan et al., 2012, Del Debbio et al., 2010, Ramachandran et al., 2011)

stem cells. This suggests that the canonical Wnt signalling is responsible for the proliferation and differentiation of these stem cells to maintain the intestinal cell homeostasis (Pinto and Clevers, 2005, Scoville et al., 2008) (Fig 5.3). HBEGF has been previously shown to protect intestinal cells from injury (El-Assal et al., 2008).

Thus, when they overexpressed HBEGF in this mice model of IR injury, it was observed that HBEGF promotes the canonical Wnt signalling, as demonstrated by the finding that HBEGF causes increase in Wnt target genes such as c-Myc, cyclin D1 and Lgr5 in ISC (Chen et al., 2014) (Fig. 5.3).

5.1.4 Interactions between the HBEGF and Notch signalling pathways

Interactions between HBEGF and Notch signalling pathways have been reported in the literature. In the zebrafish, it has been shown that injection of recombinant HBEGF into the normal zebrafish eye resulted in the upregulation of Notch signalling components such as notch1, delta and her4 (Wan et al., 2012). By contrast, the downregulation of HBEGF using morpholinos in this species suppressed activation of the Notch target genes. Furthermore, it was highlighted that the overexpression of Notch signalling in the injured zebrafish retina caused downregulation of the expression of HBEGF and subsequently reduced the progenitor proliferation effect of HBEGF (Wan et al., 2012).

Under hypoxic conditions *in vitro*, it was found that in cancer cells the expression of HBEGF is upregulated in a Notch dependent manner. This was illustrated by the demonstration that hypoxia increased the expression of HBEGF and Notch components including ADAM12 (a ligand of EGFR which mediates ectodomain

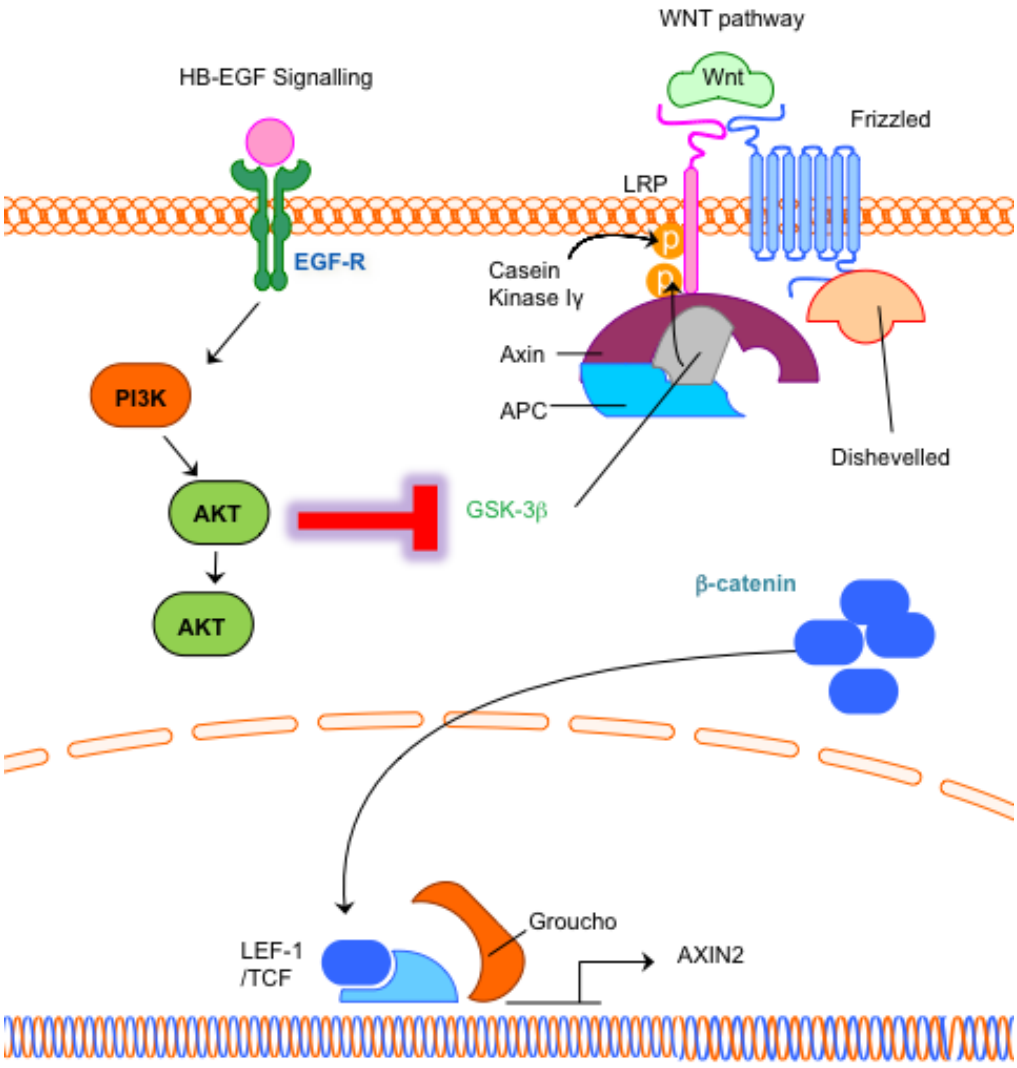

Figure 5.3 Interaction between HBEGF and WNT signalling pathway. HBEGF results in the activation of PI3K via the EGF receptor (EGF-R) and subsequently the activation of AKT. In addition, PI3K/AKT signalling phosphorylates GSK3β and inhibits it resulting in β-catenin available for canonical Wnt signalling. Adapted from (Karrasch et al., 2011, El-Assal and Besner, 2005)

shedding of HBEGF) and the Notch ligand JAG2 respectively. Furthermore, using alkaline phosphatase-HBEGF-expressing cells, it was found that upon Notch inhibition there was a decrease in the hypoxia induced shedding of HBEGF compared to cell cultured in hypoxia alone. In addition, When ADAM12, JAG2 were knocked down using siRNA, a decrease in the shedding of HBEGF was observed (Diaz et al., 2013). In mice astrocytes cultured with HBEGF under 2D and 3D cell culture conditions, it was found that there was an upregulation in the mRNA expression of the Notch pathway activator Dll1 compared to control cells. In addition, there was downregulation in the mRNA expression of Hes1 in 3D cell culture whilst no changes were observed in the 2D cell culture system (Puschmann et al., 2014)

5.2 Objectives

HBEGF has been shown to be able to initiate Müller glia derived retinal regeneration in the zebrafish (Wan et al., 2012). In addition, in human Müller glia stem cells in culture, it was found that HBEGF promoted proliferation of these cells in a similar manner as that shown in the zebrafish (Hollborn et al., 2005). HBEGF has been also shown to interact with the Wnt signalling and Notch signalling pathways during zebrafish retinal regeneration (Wan et al., 2012). However, it is not known whether similar interaction between HBEGF and Wnt or Notch signalling pathways occur in human Müller stem cells *in vitro*. On this basis, the aims of this study were as follows:

1. To examine the effect of the photoreceptor differentiation factors FGF-2, taurine, retinoic acid and Insulin-like Growth factor-1 (FTRI) on HBEGF expression.
2. To investigate the effect of HBEGF on the canonical Wnt signalling and Notch signalling components in human Müller stem cells
3. To examine the effect of HBEGF with or without a specific inhibitor on photoreceptor differentiation, and of HBEGF on RGC differentiation.
4. To investigate the effect of Notch and Wnt inhibition on HBEGF expression in human Müller stem cells.

To achieve these objectives, the following experiments were conducted:

9. Human Müller stem cells were cultured in the presence or absence of HBEGF and gene and protein expression of components of the canonical Wnt signalling pathway were examined. Selective gene components of the canonical Wnt signalling, including WNT2B, β-catenin, DKK1, WISP-1 and

AXIN2 were identified by RT-PCR of isolated mRNA, whilst intracellular protein was identified by western blot analysis of isolated protein.

10. hMSC were cultured in the presence of FTRI alone or FTRI combined with tankyrase inhibitor XAV-939 to examine gene and protein expression of HBEGF. RT-PCR was used to examine mRNA expression, whilst protein expression was identified by quantification of cells immunostaining for this factor.
11. Furthermore, to investigate the effect of HBEGF on the photoreceptor differentiation of hMSC, cells were cultured with FTRI in the presence or absence of this factor as well as in the presence of FTRI combined with a specific HBEGF inhibitor CRM197. Gene expression of the photoreceptor markers NR2E3, and recoverin were examined by RT-PCR.
12. To investigate the effect of HBEGF on the components of the Notch signalling pathway, human MSC were cultured in the presence or absence of HBEGF to examine gene and protein expression of HES1 and the Notch intracellular cytoplasmic domain (NICD). RT-PCR was performed in isolated mRNA, whilst intracellular protein was identified by western blot analysis of the whole cell lysate.

5.3 Results

5.3.1 HBEGF downregulates the canonical Wnt signalling pathway

HBEGF has been shown to interact with the components of the Wnt signalling pathway in species such as the zebrafish during Müller glia mediated retinal regeneration (Wan et al., 2012). Therefore, in order to investigate the effect of HBEGF on the components of Wnt signalling pathway in human Müller stem cells, Cell lines MIO-M1 and 6387 were cultured in the presence of recombinant soluble HBEGF (100 ng/ml) for 7 days. The western blot analysis of whole cell lysates from both cell lines showed that HBEGF induced a decrease in the protein expression of the active form of β-catenin, a canonical Wnt signalling component important for transcription of Wnt target genes, in both the MIO-M1 ($p<0.01$) and 6387 ($p<0.05$) cells, as compared to control cells cultured with medium alone (Fig 5.4 A and B).

In addition, HBEGF also caused a decrease in the mRNA expression of the canonical Wnt signalling ligand WNT2B ($p<0.05$) and canonical Wnt signalling target genes WISP-1 ($p<0.01$) and DKK1 ($p<0.05$) in these hMSC (Fig 5.5). Also, a decrease in the non-canonical Wnt signalling ligand WNT5B ($p<0.05$) was observed in these cells (Fig 5.5). Therefore, these results suggest that HBEGF downregulates the canonical Wnt signalling pathway in human Müller stem cells.

