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FACULTÉ DES ÉTUDES SUPÉRIEURES
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FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES

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Ph.D. (Microbiology and Immunology)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role of Wnt Signaling Pathway in Mammalian Retinal Development

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The Role of Wnt Signaling Pathway in Mammalian Retinal Development

By
Hong Liu

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfilment of the requirements
For the degree of Ph.D.

Department of Biochemistry, Microbiology and Immunology
Human and Molecular Genetics
Faculty of Medicine
University of Ottawa

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395 Wellington Street
Ottawa ON K1A 0N4
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395, rue Wellington
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Your file Votre référence

ISBN: 978-0-494-32411-0

Our file Notre référence

ISBN: 978-0-494-32411-0

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STATEMENT

The figures and major part of the text presented in Chapter 3 was published in *Developmental Dynamics*: Liu H, Mohamed O, Dufort D, and Wallace VA (2003). Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *Dev Dyn* 227, 323-334. The material is reproduced with permission from Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc..

The figures and major part of the text presented in Chapter 4 was published in *Investigative Ophthalmology Vision Science*: Liu H, Thurig S, Mohamed O, Dufort D and Wallace VA (2006). Mapping canonical Wnt signaling in the developing and adult retina. *Invest Ophthalmol Vis Sci* 47(11):5088-5097. The material is reproduced with permission from the Association for Research in Vision and Ophthalmology.

Part of the text and figures presented in Chapter 5 and Chapter 6 will be published in *Developmental Biology*: Liu H, Xu S, Wang Y, Mazerolle C, Thurig S, Coles B. L. K., Ren J-Ch, Taketo M, van der Kooy D and Wallace V A (2007). Ciliary margin transdifferentiation from neural retina is controlled by canonical Wnt signaling. *Dev Biol* (in press). The material is reproduced with permission from Elsevier Limited, UK.

ABSTRACT

Intercellular communication via secreted signaling molecules is essential for the generation of a normally patterned central nervous system (CNS). The mammalian retina, which is neuroectoderm derived, is an excellent model system in which to study signaling events in patterning, cell proliferation and diversification in the CNS. Although the identity and function of some signaling molecules that regulate retinal development are known, the function of many others, especially members of the Wnt family, has yet to be well characterized. Wnt ligands have been established as critical regulators of multiple developmental processes in a variety of organs and tissues during embryogenesis.

To ascertain the function of Wnt signaling in the context of retinal development, I examined the expression of Wnt pathway components in the mouse neural retina. I showed that *Wnt2b* (formerly known as *Wnt13*), Wnt receptors and Wnt antagonists are expressed within and adjacent to the distal part of the eyecup, the ciliary margin (CM). β -gal staining in the eyes of *TCF/Lef-LacZ* mice, a canonical Wnt pathway reporter mouse strain, revealed the highest level of reporter activity in the CM throughout retinal development. Thus, I hypothesized that canonical Wnt signaling plays a role in the specification and formation of the CM and its derivatives.

To test the above hypothesis, I used several approaches to activate Wnt signaling in retinal explants from *TCF/Lef-LacZ* reporter mice. Ectopic expression of *Wnt2b* did not activate Wnt reporter activity, while over expression of *Wnt3a*, a well-characterized

canonical Wnt pathway activator, induced reporter gene expression in the CM, but after a considerable delay. Treatment with lithium, a well-known Wnt pathway agonist, resulted in rapid upregulation of Wnt reporter activity, and alterations in morphology and gene expression that were consistent with induction of a CM identity. The expression of stabilized β -catenin, the key mediator of canonical Wnt pathway activation, was then targeted to the peripheral retina by using a Cre-loxP approach. These transgenic mice exhibited a dramatic increase in β -catenin-dependent signaling, as well as an expansion of the CM/ciliary epithelium in Cre-expressing region of the retina. Taken together, the findings presented in this thesis indicate that β -catenin-mediated signaling can promote the development of the CM and its derivatives.

ACKNOWLEDGEMENTS

During my graduate study in the department of BMI and in OHRI, so many people have generously and kindly supported me to make the success of this project possible. I would like to present my sincere acknowledgements, for the first, to my supervisor, Dr. Wallace for providing me with guidance, encouragement and the great opportunity to explore the sea of knowledge. Training that I obtained from Valerie in both science and language skill will forever benefit me in the future. Thanks must go to Dr. Kothary and Dr. Rudnicki, my committee members, for precious suggestions and advice throughout my study. Thanks must also go to members of the Wallace lab, including Katrina, Yaping, Gabriel, Chantal, Marosh, Sherry, Chuan, Dana, Shawn and Kowsala et al, as well as members of the OHRI, in particular members of the Kothary, Rudnicki and Picketts labs, who all together made a wonderful positive environment for me to grow up. I am very grateful to Yves De Repentigny, Chantal Maerolle, Drs. Kuroda and Mohamed for collaborations. I am also grateful to Drs. Dufort, Gruss and Taketo for providing us the transgenic mouse strains, Drs. McMahon, Zakin, Hsieh and Rattner et al for the plasmids, Dr. Bremner for antibodies, and Dr. Fuhrman for technical advices. This work was supported in part by grants from the National Cancer Institute of Canada, the Canadian Institutes of Health Research and the Stem Cell Network of Canada. I thank Foundation Fighting Blindness Canada and University of Ottawa for providing me scholarships. I must especially acknowledge my parents, brothers, sisters, my husband and my daughters, who have always been the source of drive to support me in the completion of my study and thesis writing.

TABLE OF CONTENTS

Statement	Page
Abstract	iia
Acknowledgements	iib
Table of Contents	iv
List of Abbreviations	v
List of Figures	ix
List of Tables	xi
1.0 – Introduction	xv
1.1 – Ocular development	1
1.1.1 – Formation of the eyecup	1
1.1.2 – Neural retina development	4
1.1.3 – Formation of the non-neural derivatives, ciliary body and iris	7
1.2 – Transcription factors and signaling molecules controlling eye development	13
1.2.1 – Transcription factors	13
1.2.2 – Signaling molecules	15
1.3 – Wnt signalling	17
1.3.1 – <i>Wnt</i> genes and Wnt proteins	18
1.3.2 – Wnt signaling pathway	20
1.3.3 – Components of the Wnt signaling pathway	23
1.4 – Gene expression and function of the Wnt signaling pathway in the eye	27
1.4.1 – Components of the Wnt signaling pathway are expressed in vertebrate eye	28
1.4.2 – Aberrant Wnt function is associated with eye diseases	28
1.4.3 – Wnt function in ocular structure formation and development of the neural retina	29
1.4.4 – Wnts role in the CM and its non-neural derivatives, CB/iris	31
1.5 – Rationale, hypothesis and research objectives	31
2.0 – Materials and Methods	34
2.1 – Animals	34
2.1.1 – Imported transgenic mice	34
2.1.2 – <i>Msx1mpWnt2bHA</i> transgenic mice	34
2.1.3 – Genotyping	35
2.2 – β -galactosidase activity detection by X-gal staining	35
2.3 – Cell Culture	35
2.3.1 – Generation of retrovirus in 293FT cells	36

2.3.2 – TOPFlash luciferase assay	36
2.4 – DNA constructs and construction	37
2.4.1 – pHAN/puro	37
2.4.2 – pHIT4-IRES-hrGFP (HA/FLAG) (referred to as pHIT4)	37
2.4.3 – Constitutively active β -catenin	37
2.4.4 – Wnt2b constructs	38
2.4.5 – Wnt3a constructs	39
2.5 – Immunohistochemistry	40
2.6 – <i>In situ</i> hybridization	41
2.7 – Retinal explant culture	42
2.7.1 – Retinal explants	42
2.7.2 – Dissociated retinal cell counting	42
2.7.3 – Electroporation in retinal explants	43
2.7.4 – Treatment with lithium or Wnt3a recombinant protein	43
2.8 – RT-PCR and quantitative RT-PCR	43
2.8.1 – Oligonucleotide primers	43
2.8.2 – RT-PCR	44
2.8.3 – Quantitative RT-PCR	44
2.9 – SDS-PAGE and Western blot	46
2.9.1 – SDS-PAGE	46
2.9.2 – Western blot	46
3.0 – Characterization of Wnt Signaling Components and Activation of the Wnt Canonical Pathway in the Murine Retina	47
3.1 – Results	48
3.1.1 – Expression of Wnt pathway genes in the embryonic retina	48
3.1.2 – TCF/Lef-LacZ reporter transgene activity in the developing retina ...	58
3.1.3 – Expression of Wnt pathway genes in perinatal and adult retina	58
3.1.4 – Expression of Wnt pathway genes in the eyelid, cornea and lens	63
3.2 – Discussion	64
3.2.1 – Wnt signaling in the developing neural retina	64
3.2.2 – Wnt-13 signaling at the ciliary margin	67
3.2.3 – Wnt signaling in the perinatal and adult retina	68
3.2.4 – Wnt signaling in the developing lens and optic disc	69
4.0 – Mapping Canonical Wnt Signaling in the Developing and Adult Retina	71
4.1 – Results	72
4.1.1 – Lithium treatment induces TCF/Lef-LacZ reporter gene activity in retinal explants	72
4.1.2 – Activation of the canonical Wnt signaling in the developing retina	73

4.1.3 – Identification of <i>TCF/Lef-LacZ</i> reporter positive cells in the outer neuroblast layer of the developing retina	80
4.1.4 – Dynamic expression of <i>TCF/Lef</i> family factors in the developing retina	82
4.1.5 – Activation of <i>TCF/Lef-LacZ</i> reporter gene in the postnatal and adult retina	86
4.2 – Discussion	90
4.2.1 – Canonical Wnt signaling in the development of the ciliary margin/ciliary epithelium	90
4.2.2 – A possible role of canonical Wnt signaling in RGC axon guidance	91
4.2.3 – Conserved expression of <i>TCF/Lef</i> transcription factors in the vertebrate retina	92
5.0 – Activation of Canonical Wnt Signaling Promotes Ciliary Margin Identity and Controls Neuronal Differentiation in Retinal Explants	94
5.1 – Results	96
5.1.1 – Explants represent a loss of function model with respect to canonical Wnt signaling in the ciliary margin	96
5.1.2 – Ectopic expression of <i>Wnt2b</i> in the CM fails to activate <i>TCF/Lef-LacZ</i> reporter in retinal explants	98
5.1.3 – Ectopic <i>Wnt3a</i> activates canonical Wnt signaling in the retinal explants	105
5.1.4 – Constitutively active β -catenin up regulated Wnt reporter in the retinal explants	110
5.1.5 – Effects of lithium-induced canonical Wnt pathway activation in retinal explants	112
5.2 – Discussion	126
5.2.1 – Canonical Wnt signaling promotes CM character in the murine retina	126
5.2.2 – Proliferation and cell cycle effect of Wnt canonical pathway in the vertebrate retina	127
5.2.3 – A role of the canonical Wnt signaling in neurogenesis suppression	129
5.2.4 – Differences of <i>Wnt2b</i> expression and function in the retina in chick and mouse	130
6.0 – Canonical Wnt signaling drives ciliary margin specification in the murine retina	132
6.1 – Results	134
6.1.1 – Expression of stabilized β -catenin results in expansion of	

the CM	134
6.1.2 – Phenotypes in the earlier eyecup suggest transdifferentiation of NR to CM fated tissue	140
6.1.3 – No evidence of selective proliferation in the β -catenin stabilized region of the compound transgenic mice	140
6.1.4 – Expression of stabilized β -catenin down regulates retinal progenitor gene <i>Chx10</i> and <i>Pax6</i>	143
6.1.5 – Maintained CE/CB identity with abnormal neurogenesis in the peripheral eye of postnatal mice expressing stabilized β -catenin	145
6.1.6 – Stabilization of β -catenin results in a reduction of mature neurons in the central retina of the postnatal eye	150
6.2 – Discussion	156
6.2.1 – Activation of canonical Wnt signaling converses the NR into CM	156
6.2.2 – Elevated levels of β -catenin results in phenotype different from that caused by loss of <i>Chx10</i> or <i>Pax6</i>	156
6.2.3 – Possible mechanism underlying the small eye phenotype	158
6.2.4 – Reduction of neurogenesis in the postnatal eye likely due to improper differentiation or cell death	158
7.0 – Conclusions and Remarks	160
8.0 – References	165
9.0 – Contributions of Collaborators	185
10.0 – Appendices	186
Appendix A. Oligonucleotides	186
Appendix B. Number of embryos and pups delivered after pronuclear injection of the <i>Msx1mp-Wnt2bHA</i> construct	189
Appendix C. Summary of transcription factors and cellular markers examined to evaluate the effects of ectopic Wnt signaling on retinal patterning	190
Appendix D. Plasmid map for <i>Wnt2bHA/pHIT4</i>	191
11.0 – Curriculum Vitae	192

LIST OF ABBREVIATIONS

APC	adenomatous polyposis coli
arm	armadillo
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
CA	constitutively active
CB	ciliary body
CBP	cAMP response element binding protein
CE	ciliary epithelium
CM	ciliary margin
CMZ	ciliary marginal zone
CNS	central nervous system
CRD	cysteine rich domain
Ct	threshold cycle
Dkk	dickkopf
E	embryonic day
FBS	fetal bovine serum
FEVR	familial exudative vitreoretinopathy
Fzd	Frizzled
GSK	glycogen synthesis kinase
HD	homeodomain
IHC	immunohistochemistry
iIPE	inner layer of IPE
INL	inner nuclear layer
IPE	iris pigment epithelium
IPL	inner plexiform layer
ISH	<i>in situ</i> hybridization
JNK	Jun-N-terminal kinase

Li ⁺	lithium
Lef	lymphoid enhancer-binding factor
LRP	low-density lipoprotein receptor-related protein
Mfz	mouse Frizzled
mp	minimal promoter
NDP	Norrie disease gene
nPCE	non-pigmented ciliary epithelium
NR	neural retina
oIPE	outer layer of IPE
ONL	outer nuclear layer
OPL	outer plexiform layer
OPPG	osteoporosis-pseudoglioma syndrome
P	postnatal
PCE	pigmented ciliary epithelium
PCP	planar cell polarity
PFA	paraformaldehyde
PKC	protein kinase C
PLC	phospholipase C
qPCR	quantitative RT-PCR
RGC	retinal ganglion cell
RPC	retinal progenitor cell
RPE	retinal pigment epithelium
RSC	retinal stem cell
RT-PCR	reverse transcriptase-polymerase chain reaction
RTK	receptor tyrosine kinase
Sfrp	secreted-frizzled-related protein
Shh	Sonic hedgehog
SMA	smooth muscle actin
TCF	T cell specific transcription factor
TGF-β	Transforming growth factor β
Wg	wingless
WIF	Wnt-inhibitory factor

LIST OF FIGURES

Fig. 1.	Schematic diagram of mouse eye development	3
Fig. 2.	Structure of the vertebrate retina	5
Fig. 3.	Schematic diagram of the anterior segment of the adult mammalian eye	9
Fig. 4.	Schematic diagram of the main branches of Wnt signaling pathway	21
Fig. 5.	RT-PCR analysis of <i>Wnt</i> gene expression in the developing and adult NR	49
Fig. 6.	ISH for <i>Wnt</i> gene expression in the developing retina	50
Fig. 7.	ISH for <i>Mfz</i> gene expression in the developing retina.....	54
Fig. 8.	ISH for <i>Sfrp</i> gene expression in the developing retina.....	56
Fig. 9.	Detection of β -gal activity in the developing eye of <i>TCF/Lef-LacZ</i> transgenic mice	59
Fig. 10.	<i>Wnt</i> and <i>Mfz</i> expression in the P7 and adult retina	61
Fig. 11.	<i>Sfrp-1</i> , <i>Sfrp-2</i> and <i>Sfrp-3</i> expression in the P7 and adult retina	62
Fig. 12.	Activation of the <i>TCF/Lef-LacZ</i> reporter transgene in response to Li^+ treatment in E14.5 retinal explants	74
Fig. 13.	Differential expression pattern of <i>TCF/Lef-LacZ</i> reporter gene, <i>Wnt2b</i> and <i>Otx1</i> along the dorsal-ventral axis of the optic vesicle	75
Fig. 14.	Expression of <i>TCF/Lef-LacZ</i> reporter gene in the developing eye	78
Fig. 15.	Analysis of the <i>TCF/Lef-LacZ</i> reporter positive cells located in the apical neuroblast layer	81
Fig. 16.	Expression pattern of <i>TCF/Lef</i> transcription factors in the optic	

vesicle	83
Fig. 17. Expression pattern of <i>TCF/Lef</i> transcription factors in the developing eyecup	84
Fig. 18. Asymmetric expression of <i>Lef1</i> and <i>TCF/Lef-LacZ</i> reporter gene in the temporal retina of the developing eye	85
Fig. 19. Activation of <i>TCF/Lef-LacZ</i> reporter gene in the postnatal and adult eye	87
Fig. 20. Cell type identification of the <i>TCF/Lef-LacZ</i> reporter positive cells in the adult retina	89
Fig. 21. Retinal explants represent a loss of function with respect of canonical Wnt signaling in the CM	97
Fig. 22. Detection of the HA tag and accumulation of β -catenin in Wnt2bHA-transfected cells	99
Fig. 23. Wnt2bHA or untagged Wnt2b construct fails to activate the TOPFlash reporter	101
Fig. 24. Detection of the HA protein tag in Wnt2bHA-transfected retinal explants	103
Fig. 25. Up regulation of <i>Bmp4</i> and <i>Lef1</i> expression induced by electroporation	104
Fig. 26. Activation of <i>TCF/Lef-LacZ</i> reporter gene in the CM in retinal explants electroporated with Wnt3a and cultured for 6 days	108
Fig. 27. Activation of <i>TCF/Lef-LacZ</i> reporter gene in the CM in retinal explants electroporated with Wnt3a and cultured for 10 days	109
Fig. 28. Constitutively active β -catenin activates <i>TCF/Lef-LacZ</i> reporter gene in retinal explants	111
Fig. 29. Li^+ treatment up regulates the expression of the <i>TCF/Lef-LacZ</i> reporter	

	gene and <i>Otx1</i> , and down regulates <i>Crx</i> and <i>Math3</i>	114
Fig. 30.	Quantitative RT-PCR analysis of gene expression in Li ⁺ -treated retinal explants	116
Fig. 31.	Short-term Li ⁺ treatment alters the expression pattern of Chx10 and Pax6	117
Fig. 32.	Effects of Li ⁺ treatment on nuclear morphology and proliferation	120
Fig. 33.	Li ⁺ treatment induces cell apoptosis	122
Fig. 34.	Long-term Li ⁺ treatment in retinal explants alters the expression pattern of Chx10 and Pax6	124
Fig. 35.	Persistence of S-phase cells and reduced formation of photoreceptor and amacrine cells in long-term Li ⁺ treated explants	125
Fig. 36.	Activation of canonical Wnt signaling in the peripheral retina of E14.5 β -catenin compound transgenic mice	135
Fig. 37.	Expansion of CM marker expression in the β -catenin-stabilized peripheral retina at E14.5	136
Fig. 38.	Loss of NR marker expression in the β -catenin-stabilized peripheral retina at E14.5	138
Fig. 39.	Changes in the generation of RGCs and formation of the hyaloid vasculature in the eyes expressing stabilized β -catenin	139
Fig. 40.	Activation of canonical Wnt signaling in the β -catenin-stabilized retina at E11.5	141
Fig. 41.	Stabilized β -catenin specifies a CM gene expression profile in the peripheral retina at E11.5	142
Fig. 42.	Stabilized β -catenin alters nuclei morphology with no effect on cell proliferation in the developing eye	144
Fig. 43.	Loss of <i>Chx10</i> and <i>Pax6</i> expression in the β -catenin stabilized region of the embryonic eye	146