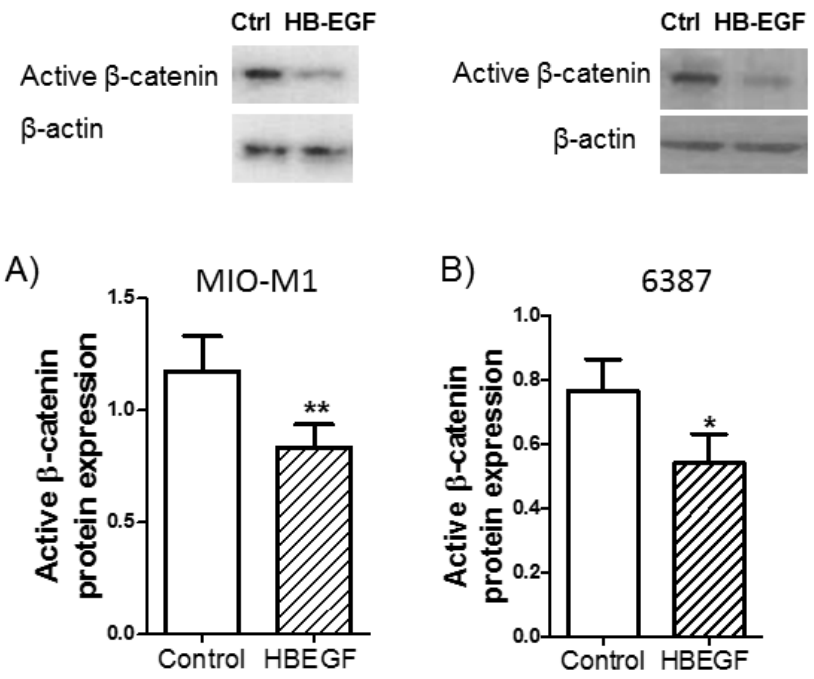

Figure 5.4 HBEGF causes downregulation of the canonical Wnt signalling component β-catenin in hMSCs. A) Western blotting analysis showed that MIO-M1 cells cultured with HBEGF induced a decrease in protein expression of β-catenin as compared to control cells; n=11. Student's t- test. **p<0.01 v. control. B) Similarly, culture of the Müller cell line 6387 with HBEGF also caused a decrease in the protein expression of β-catenin as compared to control cells; n=3. Student's t-test. *p<0.05 v. control Histograms represent the mean $\pm$ SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

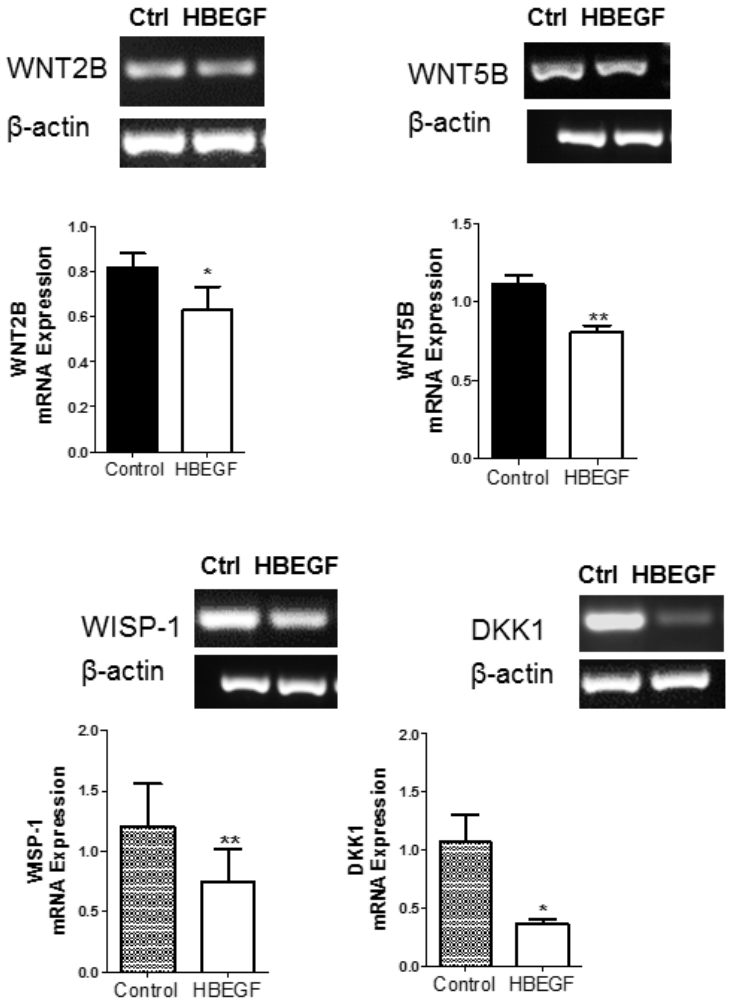

Figure 5.5 HBEGF causes downregulation of canonical Wnt signalling components in MIO-M1 cells. A) Culture of MIO-M1 cells with HBEGF caused a decrease in mRNA expression coding for the Wnt signalling components WNT2B; n=4, WNT5B; n=5, WISP-1; n=6 and DKK1; n=4 as compared to control cells. Student's t- test. *p<0.05v. control; **p<0.01 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

5.3.2 Factors that induce photoreceptor differentiation of hMSC upregulate HBEGF expression

Having shown the downregulation of canonical Wnt signalling by HBEGF, the effect of canonical Wnt signalling activation on HBEGF was investigated. To examine this effect, hMSC were cultured with recombinant canonical Wnt ligand WNT2B (100 ng/ml) for 7 days. It was found that addition of WNT2B to hMSC cultures did not modify the expression of mRNA coding for HBEGF when compared to control cells cultured with medium alone (Fig 5.6A). Furthermore, the effect of WNT2B was also examined under conditions that was previously shown to promote hMSC photoreceptor differentiation (Chapter 4) using a combination of growth factors; FGF-2, taurine, retinoic acid and Insulin-like Growth factor 1 (FTRI) as well as shown to upregulate the canonical Wnt signalling pathway. Culture of hMSC cells undergoing photoreceptor differentiation significantly increased HBEGF mRNA expression ($p<0.05$) when compared to control cells (Fig 5.6B). However, the addition of WNT2B ligand to these cells undergoing photoreceptor differentiation did not cause any changes to the mRNA expression of HBEGF as compared to FTRI alone (Fig 5.6B).

Interestingly, when tankyrase inhibitor XAV-939 (10 nM), known to downregulate β-catenin by stabilizing Axin proteins (Huang et al., 2009), were added to hMSC cultures undergoing photoreceptor differentiation, the upregulation in the HBEGF mRNA expression caused by FTRI ($p<0.05$) was inhibited in these cells as compared to cells cultured with FTRI alone (Fig 5.7A). This inhibition was further confirmed by immuno-cytochemical analysis, which also showed a significant decrease in the number of cells expressing HBEGF ($p<0.05$) proteins in hMSC cultured with FTRI in the presence of XAV-939 ($p<0.05$) (Fig 5.7B). Therefore, the

present results suggest that HBEGF is upregulated during hMSC photoreceptor differentiation and that the downregulation of canonical Wnt signalling downregulates it in hMSC.

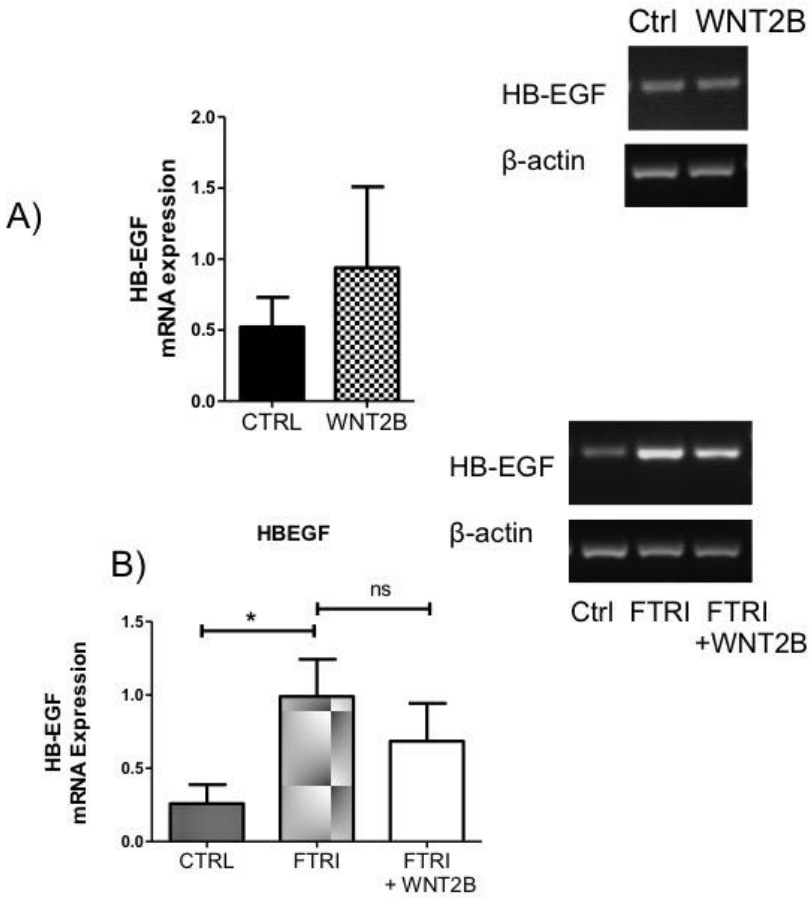

Figure 5.6 Effect of soluble WNT2B on the expression of HBEGF by hMSC cultured in the presence or absence of FTRI A) hMSC cultured with recombinant WNT2B (100 ng/ml) did not show differences in the expression fo HBEGF mRNA as compared to control cells; n=4. B) Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of HBEGF mRNA, and addition of WNT2B to cells cultured with FTRI did not modify the effect of these factors on the mRNA expression of this factor; n=3. Histograms represent the mean $\pm$ SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

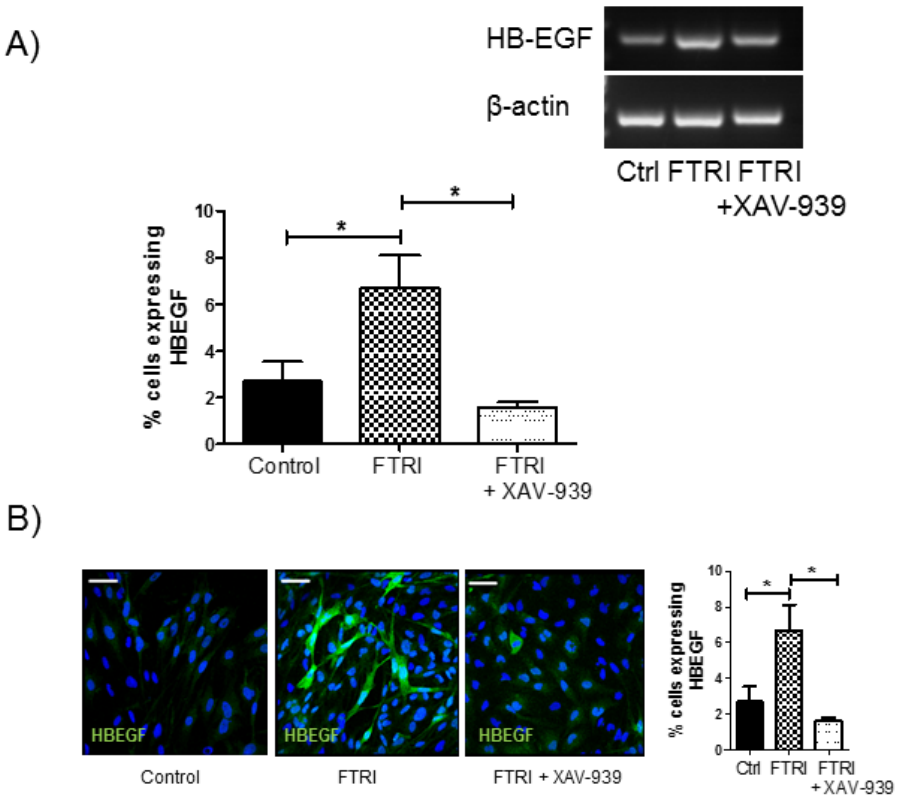

Figure 5.7 Effect of β-catenin inhibition on HBEGF expression during hMSC photoreceptor differentiation (A) As previously shown, culture of hMSC with FTRI for 7 days caused a significant increase in the expression of HBEGF mRNA. Addition of tankyrase inhibitor XAV-939 which blocks β-catenin, to cells cultured with FTRI decreased the upregulation of HBEGF; n=6.Student's t- test. ***p<0.01 v. control; *p<0.05 FTRI v. FTRI+XAV939. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms. **(B)** Confocal images confirmed the increase in HBEGF protein expression in hMSC cultured with FTRI, whilst this increase in HBEGF proteins was blocked when XAV-939 was cultured with the presence of FTRI in hMSC. (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm. Histograms on the right represent the proportion of cells immunostaining for each of the markers following 7 day culture under the different conditions; n=3. ANOVA test, *P<0.05.