Fig. 44.	Expression of stabilized β -catenin results in morphological changes in the eye of the postnatal mice	148
Fig. 45.	Activation of Wnt signaling induces CM/CE gene expression in the peripheral retina of the postnatal mice	149
Fig. 46.	Inhibition of neurogenesis in the peripheral retina of postnatal mice expressing stabilized β -catenin.	151
Fig. 47.	Changes in Wnt responding cells and persistence of dividing cells in the central retina of postnatal mice expressing stabilized β -catenin	153
Fig. 48.	Stabilized β -catenin reduces mature RGCs and amacrine cells in the central retina of the postnatal mice	154
Fig. 49.	Stabilization of β -catenin in the central retina resulted in non cell autonomous changes in bipolar and horizontal cells in the postnatal eye	155

LIST OF TABLES

Table 1.	<i>Wnt</i> transcript localization in the eye from E12.5 to E14.5	51
Table 2.	<i>Mfz</i> expression in the eye from E12.5 to E18.5	55
Table 3.	<i>Sfrp</i> expression in the developing eye and adult neural retina	57
Table 4.	Comparison of <i>TCF/Lef-LacZ</i> reporter gene activation and expression of <i>TCF/Lef</i> transcription factors during ocular development from E9.5 to E14.5	76
Table 5.	Activation of <i>TCF/Lef-LacZ</i> reporter gene in the developing retina from E15.5 to P7 and in the adult retina	79

1.0 Introduction

All of the neurons and glia in the adult central nervous system (CNS) are derived from immature neuroepithelial cells that line the embryonic neural tube in vertebrates. During CNS development these progenitor cells proliferate, diversify, migrate and establish synaptic connections that are required for normal brain function. These events are controlled by a well-orchestrated series of cell-cell interactions; however, in many instances these interactions, as well as the molecules that mediate them, have not been well characterized. The vertebrate retina is an excellent model system in which to study the role of signaling molecules in patterning and neuronal diversification, as it is very accessible and the differentiation of neuroepithelium-derived optic vesicle into the highly specified retina largely mirrors the developmental processes of the CNS. Moreover, the different retinal cell types are well characterized and can be identified with cell-type-specific markers and by their position within the retinal laminae. Among with other signaling molecules, Wnt signaling is frequently involved in CNS development, and also plays an essential role during mammalian retinal development.

1.1 Ocular development

1.1.1 Formation of the eyecup

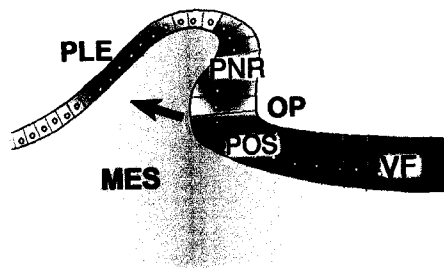
Vertebrate ocular development involves a series of inductive interactions between

neural ectoderm, surface ectoderm and periocular mesenchyme. Beginning at embryonic day (E) 8 to 8.5 in the mouse, the neuroepithelium of the diencephalon thickens and evaginates towards the surface ectoderm, forming the optic vesicles with the optic stalks connected to the forebrain (Fig.1) (125, 226). The optic vesicles grow laterally towards and eventually make contact with the surface ectoderm, inducing a thickening, called the lens placode in this tissue (reviewed by 188). At E10, the optic vesicle invaginates to form the bi-layered optic cup followed by the invagination of the lens placode at E11 to form the prospective lens (Fig.1). The outer layer of the optic cup, which is destined to become the retinal pigment epithelium (RPE), does not grow at this stage. The inner layer undergoes rapid proliferation to form multi-cellular layer that will give rise to the neural retina (NR) (125, 226). The invagination of the optic cup flips the retinal neuroepithelium to an opposite orientation of that in the CNS, with the apical surface of the epithelium oriented towards the prospective RPE. The space between the lens vesicle and the future NR is vitreous that will be filled with the vitreous humor at later stages (Fig.1). The optic stalk, which connects the optic cup to brain, remains open before E11. Later on, a groove forms at the inferior aspect of the optic cup and optic stalk, known as the embryonic fissure (39). Through the embryonic fissure, hyaloid artery enters the optic cup and branches anteriorly to connect with the anterior ciliary vessels. The embryonic fissure closes completely by late E12 (226). The interface between the optic stalk and the retina develops and gives rise to the optic disc, through which axons from developing retinal ganglion cells (RGCs) grow towards the presumptive optic nerve and exit the optic cup (reviewed by 114).

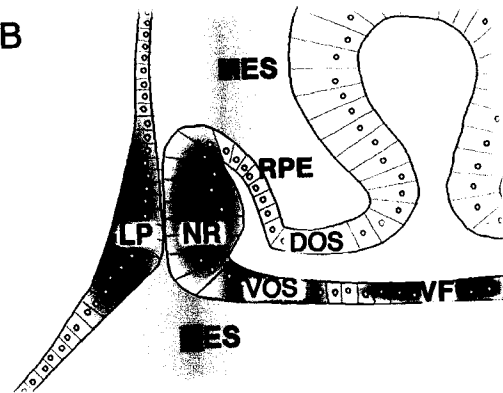
Fig. 1. Schematic diagram of mouse eye development.

(A) Formation of the optic vesicle is initiated by an evagination (arrow) of the presumptive forebrain region resulting in the formation of the optic pit (OP), which can be divided into dorsal-distal region (green), containing the presumptive NR (PNR) and RPE (not shown), and the proximal-ventral region that contains the presumptive ventral optic stalk (POS) (purple); PLE, presumptive lens ectoderm (blue); MES, mesenchyme (grey); VF, ventral forebrain (red). (B) Continued growth of the optic vesicle culminates with a period of close contact with the surface ectoderm during which important inductive signals likely exchange, resulting in the formation of lens placode (LP) and the presumptive NR: RPE, presumptive retinal pigmented epithelium (yellow); VOS, ventral optic stalk; DOS, dorsal optic stalk. (C) Invagination of the optic vesicle results in the formation of bi-layered optic cup and invagination of the lens placode gives rise to the lens vesicle (LV). The junction between the NR and RPE at the distal tip of the eyecup is the ciliary margin (CM) that will give rise to the epithelia of the ciliary body and iris. Surface ectoderm overlying the lens and migrating mesenchymal cells will form the cornea (c). (D) Mature eye: C, cornea; LE, lens epithelium; LF, lens fiber cells; I, iris; CB, ciliary body; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; ON, optic nerve; RPE, retinal pigment epithelium; V, vitreous. This figure is modified from (39).

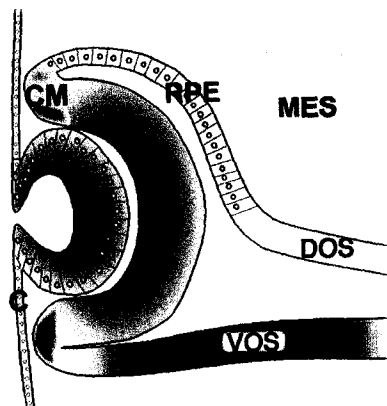
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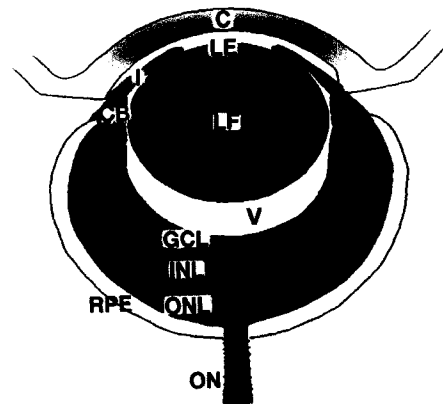
B



C



D



At the same time, cells from neural ectoderm, surface ectoderm and mesenchymal cells of migratory cranial paraxial mesoderm and neural crest contribute to the other ocular tissues, such as iris, ciliary body, cornea, conjunctiva, sclera and eyelid (reviewed by 114, 226).

1.1.2 Neural retina development

The vertebrate NR consists of six distinctive neuronal cell types (ganglion cells, amacrine cells, horizontal cells, bipolar cells, cones and rods) and one glial cell type (Müller glia) that are of neuroepithelial origin (Fig.2) (reviewed by 59). These retinal cells are organized into three cellular layers, separated by plexiform layers that contain axons and dendrites. The layers, from the apical to basal direction are the photoreceptor outer and inner segment layer, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and RGC layer (Fig.2).

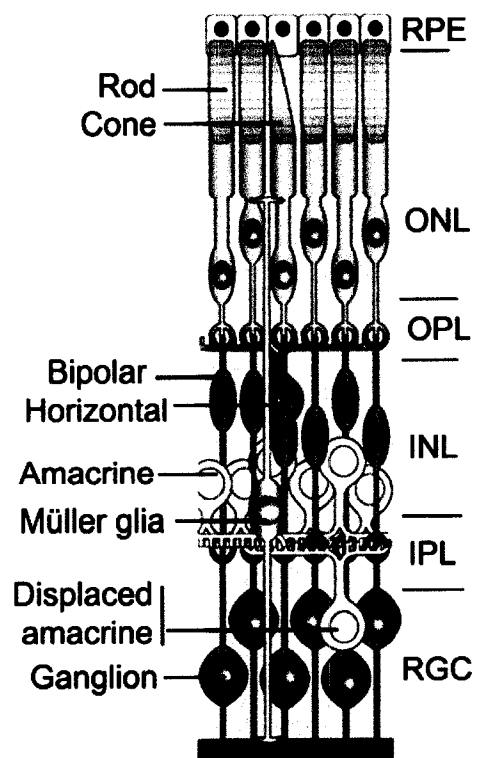
Development of the inner neuroepithelial layer of the optic cup into the well-laminated NR involves extensive proliferation, cell fate specification and differentiation events.

Growth of the neural retina

The inner neuroepithelial layer of the optic cup, the future NR, is composed of identical retinal progenitor cells (RPCs), with processes that extended from the apical to the basal surface. As in the CNS, the progenitor cell nuclei undergo interkinetic nuclear movement, traveling within the retinal neuroepithelium according to their cell-cycle phase;

Fig. 2. Structure of the vertebrate retina.

The seven main classes of cell types found in the vertebrate retina (rod and cone photoreceptors, bipolar cells, ganglion cells, amacrine cells, horizontal cells and Müller glia) are organized into three distinct cellular layers. Photoreceptor cell bodies make up the outer nuclear layer (ONL) of the retina. When stimulated, rods and cones transmit their signals through bipolar cells, whose cell bodies are found in the inner nuclear layer (INL), to the ganglion cells. From the ganglion cell layer (RGC), these signals make their way along the optic nerve to the brain. Horizontal and amacrine cells in the INL make lateral connections and modulate the direct signalling pathway from photoreceptors to ganglion cells. Amacrine cells also make up a significant proportion of the cells in the RGC and are referred to as displaced amacrine cells. Müller glial cell bodies lie in the centre of the INL and their processes span all three cellular layers of the retina. These specialized glial cells are believed to be involved in the protection and/or repair of retinal neurons. Processes of retinal cells form two intermediate histological layers, the outer plexiform (OPL) and the inner plexiform layer (IPL). This figure is modified from (59).



mitosis occurs near the apical surface and S phase occurs near the basal surface. Nuclei move basally during G1 and apically in the G2 phase of the cell cycle. After terminal division, postmitotic cells (G0 phase) migrate from the apical surface to locations appropriate to their fate (30, reviewed by 59, 203). In the early embryonic retina, RPCs divide to give rise to two progenitors, or one progenitor cell and one cell that will exit cell cycle, or two cells for further specification that are unable to divide again, providing increasing numbers of cells for the expanding neural retina (reviewed by 198). In the perinatal retina, however, the symmetric division in a plane horizontal to the RPE, tends to produce daughters that become the same cell types; whereas vertical division, in a plane perpendicular to the RPE, tends to produce daughters that become different (29). The symmetric or asymmetric distribution of Numb, which has been proposed as a cell-fate determinant (216), was shown to influence the plane of cell division and thus, the identity of progeny (29); however, this hypothesis was not supported by other studies (221). Considering the complexity of the nuclear movement and the plane of cell division, proliferation of the RPCs must be precisely regulated such that growth of the eyecup and generation of different cell types can be well balanced to give rise to the highly organized neural retina.

Retinal neurogenesis

Similar to other areas of the CNS, an essential feature of NR development is the highly conserved birth order of different types of retinal neurons and glia. RGCs, cone photoreceptors, horizontal cells and half of the amacrine cells are generated firstly, followed by rod photoreceptors, bipolar cells, Müller glia and the remaining amacrine cells generated

in a second wave of histogenesis, which extends into the postnatal period (281). Notably, many birthdating studies also indicate that there is considerable overlap in the times at which these cells are produced, in particular, the late born cell types (230, 280). Neuronal differentiation begins in central regions of the retina and propagates in a wave-like fashion towards the peripheral regions (105, 164). It is remarkable that the pattern of central to peripheral differentiation makes the periphery the least mature region (reviewed by 249), which is a distinct area in the retina in terms of development and function, and is most relevant to this study, as will be discussed later.

Lineage-tracing studies using retrovirus and direct injections of tracers into progenitor cells have shown that RPCs, like other CNS progenitors, are multipotent, in that the progeny of a single progenitor cell can differentiate into different cell types as diverse as neurons and glia (reviewed by 59). Meanwhile, heterochronic mixing and birthdating studies indicate that a particular progenitor cell can generate only a limited number of cell types at any given time (reviewed by 151). These findings, together with the strict birth order of retinal cells, led to the proposal of the competence model of retinal development. In this model, progenitors pass through a series of competence states, during each of which they are competent to produce a subset of retinal cell types (reviewed by 32). During this process, intrinsic cues and extrinsic molecules are well coordinated in that an intrinsic program controls competence stage of a progenitor, however, within a certain competence stage, extrinsic signals regulate generation of a particular type of retinal cell (reviewed by 151).

1.1.3 Formation of the non-neural derivatives, ciliary body and iris

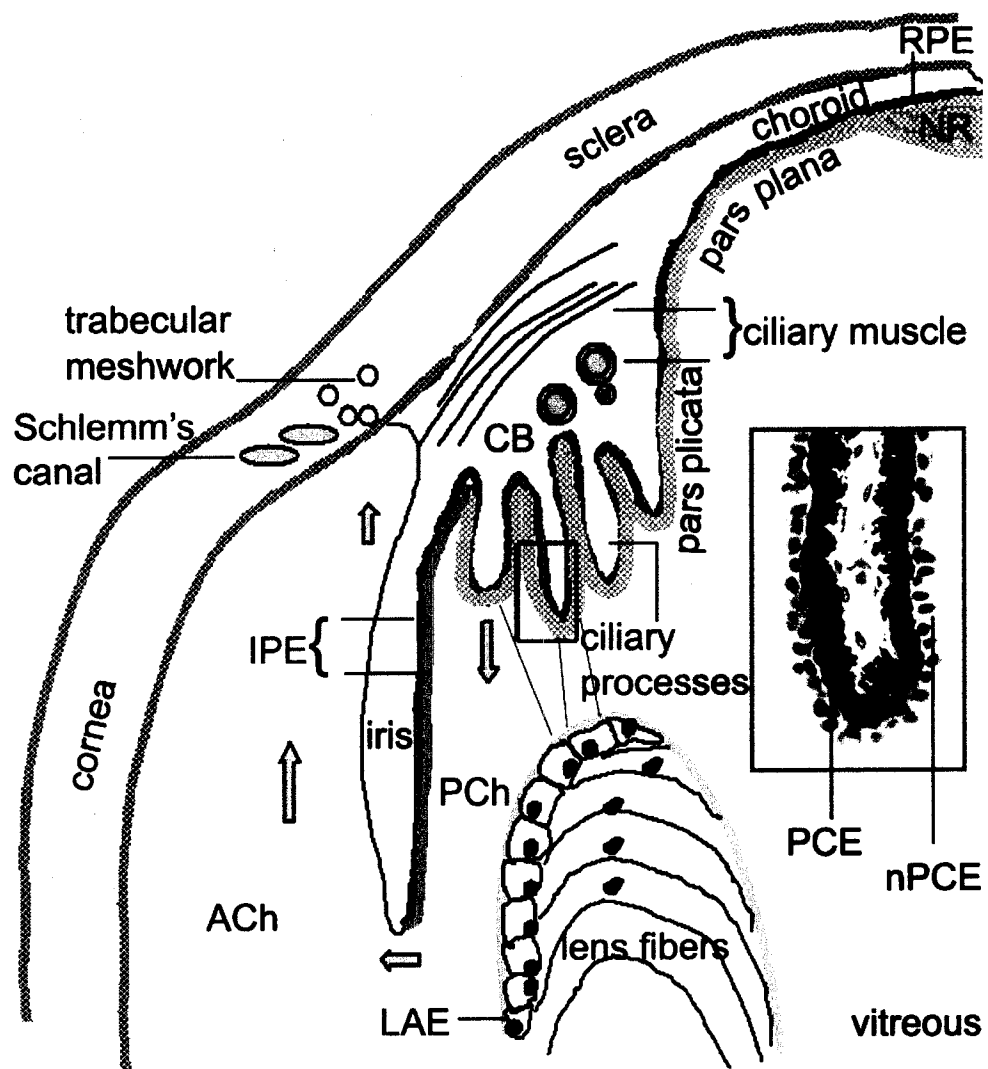
The optic cup becomes further compartmentalized as development proceeds, such that the ciliary margin (CM) located at the peripheral region of the optic cup, differentiates distally into the iris epithelium and more proximally into the epithelium of the ciliary body (CB) (124). Thus, although the CM is neuroepithelial in origin, it gives rise to non-neural derivatives. This represents one of the very rare situations of neural derived non-neural tissues in the vertebrates, the mechanism underlying which is mostly unknown.

Structure of the ciliary body and iris

The CB and iris are essential components of the anterior segment of the mammalian eye. Additional components of this structure include the cornea, lens, ocular drainage structures (trabecular meshwork and Schlemm's canal), as well as the anterior (space between the iris and cornea) and posterior (space between iris and lens) chambers (Fig.3). The anterior segment is critical for normal function of the eye and developmental defects of the tissues of this region results in eye diseases, such as glaucoma (70). To be consistent with the term of anterior and posterior chambers in the eye, I will describe the adult eye in an anterior/posterior orientation, which corresponds to the distal/proximal eye at embryonic stages. The CB can be subdivided into two regions, the posterior pars plana that is flat and the more anterior pars plicata that is folded into finger-like ciliary processes (Fig.3). The pars plicata consists of a stroma and an epithelium (reviewed by 11, and by 62). The stroma can be divided into two layers, the outer ciliary muscle and the inner vascular region. The ciliary epithelium (CE) consists of two epithelial layers, an outer pigmented ciliary epithelium (PCE) layer that is continuous with the RPE and an inner non-pigmented ciliary epithelium (nPCE)

Fig. 3 Schematic diagram of the anterior segment of the adult mammalian eye.

The insert shows a large view of the boxed area, the ciliary process. The anterior region of the ciliary body (CB), the pars plicata, is folded to form finger-like ciliary processes lined with double layered ciliary epithelium, the pigmented (PCE) (dark brown) and the non-pigmented epithelium (nPCE) (purple). The iris contains stroma (orange) and two layers of iris pigmented epithelial (IPE), the inner and outer IPE (light and dark brown), which are continuous with the ciliary epithelium, the neural retina (NR) and the retinal pigmented epithelium (RPE). Arrows indicate that aqueous humor secreted from the ciliary epithelium flows from the posterior chamber (PCh) into the anterior chamber (ACh) and ends at the iridocorneal angle where the iris and cornea meet. This figure is adapted and modified from (260, 261).



layer that is continuous with the NR (Fig. 3) (reviewed by 11, and by 223). The nPCE layer produces aqueous humor, which is secreted into posterior chamber and flows through the pupil into the anterior chamber to bathe and nourish the lens and cornea, and components of the vitreous (Fig. 3). The iris projects anteriorly from the CB, and runs radially around the anterior surface of the lens, with the pupil in the center of the iris. It consists of stroma and connective tissue anteriorly, and the iris muscle posteriorly, including the sphincter muscle and smooth muscle, which are formed from the double-layered iris pigment epithelium (IPE) (Fig. 3) (reviewed by 239). Dilation of the pupil through contraction of the iris muscle controls the amount of light admitted into the eye. The angle between the iris and the cornea is filled with a connective tissue, the trabecular meshwork, responsible for the drainage of the aqueous humor (Fig. 3). The stroma of the CB and iris are derived from mesenchymal cells of cranial paraxial mesoderm and neural crest (226).

Ciliary margin development

CM differentiates and becomes non-neural derivatives, the epithelia of the CB and iris (124). The most distal region of the CM gives rise to the IPE. More proximally, the inner layer of the CM gives rise to the nPCE and the outer pigmented layer of the CM gives rise to the PCE of the CB (reviewed by 239). CM is distinctive from the rest of the optic cup as early as E12.5 in terms of marker gene expression, such as *Msx1* (172), *Otx1* (162), *Tmsb4* and *Tgfbli4* (240), which are expressed in the CM but excluded from the remainder of the optic cup. From about E14.5, the CM elongates and extends distally, providing a support for the migrating periocular mesenchymal cells that will form the stroma of iris and CB

(reviewed by 82). The morphological change of the CM makes it easily recognized from the rest of the NR by E16.5. By postnatal day (P) 2 to P4, the iris and CB are clearly distinguishable from each other (reviewed by 82). The developmental process to give rise to iris and CB is complete by the third week after birth in mouse (reviewed by 239). The exact developmental stage when nPCE of the CB starts to produce aqueous humor is still unclear; however, it is likely that nPCE is functional and transporting fluid before it can be morphologically identified (reviewed by 107), that is to say, before E16.5 in mouse.

Studies in chick and mammals have provided pieces of evidence for a role of the lens in inducing the formation of CB from the CM (reviewed by 107), while the CB, through its secreted molecules, is involved in coordinating the growth and morphogenesis of the optic cup, retinal development and maintenance of the functional eye (reviewed by 11, 85, 86).

Comparison of CMZ and CB

In lower vertebrates, including fish and amphibians, the eyes grow throughout life (118, 231) with the new cells generated from specific germinal zones; the lens equator for lens growth and the ciliary marginal zone (CMZ) (also called the *ora serrata*) for retinal growth (reviewed by 89). The CMZ is a circumferential zone of undifferentiated cells, located at the distal tip of the retina. Thus the CMZ in lower vertebrates, due to its extreme peripheral position, is topologically analogous to the CB of the eye in mammals (reviewed by 190). Cell lineage analysis has identified multipotent precursors in the CMZ (266). BrdU (bromodeoxyuridine) or tritiated thymidine labeling studies have revealed that the postmitotic descendants of the CMZ remain in place while the later generated cells are added

to the retina from the edge, forming a ring of retinal cells generated during the labeling period, like the pattern of tree rings (reviewed by 89). Moreover, there appear to be two populations of cells at the CMZ that incorporate thymidine, a fast-cycling population and a slow-cycling one (198). Gene expression profiling further demonstrated that the CMZ can be divided into several zones, from distal to proximal: the deep stem cell zone, the mitotic retinoblast zone and the postmitotic undifferentiated cell zone, representing a spatial recapitulation of retinal development (reviewed by 89). Studies on posthatch chick also discovered progenitors at the retinal margin that resemble the CMZ cells of fish and amphibians in that they proliferate and produce neurons that are integrated into the peripheral edge of the retina (64). A comparative study of several other species of vertebrates, ranging from quail to opossum and mouse, suggested that the CMZ cells in the postnatal retina have gradually diminished during vertebrate evolution (137). The quail has a reduced CMZ, compared with chicken, while the opossum has only a few cells likely related to the CMZ and there is no evidence of CMZ cells at the margin of the mouse retina (137).

Although the adult rodent retina does not appear to have a CMZ that is actively contributing new neurons to the retina, as is the case in lower vertebrates, the PCE of the CB in adult rodent, monkey and human eye contains cells that are capable of clonal growth in sphere cultures and differentiation into retinal neurons (4, 62, 243). Similarly, the nPCE of the chicken CB, which is a distinct anatomical structure to the chick CMZ, has the capacity to generate retinal neurons (63). These studies indicate that there has been, to some degree, an evolutionary conservation in the capacity of the CMZ and CB to support stem cells in the

vertebrate eye; however, this function is severely attenuated at the higher end of the evolutionary scale.

1.2 Transcription factors and signaling molecules controlling eye development

Morphogenesis of the vertebrate eye parallels with the CNS development chronologically and employs similar regulatory machinery in modulating developmental events, which consists of early eye field specification, initial extensive proliferation of retinal progenitors and subsequent neurogenesis/gliogenesis. These processes are under precise temporal and spatial control coordinated by both intrinsic determinants, such as transcription factors and extrinsic cues, including secreted signaling molecules.

1.2.1 Transcription factors

Control of retinal development at the transcriptional level involves both homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors. The HD and bHLH factors that are most relevant to the present study will be highlighted below. The Pax6 HD transcription factor is a member of the large family of PAX proteins that each contains two DNA-binding motifs, a paired box and a paired-like homeobox (reviewed by 39). Pax6 is the vertebrate homologue of *Drosophila* eyeless (*ey*), which derives its name from the “eyeless” phenotype that is caused by eye-specific, loss-of-function alleles of the *ey* gene in flies (reviewed by 39). Loss- and gain-of-function phenotypes have suggested that Pax6/*ey* is one of the most important and evolutionarily conserved transcription factors in multiple aspects of eye development in both vertebrates and invertebrates (reviewed by 192, and by

222). In vertebrates, *Pax6* expression is first detected in the anterior neural tube, the future eye domain; however, *Pax6* is not required for optic vesicle outgrowth or specification, as optic vesicles form in *Pax6* null mutants (98). Later on, *Pax6* is expressed in RPCs throughout the developing optic cup and is required within retinal progenitors for the maintenance of the multipotential state to generate all retinal cell types, as retina-specific ablation of *Pax6* results in the exclusive production of amacrine cells (161, 255). In the adult eye, *Pax6* expression is maintained in the CM-derived epithelia of the CB and iris (64, 115) and in three types of retinal neurons: RGC, amacrine and horizontal cells (101, 195, 255).

Chx10 and its additional vertebrate orthologues, *Vsx2* and *Alx1*, are members of the paired-like HD class of homeobox genes. *Chx10* is among the earliest and most specific markers of vertebrate RPCs. From early stage of optic cup formation throughout retinal development, *Chx10* expression is maintained in the RPCs of the NR, including the adjacent proximal CM (207). In the adult retina, *Chx10* is expressed in all bipolar neurons and possibly in Müller glia as well (13, 25, 206). *Chx10* may regulate proliferation in RPCs (reviewed by 144), but this effect seems to be restricted to the early retinogenesis, as *Chx10* has been shown dispensable for late-stage RPC proliferation (152). *Chx10* also appears to be absolutely required for differentiation of bipolar cells (reviewed by 144) and has been suggested to inhibit rod differentiation (55, 152). Several other transcription factors that pattern the anterior neural plate of the early vertebrate embryo, including *Rx/Rax*, *Six3* and *Lhx2*, are also essential for normal eye formation. See (39, 144) for a detailed review.

Control of retinal development, in particular retinal cell fate determination, also

involves the antagonistic effect of multiple bHLH transcriptional activators and repressors. bHLH repressors, such as *Hes1* and *Hes5*, homologues of *Drosophila Hairy and Enhancer of Split* genes, are expressed in RPCs and promote maintenance of progenitors in the embryonic retina and generation of glial cells in the postnatal retina (reviewed by 31, and by 90). bHLH activators, such as *Xash/Cash/Mash1*, orthologues of *Drosophila acheate-scute*, and *Xath5/Math3, 5*, *NeuroD* and *Neurogenin (Ngn)*, orthologues of *Drosophila atonal*, are generally expressed in a salt-and-pepper like pattern in sub-sets of RPCs (reviewed by 31, and by 160). bHLH activators override activities of bHLH repressors and promote neuronal differentiation (reviewed by 90), thus are also referred to as proneural genes or neurogenic transcription factors (reviewed by 160, and by 189). The prevailing idea is that bHLH factors work in combination with HD transcription factors to specify retinal cell fate (reviewed by 90, and by 160). In the developing eyecup, the first genes expressed are usually the HD transcription factors, which regulate the layer specificity, while the bHLH factors are followed by and involved in specifying the neuronal fate within the HD factor-defined layers (reviewed by 90). Moreover, the expression of some bHLH activators is maintained in differentiated retinal cells, although their function in mature neurons is unknown (reviewed by 189).

1.2.2 Signaling molecules

In addition to the intrinsic cues, such as determined by the above transcription factors, intercellular communication mediated by extrinsic signaling factors is also crucial for normal patterning during development of both vertebrate and invertebrate eyes. The most studied

signaling pathways include those mediated by diffusible molecules, such as Hedgehog, Transforming growth factor (TGF)- β /bone morphogenic protein (BMP) and Wnt, as well as the Notch receptor pathway and receptor tyrosine kinase mediated pathways. Not surprisingly, all of these signaling systems appear to play a role in patterning the vertebrate eyecup and retinal precursor cell proliferation and differentiation. Due to space limitations, hereafter I will focus on Wnt signaling and on the possible roles of a few pathways in peripheral eye development; however, it is clear that other pathways are playing important roles in retinal histogenesis.

Signaling molecules involved in the development of CM and its derivatives

A number of signaling pathways have been suggested for an important role in the development of the CM of the eyecup into the epithelia of the CB and iris. BMPs, members of the TGF- β super family have been implicated in CB development (reviewed by 278). Lens-specific expression of Noggin, a BMP antagonist, can block BMP signaling and inhibits formation of the CB, while co-expression of transgenic BMP7 restores normal development of the CE, indicating that BMP signaling is critical for CB formation in the murine eye (283). In chick retina, *BMP4* and *BMP7* are expressed at an appropriate time and place to regulate not only ciliary epithelium but also iris muscle development (115). Expression of several components of another signaling pathway, the Notch receptor pathway, has also been shown in the anterior segment of the eye. Notch receptor binding with its membrane-linked ligands of the Delta/Serrate/Lag2 family activates the Notch receptor pathway and up regulates the expression of target genes, such as *Hes1/Hes5*, which then

negatively regulate the expression of proneural bHLH transcription factors and inhibit neuronal differentiation (reviewed by 142). Notch2 is expressed in the non-neuronal derivatives of the optic cup, including the RPE, optic stalk, and CB (9). The homologue of the *Delta* ligand, *Jagged*, is expressed in distinct regions within the optic vesicle, CB, and lens, with patterns that change over time (9). Although Notch pathway members are expressed in the CB, the functional significance is still unknown. Indeed, there has been no reported study that targets this pathway in the eye while specifically examining the CB.

Of particular interest, the role of Wnt signaling in the context of CM/CB development will be discussed in section 1.4.3.

1.3 Wnt signaling

Among with other signaling pathways, Wnt signaling has also been well recognized as a key player in a number of important cellular and biological processes in vertebrate and invertebrate development. The first *Wnt* gene, *Wnt1* (named from *wingless*, the *Drosophila* homologue and *int-1*, the gene name when it was firstly described in mouse), was discovered in 1982 as a proto-oncogene that was activated by integration of mouse mammary tumour virus in mammary tumours (180). The potential involvement of *Wnt* genes in cancer formation was the primary focus of Wnt research in the 1980s (reviewed by 181). Soon after, the *Drosophila* segment polarity gene *wingless* (*wg*) was identified as the orthologue of *Wnt1* (27, 202) and the phenotypic analysis of *Wnt1* mutations in the mouse (166, 238) started a new page of Wnt study in development. After two decades of extensive study in various

organisms, it has been well recognized that Wnt proteins play key roles as intercellular signals to regulate cell proliferation and cell fate decisions, cell polarity and movement, and programmed cell death during development (reviewed by 40, and by 271). Wnts also play roles in regulation of self-renewal of stem cells (reviewed by 200) and in adult tissue (reviewed by 40, and by 169). Mutations of downstream components of the Wnt pathway have been implicated in tumorigenesis and human genetic diseases (reviewed by 12), including a variety of ocular diseases, such as familial exudative vitreoretinopathy (FEVR), retinal degenerations, cataract, ocular tumors and various congenital ocular malformations (reviewed by 49).