5.3.3 Downregulation of HBEGF required for photoreceptor differentiation inhibits hMSC photoreceptor differentiation.

After observations that FTRI, which induces photoreceptor differentiation of hMSC, causes upregulation of HBEGF (Fig 5.7 and Fig 5.8A), we investigated whether HBEGF is essential for photoreceptor differentiation in hMSC. The effect of a specific HBEGF inhibitor CRM197 (Dateoka et al., 2012) was examined on hMSC cultured with FTRI . It was observed that 10 µg/ml of CRM197, a concentration chosen based on previous reports published in the literature (Hu et al., 2015) caused a significant decrease in the mRNA expression of HBEGF ($p<0.05$) in these cells as compared to cells treated with FTRI alone (Fig 5.8B). Furthermore, when the HBEGF inhibitor CRM197 was cultured with hMSC undergoing photoreceptor differentiation, a decrease in the mRNA expression of photoreceptor markers NR2E3 ($p<0.05$) and recoverin ($p<0.05$) were observed in these cells, as compared to control cells (Fig 5.9A and Fig 5.9B). In addition, a decrease in the immuno-cytochemical staining for recoverin protein in hMSC cultured with CRM197 in the presence of FTRI was observed when compared to FTRI alone (Fig 5.9C). In contrast, culture of these cells undergoing photoreceptor differentiation with recombinant HBEGF (100ng/ml) resulted in a significant upregulation of these photoreceptor markers ($p<0.05$ for NR2E3 and recoverin respectively) when compared to control cells (Fig 5.10A and Fig 5.10B). This was confirmed by an increase in the immune-cytochemical staining for recoverin protein under these conditions (Fig 5.10C). Therefore, the present results suggest that HBEGF is essential for photoreceptor differentiation of human Müller stem cells.

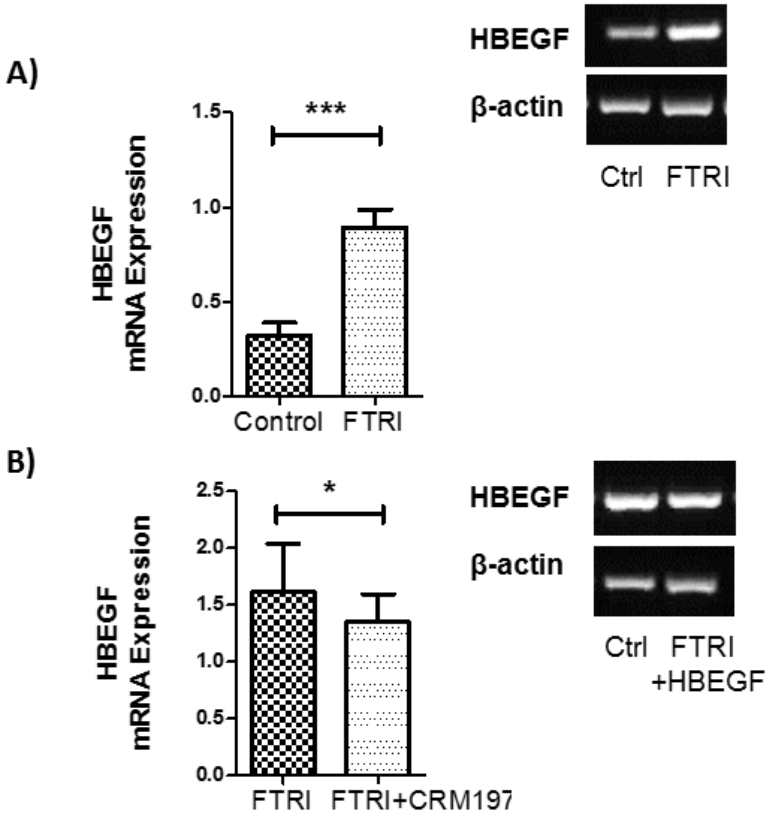

Figure 5.8 Effect of FTRI and HBEGF inhibitor CRM197 on HBEGF mRNA expression in hMSC. A) Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of HBEGF mRNA; n=6-11. Student's t- test. ***p<0.001 v. control. B) Addition of CRM197 to cells cultured with FTRI caused a significant decrease in the HBEGF mRNA expression. Student's t- test. *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

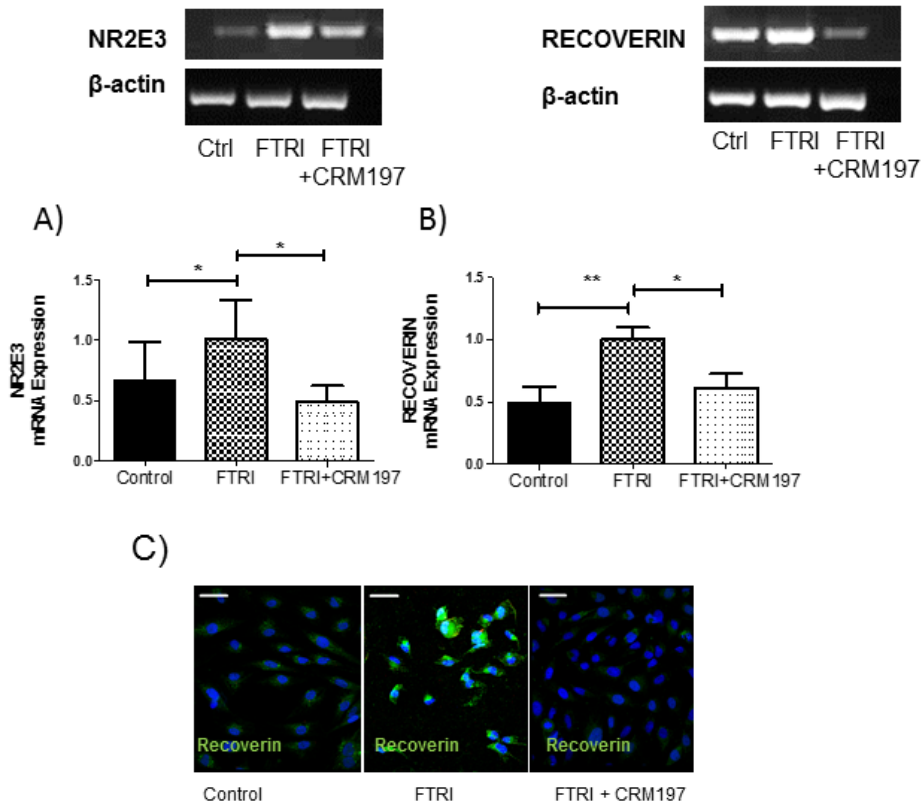

Figure 5.9 Effect of the HBEGF inhibitor CRM197 on the photoreceptor differentiation of hMSC. (A) Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of NR2E3 mRNA, whilst addition of HBEGF inhibitor CRM197 in the presence of FTRI decreased the upregulation of HBEGF; n=6. Student's t- test. *p<0.05 v. control; *p<0.05 FTRI v. FTRI+CRM197. **(B)** Similarly, hMSC cultured with FTRI for 7 days caused a significant increase in the expression of recoverin mRNA, whilst addition of HBEGF inhibitor CRM197 in the presence of FTRI decreased the upregulation of recoverin; n=6. Student's t- test. **p<0.01 v. control; *p<0.05 FTRI v. FTRI+CRM197. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms. **(C)** Confocal images confirmed that addition of CRM197 to hMSC cultured in the presence of FTRI caused a decrease in the expressions of recoverin which is upregulated by FTRI alone (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm

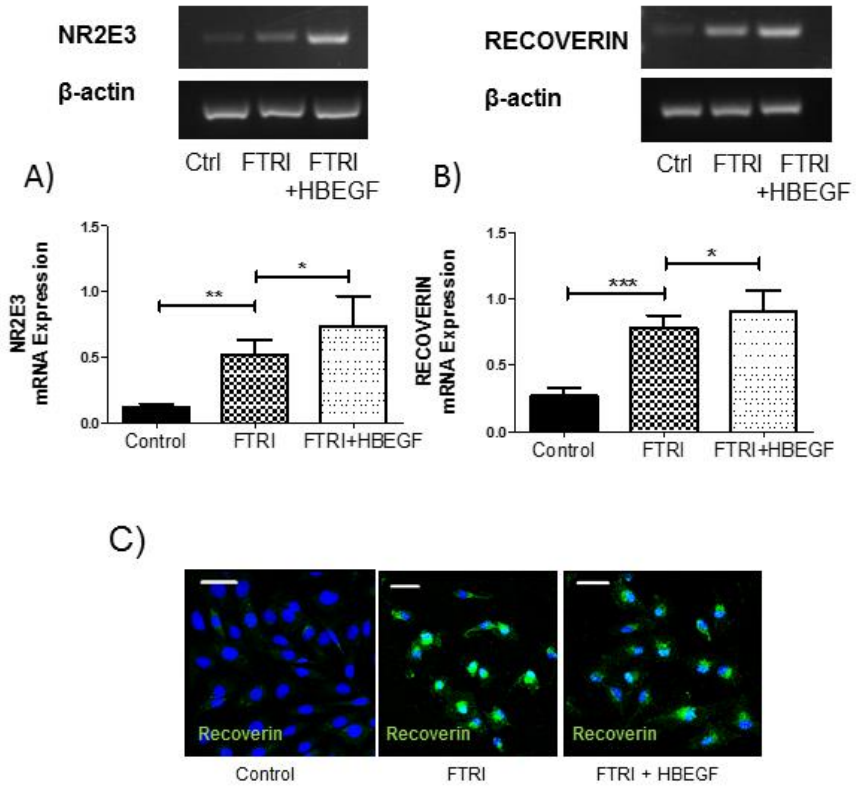

Figure 5.10 Effect of HBEGF on photoreceptor differentiation in hMSC. **(A)** Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of NR2E3 mRNA, whilst addition of exogenous HBEGF in the presence of FTRI significantly increased NR2E3 mRNA expression as compared to FTRI alone; n=6-11. Student's t- test. **p<0.01 v. control; *p<0.05 FTRI v. FTRI+HBEGF. **(B)** Culture of hMSC with FTRI for 7 days caused a significant increase in the expression of recoverin mRNA, whilst addition of exogenous HBEGF in the presence FTRI also increased the mRNA expression of recoverin further as compared to FTRI alone; n=6-11. Student's t- test. ***p<0.001 v. control; *p<0.05 FTRI v. FTRI+HBEGF. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms. **(C)** Confocal images showed an increase in the expressions of recoverin in hMSC cultured in the presence of FTRI alone or with HBEGF as compared to the controls. (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm.

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5.3.4 HBEGF upregulates components of the Notch signalling pathway

The interaction between HBEGF and the Notch signalling pathway has been shown in zebrafish and cancer cell lines (Wan et al., 2012, Diaz et al., 2013). As both of these signalling pathways play important roles during Müller glia induced retinal regeneration in zebrafish, we examined the effect of HBEGF on the mRNA and protein expression of components of the Notch signalling pathway. Culture of two different Müller glia cell lines MIO-M1 and 6387 in the presence of HBEGF (100 ng/ml) induced an increase in the mRNA expression of the Notch downstream signalling target HES1 ($p<0.01$ for MIO-M1 and $p<0.05$ for 6387 cell lines) as compared to control cells (Fig 5.11A and Fig 5.11B). Culture of these cells with HBEGF also caused an increase in the protein expression of HES1 ($p<0.01$) and the Notch intracellular cytoplasmic domain (NICD) ($p<0.05$) in these two cell lines respectively (Figs 5.12A and Fig 5.12B). In addition, this was further confirmed by the immuno-cytochemical results that showed an increase expression of HES1 proteins in hMSCs cultured with HBEGF (100 ng/ml) as compared to control cells cultured with medium alone (Fig 5.12C).