1.3.1 *Wnt* genes and Wnt proteins

Since the discovery of the first vertebrate *Wnt* gene, close to 100 *Wnts* have been identified from a wide range of vertebrate and invertebrate species. At this moment, 19 *Wnt* genes have been identified in mammals (view a comprehensive list of Wnt genes on 262). *Wnt* genes encode a large family of secreted cysteine-rich glycoproteins that are generally about 39-46kD in size (reviewed by 169). The most remarkable feature of Wnt proteins is the high conservation of 22-24 cysteine residues spaced in a nearly invariant pattern, which is the most important criteria for assigning a new gene as a Wnt (reviewed by 169, and by 181). Wnt precursor proteins mostly contain a hydrophobic N-terminus that serves as a signal peptide and targets precursors to the secretory pathway (reviewed by 181, 218). It has been suggested that the N-terminal region of the Wnt protein may mediate interaction with Wnt receptors but requires the C-terminus to activate these receptors (reviewed by 169). Wnt

proteins are notoriously insoluble and usually accumulate on the cell surface or extracellular matrix when expressed in cell lines, making it difficult to produce soluble and biologically active Wnt proteins (20, 26, 186). Only in rare instances, such as Wnt1/wg (21, 199, 247) and Wnt3a (217, 269), are relatively large amounts of Wnt protein secreted into the culture medium. Another remarkable feature of Wnt proteins is the palmitoylation on a conserved cysteine residues, firstly identified in Wnt3a (269). Palmitoylation is required for secretion of *Drosophila* Wg (but not mouse Wnt3a) and also essential for the signaling activity of mouse Wnt3a (170), as mutation of palmitoylation site of Wnt3a does not interfere with the folding and secretion of this protein, but does interfere with its biological activity (269).

Wnt proteins can be defined into distinct subgroups by assaying their biological activities. The most convenient and informative method, the *Xenopus* axis duplication assay, has divided Wnts into two distinct classes, the Xwnt-8 class (among them Wnt-1) that are active in the axis duplication assay and the Xwnt-5a class that are not (reviewed by 271). Two functional classes of Wnt proteins can also be distinguished when assayed for their ability to transform mammary epithelial cells. One class, including Wnt1, 2, 3, 3a and 7a are strong transformers, and the other class, including Wnt4, 5a, 5b, 6, 7b and 11 do not transform cells (reviewed by 224, and by 271). Notably, the classes as defined by the two assays described above are similar; moreover, axis duplication caused by the Wnt1/Wnt8 subgroup has been shown to depend on canonical Wnt/ β -catenin signaling (56), the pathway that will be discussed later.

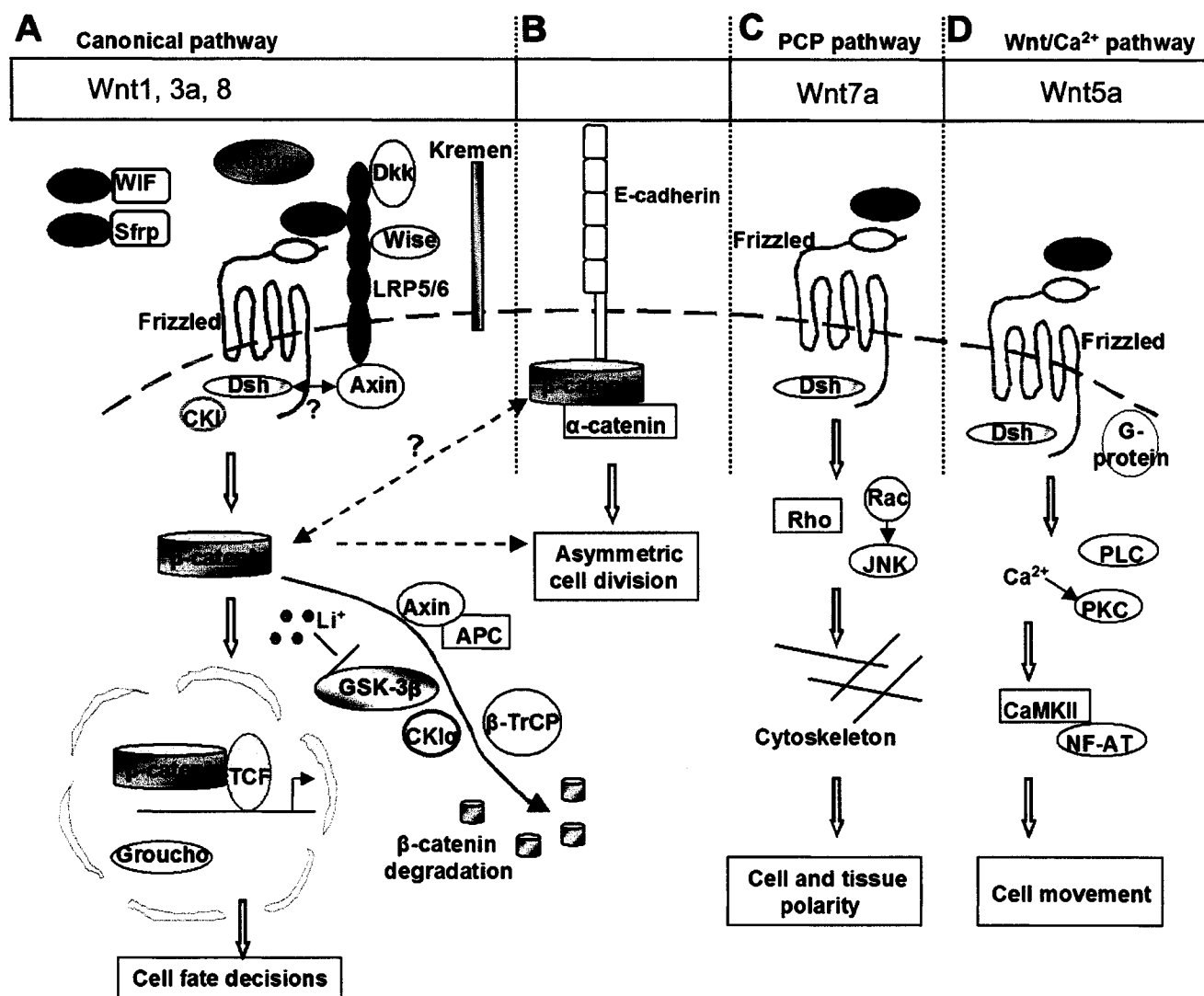
1.3.2 Wnt signaling pathway

Wnt ligands signal through the Frizzled receptors and activate a number of distinct intracellular signaling cascades: [1] The β -catenin dependent canonical Wnt pathway (known as wingless or wg signaling pathway in *Drosophila*), which activates target genes in the nucleus and primarily regulates cell fate determination during development; [2] The planar cell polarity (PCP) pathway, which involves Jun-N-terminal kinase (JNK) and cytoskeletal rearrangements; [3] The Wnt/ Ca^{2+} pathways, which involve activation of phospholipase C (PLC) and protein kinase C (PKC) and [4] A pathway that regulates spindle orientation and asymmetric cell division (Fig.4) (reviewed by 40, and by 106, and by 132). In contrast to the canonical Wnt pathway, the majority of research on the remaining non-canonical Wnt signaling pathways, has implicated activation of these pathways in cell behaviour but not cell fate decisions (reviewed by 250).

In the canonical Wnt pathway (Fig.4), Wnt binding Frizzled receptors and its co-receptor LRP5/6, members of the low-density lipoprotein receptor-related protein family (LRP) (193, 235), activates Dishevelled protein and Casein kinase I ϵ , resulting in the stabilization and accumulation of cytoplasmic β -catenin (reviewed by 106, and by 154). β -catenin translocates to the nucleus and associates with TCF/Lef (T cell specific transcription factor and lymphoid enhancer-binding factor) transcription factors (94, 245) to activate TCF/Lef-dependent gene transcription. Recently, a variety of coactivators and corepressors, such as cAMP response element binding protein (CBP)-p300, and other molecules involved in chromatin-remodelling, have been identified to modulate the transcription of Wnt/ β -

Fig.4. Schematic diagram of the main branches of Wnt signaling pathway.

The box shows members of the Wnt family that belong to each signaling branch (see details in text on P. 19-20). (A) In the canonical Wnt/ β -catenin pathway, binding of Wnt to the Frizzled and LRP5/6 receptor complex activates Dishevelled (Dsh), resulting in the stabilization of β -catenin, which is otherwise kept at low level in the cytoplasm by the degradation complex including Axin, APC, Gsk-3 β and CKI α . Stabilized β -catenin accumulates and translocates into nucleus and binds with TCF/Lef transcription factor to activate target gene expression and regulate cell fate decisions. Extracellular antagonists of Wnt ligand include WIF and Sfrp that associate with Wnt directly, and Dkk and Wise that act through binding with coreceptor LRP5/6. Lithium (Li⁺) can inhibit Gsk-3 β activity and thus is an agonist of this pathway. Norrin, an additional ligand for Frizzled and LRP5/6 receptor complex, can activate the canonical Wnt pathway as well. See text for more details. (B) β -catenin binds with α -catenin and E-cadherin, the important components of the cell adhesion complex, and is involved in asymmetric cell division, which might also be linked with a divergent canonical Wnt pathway. Switching between the two roles of β -catenin can be regulated by tyrosine phosphorylation. (C) Planar cell polarity (PCP) pathway signals through Frizzled to activate Dsh. PCP pathway involves Rho/Rac GTPases and JNK, inducing changes in cytoskeleton organization and primarily regulate cell and tissue polarity. (D) Wnt/Ca²⁺ pathways also signal through Frizzled to activate Dsh, inducing the release of Ca²⁺ and activation of PKC or PLC. Wnt/Ca²⁺ pathways are primarily implicated in cell movement. This figure is adapted from (40, 106, 154, 169, 267).



catenin target genes (reviewed by 22). In the absence of Wnt signaling, β -catenin is present in a protein complex containing Axin and adenomatous polyposis coli (APC). The protein complex functions as a scaffold for glycogen synthesis kinase (GSK)-3 β and Casein kinase I α , which phosphorylate β -catenin at the N-terminal serine-threonine residues (reviewed by 22, and by 154, and by 267). Phosphorylated β -catenin is ubiquitinated and associated with the F-box protein β -TrCP, a component of an E3 ubiquitin ligase complex (reviewed by 22, and by 154). The ubiquitin tag marks β -catenin for subsequent degradation by the proteasome. Controlling levels of β -catenin is essential in the transduction of canonical Wnt signalling, and thus increasing β -catenin by whatever method, such as by inhibiting negative regulators or introducing activating mutations in β -catenin, is sufficient in most cases to activate this pathway (reviewed by 22, and by 154). In this regard, more details will be discussed in section 1.3.3.

In addition to Wnt ligands, Frizzled can also interact with another ligand, Norrin. Norrin is a secreted protein that has no discernable sequence similarity to the Wnts; however, it binds with high affinity to the Frizzled4 cysteine rich domain (CRD) (275). Together with LRP, Frizzled4 and Norrin can activate the canonical Wnt pathway (275), indicating that ligands unrelated to Wnts can act through Frizzled receptors, thereby bringing up the issue of the complexity of the system. Mutations in the Norrie disease gene (*NDP*), which encodes Norrin, are the cause of several types of congenital blindness in humans, including the Norrie disease and FEVR (15).

1.3.3 Components of the Wnt signaling pathway

Some components of the Wnt signaling pathway will be discussed below with particular emphasis on those involved in canonical pathway activation, including Wnt receptors/coreceptors, β -catenin, TCF/Lef transcription factors and Wnt antagonists.

Receptors and coreceptors

Wnt receptors, encoded by members of the *Frizzled* gene family, are 7-transmembrane-domain proteins (17, 252, 258). Twelve *Frizzled* genes that belong to the *Frizzled* gene family, have been discovered in mouse (262). Frizzled proteins harbour a long N-terminal extracellular extension, containing 10 highly conserved cysteine residues, the CRD, which is the site of interaction between Wnt and Frizzled proteins (reviewed by 271). There is evidence that an individual Frizzled receptor can have more than one physiological ligand, and a Wnt may bind to more than one type of Frizzled receptor (reviewed by 271). To date, it is still difficult to measure ligand-receptor binding specificities owing to the difficulty to purify Wnt proteins (reviewed by 267). There is another type of transmembrane Wnt receptor, Derailed, which is a tyrosine kinase (reviewed by 154). Although Derailed is entirely distinct from the Frizzled protein, it can bind to DWnt5 and is likely involved in axon guidance in *Drosophila* CNS (reviewed by 154).

Vertebrate LRP5/6 (193, 235), as well as the *Drosophila* homologue, arrow (265), are single-pass transmembrane molecules of the LRP family. It has been proposed that Wnt molecules bind to LRP5/6 and Frizzled to form a receptor trimeric complex (Fig. 4) (reviewed by 154). LRP5/6 appears to be specific to the canonical Wnt/ β -catenin signaling,

whereas Frizzled is used in both the canonical and non canonical Wnt pathways (reviewed by 40, and by 284).

β -catenin

β -catenin is an essential mediator of the canonical Wnt signaling. An elevation of cytoplasmic β -catenin levels is a hallmark of canonical Wnt pathway activation and thus manipulation of Wnt signaling components to stabilize β -catenin is the most widely utilized approach to activate this pathway (reviewed by 22, and by 106). The central domain of β -catenin consists of 12 armadillo repeats (arm, the *Drosophila* homologue of β -catenin) (131) that interact with APC, TCF and E-cadherin respectively (reviewed by 270). The N-terminal serine-threonine residues can be phosphorylated by GSK-3 β , leading β -catenin to degradation (Fig. 4) (reviewed by 22), and thus are critical for regulating the stability of β -catenin. Mutation of these residues allows β -catenin to escape modification by GSK3 β degradation complex and enter the nucleus, resulting in constitutive transactivation of targets (reviewed by 22, and by 154). The activity of GSK-3 β can be inhibited pharmacologically with lithium (Li⁺) (Fig. 4) (130), which leads to stabilization of β -catenin and activation of Wnt/ β -catenin signaling in various cellular contexts (16, 34, 95, 129, 228). Regulation of β -catenin likely also involves another mechanism, in which reception of a Wnt signal triggers the recruitment of Axin to the plasma membrane, thus removing Axin from the destruction complex to promote β -catenin stability (154). Notably, miscontrolled Wnt signaling, for example, through mutations causing activated β -catenin or inactivated APC, is often associated with tumours (reviewed by 76). Approximately 90% of familial and sporadic

colorectal cancer is marked by elevated levels of β -catenin (reviewed by 22). The C-terminus of β -catenin functions as a transcriptional activation domain (reviewed by 270). It is thus possible to block β -catenin signaling with truncated β -catenin, in which the N- and C-terminal domains required for coactivation of transcription are deleted, leading to dominant negative β -catenin (229).

In addition to its role in Wnt signaling, β -catenin is also an important component of cell adhesion through associating with E-cadherin and α -catenin at the plasma membrane (Fig. 4) (reviewed by 22, and by 176). The ability of β -catenin to participate in both transcriptional signaling and cell adhesion is likely the result of β -catenin interacting with a variety of proteins in a mutually exclusive fashion, thus giving rise to pools of β -catenin molecules that are restricted to a single process (reviewed by 270). It is also likely that two different molecular forms of β -catenin act in adhesion and transcriptional function respectively (81). Recently, increasing evidence has shown that there are likely some direct connections between these two pathways. Two tyrosine residues in β -catenin are essential in regulating its function in cell adhesion, and phosphorylation of these two residues leads to the disassembly of the adhesion complex, resulting in increased β -catenin dependent transcription (reviewed by 22, 176). So far evidence of whether an activated Wnt/ β -catenin pathway has an effect on cell adhesion is still lacking.

TCF/Lef transcription factors

TCF/Lef transcription factors have been identified in a number of vertebrate species, including frog, fish and chicken (52, 54, 126, 128, 251, 279). Four members of TCF/Lef

family have been identified in mammals, including Tcf1, Lef1, Tcf3 and Tcf4 (134, 242, 246). Generally, TCF/Lef transcription factors contain an N-terminal β -catenin interaction domain and a C-terminal DNA-binding domain, the high mobility group (HMG box) (reviewed by 156). There is an additional domain, termed the Groucho binding domain, that is located upstream of the HMG box and encoded by an alternatively spliced exon in some of the TCF/Lef factors (reviewed by 12). TCF/Lef acts as a transcriptional repressor when bound to transcriptional corepressors, such as CBP or members of the Groucho family, whereas TCF/Lef transactivates targets when association of β -catenin converts it into an activator (reviewed by 12, and by 106). The repressive effect of Groucho is mediated by interaction with histone deacetylases, and removal of this repression involves displacement of Groucho and recruitment of histone acetylase, as well as components of a chromatin remodelling complex (reviewed by 154). A TCF/Lef responsive element, the TCF/Lef consensus motif (CCTTTGATC), has been identified in Wnt target genes (75, 246) and has been used to drive reporter transgene expression in mice and fish (46, 53, 97, 159, 171).

Antagonists

Wnt antagonists, such as members of the secreted-frizzled-related protein (Sfrp) family, regulate Wnt signaling at the extracellular level. Identical to that of Frizzled proteins, Sfrps contain CRDs, through which Sfrps bind with Wnts and prevent them from interacting with Frizzled (reviewed by 271). Six Sfrps have been identified in mouse (262). The *Xenopus* axis duplication assay has uncovered some specificity between Sfrps and Wnts (reviewed by 271). Sfrp1 and Sfrp3 block the activities of Wg, Wnt1 and 8, which are

members of the Wnt1 functional class; however, they do not efficiently inhibit Wnt3a, which also belongs to the Wnt1 class (reviewed by 23). Very recently, Sfrp1 and 2, but not Sfrp3, have been shown to inhibit Wnt3a activity in different assays in cultured cells and in chick (73).

In addition to Sfrps, there are other secreted proteins that function as Wnt inhibitors, including Wnt-inhibitory factor (WIF)-1 (104), Cerberus (191), Wise (112) and Dickkopf (Dkk) (77). WIF-1 and Cerberus are thought to antagonize Wnt function, similar to Sfrps, by preventing their interaction with Frizzled receptors (reviewed by 169). Dkks do not bind Wnts, instead they form a ternary complex with Dkk receptor Kremen (Fig. 4) (158) and interact with the extracellular domain of LRP5/6 coreceptors, thereby blocking activation of Wnt signaling at the level of the cell membrane (8, 215). As LRP5/6 seem to be specific to the canonical Wnt/ β -catenin signaling pathway, Dkks are thought as pure antagonists for this pathway and may play an important role in determining which of the Wnt pathways is activated in response to a Wnt signal (reviewed by 284).

1.4 Gene expression and function of the Wnt signaling pathway in the eye

Wnt signaling molecules play an important role in the development of many regions of the vertebrate embryo, in particular, the CNS. In the CNS, Wnts function in a variety of cellular and developmental processes, including cell cycle control, cell fate specification, proliferation effect, patterning of the CNS and regulation of neuronal connectivity (reviewed

by 40, and by 208). Not surprisingly, development of the ocular system, which belongs to the CNS, also involves Wnt signaling.

1.4.1 Components of the Wnt signaling pathway are expressed in vertebrate eye

The expression of a number of Wnt receptor, *Frizzled* and Wnt antagonist, *Sfrp* genes in the frog, chick and mouse eye has been reported (reviewed by 248, 249). In the murine eye, mouse *Frizzled* (*Mfz*) 3, 4, 6, 7 and *Sfrp1*, 2, 3 genes were expressed in a dynamic fashion in the NR, CM and RPE throughout retinal development, and in the postnatal and adult retina (148). Expression of several *Wnt* genes was detected in the mouse and chicken eye (reviewed by 249). *Wnt2b* (formerly known as *Wnt13*) is expressed in the RPE overlying CM in both of the mouse and chick eye, and in the lens epithelium in chick (113, 135, 148). These observations suggest a role for the Wnt signaling pathway in the development of the retina as well as in the lens of the vertebrate eye. Moreover, members of the *TCF/Lef* transcription factor family are expressed in the developing vertebrate eye (135, 149, 205, 251). *TCF/Lef-LacZ*, the canonical Wnt reporter transgene is activated in a dynamic pattern in several regions of the developing optic cup and in the adult eye (148, 149), indicating multiple roles of this β -catenin dependent pathway in the mammalian eye.

1.4.2 Aberrant Wnt function is associated with eye diseases

Mutations in the Wnt signaling pathways have been associated to various ocular diseases, such as FEVR, retinal degenerations, cataract, ocular tumours and congenital ocular malformations (49). Only a few of the diseases that the role of Wnt signaling is most characterized are discussed below.

Mutations in Frizzled (Fzd) 4 have been found in patients with FEVR, which is characterized by disruption of retinal angiogenesis, causing a failure of peripheral retinal vascularization, retinal detachment and microphthalmia (49). It has been reported that Fzd4 likely acts through Wnt/Ca²⁺ signaling pathway to play a role in retinal angiogenesis (204); however, the Fzd4-null mice develop progressive cerebella and auditory dysfunction, but no eye phenotype has been reported (257). Altered regulation of Wnt function has also been implicated in retinitis pigmentosa, a disease characterized by the progressive death of photoreceptors due to apoptosis, as expression level of *Sfrp1*, 2, 3 and 5 has been found to be up regulated in retinitis pigmentosa patients (119, 120). Consistent with the possibility of aberrant Wnt signaling in retinal degeneration, *Mfrp*, a member of the mouse *Sfrp* family, is expressed in the RPE and CE, and mutated in the mouse retinal degeneration 6 (*rd6*) (121).

Mutation in LRP5, a Wnt coreceptor, causes osteoporosis-pseudoglioma syndrome (OPPG) (80), an autosomal recessive disorder in human. OPPG patients display ocular pathology, which is thought to be caused by a failed regression of the primary vitreal vasculature during fetal growth (79). Consistent with this finding in humans, Wnt7b, presumably through the canonical Wnt pathway, mediates macrophage-induced apoptosis in patterning the vasculature in mouse retina (153). LRP5 or Lef1 mutant mice display persistent embryonic eye vascularization as a consequence of reduced vascular endothelial cell apoptosis (123, 153). All together, these studies support a critical role of Wnt canonical signaling in retinal vascularization.

1.4.3 Wnt function in ocular structure formation and development of the neural retina

Recently, functional studies carried out in a number of model species have revealed multiple roles of canonical Wnt signaling in the context of eye development, such as eye field formation (28, 61), induction of retinal lamination (175), lens development (193, 225, 232) and transition of retinal progenitor cells to neural precursors (248).

Activation of Wnt canonical signaling has been shown to inhibit retinal differentiation. Expression of several mature NR markers is down regulated in the presence of cWnt2b in chick retinal explants (135, 175). This effect was proposed to act through the β -catenin dependent canonical Wnt pathway based on the ability of cWnt2b to stabilize β -catenin in culture (135). Kubo et al have reported that a dominant negative Lef1 induces the differentiation of premature RGCs (135). Moreover, it was reported that inhibition of RPC differentiation by cWnt2b is Notch independent, but involves downregulation of proneural genes (136). In keeping with this, it has been shown that activated canonical Wnt signaling reduces cell differentiation associated with downregulation of proneural gene expression in the mouse retina (Chapter 5.0 and 6.0 in this thesis).

The canonical Wnt pathway likely also has an effect on precursor proliferation; but it is not consistent across species. cWnt2b was shown to promote proliferation of the CMZ-derived progenitor cells in culture (135) and induce continuous growth of the retinal explants (136). Blocking canonical Wnt signaling reduces cell proliferation in *Xenopus* retina (248). However, studies from another research group and this thesis do not support a role of Wnt canonical pathway in promoting cell proliferation (38) (Chapter 5.0 and 6.0).

1.4.4 Wnts role in the CM and its non-neural derivatives, CB/iris

In particular, gene expression patterns suggest that Wnt signaling plays a role in CM development and the formation of the CB and iris. *Wnt2b* is exclusively expressed within the dorsal optic vesicle, the prospective CM, and in pigmented CM at the optic cup stage in both chick and mouse (38, 149, reviewed by 249). At later stages, *Wnt2b* is expressed solely in the iris epithelium, both in the pigmented and non-pigmented layers, but not in the RPE in chick eye (38). The reporter assays indicate that high level of signaling through the canonical Wnt pathway occurs in the dorsal optic vesicle and CM of the optic cup in a pattern that is overlapping and adjacent to *Wnt2b* expression, in the epithelium of CB/iris in the postnatal and adult mouse eye (148, 149), as well as in the CM of developing zebrafish eye (53).

Evidence from functional studies strengthens the case for a role of canonical Wnt signaling in the development of CM and its derivatives. Increased canonical Wnt signaling via ectopic expression of constitutively active (CA) β -catenin results in ectopic expression of peripheral eyecup markers and morphological changes characteristic of CB/iris epithelial cells in chick retinal explants (38), while inhibition of Wnt signaling interferes with the development of the iris in chick retina (38). Consistent with these results in the chick, it has been found that activated Wnt signaling promotes CM/CB development in mouse retinal explants and transgenic mice (Chapter 5.0 and 6.0 in this thesis), demonstrating a crucial function of Wnt canonical signaling in the periphery of the mammalian eye.

1.5 Rationale, hypothesis and research objectives

Expression pattern of Wnt component genes and activation of Wnt reporter in the eye and association of eye diseases with aberrant Wnt signaling have suggested that this pathway performs an important function in eyes. Thus I hypothesized that:

Wnts play a role in mammalian retinal development, particularly in the development and formation of the CM and its derivatives

Objective 1. To determine the spatial and temporal expression patterns of *Wnt*, *Frizzled* receptor and *Sfrp* antagonist in the developing and adult retina.

At the outset of this thesis project, little was known regarding the function of Wnt signaling in mammalian eye patterning and neurogenesis and a comprehensive analysis of Wnt component gene expression in the mammalian retina was lacking. As a first step to identify the role of Wnt signaling pathway in the context of mammalian retinal development, I surveyed the expression pattern of components of this pathway in the eye.

Objective 2. To characterize the temporal and spatial pattern of canonical Wnt signaling activation and the expression pattern of *TCF/Lef* family members in the developing and adult retina.

While it is likely that the non-canonical Wnt pathways are also operating in the retina, given the evidence linking canonical Wnt signaling with cell fate determination, this pathway has been set as the focus in exploring its role in the context of retinal development in this thesis project. To gain insight about the timing and retinal regions that are activated by

canonical Wnt signaling, I carried out a comprehensive analysis on *TCF/Lef-LacZ*, a canonical Wnt reporter transgenic mouse strain and compared activation of this reporter transgene with the expression of *TCF/Lef* transcription factors and cell type specific markers.

Objective 3. To activate the Wnt canonical pathway by manipulating Wnt component genes in retinal explants and transgenic mice, and investigate how this signaling pathway is involved in diverse developmental events in the retina.

Studies on objective 1 and 2 revealed overlapping or exclusive expression pattern of *Wnt2b*, Wnt receptors and antagonists in the CM at the optic cup stage and high level activation of canonical Wnt signaling in the CM and the epithelial layers of the CB/iris, suggesting a particular role of this pathway in the development and function of the peripheral eye. To address this possibility, the Wnt canonical pathway was activated by using *in vitro* and *in vivo* approaches and it was explored how this signaling pathway plays a role in the development of CM and NR in the mammalian eye.

2.0 Materials and Methods

2.1 Animals

All animal-related experiments were performed in accordance with the University of Ottawa Animal Care and Veterinary Service. Unless otherwise indicated, animals were purchased from Charles River Laboratories, Inc.

2.1.1 Imported transgenic mice

α -Cre-GFP transgenic mice were obtained from Peter Gruss (Max-Planck-Institute of Biophysical Chemistry, Germany) (161); $Catnb^{+/lox(ex3)}$ transgenic mice were obtained from Makoto Taketo (University of Tokyo, Japan) (88). The above two mouse strains were maintained on a C57BL/6 background. TCF/Lef-LacZ transgenic mice were obtained from Daniel Dufort (McGill University, Canada) (171) and were maintained on a CD1 background.

α -Cre mice were crossed to TCF/Lef-LacZ mice to generate α -Cre;TCF/Lef-LacZ double transgenic mice. Heterozygous α -Cre;TCF/Lef-LacZ mice were crossed with heterozygous $Catnb^{+/lox(ex3)}$ mice to generate the β -catenin compound transgenic mice with Wnt reporter, α -Cre; $Catnb^{+/lox(ex3)}$;TCF/Lef-LacZ (referred to as $Catnb^{\Delta ex3}$;LacZ), or without Wnt reporter, α -Cre; $Catnb^{+/lox(ex3)}$ (referred to as $Catnb^{\Delta ex3}$). In all the experiments regarding compound transgenic mice, α -Cre⁺ littermates with or without Wnt reporter were used as controls, α -Cre; $Catnb^{+/+}$;TCF/Lef-LacZ (referred to as $Catnb^{+}$;LacZ) or α -Cre; $Catnb^{+/+}$ (referred to as $Catnb^{+}$).

2.1.2 *Msx1mpWnt2bHA* transgenic mice

Transgenic mice were generated through pronuclear microinjection as described

previously (50). Briefly, hybrid C57BL/6-C3H F1 mice (produced by crossing C57B/6 female mice with C3H male mice) were used as donors for fertilized one-cell embryos. 3ng/μl purified DNA fragment, *Msx1mpWnt2bHA* [Materials and Methods/2.4], was used for pronuclear microinjection. Zygotes were cultured overnight at 37°C in M16 medium under oil. The following day, two-cell stage embryos were subjected to oviduct transfers in pseudopregnant female CD-1 mice.

2.1.3 Genotyping

Genotyping for the α -*Cre*, *TCF/Lef-LacZ* transgene, conditional β -*catenin* allele and *Msx1mpWnt2bHA* transgene was performed by "PCR on total genomic DNA extracted from embryonic tissue or tail samples. Sequences and PCR conditions of primer pairs are listed in [Appendix A].

2.2 β -galactosidase activity detection by X-gal staining

Preparation and X-gal staining of postnatal day 7 (P7) and adult tissues were performed as described previously (58, 148, 254). After X-gal staining, slides were rinsed in PBS for 30 minutes and mounted or processed for immunohistochemistry. Preparation and X-gal staining of embryonic material were essentially the same as above with the following modifications: Whole heads or dissected eyeballs were fixed in 4% paraformaldehyde (PFA) for varying length of time: 5 (E9.5-E10.5), 10 (E11.5-E13.5), 15 (E14.5-E15.5) or 15~20 minutes (dissected eyeballs of E16 and older tissue).

2.3 Cell Culture

293FT cells (Invitrogen) and NIH3T3 cells were cultured in 10% fetal bovine serum

(FBS)/DMEM/ Streptomycin&Penicillin (Sigma). 293FT cells are a clonal derivative of the human kidney 293T cell line, which is a variant of 293 cells containing a temperature sensitive SV40 large T antigen (57). To maintain the expression of large T antigen, 500µl/ml G418 (Sigma) was added to the media during routine culture, but was removed before transfection.

2.3.1 Generation of retrovirus in 293FT cells

Wnt2b expressing retrovirus was produced by calcium phosphate transient cotransfection of gag-pol (pHIT60), env (pHIT456), and expression (Wnt2bHA/pHIT4) plasmids [Materials and Methods/2.4] in 293FT cells as described previously (110). 36-48 hours after transfection, culture media containing virus was harvested and filtered (0.45µm), and aliquoted and stored at -70°C. Virus stock was then titered on NIH3T3 cells according to protocol available online (264). Briefly, approximately 12-18 hours prior to infection, 4.5×10^5 NIH3T3 cells were plated in a 6-well plate in standard media. Cells were approximately 80% confluent prior to infection. Serially diluted infection solution containing 4µg/ml polybrene was prepared. Media was removed from the NIH3T3 cells and replaced with the 1ml virus solution, one concentration of infection solution for one well. After 4 hours, an additional 2 ml of media was added to the cells. 48 hours post infection, the monolayers were stained with anti-GFP antibodies. GFP⁺ colonies were counted in each well (the dilution factor at 10^n) and scored: titre=number of colonies x 10^n CFU/ml.

2.3.2 TOPFlash Luciferase Assay

Luciferase assay was performed according to the protocol described previously with

minor modification (139). Briefly 293FT cells seeded in 24-well plates were transfected with a combination of testing plasmid (33ng/well), pTOPFlash (170ng/well), pRL-PGK (17ng/well) and pcDNA3 (117ng/well) using Lipofectamine (Invitrogen). Transfected cells were harvested 24 hours after transfection. Luciferase activity in the cell extracts was measured by Dual-luciferase Reporter Assay System (Promega) in a luminometer (Microplate luminometer, Berthold) according to the manufacturer's instructions and firefly luciferase activity was normalized to renilla luciferase activity in each sample. All experiments were repeated at least three times. To evaluate statistical significance, Student's t-test was performed.

2.4 DNA constructs and construction

2.4.1 pHAN/puro

This vector was kindly provided by Michael Rudnicki (University of Ottawa and Ottawa Health Research Institute, Canada). It was modified from the retroviral expression vector pBABE/puro by replacing the original LTR promoter with a hybrid CMV/LTR promoter.

2.4.2 pHIT4-IRES-hrGFP (HA/FLAG) (referred to as pHIT4)

Generated and kindly provided by Jeff Ishibashi (Rudnicki lab, University of Ottawa and Ottawa Health Research Institute, Canada). This is a retroviral expression vector based on pHAN/puro. It was generated by combining pHAN/puro backbone with pIRES-hrGFP-1 α (Stratagene) and was modified to carry both HA and Flag epitope tags (110).

2.4.3 Constitutively active β -catenin

(a) N57 β cat (N57 β -catenin-HA/pCGN): Generated and kindly provided by Marina Glukhova, Institute Curie, France (237).