When these hMSC were cultured with γ-secretase inhibitor RO429097 (0.5 µM), it was observed that this inhibitor significantly decreased the mRNA expression of HES1 ($p<0.01$) as compared to control cells (Fig 5.13A). Furthermore, It was also shown that the Notch inhibition in these hMSC caused downregulation of HBEGF mRNA expression ($p<0.01$) as compared to control cells (Fig 5.14B). Addition of HBEGF (100 ng/ml) to hMSC cultured with RO4929097 did not modify the downregulation of HES1 mRNA expression caused by the Notch inhibitor alone (Fig 5.14B). Similar result where there was no change in protein expression was obtained

from immune-cytochemical staining for HES1 in hMSC cultured with HBEGF in the presence of RO4929097 as compared to RO4929097 alone (Fig 5.14B). Taken together, these findings indicate that, whilst HBEGF alone promote HES1 upregulation, it is unable to modify HES1 downregulation in the presence of a Notch inhibitor in hMSC.

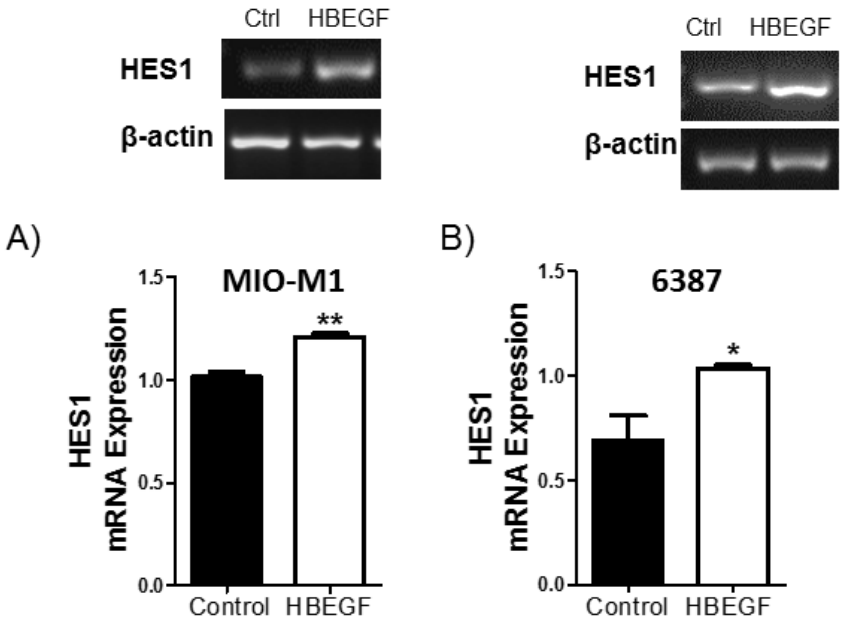

Figure 5.11 The effect of HBEGF on the mRNA expression of the Notch downstream target HES1 in hMSCs. **(A)** Culture of MIO-M1 cells with HBEGF caused an increase in mRNA coding for the Notch downstream target HES1 as compared to control cells; n=3. Student's t- test. **p<0.01 v. control. **(B)** Culture of 6387 cells with HBEGF also caused an increase in mRNA expression of HES1 as compared to control cells; N=3. Student's t-test. *p<0.05 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

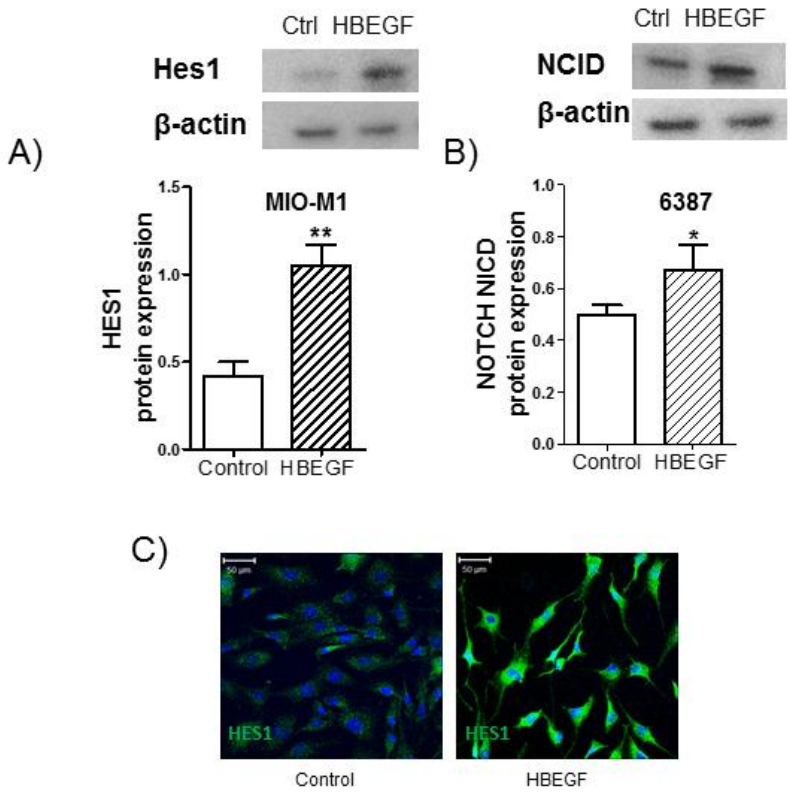

Figure 5.12 Effect of HBEGF on the Notch downstream target HES1 and the Notch intracellular domain (NICD) in hMSCs. (A) Western blot analysis of MIO-M1 cells cultured with HBEGF showed an increase in protein expression coding for the Notch downstream target HES1 as compared to control cells; n=4. Student's t- test. **p<0.01 v. control. **(B)** Similarly, culture of 6387 cells with HBEGF also caused an increase in expression of NICD protein as compared to control cells; N=3. Student's t-test. *p<0.05 v. control Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms. **(C)** Confocal images confirmed that hMSC cultured in the presence of HBEGF showed an increase in the expression of HES1 protein expression (Alexa 488, green). Scale bars 50μm.

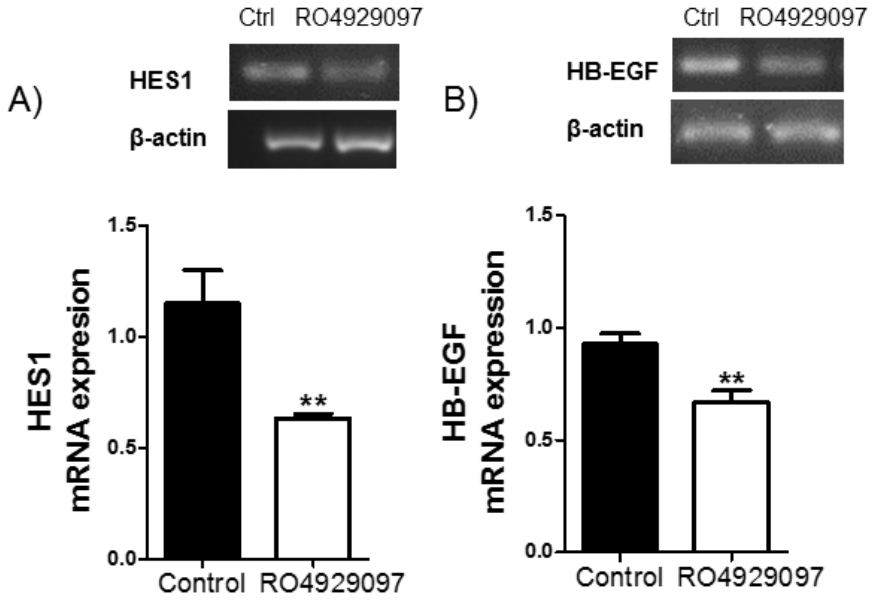

Figure 5.13 The effect of Notch inhibition on HBEGF mRNA expression in hMSC. A) Culture of MIO-M1 cells with R0492907 caused a decrease in mRNA expression coding for the HES1 as compared to control cells; n=4. Student's t- test. **p<0.01 v. control. B) MIO-M1 cells cultured with R0492907 caused a decrease in HBEGF mRNA expression as compared to control cells; n=4. Student's t- test. **p<0.01 v. control. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms.

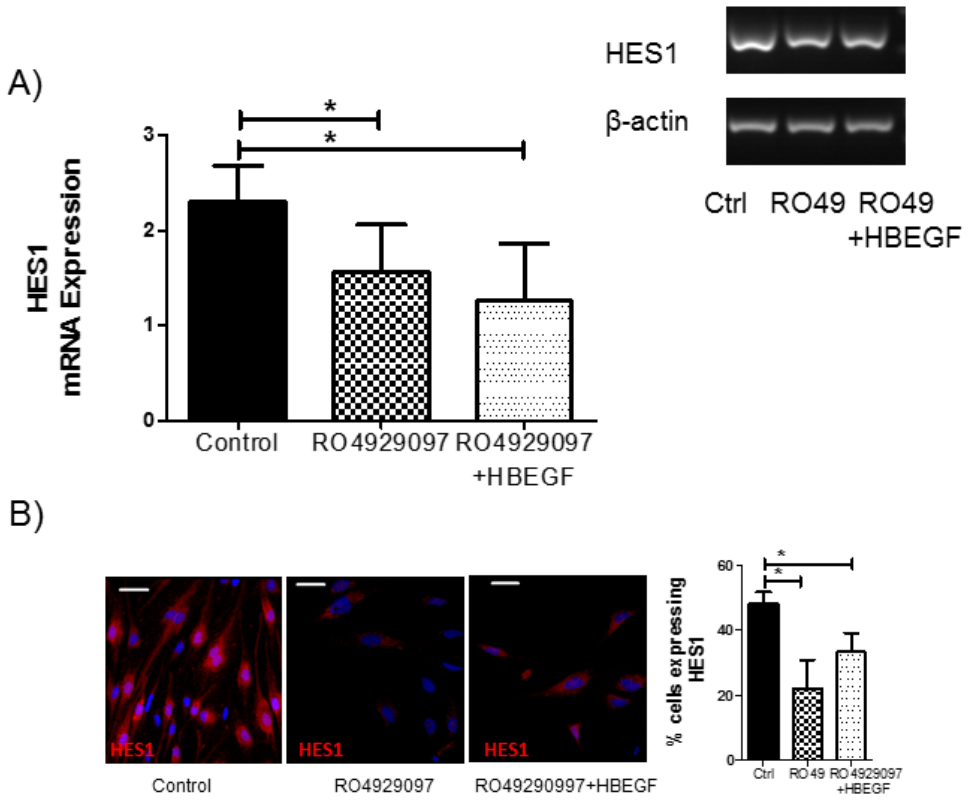

Figure 5.14 Effect of HBEGF on HES1 expression in hMSC. (A) Culture of hMSC with RO4929097 for 7 days caused a significant decrease in the expression of HES1 mRNA, whilst addition of HBEGF in the presence of RO4929097 did not modify the effect of RO2929097 on the expression of this gene . n=3. Student's t- test. *p<0.05 v. control; *p<0.05 FTRI v. FTRI+CRM197. Histograms represent the mean ± SEM of the optical density of gel bands normalized to β-actin. Representative gel bands are shown on the right side above the histograms. **(B)** Confocal images showed that addition of HBEGF to hMSC cultured in the presence of RO4929097 did not modify a decrease in the HES1 protein expression which is induced by RO4929097 alone (Alexa 488, green). Cell nuclei counterstained with DAPI (blue). Scale bars 50μm. Histograms on the right represent the proportion of cells immunostaining for each of the markers following 7 day culture under the different conditions; n=3. ANOVA test, *P<0.05.

5.4 Discussion

HBEGF has been shown to play an important regenerative role in the adult zebrafish by initiating retinal regeneration following injury (Wan et al., 2014, Wan et al., 2012). HBEGF promotes Müller glia progenitor proliferation in the damaged retinae across species including zebrafish (Wan et al., 2012), chick and mice *in vivo* (Todd et al., 2015), as well as in human cells *in vitro* (Hollborn et al., 2005)

Furthermore, various studies in the literature have highlighted the interaction between HBEGF and Wnt or Notch signalling pathways in various cell types, including intestinal stem cells, zebrafish Müller glia and cancer cells (Chen et al., 2014, Wan et al., 2012, Diaz et al., 2013). However, there have not been any studies investigating the interaction of these pathways on hMSCs.

The present results in this chapter demonstrated that the addition of exogenous recombinant HBEGF to two different hMSC lines in culture (MIO-M1 and 6387) resulted in downregulation of the expression of canonical Wnt signalling pathway components in these cells. This was shown by a decrease in the expression of active β –catenin and mRNA expressions of the Wnt target genes WISP-1 and DKK1 (Fig 5.4 and 5.5). This contrasts with the observations by others in the literature that injured adult zebrafish retina increased expression of HBEGF and that this led to the promotion of Wnt signalling components such as β -catenin and subsequent retinal progenitor proliferation (Wan et al., 2012). One of the possible reasons for the differences in the regulation of Wnt signalling pathway by HBEGF in hMSCs as compared to that of zebrafish could be attributed to the fact that these cell lines are already found in a proliferative stem cell-like state *in vitro* (Lawrence et al., 2007). Thus, there is no need for HBEGF to induce canonical Wnt signalling for proliferation of these cell lines. In contrast, following retinal injury, the adult zebrafish Müller glial

cells require proliferation of these MG derived progenitors in order to generate and replace the damaged retinal cells (Wan et al., 2012).

When human Müller stem cells were cultured in the presence of combinatory factors that induce photoreceptor differentiation; ie, FGF-2, taurine, retinoic acid and Insulin like growth factor-1 (FTRI), an upregulation in the mRNA expression of HBEGF was observed (Fig 5.8). Retinoic acid has been shown to upregulate HBEGF mRNA expression as reported in studies of retinoid induced epidermal hyperplasia in human skin *in vivo* (Rittie et al., 2006). Furthermore, IGF-1 has also been shown to interact with HBEGF, resulting in shedding of the extracellular domain of HBEGF and subsequently transactivating the EGFR (Zhou et al., 2006). Addition of WNT2B alone or in the presence of FTRI did not modify the mRNA expression of HBEGF in these cells (Fig 5.6). However, when β-catenin was inhibited by the tankyrase inhibitor XAV-939 in the presence of FTRI, the increased in HBEGF mRNA expression induced by FTRI was diminished. Wan et al., observed that the inhibition of canonical Wnt signalling by pharmacological inhibitor pyrvinium prevented the HBEGF induced formation of Müller glia progenitors in the zebrafish. This is in agreement with the present result which show that photoreceptor differentiation of hMSCs require both HBEGF and canonical Wnt signalling pathway (Fig 5.7). Furthermore, when HBEGF was inhibited using a specific inhibitor CRM197 in hMSC undergoing photoreceptor differentiation, a decrease in the expression of photoreceptor markers NR2E3 and recoverin were observed (Fig 5.9), whilst addition of HBEGF to these cells increased the mRNA expression of these markers (Fig 5.10).

In addition, it was found that the addition of HBEGF to hMSC cultures induced an upregulation of components of the Notch signalling pathway (Fig 5.11 and Fig 5.12).

This was shown by the increase in the protein and gene expressions of the Notch signalling target HES1 and the Notch intracellular cytoplasmic domain (NICD) in different hMSC lines. This result was in agreement to reports published in the zebrafish studies where it has been shown that both Wnt and Notch signalling pathways are downstream of the HBEGF and that components of the Notch signalling are upregulated when HBEGF is overexpressed during retinal damage (Wan et al., 2012). However, when HBEGF was added to these human Müller stem cells undergoing Notch inhibition, under these conditions HBEGF did not prevent the downregulation of the Notch target gene HES1 mRNA expression. This may suggest that the inhibition of Notch by γ-secretase inhibitors RO429097 may be more dominant than the effect induced by HBEGF as the concentration of RO4929097 used in these experiments were higher than that of HBEGF or perhaps it may indicate that these two molecules trigger different pathways of activation at the receptor level.

In summary, based on the results of this chapter there is an interaction between HBEGF and Wnt or Notch signalling pathways in human Müller stem cells *in vitro* as previously observed in the zebrafish (Wan et al., 2012). HBEGF downregulated the canonical Wnt signalling pathway in these hMSC, whilst activated canonical Wnt signalling by WNT2B ligand did not modify the mRNA expression of HBEGF. More importantly, it was observed that the presence of HBEGF was as essential as the canonical Wnt signalling pathway during the hMSC photoreceptor differentiation, as the inhibition of HBEGF by specific inhibitor CRM197 prevented this differentiation of hMSC. In addition, HBEGF upregulated the Notch signalling pathway in these hMSC similar to that seen in the zebrafish (Wan et al., 2012).

Chapter 6: General Discussion

Endogenous regeneration is not seen in adult mammals, although it can be induced in small rodents in early postnatal life by intraocular injection of growth factors (Del Debbio et al., 2010). A population of Müller glia with neural stem cell characteristics can be isolated from the adult human retina (Lawrence et al., 2007) and through the application of growth factors and suitable medium, they can be induced to differentiate into retinal neurons *in vitro* (Singhal et al., 2012). These findings suggest that these hMSC may have the potential to regenerate the retina *in vivo*, but that mechanisms which prevent this ability may have developed in adult mammals.

Individually, Wnt and Notch signalling play important roles in retinal development and the maintenance of adult MSCs across species. Wnt signalling inhibition results in CMZ retinal progenitor differentiation (Kubo et al., 2003), and continuous stimulation of the canonical Wnt signalling pathway leads to larval zebrafish photoreceptor differentiation (Meyers et al., 2012). Notch signalling inhibition results in Müller glia derived neuronal differentiation in both the zebrafish *in vivo* (Wan et al., 2012) and in hMSC *in vitro* (Singhal et al., 2012). Furthermore, co-stimulation of Notch and Wnt signalling pathways promote Müller glia derived progenitor proliferation and differentiation in the adult rat eye (Del Debbio et al., 2010). On this basis, one of the main aims of this thesis was to clarify the involvement of these pathways in the differentiation of human Müller stem cells into RGC and photoreceptors *in vitro*.

Factors such as TGFβ and HBEGF have been shown to play important roles during zebrafish retinal regeneration. In the injured zebrafish, HBEGF was identified to initiate MG derived retinal regeneration and to promote activation of both the Notch and Wnt signalling pathways (Wan et al., 2012). By contrast, increased in TGFβ signalling prevented this regenerative process, suggesting that expression levels of

either of these factors may interfere with the regenerative ability of MG in the zebrafish (Wan et al., 2012, Lenkowski et al., 2013, Qin et al., 2009). Therefore, it was important to understand the role of these factors on the Notch and Wnt signalling pathways in human Müller stem cells, and on the induction of neuronal differentiation of these cells *in vitro.*

6.1 Effect of Notch inhibition on Wnt signalling and the expression of RGC markers in hMSC

The results provided in this thesis demonstrate that the γ-secretase inhibitors RO429097 or DAPT induce downregulation of the Notch signalling pathway in most hMSC cultures. This was demonstrated by a decrease in the expression of mRNA and protein coding for the Notch primary target HES1. This was further accompanied by upregulation of the RGC marker BRN3A/B, which is in agreement with previous reports that Notch inhibition causes RGC differentiation of these cells (Singhal et al., 2012, Becker et al., 2013). However, addition of TGFβ to hMSC MIO-M1 cultures undergoing RGC differentiation did not modify the expression of Notch target gene HES1 and RGC marker BRN3A/3B. Thus, as a future work, other hMSC cell lines undergoing RGC differentiation could be cultured with TGFβ1. Given the importance of TGFβ and Notch signalling during Müller glia derived neural differentiation in other species, it is possible that both these pathways may regulate hMSC RGC differentiation by interacting with other signalling pathways such as the Wnt pathway. Thus, the effect of TGFβ on hMSC RGC differentiation by interacting with other signalling pathways could be explored in future studies. Also, TGFβ signalling could be more important during early stages of hMSC RGC differentiation to regulate hMSC cell proliferation as reported in the zebrafish (Qin et al., 2009, Lenkowski and Raymond, 2014). Therefore, microarray studies using hMSC undergoing RGC

differentiation cultured with TGFβ at various time points can be performed to understand the expression pattern in these cells.

The γ-secretase inhibitor RO4929097 downregulated the components of the canonical Wnt signalling including WNT2B, WISP-1, AXIN2 or DKK1 in hMSC cell lines MIO-M1 and 6387 respectively, agreeing with the results previously observed in the zebrafish (Ramachandran et al., 2012). However, no changes in WNT2B occurred in cell lines 6426 and 6391. Similarly, non-canonical Wnt ligand WNT5B in hMSC was only downregulated in MIO-M1 cells, but not in 6387. The variability observed in the effect of γ-secretase inhibitor on various hMSC lines may be attributed to the different condition of the donor tissues when used for isolating these cells. Therefore, although all hMSC lines retain various signalling pathways including the Wnt pathway, involved in stem cell differentiation and proliferation, but each cell line may possibly vary in the way these pathways can be modulated within these different hMSC lines. Taken together, the present results suggest the possible existence of a crosstalk between the components of Wnt and Notch signalling pathways in cultured hMSC. Moreover, the downregulation of the canonical Wnt signalling pathway may play an important role during hMSC RGC differentiation. Further experiments using canonical Wnt inhibitors to hMSC cultured with RO4929097 will be required to test this hypothesis. Additionally, as a future study, it would be interesting to use this inhibitor and growth factor to retinal explants and analyse RGC or photoreceptor markers in retinal sections. Furthermore, these factors could also be injected in the damaged retina of newly born mice and the retinal cell markers analysed.

6.2 TGFβ modulates the expression of WNT signalling components in human Müller stem cells

Most retinal degenerative conditions that lead to blindness, including inflammatory, pro-angiogenic and dystrophic retinal diseases, have been associated with abnormal proliferation of Müller glia that does not lead to repair but to the formation of glial scarring (Bringmann et al., 2009). Many of these conditions are also accompanied by local increased production of pro-inflammatory factors such as TGFβ (Guo et al., 2014, Wang et al., 2013, Paine et al., 2012), which may potentially modify the neural progenicity of hMSC. During early development, TGFβ has been shown to synergize or antagonize with Wnt proteins, a family of highly conserved secreted signalling molecules that regulate cell-to-cell interactions (Nishita et al., 2000, Satterwhite and Neufeld, 2004, Tuli et al., 2003).

Activation of the canonical Wnt signaling by TGFβ has been shown to mediate fibrosis (Akhmetshina et al., 2012), and cooperation between TGFβ and Wnt signaling pathways are known to play a role in controlling developmental events such as the regulation of osteoblast differentiation of human mesenchymal stem cells (Zhou, 2011). Although interaction of these signalling pathways in fish and amphibians as well as small mammals during development and adult regeneration are documented, there is no knowledge about the interaction of these factors in the human Müller stem cells. On this basis, we investigated the role of TGFβ1 on the regulation of the Wnt signalling pathway in hMSC by examining the effect of this factor on the expression of the DKK1 and WNT2B and WNT5B ligands, previously shown to be expressed by mammalian Müller glial cells (Yi et al., 2007), as well as on the phosphorylation of β-catenin.