(b)N89 β cat (Myc-N89 β -catenin-IRES-Lef1/pHAN): Generated and kindly provided by Kazuki Kuroda (Rudnicki lab, University of Ottawa and Ottawa Health Research Institute, Canada). It was generated by sub cloning the PmeI fragment containing Myc-N89 β -catenin-IRES-Lef1 (44) into pHAN/puro at the blunted BamHI site.

2.4.4 Wnt2b constructs

(a) Wnt2b/pHIT4 (lab code O-211): The sequence of full length Wnt2b cDNA (1170bp) was generated by RT-PCR from total RNA extracted from E14.5 RPE with restriction-site-tagged primers [Appendix A/Wnt2b set 2]. After digestion and sequence confirmation, the PCR product was inserted into pZErO-2 cloning vector (Invitrogen) at the Hind III/EcoR I sites, and then released at the Hind III/Xho I sites and sub cloned into pCMV-Tag2B expression vector (Stratagene) in frame downstream of the Flag epitope to generate FlagWnt2b/pCMV (lab code O-152). Full length Wnt2b cDNA was released from FlagWnt2b/pCMV by EcoRI digestion and then sub cloned into pHIT4 expression vector at the EcoR I sites. Note that the HA or FLAG tag is invalid as the insertion was not in frame.

(b) Wnt2bHA/pHIT4 (lab code O-166): A C-terminal HA-tagged Wnt2b cDNA was sub cloned upstream of the IRES-hrGFP sequences in the pHIT4 expression vector. Briefly, the following three-way ligation and cloning strategy was applied: Insert one, the EcoR I/Kpn I fragment was released from FlagWnt2b/pCMV; Insert two, the Kpn I/Sal I fragment was generated from a PCR product amplified from FlagWnt2b/pCMV by using Sal I-tagged

Wnt2b primers [Appendix A/Wnt2b set 3] and then digested with Kpn I/Sal I; The above two inserts were sub cloned into pHIT4-IRES-hrGFP (HA/FLAG) at the EcoR I/ Sal I sites in frame upstream of the HA epitope [map in Appendix D]. Note that the FLAG tag is invalid as the insertion was not in frame for FLAG. The sequence of Wnt2bHA/pHIT4 was confirmed by sequencing. The N-terminus of the deduced protein contains a high hydrophobic domain, which is usually considered as a signal peptide (169).

(c) Msx1mp-Wnt2bHA: the sequence (from 1115 to 1415 of Msx1 genomic DNA, Genebank accession number S73812) containing Msx1 minimal promoter (Msx1mp) (-165/+106) was amplified by PCR reaction with specific primers [Appendix A] on total genomic DNA from E14.5 C57Bl/6 embryos. The PCR product was sub cloned into pCR2.1 expression vector directly at the EcoRI sites to give rise to Msx1mp/pCR2.1. EcoRI digestion released Msx1mp, which was sub cloned into Wnt2bHA/pHIT4 expression vector to generate Msx1mp-Wnt2bHA/pHIT4. To have a better polyA addition signal, NotI/MscI fragment from Msx1mp-Wnt2bHA/pHIT4 was then subcloned into pIRES.hrGFP.1 α (Stratagene) to give rise to Msx1mp-Wnt2bHA/ pIRES.hrGFP.1 α (lab code O-203). The 3.3kb NotI/MluI fragment released from this expression vector was used for microinjection to generate transgenic mice.

2.4.5 Wnt3a constructs

(a) Wnt3a/pHAN: Generated and kindly provided by Kazuki Kuroda (Rudnicki lab, University of Ottawa and Ottawa Health Research Institute, Canada). In this Wnt3a expression vector, full length sequence of Wnt3a was sub cloned into pHAN/puro at BamHI

sites.

(b) Wnt3a/pHIT4 for ribonucleotide probe (V2): A full length Wnt3a cDNA was inserted in antisense orientation into pHIT4 expression vector. Briefly, the full length Wnt3a cDNA was released from Wnt3a/pHAN expression vector by BamH I digestion and sub cloned directly into pHIT4 expression vector at the BamH I sites. To synthesize antisense probe, the plasmid was linearized with EcoR I, Sph I or Sal I and transcribed with T3 RNA polymerase.

2.5 Immunohistochemistry

Immunohistochemistry (IHC) was performed according to the protocol for fluorescent detection or DAB reaction described previously (45, 116) with the following primary antibodies: mouse monoclonal anti- β -catenin (BD Bioscience); rabbit anti- β -gal (Molecular Probes); mouse monoclonal anti- β -tubulin (E7, Developmental Studies Hybridoma Bank); mouse monoclonal anti-BrdU (BD Bioscience); mouse monoclonal anti-Brn3A (Clone 14A6, Santa Cruz Biotechnology); goat polyclonal anti-Brn3B (Clone C-13, Santa Cruz Biotechnology); mouse monoclonal anti-calbindin (Sigma); rabbit monoclonal anti-calretinin (Swant); goat anti-Chx10 (a kind gift from Dr. R. Bremner, University of Toronto, Canada); rabbit polyclonal anti-collagen IV (Biogenesis); rabbit polyclonal anti-GFP (Molecular Probes); mouse monoclonal anti-HA (Sigma HA-7, for WB1:1000); mouse monoclonal anti-Ki67 (BD Bioscience); mouse monoclonal anti-Pax6 (Developmental Studies Hybridoma Bank); rabbit polyclonal anti-phospho-histone H3 (anti-phosphoH3) (Upstate); mouse monoclonal anti-rhodopsin (B630) (Developmental Studies Hybridoma

Bank); mouse monoclonal anti-smooth muscle actin (SMA) (clone 1A4, Sigma); mouse monoclonal anti-syntaxin (Clone HPC-1, Developmental Studies Hybridoma Bank). Sections were viewed under a Zeiss Axioplan microscope and digital images were captured using an Axio Vision 2.05 (Zeiss) camera and processed with Adobe Photoshop®.

2.6 *In situ* hybridization

Embryonic and adult retinal tissues for *in situ* hybridisation (ISH) were prepared, sectioned and processed for ISH, as described previously (116, 148) with the following DIG-labelled antisense riboprobes: *Bmp4* (a kind gift from John M. Wozney, Harvard University, USA), *Chx10* (a kind gift from J. Horsford, The Hospital for Sick Children, Toronto, Canada), *Crx* (a kind gift from Connie Cepko, Harvard Medical School, USA), *CyclinD1* (a kind gift from Gordon Peters, London Research Institute, Cancer Research United Kingdom, UK), *CyclinD2* (a kind gift from Nigel Pringle, University College London, UK), *GFP* (pIRES-hrGFP-1a, Stratagene), *Math3* (a kind gift from Tom Glaser, University of Michigan, USA), *Mfz-3*, *-4*, *-6* and *-7* (a kind gift from Jen-Chin Hsieh, Johns Hopkins University School of Medicine, USA), *Msx1* (a kind gift from Yi-Hsin Liu, Keck School of Medicine, USA), *Pax6* (a kind gift from V. van Heyningen, Medical Research Council Human Genetics Unit, UK), *Otx1*, *Otx2* (a kind gift from Masayo Takahashi, Kyoto University, Japan), *Sfrp-1*, *-2*, *-3* and *-4* (a kind gift from Amir Rattner, Johns Hopkins University School of Medicine, USA), *Tcf1*, *Lef1*, *Tcf3* and *Tcf4* (a kind gift from Johannes Meeldijk, University of Utrecht, The Netherlands), *Wnt-1*, *-2*, *-3*, *-4*, *-5a*, *-5b*, *-6*, *-7a* and *-7b* (a kind gift from Andrew P. McMahon, Harvard University, USA), *Wnt2b* or *Wnt-13* (a kind gift from Lise Zakin,

University of California, USA), *Wnt3a* version 1 (V1): the cDNA sequence locates at the 3' UTR of *Wnt3a* mRNA and it was a kind gift from Andrew P. McMahon, Harvard University, USA, *Wnt3a* (V2) was generated as described in [Materials and Methods/2.4.5 (b)].

2.7 Retinal explant culture

2.7.1 Retinal explants

Retinal explants from embryonic mice were established by removing the RPE and placing the globe with lens side down on a polycarbonate filter (pore size: 0.8µm; Nucleopore). The globe was opened at the optic nerve head and the pieces of the retina (still attached to the lens) were flattened to the filter. Globes were dissected in MEM (Sigma). Explants were cultured under serum free conditions as described previously (259) .

2.7.2 Dissociated retinal cell counting

Retinal explants were rinsed in Ca²⁺ free PBS and left in 250µl PBS in a 1.5ml eppendorf tube. 10µl 2.5mg/ml trypsin stock was added to the sample and put in 37°C water bath. After 12 minutes, 500µl stop solution (50µl DNase I in 10% FBS/MEM) was added into the sample. Then the sample was gently mixed with pipette for 8 times and spun at 1000rpm for 5 minutes. The pellets were resuspended in 30-50µl sitting solution (5µl/ml insulin/MEM) and the cell number was counted on counting chamber.

For IHC and cell type counting, 10µl retinal cell suspension (10^{2-3} cells) was loaded on cryostat slide and incubated in a humidified box at 37°C for 45min. Then the sample was fixed in 4%PFA for 5 minutes, rinsed in PBS and air dried at RT. For IHC, the sample was

processed by following the IHC protocol described in [Materials and Methods/2.5]. Hoechst staining was used to reveal nuclei. Immunopositive cells were scored under the microscope.

2.7.3 Electroporation in retinal explants

Embryonic eyeballs were dissected in MEM and the RPE and sclera were removed. The eyecup was then transferred to a micro electroporation chamber (Nepagene, model CUY532, 3 mm/10 mm/5 mm) filled with 150µl DNA solution. The concentration of DNA solution was at 700-1000 ng/µl in Tris buffer. Five square pulses (30 V) of 50 millisecond duration with 950 millisecond intervals were applied by using pulse generator CUY21 (Nepagene). Electroporated retinæ were then flattened on polycarbonate filters and cultured in serum-free culture medium as described in [Materials and Methods/2.7.1].

2.7.4 Treatment with lithium or Wnt3a recombinant protein

To activate the Wnt pathway with Li^+ in retinal explants, 20mM LiCl was added to the media and 20mM NaCl was added to the control explant media. To activate the Wnt pathway with Wnt3a, Wnt3a recombinant protein (R&D system, Inc. #1324-WN-002) was prepared as a stock solution of 10µl/ml in sterile PBS containing 0.1% BSA and added to the culture media at the concentration indicated in the text. 0.1% BSA/PBS was used as the control condition.

2.8 RT-PCR and quantitative RT-PCR

2.8.1 Oligonucleotide primers

Primer pairs were designed using Primer3 software. The primers shared 100% homology with the target sequence, but no significant homology with other sequences, as

determined by BLAST search available online (National Center for Biotechnology Information) (263). Primers for quantitative RT-PCR were specially designed so that they cross the boundary of the adjacent exons to avoid amplification of genomic DNA and the amplicon sizes ranged from 80 to 150 bp. Sequences of primers are listed in [Appendix A].

2.8.2 RT-PCR

To harvest embryonic and adult NR, care was taken during the dissection to remove the RPE and all other ocular structures. Total RNA was extracted by using Tri Reagent (Sigma) and was reverse transcribed using oligo-dT primer and Thermoscript reverse transcriptase (Invitrogen), following the manufacturer's instructions. The resultant first strand cDNA was used for PCR analysis with the primer pairs, as indicated. Amplification of untranscribed RNA was used as a negative control and amplification of cDNA with a primer pair for the *mHPRT* gene served as a positive control. The cycling parameters were as follows: 2 minutes at 94°C, followed by 30 cycles consisting of 1 minute at 94°C, 1 minute at the annealing temperature (determined for each Wnt primer pair), 45 seconds at 72°C, followed by a final 7 minutes extension at 72°C.

2.8.3 Quantitative RT-PCR

Total RNA extracted from retinal explants was reverse transcribed and subsequent quantitative RT-PCR (qPCR) was performed using the Mx4000 machine (Stratagene) according to the manufacturer's instructions. Briefly, pooled total RNA was extracted from 5-7 retinal explants by using Tri Reagent (Sigma) according to the manufacturer's recommendations. DNase I was added to remove trace DNA contamination and was heat-

inactivated prior to cDNA synthesis. 4 µg of RNA was used as template for first-strand cDNA synthesis by using Superscript II reverse transcriptase reaction kit (Invitrogen) in a 60µl reaction volume following the manufacturer's instructions. Both random hexamer primers and oligo-dT primers were used to prime the RT reaction. The qPCR was performed in a total volume of 25 µl containing 1 µl of RT product, 1µl of each primer (10µM) and 1µl of dye mix (SYBR green: Molecular probes / Passive reference dye: Stratagene). The cycling parameters were as follows: 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 min. The amount of the PCR product was monitored by the fluorescence intensity of SYBR green on the Mx4000 machine. All reactions were performed in triplicate. For each primer pair, serial dilutions of cDNA reverse transcribed from P0 retina total RNA were used to generate standard curve and assess linearity and efficiency. Controls for each reaction included non-reverse-transcribed cDNA (No RT) and no template control (NTC). The house-keeping gene *HPRT* and *β-actin* were amplified in separate wells in each qPCR run as a cDNA loading control. Fold change in expression of each gene was calculated by the 2^{-Ct} method adjusted with the amplification efficiency of each primer pair and normalized to house-keeping gene. Fold change = $(1+E_{\text{target}})^{-\Delta C_{t \text{ target}}}/(1+E_{\text{norm}})^{-\Delta C_{t \text{ norm}}}$. In the above equation, target=each specific target gene; norm=the normalizing house-keeping gene; E=PCR amplification efficiency obtained from the standard curve; Ct=threshold cycle; $\Delta C_{t \text{ target}}=C_{t \text{ control}}-C_{t \text{ Li+}}$. The qPCR experiments were performed with 6 independent batches of cDNA. To evaluate statistical significance, Student's t-test was performed.

2.9 SDS-PAGE and Western blot

2.9.1 SDS-PAGE

It was performed on mini electrophoresis apparatus according to the standard protocol (210). Briefly, 5% acrylamide stacking gel and 10% resolving gel was prepared and filled into the apparatus. The protein samples were prepared by adding the same amount of 2x gel loading buffer to the supernatant of cell lysate. The samples were loaded at 10-40 μ l/well to achieve a final DNA amount at 25 μ g/well. The voltage was set at 110V for stacking gel and 125V for resolving gel. The electrophoresis was stopped when the dye came out from the bottom of the gel.

2.9.2 Western blot

It was performed according to the standard protocol (210). Briefly, the 3MM paper and the transfer membrane were prepared at the exact size of the gel. The 3MM paper, membrane and gel were all soaked in ddH₂O and then in transfer buffer for 2min, and packed in the transfer cassette. The voltage was set up at 50V for transfer. After 2 hours, the membrane was taken out from the cassette and air dried for 2 minutes. The membrane was blocked in 5% milk/PBS-T at room temperature for one hour and incubated with primary antibody (prepared in 5% milk/PBS-T) at 4°C for overnight. Then the membrane was incubated with HRP-conjugated secondary antibody (prepared in 5% milk/TBS-T) at room temperature for one hour. After quick and thorough washing with TBS-T, the sample was detected using ECL detection kit (GE Healthcare) following the manufacturer's instructions.

3.0 Characterization of Wnt Signaling Components and Activation of the Wnt Canonical Pathway in the Murine Retina

The neuroepithelial layer of the developing eyecup contains multipotent precursor cells that give rise to all of the neurons and the one glial cell type present in the adult retina. Patterning within the retinal neuroepithelium is regulated by cell-intrinsic as well as cell-extrinsic mechanisms. Although the identity of some of the signaling molecules that regulate retinal development is known, the function of many others, especially members of the Wnt family, has yet to be characterized in the context of retinal development. The expression of members of the *Wnt*, *Frizzled* receptors and antagonist *Sfrp* gene families has been documented in the eyes of several vertebrate species (reviewed by 249); however, a comprehensive analysis of the expression patterns of these genes in the developing and adult mammalian retina has been lacking. As a first step towards understanding the function of Wnt signaling in eye development, I performed RT-PCR and ISH analyses to determine when and where components of the Wnt signaling pathway are expressed in the developing and adult murine eye. I also examined *TCF/Lef-LacZ* reporter gene activation in the eyes of *TCF/Lef-LacZ* transgenic mice, a canonical Wnt reporter strain. In this reporter strain, LacZ expression is under the control of the heat shock protein 68 minimum promoter and six copies of TCF/Lef responsive element (171); thus, detection of β -gal activity indicates the location of TCF/Lef-dependent gene expression within a tissue. Components of the Wnt signaling pathway are expressed in a dynamic pattern during eye development and in adult

retina. The canonical Wnt pathway is activated at a high level at the peripheral region, the CM, in the developing eye. The results implicate multiple roles of Wnt signaling during retinal development and homeostasis, and suggest that the canonical Wnt signaling plays a role in the development of non-neural derivatives of the optic cup.