The results showed that TGFβ caused *in vitro* downregulation of the canonical Wnt signalling pathway in hMSC. This was demonstrated by a decrease in the expression of canonical Wnt signalling components WNT2B, DKK1 and active β-catenin cultured with this factor. Activation of the TGFβ and Wnt signalling pathways require the expression of specific receptors on the cell surface, and as previously shown, mammalian Müller glia express TGFβ and Wnt receptors and their ligands (Ikeda et al., 1998, Yafai et al., 2014, Liu et al., 2013, Yi et al., 2007), for which it is possible that activation of these pathways may trigger the neurogenic properties of human Müller glia as observed in other species. By contrast, non-canonical Wnt component WNT5B mRNA and protein expressions were increased by TGFs. The non-canonical Wnt signalling mediated by ligands such as WNT5B is known to inhibit canonical Wnt signalling by acting on β-catenin (Stoick-Cooper et al., 2007). Thus, in hMSCs it may be possible that TGFβ signalling can regulate the canonical Wnt signalling pathway by increasing the expression levels of non-canonical Wnt signalling ligands such as WNT5B and subsequently inhibiting the canonical Wnt signalling pathway. Therefore, further investigations into the effect of non-canonical Wnt signalling ligands such as WNT5B on the downstream canonical Wnt signalling components in hMSC may give light into the involvement of this pathway. Furthermore, to understand whether these findings are limited to *in vitro* conditions, the next step would be carrying out similar experiments under ex-vivo conditions or in animal models of retinal degeneration.

Furthermore, the effect of inhibition of TGFβ signalling on the Wnt signalling pathway was examined by using SB431542, an inhibitor of TGFβ type I receptor (ALK5) which selectively blocks the SMAD 2/3 dependent pathway (Inman et al., 2002) and

JNK inhibitor SP600125, which blocks SMAD independent pathway mediated through JNK transcription factor (Bennett et al., 2001). SB431542 antagonized the effect of this factor on the downregulation of WNT2B and the upregulation of WNT5B, whilst there was no observed alteration on the modulation of both ligands by TGFβ1 following the addition of the JNK inhibitor. These results indicate that modulation of the expression of WNT2B and WNT5B by TGFβ1 is caused by SMAD signalling activation. These findings suggest that TGFβ signalling may regulate the neurogenic ability of human Müller stem cells by modulating the components of the Wnt signalling pathway.

6.3 Role of Wnt Signalling on the photoreceptor differentiation of human Müller glial stem cells and their modulation by TGFβ1

The spontaneous retinal regeneration observed in zebrafish has been ascribed to the ability of a population of Müller glia to de-differentiate and become progenitors that give rise to retinal neurons (Bernardos et al., 2007). Although Müller glia de-differentiation into retinal progenitors has not been demonstrated *in vivo* in the human eye, a population of Müller glia able to proliferate indefinitely *in vitro* has been identified (Lawrence et al., 2007). Unlike their inability to regenerate the human retina *in vivo*, when cultured with selective growth and differentiation factors these cells can be induced to acquire characteristics of retinal neurons (Lawrence et al., 2007, Singhal et al., 2012) The reasons why Müller glia do not regenerate the adult human retina are not known, but it is possible that factors produced in adult life or during degenerative diseases may prevent these cells from exerting these functions *in vivo*.

The results obtained in this thesis show that culture of human Müller stem cells with a combination of factors, FGF2, taurine, retinoic acid and Insulin-like growth factor 1; FTRI induces a differentiation of human Müller stem cells towards photoreceptors, which is in accordance with previous reports (Jayaram et al., 2014). This was demonstrated by mRNA as well as protein expression by immuno-cytochemical staining of the photoreceptor markers NR2E3 and recoverin. Furthermore, the result showed that FTRI upregulated the expression of genes and proteins of canonical Wnt signalling pathway components such as WNT2B, β–catenin and DKK1. The involvement of canonical Wnt signalling during hMSC photoreceptor differentiation was demonstrated by inhibiting this pathway using pharmacological inhibitor XAV-939. This prevented hMSC photoreceptor differentiation, agreeing to reports in other species, where continuous activation of canonical Wnt signalling induce generation of rhodopsin positive cells from Müller glia in mice (Liu et al., 2013). This is further supported by the demonstrations that continuous activation of canonical Wnt signalling pathway after acute injury in larval zebrafish also promotes the generation of neuronal progenitors from Müller glia (Meyers et al., 2012). Furthermore, addition of TGFβ to cells undergoing human MG photoreceptor differentiation prevented their differentiation, as shown by lack of NR2E3 and recoverin mRNA expression, as well by the lack of immunostaining for these molecules. The present results are in agreement with that observed by Lenkowksi et al., 2013 in the adult zebrafish, where TGFβ signaling, mediated through Smad 2/3 and controlled by the transcriptional co-repressors tgif1 and six3b, has also been demonstrated to downregulate Müller glia derived photoreceptor regeneration (Lenkowski et al., 2013). Therefore, further investigations into the crosstalk between the Wnt signalling and TGFβ signaling in the regulation of photoreceptor differentiation are merited. By building upon the *in vitro* results generated in this study, it would be important to examine whether similar effects can be induced in animal model studies or *ex-vivo* retinal explant cultures. In

addition, it would be important to investigate the downstream pathways common to both Wnt and TGFβ signalling pathways and identifying targets by selective downregulation using pharmacological inhibitors or small interfering RNAs. Furthermore, it would be interesting to examine how these hMSCs photoreceptor differentiation would be affected if the Wnt signalling pathway was ablated or whether TGFβ signalling would have any effect on the photoreceptor differentiation under these conditions.

6.4 Effect of HBEGF on the Wnt and Notch signalling pathways in human Müller stem cells

HBEGF is an important growth factor that act in an autocrine or paracrine manner using the EGF receptor (Hollborn et al., 2005) and induces Müller glia mediated progenitor proliferation in the damaged retinae across species such as zebrafish (Wan et al., 2012) and, chick and mice *in vivo* (Todd et al., 2015). HBEGF is highly expressed after retinal diseases such as Proliferative vitreoretinopathy (Hollborn et al., 2005). Moreover, in the retina injured zebrafish, HBEGF interacts with Wnt and the Notch signalling pathways to drive the Müller glial cells mediated retinal regeneration (Wan et al., 2012).

Addition of recombinant HBEGF into hMSC culture decreased the canonical Wnt signalling pathway as judged by the decreased mRNA expression of WIN2B, WISP-1 and DKK1 and β-catenin protein expression. As activation of canonical Wnt signalling was shown to promote hMSC photoreceptor differentiation (chapter 4), examinations of HBEGF mRNA expression under these conditions were performed. When canonical Wnt signalling was activated by WNT2B ligand, HBEGF mRNA

expression was not altered. However, FTRI induced hMSC photoreceptor differentiation that was shown to increase the canonical Wnt signalling (Chapter 4), also increased the HBEGF mRNA expression. By contrast, when hMSC photoreceptor differentiation was blocked using tankyrase inhibitor XAV-939 (inhibits β-catenin) in hMSC undergoing photoreceptor differentiation, it also decreased the HBEGF mRNA expression upregulated by FTRI. Thus, the result suggests that although HBEGF downregulates canonical Wnt signalling in hMSC cultured with medium alone, HBEGF is upregulated when there is activation of canonical Wnt signalling during hMSC photoreceptor differentiation and is downregulated when canonical Wnt signalling is blocked. Therefore, future studies could be performed to identify targets the Wnt signalling pathway uses to regulate HBEGF mRNA expression during hMSC photoreceptor differentiation. Interestingly, when a specific HBEGF inhibitor CRM197 was cultured with hMSC undergoing photoreceptor differentiation, it also prevented hMSC photoreceptor differentiation induced by FTRI. By contrast, addition of HBEGF to these cells undergoing differentiation promoted photoreceptor differentiation *in vitro.* Taken together, the results suggest that the activation of both HBEGF and canonical Wnt signalling are required for hMSC photoreceptor differentiation. Using these results in conjunction with that of TGFβ signalling on hMSC photoreceptor differentiation, HBEGF along TGFβ inhibitors could be injected individually or in-combination in early postnatal P23H rats with retinal damage, animal model of primary photoreceptor degeneration to examine their effect on endogenous Müller glia derived photoreceptor regeneration.

Addition of exogenous HBEGF alone cultured with hMSC increased the mRNA and protein expression of Notch signalling pathway components. This study is in agreement to other studies suggesting HBEGF acts upstream of the Notch signalling

pathway and activates Notch pathway through Ascl1a (Wan et al., 2012). However, when recombinant HBEGF was added to hMSC cultured with the γ-secretase inhibitor RO429097, no changes in the expression of these Notch components were observed. It is possible that the γ-secretase inhibitor RO429097 blocks cleavage of the Notch intracellular domain (NICD) longer period than effect induced by recombinant HBEGF. Thus, HBEGF may be unable to promote HES1 mRNA expression, which requires cleavage of NICD and translocation into the nucleus for gene transcription. Additionally, there is a possibility of the upstream activation of these pathways may involve sharing of molecules that once activated may not be immediately resynthesized to potentiate activation. Thus, future studies are required to confirm these hypotheses.

Conclusion

This study demonstrated the existence of crosstalk between the components of the Notch and Wnt signalling pathways in hMSC as that seen in other species such zebrafish and mice during retinal development and after injury. Thus, this study further adds evidence to the potential ability of hMSC to regenerate retina *in vivo.* In addition, the results suggest that TGFβ or HBEGF signalling can modulate the Notch or Wnt signalling pathways in human Müller stem cells. This study also suggest that through crosstalk and regulation of these pathways, neural differentiation of these human Müller stem cells induced by combination of growth factors, FTRI can be modulated. Further studies on the regulation of activation or inhibition of these

transcriptional regulators by factors may lead to the identification of small molecules with the ability to promote regeneration of the human retina.

Chapter 7: MATERIALS AND METHODS

7.1 Cell culture

7.1.1 Müller stem cell culture and cryopreservation

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) containing 10% foetal calf serum (FCS) (Gibco-BRL) and penicillin/streptomycin (Cat no: 15140-122, Invitrogen, 5mls of solution containing 10,000 units penicillin and 10,000 ug streptomycin per 500mls of media) in a 37°C incubator with 5% CO_2. Existing established Müller stem cell lines (MIO-M1, 6426, 6391 and 6387) at the institute of Ophthalmology were used (Lawrence et al., 2007). Cells were passaged by removing the media, while the cell monolayer was dissociated by incubation for 5 minutes at 37°C with 1ml of TrypLE (Invitrogen). At the end of the incubation, the cells were check under the light microscope to confirm dissociation (dissociated cells float). The cells were centrifuged at 1400 rpm for 5 minutes and then the supernatant was discarded. The cell pellet was suspended in 10% FCS containing DMEM (Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, Invitrogen) to inactivate the TrypLE. Confluent monolayers were passaged in a 1 in 5 dilution and maintained by similar passages once a week. T-75 flask tissue culture flasks (BD Biosciences) were used.

Cryopreservation was carried out by resuspending the cell pellet from a T-75 flask in 1ml of a freezing mix containing 40% FCS and 10% Dimethyl Sulfoxide (DMSO, Sigma-aldrich, UK) in DMEM. Cells were transferred to a cryovial and allowed to undergo controlled freezing in an isopropanol freezing container at -80°C for 24 hours. The frozen cells were then transferred to liquid nitrogen (-150°C) for long-term storage.

7.1.2 Culture of Müller stem cells with various growth factors

7.1.2.1 Photoreceptor Differentiation

Photoreceptor differentiation of hMSC was induced from passages 7 to 33 for the experiments. 2 x 10^5 cells were cultured on T-25 flask with the bottom coated with extracellular matrix proteins (ECM gel from Engelbreth-Holm-Swarm murine sarcoma, Sigma-Aldrich, UK). ECM was reconstituted to stock concentrations as per manufacturer's instructions and stored at -20°C. Stock solutions were then diluted in a sodium bicarbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) to achieve solutions of working concentrations (50 µg/ml ECM gel). 1 ml of ECM gel solution was used per T-25 flask to cover the entire surface. Then, the flasks were either incubated overnight at 4°C or for 2 hours at 37°C. If incubated at 4°C, the plates were transferred to 37°C for an hour prior to use. The protein buffer was then completely aspirated and cells were cultured on this prepared surface. hMSC cells were cultured for 5-6 days in ECM gel coated flasks in the presence of 20ng/ml FGF2, 20µM taurine (Sigma Aldrich, UK), 5µM retinoic acid (Sigma Aldrich, UK) and 100ng/ml IGF-1 (PeproTech, UK) (FTRI) (Jayaram et al., 2014).

To investigate the effect of TGFβ1 on photoreceptor differentiation of hMSC, cells were cultured for 7 days in flasks coated with ECM gel (Sigma-Aldrich, UK) in the presence or absence of TGFβ1 (50 ng/ml) (PeproTech, USA) and factors known to induce the expression of photoreceptor cell markers .

7.1.2.2 Inhibition or activation of the TGFβ and Wnt signalling pathways

In selected experiments, hMSCs were stimulated with recombinant human TGFβ1

(PeproTech, USA) alone, or in combination with inhibitors of the TGFβ signalling pathway or β–catenin at the following concentrations: 10 μM TGFβ type I receptor (ALK5) inhibitor SB431542 (Selleckchem, UK), 20 μM JNK inhibitor SP600125 (Sigma-Aldrich, UK) and 10 nM tankyrase inhibitor XAV-939 (Selleckchem, UK). Furthermore, in order to investigate the effect of WNT2B and WNT5B on the expression of DKK1, hMSC were cultured with these recombinant proteins for 7 days at concentrations of 100 ng/ml (WNT2B, Abnova, UK) and 500 ng/ml (WNT5B, R&D Systems, UK).

To assess the effect of HBEGF, cells were cultured for 7 days in flasks coated with ECM gel in the presence or absence of recombinant human HBEGF (100 ng/ml) (Peprotech, UK). To further investigate the role of this factor during photoreceptor differentiation, recombinant human HBEGF was added to hMSC undergoing photoreceptor differentiation by FTRI for 5-6days. In selected experiments, hMSCs were stimulated with FTRI in the presence of specific HB-EGF inhibitor CRM197 (10 μg/ml) (Reagent Proteins, USA).

7.1.2.3 RGC Differentiation

RGC differentiation was induced using hMSCs passages 7 to 33 for the experiments. Similar to the photoreceptor differentiation protocol, 2 x 10^5 cells were cultured on T-25 flask coated with ECM gel (Sigma-Aldrich, UK). hMSCs were cultured doe 7 days in DMEM containing 2% FCS in the presence of the γ-secretase inhibitors 0.5 μM of RO4929097 (Selleckchem, UK) or 50 μM of DAPT (Sigma-Aldrich, UK), previously shown to promote RGC differentiation (Singhal et al., 2012). Further, hMSCs were also cultured for 7 days in DMEM containing 2% FCS in the presence or absence of γ-secretase inhibitor RO4929097 (Selleckchem, UK) at $\log_{10}$

concentrations ranging from 0.0005-50 µM to investigate the effect of Notch inhibition at different concentrations. In selected experiments, these cells were also stimulated with recombinant human TGFβ 1 (50 ng/ml) (PeproTech, USA) in the presence of γ-secretase inhibitor RO4929097 (0.5 µM) .To assess the effect of TGFβ1, cells were cultured for 7 days in the presence or absence of human recombinant TGFβf (PeproTech, USA) at $\log_{10}$ concentrations ranging from 0.1-100 ng/ml. In addition, to examine the effect of HBEGF on hMSC on Notch inhibition and RGC differentiation, hMSCs undergoing RGC differentiation were cultured for 7 days with HB-EGF.

7.1.3 Cell proliferation Assay

In order to measure the effect of different growth factors or inhibitors on human Müller stem cell proliferation, the hexosaminidase assay was carried out which determines hexosaminidase levels (Landegren, 1984). 96 well plates were seeded with 3,000 cells per well (cell number obtained by growing cells on medium only) and cultured for 7 days at 37 recombiO2 in the presence of different factors or inhibitors. At day 7, the media was aspirated from the plates and rinsed with PBS. 60µl of hexosaminidase substrate was transferred to each well and incubated for 4 hours at 37°C. Addition of 90 µl of 0.1M glycine-sodium hydroxide buffer, pH 10.4 was used to stop the reaction and the absorbance was read at 405nm in a Spectrophotometer with a blank reading taken at 620 nm. The absorbance values obtained at 405 nm (after taking into account blank reading at 620) were used as proliferation activity of the examined hMSC and were plotted against various concentrations of RO4929097 (0.0005-50 µM) or TGFβ1 (0.01-100 ng/ml) as a line graph. Hexosaminidase substrate was made by adding 200mg of p-nitroph-Nacetyl-

acetglucosaminide to 78ml of 0.1M Tris sodium citrate, adjusted to pH 5.0 and 78ml of 0.5% Triton X-100 in water.

7.1.4 Cell Apoptosis Assay

To investigate the effect of TGFβ1 on cell death, the Caspase-Glo 3/7 assay was performed. This test measured the activity of Caspase -3 and -7 (cysteine aspartic acid-specific protease), members of the caspase family that play a key role in mammalian cell apoptosis. 96 well plates were seeded with 3,000 cells per well (cell number obtained by growing cells on medium only) and cultured for 7 days at 37nt factors or inhibitors. At day 7, the media was aspirated from the plates and, 100 µe of a mixture containing Caspase-Glo 3/7 buffer and Caspase-Glo 3/7 substrate was transferred to each well and incubated for 1 hour at room temperature. At the end of this incubation the luminescence was read at each conditions (Control, negative control and TGFβ1(50 ng/ml)) and the obtained data values (relative luminescence unit) were presented as a column scatter plot.

7.2 RT-PCR

7.2.1 RNA isolation

RNA isolation was performed using the RNeasy mini kit (Invitrogen) according to the manufacturer's protocol. After removing the media, flasks were treated with 2 ml of TrypLE. Following 3 min incubation at 37°C, PBS was added to the flasks to facilitate cell removal. This was then transferred to a 15 ml tube. Cells were centrifuged at 1400 rpm for 5 minutes. The cell pellet was resuspended in 350ul Buffer RLT

containing 3.5 ul β mercapto-ethanol (Sigma-Aldrich, UK) (part of the RNeasy Kit) to induce cell lysis. This solution was either used to isolate RNA immediately or stored at -80°C for later use. RNA isolation was performed as per manufacturer's instructions. DNase (RNase-Free DNase Set, Qiagen, 79254) treatment was applied during RNA isolation to prevent any genomic DNA contamination. After elution of RNA from a column using RNAse free water (30 µl), the concentration of RNA was determined using a spectrophotometer (Nanodrop-1000, Thermoscientific). RNA samples were stored at -80 degrees and thawed on ice before use for RT- PCR.

7.2.2 Reverse Transcription (RT) (First strand cDNA synthesis):

RNA was transcribed into cDNA using reagents from various manufacturers. Depending on the yield, the amount of RNA used for the RT reactions varied from 500ng-1µg. In all cases, when comparing samples within a given experiment, the same amounts of RNA were used for all the RT reactions. In each reaction, RNA was used to generate cDNA as follows. A 20 µl reaction mix was prepared containing 5X First-strand buffer (Invitrogen, UK), 0.1 M DTT (Invitrogen, UK), 10mM dNTP mix (Promega, UK), oligo (dT)15 primer (Invitrogen, UK), RNase inhibitor (Promega, UK), Superscript reverse transcriptase III/ IV (Invitrogen, UK) and RNAse free water (as per kit instructions). The mix was vortexed and briefly centrifuged before transfer to a thermocycler. Incubations were carried out at 25 ^{0}C for 10 minutes, 42 ^{0}C for 60 minutes, 99^{0}C for 5 minutes and 4 ^{0}C for 5 minutes. The resulting product was either used immediately in PCR reactions, or stored at -20 ^{0}C for future use.

7.2.3 PCR

Expand high fidelity plus PCR system (Cat no # 03300226001, Roche Applied Biosciences) was used to carry out the PCR. For these studies, 20 µl reaction mix was prepared on ice using 1 µl of cDNA and mixed with reaction buffer, dNTP mix, primers, polymerase enzyme and water as per kit instructions. All primers were obtained from Eurofins, Invitrogen or Sigma in the desalted form and reconstituted to 100 µM concentration in Nuclease free water. Final primer concentration used in the reaction was 0.4 µM/ml. The PCR mix was thermocycled at 94 ^{0}C (initial denaturation) for 2 minutes, 94^{0}C for 30 seconds, annealing temperature for 30 s, 72 ^{0}C for 1 minute (extension) and repeat from step 2 for 30 cycles before a final extension at 72 ^{0}C for 7 minutes and held at 4 ^{0}C. The cycling was performed using an Eppendorf mastercycler gradient thermocycler (#950000023, Eppendorf). Depending on the melting temperature of the primer as well as through semi-quantification from optimal expression curve PCR cycle numbers (30-36) were used.

The PCR products were ran in 2% agarose gel containing 1: 10,000 dilution of Gel Red nucleic acid stain (Cambridge Biosciences, U.K.). A 100 base-pair ladder (Promega, UK) was used to identify the product size. The agarose gels were ran at 100V for 60 minutes, visualised under a UV light and images were captured using Genensnap advanced image acquisition software (Syngene). Image J software was used to analyse the bands for semi-quantification. To reduce variability between different images captured for the same condition, each image was processed by using “inverted” and “subtract background” before semi-quantification. The optical density of each band was normalised by dividing the optical density of the sample by the optical density of its corresponding control gene (β-actin) band and expressed

as relative mRNA expression. Histograms were generated to represent the pixel intensities of each band.