3.1 Results

3.1.1 Expression of Wnt pathway genes in the embryonic retina

To examine the *Wnt* expression pattern in the neural retina, I performed RT-PCR [2.8.2] analysis using *Wnt*-specific primer pairs [Appendix A] on RNA isolated from embryonic day 14.5 (E14.5) and E18.5 NR. Care was taken to remove the retinal pigment epithelium (RPE) during the dissection process, so as to reduce the likelihood of amplifying RPE-specific products. *Wnt-1*, *Wnt-3*, *-5a*, *-5b*, *-7b* and *-13* (*Wnt-13* has been renamed as *Wnt-2b* after this study) specific gene products were amplified from the E14.5 and E18.5 NR (Fig. 5). No specific signal was detected in the NR at any age for *Wnt-2*, *-3a*, *-4*, *-6*, *-7a*, *-8b*, *-10b* and *-11* (Fig. 5).

To localize *Wnt* gene expression in ocular structures, I performed ISH [2.6] on eye tissues obtained from developing mice with DIG-labeled anti-sense riboprobes corresponding to these genes (Fig. 6, Table 1). As a control for probe specificity, I confirmed that each riboprobe demonstrated specific hybridization in tissues where their expression had been reported previously (Fig. 6 and not shown).

Fig. 5. RT-PCR analysis of *Wnt* gene expression in the developing and adult NR.

RNA from the neural retina at the indicated ages was analyzed by RT-PCR using primer pairs specific for each *Wnt* gene. Each primer pair amplified a specific product from RNA derived from control tissues that had previously been shown to express that *Wnt* gene. Amplification of the *mHPRT* gene was used to confirm that equivalent amounts of starting material were used and reactions carried out without reverse transcriptase did not yield a product (data not shown).

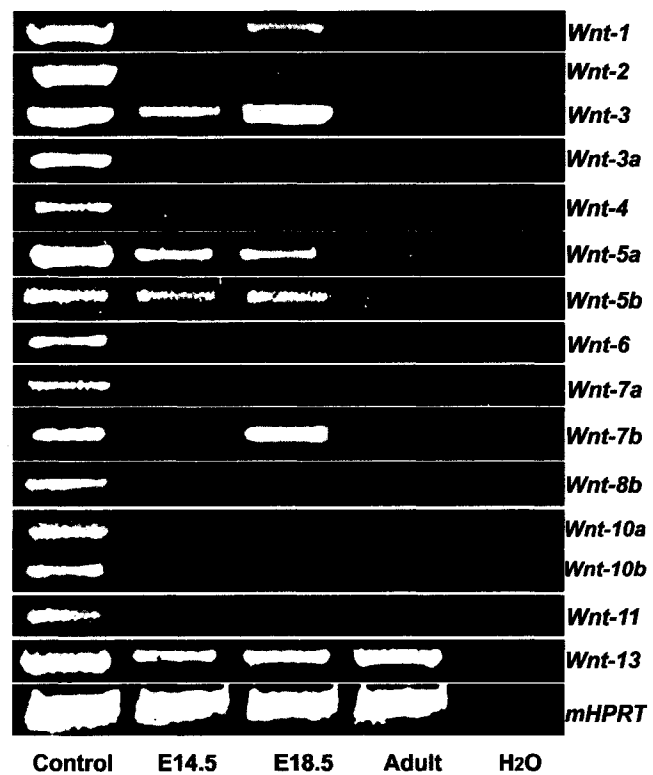


Fig. 6. ISH for *Wnt* gene expression in the developing retina.

Wnt-1, -2, -3, -4, -5a, -5b, -6, -7a, -7b and -13 expression in control tissues and horizontal sections of the eye at E12.5 and E14.5. The (>) indicates *Wnt-2*, -3, -4 and -6 expression in the corneal epithelium; asterisks indicate *Wnt-1*, -3, -4, -5b and -6 expression in the eyelid epithelia and *Wnt-5a* expression in the underlying eyelid mesenchyme; arrows indicate *Wnt-3*, -5b and -7a expression in lens epithelium, lens equator and lens fiber, respectively; arrowheads indicate the increase in *Wnt-3* and -5a expression in distal regions of the optic cup, and *Wnt-13* expression in the pigmented and non-pigmented cells in the distal ciliary margin, as well as in the RPE in the distal portion of the optic cup; the (^) indicates *Wnt-3*, -5b and -7b expression in the neural retina. Note that *Wnt-3*, -5b and -7b expression in the neural retina is downregulated by E14.5. An example of the *Wnt-13* expression pattern in the RPE at E14.5 is shown in an albino strain (CD1). CE, cornea epithelium; CM, ciliary margin; ELE, eyelid epithelium; ELM, eyelid mesenchyme; LE, lens epithelium; LEZ, lens equatorial zone; LF, lens fiber; NR, neural retina; ON, optic nerve; RPE, retinal pigment epithelium. Scale bar: 100 μ m.

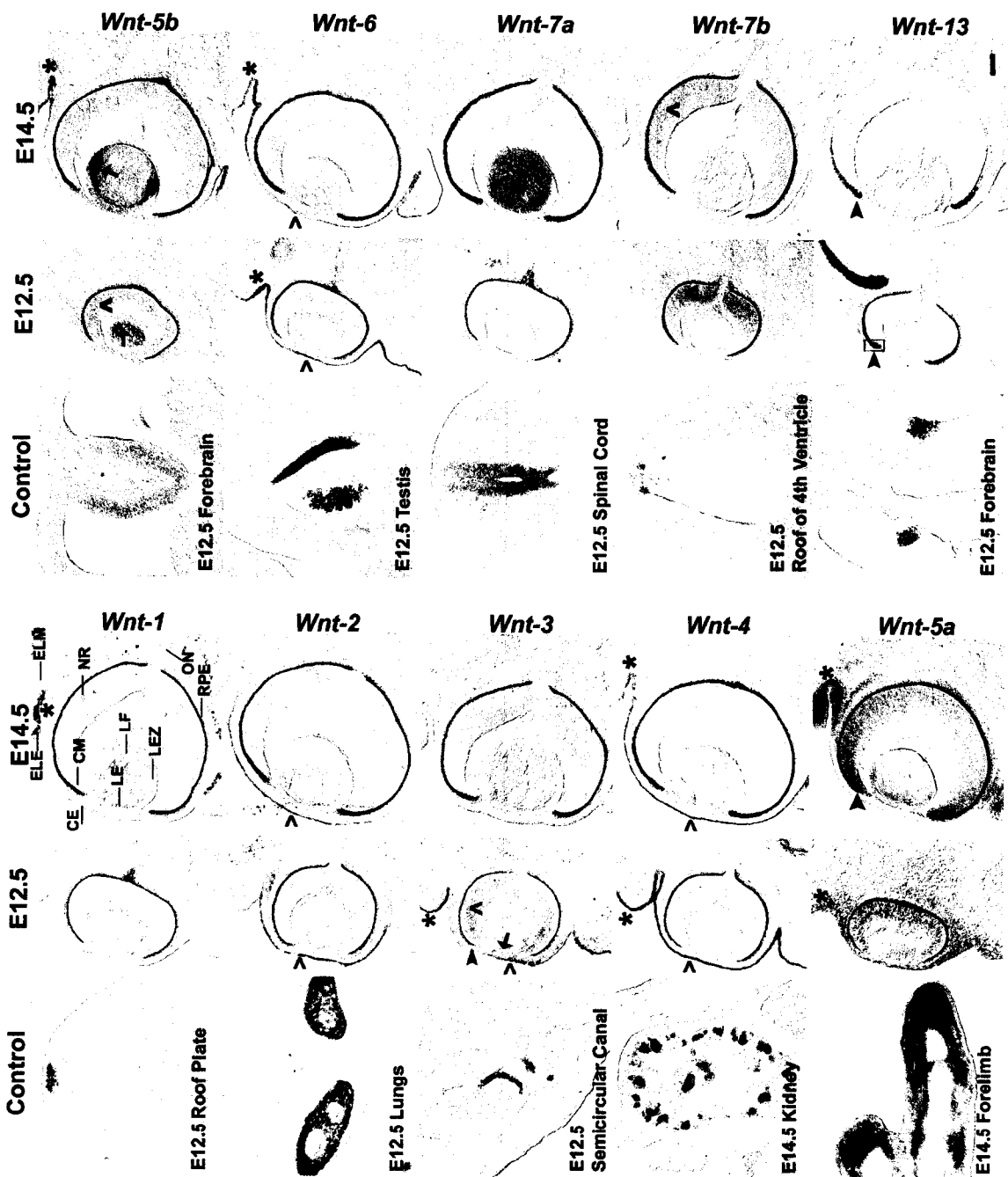


Table 1. *Wnt* transcript localization in the eye from E12.5 to E14.5^a.

Gene	NR		RPE	Lens	Cornea	Eyelid
	ISH	RT-PCR				
<i>Wnt-1</i>	-	-/+ ^c	-	-	-	+
<i>Wnt-2</i>	-	-	-	-	+	-
<i>Wnt-3</i>	+	+ ^c	-	+	+	+
<i>Wnt-4</i>	-	-	-	-	+	+
<i>Wnt-5a</i>	+ ^b	+ ^c	-/+	-	-	+
<i>Wnt-5b</i>	+ ^b	+ ^c	-	+	-	+
<i>Wnt-6</i>	-	-	-	-	+	+
<i>Wnt-7a</i>	-	-	-	+	-	-
<i>Wnt-7b</i>	+	-/+	-	-	-	-
<i>Wnt-13</i>	+	+	+	-	-	-

^a NR, neural retina; RPE, retinal pigment epithelium; ISH, in situ hybridization; RT-PCR, reverse transcriptase-polymerase chain reaction; E, embryonic day

^b Expression also detected by ISH in the P7 and adult retina.

^c Expression also detected by RT-PCR at E18.5 and adult (*Wnt-13*).

The developing eye begins as an optic vesicle, originating from the ventral diencephalon that grows towards the surface ectoderm where it invaginates to form a bilayered optic cup. The outer layer differentiates as the RPE and the inner layer differentiates as the NR. At E12.5 the NR consists of a single layer of proliferating RPCs, but by E18.5 the NR consists of two layers—the outer neuroblast layer, which contains RPCs, and the inner RGC layer, which contains the RGCs. The ISH analysis revealed that *Wnt-3*, *-5a*, *-5b* and *-7b* were expressed diffusely in the NR at E12.5, and that *Wnt-3*, *-5b* and *-7b* expression was downregulated in the central retina by E14.5 (Fig. 6). Although I detected a *Wnt-1* product by RT-PCR analysis in the NR, it is likely that this is not a physiologically relevant signal, as I was unable to localize *Wnt-1* transcripts in the developing retina by ISH (Fig. 6 and data not shown). Compared with the other Wnt transcripts, I detected high levels of *Wnt-13* transcript in the RPE located in the most peripheral part of the optic cup, which corresponds to the CM, a region of the optic cup that is fated to give rise to the iris and CB (Fig. 6). The expression of *Wnt-13* in the RPE in this region was confirmed by ISH analysis on sections from albino (CD1) mice (Fig. 6).

To complement the analysis of *Wnt* gene expression in the retina, I also examined the expression of *Mfz* receptor and *Sfrp* genes in the embryonic retina. To date, 12 *Mfz* genes have been described (262) and I examined the expression of *Mfz-3*, *-4*, *-6* and *-7* by ISH. I also confirmed the expression in the NR of a fifth *Mfz* gene, *Mfz-5*, by RT-PCR (data not shown). The ISH analysis revealed that these four *Mfz* genes were expressed in the developing eye, but that they had unique expression patterns, especially in the CM (Table 2).

At E12.5, *Mfz-3*, *-4*, *-6* and *-7* were expressed throughout the NR and CM, *Mfz-4* was also expressed in the RPE (Fig. 7 and data not shown). Differences in the expression pattern of *Mfz* genes in the CM of the optic cup were apparent by E14.5. Although low levels of *Mfz-6* expression were maintained in the CM, *Mfz-3* expression was downregulated and *Mfz-4* expression was upregulated at the distal part of the CM, a region that gives rise to the iris. *Mfz-7* expression became restricted to the proximal half of the CM, a region that gives rise to the epithelium of the CB (Fig. 7). The expression of *Mfz-4* in the CB and developing iris was maintained until at least postnatal day 7 (P7) (data not shown). At E14.5, *Mfz-3* and *Mfz-7* were also expressed in astrocyte precursor cells in the optic disc, as indicated by the intense triangular pattern of the ISH signal in this region of the retina, and in the optic nerve (Fig. 7).

I examined the expression of the four murine *Sfrp* genes (*Sfrp-1*, *-2*, *-3* and *-4*) in the developing eye by ISH. *Sfrp-4* expression was not detected in the retina at E14.5 and E18.5 and was not studied further. *Sfrp-1*, *-2* and *-3* were expressed in the retina in distinct patterns (Table 3). *Sfrp-1* was expressed throughout the NR at all stages, with higher levels of expression observed in the pigmented and non-pigmented layer of the CM as early as E12.5 until at least P7 (Fig. 8 and data not shown). Although *Sfrp-2* was highly expressed in the NR throughout the developmental stages, it was downregulated in the CM as early as E12.5 and from E14.5 onward it was downregulated in the RGC layer (Fig. 8). A high level of *Sfrp-1* and *-2* expression was also observed in astrocyte precursor cells at the optic disc at E14.5. *Sfrp-3* expression in the retina was not initiated until after E18.5 (Fig. 8), see text below.

Fig. 7. ISH for *Mfz* gene expression in the developing retina.

Mfz-3, -4, -6 and -7 expression in horizontal sections of the developing eye at E12.5, E14.5 and E18.5. The (>) indicates *Mfz*-3, -4, -6 and -7 expression in the corneal epithelium; asterisks indicate *Mfz*-3, -4, -6 and -7 expression in the eyelid epithelium and *Mfz*-7 expression in the underlying eyelid mesenchyme; arrows indicate *Mfz*-3 and -6 expression in lens equatorial zone, *Mfz*-3, -4 and *Mfz*-7 expression in lens anterior epithelium; arrowheads indicate the increase in *Mfz*-4 and -6 expression in the distal and *Mfz*-7 expression in the proximal ciliary margin; open triangles indicate *Mfz*-3 and -7 expression in the optic nerve and optic disc. Note that *Mfz*-3 was weakly expressed or absent in the ciliary margin at E14.5 and E18.5, and *Mfz*-7 expression was absent in the distal portion of ciliary margin after E14.5. OD, optic disc. Scale bar: 100 μ m.

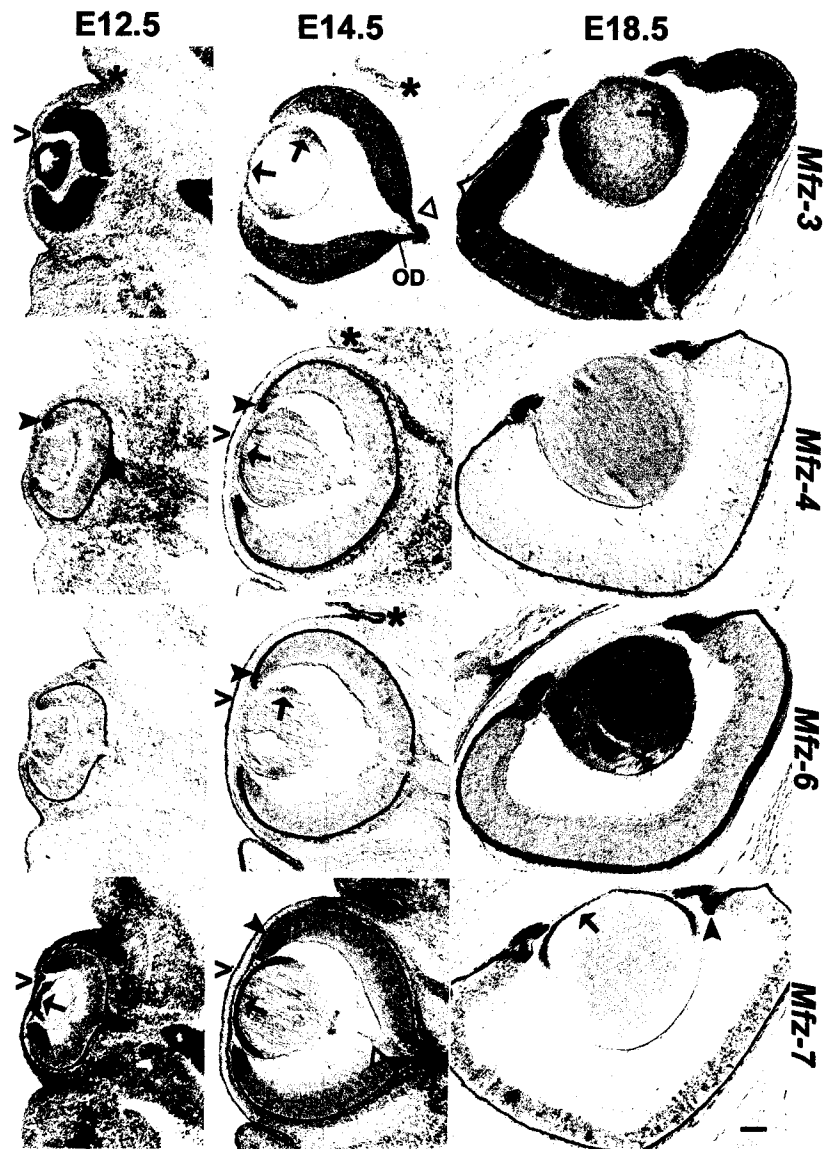


Table 2. *Mfz* expression in the eye from E12.5 to E18.5^a.