Table 7.1: Primer sequences used for RT-PCR

Gene	Accession No.	Sequence	Tm (°C)	Product size (bp)
β-actin	NM_001101	(F) CATGTACGTTGCTATCCAGGC (R) CTCCTTAATGTCACGCACGAT	60	250
WNT2B	NM_004185.3	(F) GACGGCAGTACCTGGCATAC (R) CTCCTTAATGTCACGCACGAT	58	188
WNT3A	NM_033131.3	(F) AGATGGTGGTGGAGAAGCAC (R) GTAGCAGCACCAGTGGAACA	58	290
WNT5B	NM_032642.2	(F) TTCTGACAGACGCCAACTC (R) TGACTCTCCCAAAGACAGATG	58	264
WNT8B	NM_003393.3	(F) CCATGAACCTGCACAACAA (R) TGAGTGCTGCGTGGTACTTC	58	174
WNT11	NM_004626.2	(F) TGACCTCAAGACCCGATACC (R) GCTTCCGTTGGATGTCTTGT	58	214
FZD1	NM_003505.1	(F) AGACCGAGTGGTGTGTAATGA (R) ATACTGTGAGTTGGCTTCGAT	60	253
FZD4	NM_012193	(F) AACTTTCACACCGCTCATC (R) CAGCATCATAGCCACACTTG	55	391
FZD5	NM_003468.3	(F) TTCTGGATAGGCCTGTGGTC (R) CGTAGTGGATGTGGTTGTGC	60	214
FZD7	NM_003507	(F) GCTCTTTACCGTTCTCACCTA (R) CAGGATAGTGATGGTCTTGAC	55	388
DKK1	NM_012242.2	(F) CCTTGAACTCGGTTCTCAATTCC (R) CAATGGTCTGGTACTTATTCCCG	60	138

Gene	Accession No.	Sequence	Tm (°C)	Product size(bp)
Rhodopsin	NM_000539	(F) GCTTCCCCATCAACTTCCTCA (R) AGTATCCATGCAGAGAGGTGTAG	60	156
NR2E3	NM_014249.3	(F) GGCGTGGAGTGAACTCTTTC (R) CTGGCTTGAAGAGGACCAAG	58	230
IRBP	NM_002900	(F) CTGGTCATCTCCTATGAGCCC (R) CAGGAACTCCCCCATCATGC	60	195
Recoverin	NM_002903	(F) AGCTCCTTCCAGACGATGAA (R) CAAACTGGATCAGTCGCAGA	60	150
Blimp1	NM_001198.3	(F) GCCAAGTTCACCCAGTTTGT (R) GATTCGGGTCAGATCTTCCA	58	183
HES1	NM_005524.2	(F) AAGATAGCTCGCGGCATTCCA (R) CGTTCATGCACTCGCTGAAG	54	160
HBEGF	NM_001945.2	(F) TTCTGGCTGCAGTTCTCTCG (R) AAGTCACGGACTTTCCGGTC	56	159
AXIN2	NM_004655.3	(F) AGCCAAAGCGATCTACAAAAGG (R) AAGTCAAAAACATCTGGTAGGCA	54	188
WISP-1	NM_001204870.1	(F) CTTCTCCCTTGTCCTGCTTG (R) TGGGTCTCTCAAGGCTCTGT	60	121
BRN3B	NM_004575.2	(F) CGTGGACATCGTCTCCCAG (R) CGGGATGGTATTCATAGTGTGG	54	100

7.3 Western Blotting

7.3.1 Protein isolation

Cell monolayers were treated with TrypLE and centrifuged to obtain a cell pellet. The pellet was either resuspended in lysis buffer for protein preparation or immediately frozen at -80°C for future use. For lysis, cells were treated with Radio Immuno Precipitation Assay (RIPA) buffer containing a protease inhibitor cocktail (Sigma-Aldrich, UK)-10 µl per ml of buffer, Dithiothreitol (DTT) 0.5 mM, Phenyl Methyl Sulfonyl Fluoride (PMSF) 1mM and Sodium Orthovanadate 3mM.

The cell pellet was resuspended in ice cold lysis buffer (a minimum of 50 µl, usually 100 µl for a T75 flask), pipetted thoroughly and vortexed vigorously to obtain a homogenous suspension. The suspension was placed on ice for 5 minutes to allow cell lysis to complete, followed by centrifugation for 5 minutes at 10,000 rpm to pellet cell debris. The cleared supernatant containing the proteins of interest was collected.

7.3.2 Determination of protein concentration

Using Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo fisher scientific), the protein concentration was estimated. The colorimetric reagent containing Reagent B Cupric sulfate and Reagent A with bicinchoninic acid was diluted 1 in 50. 10 µl of protein (diluted 1:2 with RIPA buffer) was added to 200 µl of the colorimetric reagent per sample in 96 well plates (Thermo fisher scientific). The plate was vortexed briefly before incubation at 37^0C for 30 minutes. The peptide bond from protein reduces Copper in Cupric Sulfate (Cu^{2+} to Cu^{+}) whilst the bicinchoninic acid

forms a complex with Cu^{+} ion producing a purple colour that has a strong absorbance at a wavelength of 562 nm. The absorbance of the resulting purple solution was then measured at 562 nm using a spectrophotometer. Also, in addition BSA standard curve was generated to be used as a reference for determining the protein concentrations of the unknown samples from the absorbance values obtained from the standard BSA (2mg/ml) diluted with water into following concentrations (0, 0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 1.5 mg/ml).

7.3.3 Protein Gel Electrophoresis

NuPAGE electrophoresis gels and buffer systems (Invitrogen, UK) were used for gel electrophoresis. 4-12% Bis-Tris mini gels (NP0315, Invitrogen, UK) were used in combination with the MOPS SDS running buffer (NP0001, Invitrogen, UK). 24 µl of loading sample was prepared with 2.4 µl of reducing agent (10X), 6 µl of loading buffer (LDS 4X) and a maximum of 15.6 µl of protein sample. In order to load equal amounts of protein, this volume of 19.5 µl was adjusted based on the protein concentrations and the remaining volume made up with water.

The loading samples were boiled at 80^{0}C for 10 minutes to denature the protein. The pre-prepared gels were removed from their packaging, washed in distilled water and fitted into the XCell SureLock™ Mini-Cell mini vertical electrophoresis system (Invitrogen, UK) after removal of the white tape seal to allow for communication of the gel with the buffer system during electrophoresis. The chamber was then locked to generate a water tight compartment for the gel and the electrodes. Into the inside compartment, running buffer containing antioxidant (500 µl/ 200mls buffer) was added. The outside compartment was filled with running buffer without antioxidant. The wells were then loaded with the protein samples as well as the ladder and run

at 180V for 60 minutes. As a protein ladder, a prestained protein marker (7-175 KDa) (New England Biolabs, UK) was used.

7.3.4 Gel Transfer

Poly VinyliDene Fluoride (PVDF) membranes (Millipore, UK) were hydrated in absolute methanol for 2 minutes, washed in distilled water and equilibrated in working concentration transfer buffer before being applied to the gel. The transfer was carried out at 10V for 30 minutes using the XCell SureLock™ Mini-Cell mini transfer set up (Invitrogen, UK).

7.3.5 Immunoblotting

Following transfer, for analysis of non-phosphorylated proteins, PVDF membranes were blocked in 5% milk and 5% FBS in TBS with 0.1% Tween 20 at room temperature for 1.5 hours. For phosphorylated proteins, PVDF membranes were blocked using phosphoprotein blocker (Millipore, UK) at room temperature for 1.5 hours. Primary antibody was diluted in blocking reagent and the membrane was incubated with this overnight at 4 ^{0}C on a shaker. The next morning the membrane was washed 4 times for 20-25 mins each with TBS containing 0.1% Tween 20. The membrane was then incubated for 1 hour at room temperature with the secondary antibody (species specific peroxidase conjugated IgG, Jackson Laboratories) diluted 1 in 10,000 in the blocking reagent. After removal of the secondary antibody, the membrane was washed 4 times over an hour with TBS containing 0.1% Tween 20. Bound antibody was then reacted with the ECL2 advanced western blotting chemiluminescent immunodetection system according to the manufacturer's instructions. Images on the x-ray film (Fujifilm, Japan) were visualised using Fujifilm imager (LAS-100; Fujifilm, Japan). X-ray film were scanned and the optical density of each band was normalised by dividing the optical density of the sample by the

optical density of its corresponding control β-actin band using Image J software (Walker, 2002) and expressed as a relative protein expression. Histograms were generated to represent the pixel intensities of each band.

Table 7.2: Antibodies and dilutions used for Western blotting

Antibody	Raised in	Manufacturer	Catologue no.	Dilution
β-actin	Mouse	Sigma-Aldrich	A2228	1:5000
WNT2B	Rabbit	Abcam	AB50575	1:1000
WNT5B	Rabbit	Abcam	AB94914	1:500
HES1	Rabbit	US biologicals	H2034-35	1:100
Phospho-β–catenin	Rabbit	Millipore	07-1651	1:1000
β–catenin	Rabbit	Abcam	AB6302	1:1000
Active β–catenin	Mouse	Millipore	05-665	1:250
Notch	Rabbit	Santa Cruz	SC6014	1:250

7.4 Immunocytochemistry

Human Müller stem cells at a density of 1 x 10^4/ml were cultured for 7 days in DMEM supplemented with 2% FCS on ECM gel-coated 13 mm glass coverslips in Costar 24 well plates with 0.5ml of media per well. Following 7 days, the media was aspirated and the cells were fixed in 200µl of 4% paraformaldehyde for 4 minutes at room temperature using a shaker. After aspirating, for cryopreservation the paraformaldehyde, it was replaced with 100 µl 30% sucrose solution for 15 minutes at room temperature. After removal of the sucrose, the slides were either used immediately for immunocytochemistry or dried and kept frozen at -20°C until use. Blocking reagent was either made up in TBS containing 0.3% Triton®-X-100 (Sigma-Aldrich) and 5% donkey serum (Jackson ImmunoResearch) or PBS containing 0.5 % Roche blocker (Roche, UK) and 5 % donkey serum. The primary antibodies were diluted at various concentrations in the blocking reagent as highlighted in the table below. Primary antibody incubation was performed overnight at 4°C on a shaker. The following day, the plates were washed 3 times with TBS in 5 minute cycles, and subsequently covered with corresponding secondary antibody diluted 1:500 in blocking reagent without the donkey serum and incubated for 3 hours at room temperature in the dark by covering the plates in aluminum foil. After completion of secondary antibody incubation, the plates were washed again as before and the nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, UK) diluted 1:2500 in PBS for 1 minute. Slides were then rinsed in distilled water and mounted with Vectashield (Vector Laboratories) onto a glass slide and sealed with nail polish.

Table 7.3: Antibodies and dilutions used for Immunocytochemistry

Antibody	Raised in	Manufacturer	Catologue no.	Dilution
HES1	Rabbit	US biologicals	H2034-35	1:200
Active β–catenin	Mouse	Millipore	05-665	1:250
NR2E3	Rabbit	Millipore	AB2299	1:50
Recoverin	Rabbit	Millipore	AB5585	1:500
CRX	Mouse	Abnova	H00001406-M02	1:250
Ki-67	Mouse	Novocastra	KI67-MM1-L-CE	1:1000
BRN3A	Mouse	Millipore	AB5945	1:250
ISL-1	Mouse	DSHB	39.4D5	1:10

7.5 Microscopy

Fluorescent images were captured with identical exposure times using a Zeiss LSM710 confocal microscope and identically processed using Carl Zeiss Zen imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany). Phase images were acquired using Leica Phase contrast microscope and Leica DC viewer software.

7.6 Enzyme-linked immunosorbent assay (ELISA)

Elisa analysis was used for quantification of secreted DKK1 (R&D Systems, UK), WNT2B and WNT5B (Cusabio, China) using the manufacturers' instructions. Human Müller stem cells (2 x 10^5 cells) were grown for 7 days in DMEM with 2% FCS on ECM gel coated flasks in the presence or absence of either human recombinant TGFng Carlng/ml) or FTRI to induce photoreceptor differentiation as indicated above (Jayaram et al., 2014). 500 µl Supernatants were collected from these cultured cells. 100 µl of ELISA assay diluent was transferred to each well of a 96 well plate. Then 100 µl of standard, control or sample was added to these wells. Following 2 hour incubation at room temperature on an orbital shaker, each well was aspirated and washed with the buffer provided in the ELISA kit four times. This was followed by transfer of 200 µl conjugate, 2 hour incubation at room temperature and aspirate/wash four times. 200 µl of substrate solution was then added to each well and incubated at room temperature for 30 minutes in the dark. The reaction was stopped by transferring 50 µl of stop solution to each well. The absorbance was read at 450 nm, with wavelength correction set at 540 nm. A standard curve was plotted of diluted standard solutions for each experiments (DKK1, WNT2B and WNT5B) using Curve Expert 1.3 software. The concentration of proteins in each of the samples was then identified by extrapolation to the generated curve.

7.7 Statistical analysis

Statistical analysis of all results was carried out using Graphpad Prism 5 software. Statistical differences were calculated using paired Student's t-test or one-way repeated measures ANOVA. The standard error of the mean (SEM) was plotted as error bars on bar charts and a probability of <0.05 was considered to be significant.

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Chapter 9: Publications