Gene	NR ^b	RPE	ON/OD	Lens	Cornea	Eyelid
<i>Mfz-3</i>	+ / Decreased in CM	-	+	+	+ (E12.5) ^d	+
<i>Mfz-4</i>	+ / Increased in distal CM	+ ^c	-	+	+ (E14.5)	+ (E14.5)
<i>Mfz-6</i>	+ / Increased in distal CM	+	-	+	+	+
<i>Mfz-7</i>	+ / Increased in proximal CM	-	+	+	+	+

^a CM, ciliary margin; NR, neural retina; OD, optic disc; ON, optic nerve; RPE, retinal pigment epithelium; E, embryonic day.

^b *Mfz-3*, -4, -6 and -7 expression also detected in the neural retina at P7 and adult stages.

^c *Mfz-4* expression also detected in the RPE at P7.

^d Embryonic stages in parentheses indicate that gene expression in this region was restricted to this developmental timepoint.

Fig. 8. ISH for *Sfrp* gene expression in the developing retina.

Sfrp-1, -2 and -3 expression in horizontal sections of the developing eye at E12.5, E14.5 and E18.5. The (>) indicates *Sfrp-1* expression in the corneal epithelium at E14.5 and E18.5; asterisks indicate *Sfrp-1* and -2 expression in the eyelid epithelium; arrows indicate *Sfrp-2* expression in lens anterior epithelium; arrowheads indicate *Sfrp-1* expression in high levels in the ciliary margin at E12.5 and E14.5, and in the ciliary body at E18.5; open triangles indicate *Sfrp-1* and -2 expression in the optic disc and optic nerve. Note that *Sfrp-1* expression in the corneal epithelium is up regulated at E18.5, and that *Sfrp-2* expression was absent in the ciliary margin and retinal ganglion cell layer at E14.5 and E18.5. OD, optic disc. Scale bar: 100 μ m



Table 3. *Sfrp* expression in the developing eye and adult neural retina^a

Gene	NR E12.5-18.5	NR P7	NR adult	RPE	ON/OD	Lens	Cornea	Eyelid
<i>Sfrp-1</i>	+/Increased in CM	+	+ INL, ONL	+ ^b	+	+	+	+
<i>Sfrp-2</i>	+/Absent in CM	+INL	-	-	+	+	-	+
<i>Sfrp-3</i>	-	+INL	+ INL	-	-	-	-	-

^a CM, ciliary margin; E, embryonic day; INL, inner nuclear layer; NR, neural retina; OD, optic disc; ON, optic nerve; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

^b *Sfrp-1* expression also detected in the RPE at P7.

3.1.2 TCF/Lef-LacZ reporter transgene activity in the developing retina

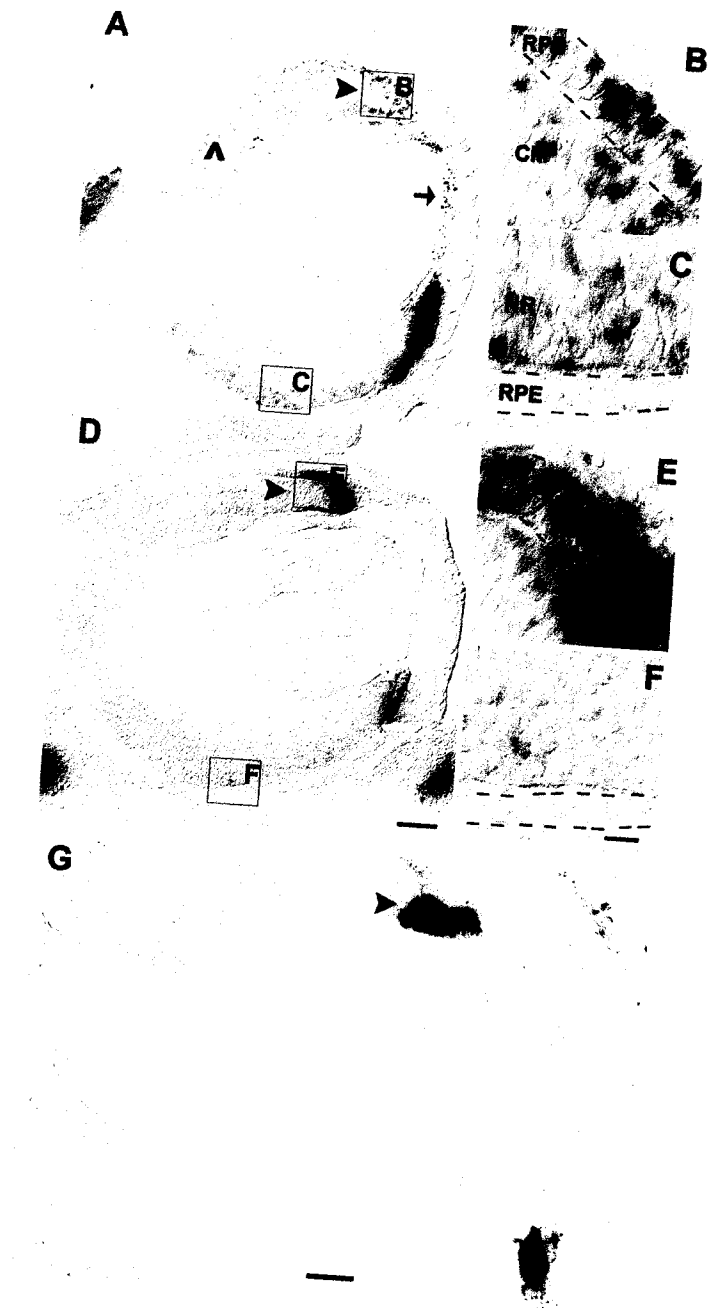
To identify those regions of the developing eye that respond to Wnt signaling via the canonical pathway, I examined reporter gene activation in *TCF/Lef-LacZ* transgenic mice. β -gal reporter activity was detected by X-gal staining [2.2]. From E11.5 to E14.5, high levels of β -gal activity were detected in the CM in both the presumptive pigmented outer layer and non-pigmented inner layer, but not in the proximal RPE at both of E13.5 and E14.5 (Fig. 9, A-F and data not shown). At E17.5 β -gal activity was detected exclusively in the future CB and iris (Fig. 9G). The pattern of β -gal activity in the CM overlapped with and was adjacent to the *Wnt-13* expression domain (Fig. 6), the only identified *Wnt* gene expressed in this region. At E13.5 β -gal activity was also detected, albeit at lower levels compared with the CM, in cells located in the outer neuroblast layer of the NR and in the anterior lens epithelium (Fig. 9A).

3.1.3 Expression of Wnt pathway genes in perinatal and adult retina

Wnt signaling is likely to be important at perinatal and adult stages of the retina, as the RT-PCR analysis revealed that *Wnt-5a*, *-5b*, *-10a* and *-13* were expressed in the adult NR (Fig.5). I, therefore, determined the localization of *Wnt-5a*, *-5b* and *-13* in the P7 and adult retina by ISH. By P7 cells in the retina are organized into 3 layers, however, retinal histogenesis is incomplete, as a number of important processes, such as photoreceptor differentiation and synaptogenesis, are occurring at this age. *Wnt-5a*, *-5b* and *-13* were expressed in the INL at P7, in what are likely to be amacrine cells, as determined by staining of serial sections with anti-Pax6 antibodies, which identify amacrine cells in this layer

Fig. 9. Detection of β -gal activity in the developing eye of *TCF/Lef-LacZ* transgenic mice.

Horizontal sections of the eye at E13.5 (A-C), E14.5 (D-F) and E17.5 (G) were stained with X-gal. The framed areas corresponding distal (B and E) and proximal (C and F) regions of optic cup in A and D are shown at higher magnification. The dashed lines indicate the location of RPE. Arrow indicates β -gal activity in the lens anterior epithelium at E13.5; the (^) indicates β -gal activity in the neuroblast layer at E13.5; arrowheads indicate β -gal activity in the ciliary margin and RPE in the distal portion of the optic cup at E13.5 and E14.5, and in the future ciliary body and iris at E17.5. Note that β -gal activity in the neuroblast layer was down regulated by E14.5. See legend to Fig. 2 for the definition of the abbreviations. Scale bars: 100 μ m in (A, D) and G, 25 μ m in (B, C, E and F).



(47, 179) (Fig. 10 and data not shown). *Wnt-13* expression was also detected in the RGC layer at P7. *Wnt-5b* and *-13* transcripts were localized to the INL in the adult retina (Fig. 10). Although I detected the expression of *Wnt-5a* in the adult retina by RT-PCR analysis, I was unable to localize *Wnt-5a* transcripts in the adult retina by ISH.

Mfz-3, *-6* and *-7* were expressed in the RGC and INL layer at P7, but their expression was restricted to the INL in the adult retina. *Mfz-4* was expressed in all three cellular layers at P7 and restricted to INL and in photoreceptors in the ONL in the adult (Fig. 10). The expression of the *Mfz* genes in the INL was diffuse, making it difficult to correctly identify the *Mfz*-expressing cells in this layer.

The *Sfrp* genes were also expressed at late stages of retinal development (Table 3). *Sfrp-1* expression was detected in all three cellular layers at P7 and in the INL and ONL in the adult retina (Fig. 11). At P7, *Sfrp-2* and *-3* were expressed in a complementary pattern: *Sfrp-2* was expressed in the INL in the peripheral retina but not in central retina and *Sfrp-3* was expressed in the INL in the central but not in the peripheral retina (Fig. 11). I also noted that *Sfrp-2* was expressed at higher levels in the nasal compared with the temporal retina at this stage. In the adult retina, *Sfrp-2* expression was downregulated and *Sfrp-3* expression was restricted to the INL, possibly in bipolar cells, as indicated by the expression of Chx-10, a bipolar cell marker (25, 91) (Fig. 11 and data not shown). The complementary distribution of *Sfrp-2* and *-3* transcripts could reflect the central to peripheral maturation gradient in the retina, with *Sfrp-2* expression marking immature and *Sfrp-3* expression marking more differentiated regions of the retina.

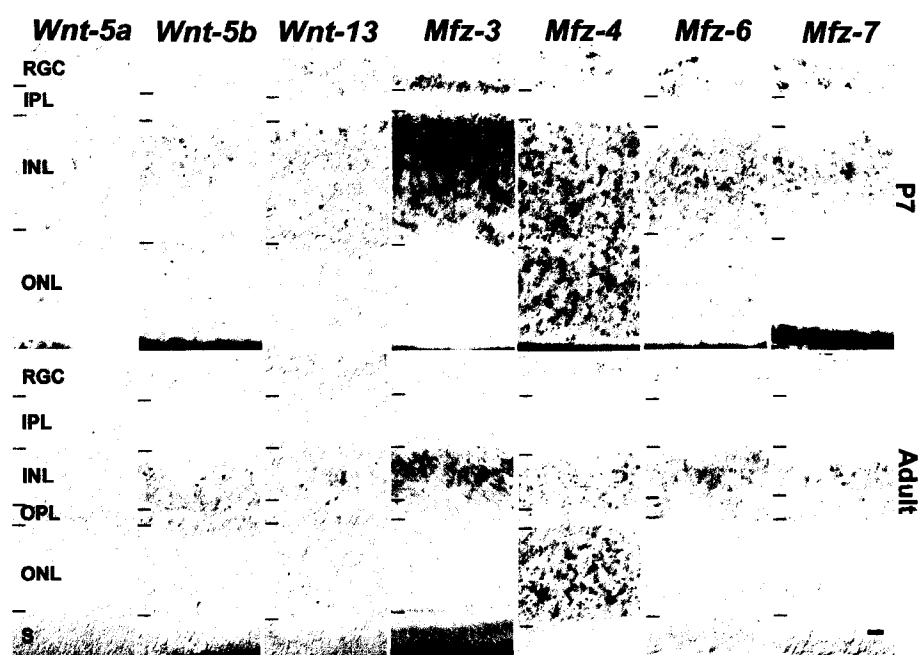
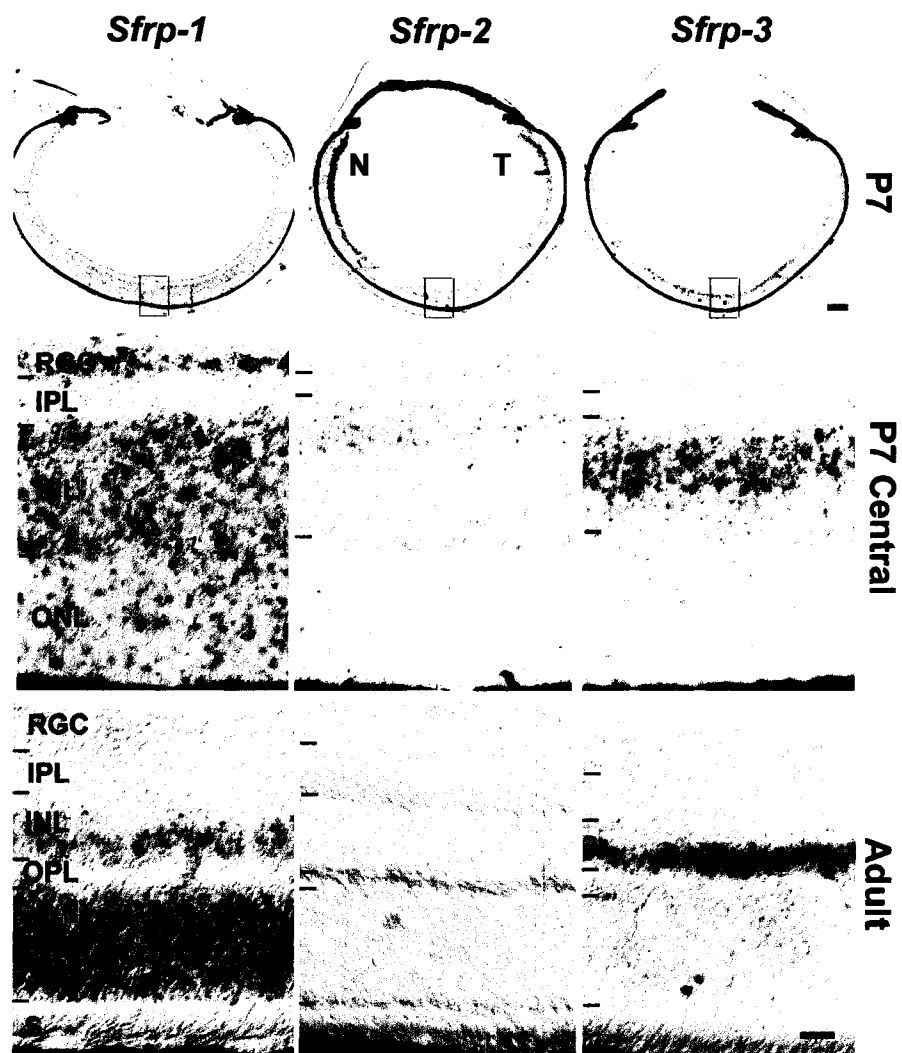


Fig. 10. *Wnt* and *Mfz* expression in the P7 and adult retina.

ISH on transverse sections of the retina for the indicated *Wnt* and *Mfz* genes. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell layer; S, photoreceptor segments. Scale bar: 10 μ m.

Fig. 11. *Sfrp-1*, *Sfrp-2* and *Sfrp-3* expression in the P7 and adult retina.

ISH on transverse sections of the retina from P7 (top and middle panels) and adult (bottom panel) mice for the indicated *Sfrp* genes. Framed areas in top panel are shown at higher magnification in the middle panel. N, nasal side; T, temporal side. See legend to Fig. 6 for the definition of the abbreviations. Scale bars: 200 μ m in top panel, 20 μ m in middle and bottom panels.



3.1.4 Expression of Wnt pathway genes in the eyelid, cornea and lens

Several of the *Wnts*, *Mfzs* and *Sfrps* were expressed in other regions of the eye, such as the eyelid, cornea and lens (Table 1 to 3), implicating a role for Wnt signaling in the development of these structures. In the developing eyelid, for example, *Wnt-1*, -3, -4, -5b, -6, *Mfz-3*, -4, -6, -7 and *Sfrp-1* and -2 were expressed in the epithelial layer, while *Wnt-5a*, *Mfz-7* and *Sfrp-2* were expressed in the underlying mesenchyme. *Wnts-2*, -3, -4, -6, *Mfz-4*, -6, -7 and *Sfrp-1* were expressed in the cornea epithelium. In the lens, *Wnt-3*, *Mfz-3*, -4, -7 and *Sfrp-1* and -2 were expressed in the epithelium, *Wnt-5b*, *Mfz-3*, -6 and *Sfrp-1* were expressed at the lens equator and *Wnt-7a* and *Sfrp-1* was expressed in the lens fibers (Fig. 6 to 8). In *TCF/Lef-LacZ* transgenic mice, β -gal activity was detected in the lens epithelium at E13.5, suggesting activation of canonical Wnt signaling in this region during development (Fig. 9 and data not shown). *Mfz-7* expression was also detected in the periocular mesenchyme at E12.5 and E14.5 (Fig. 7).

3.2 Discussion

Although the expression of members of the Wnt signaling cascade has been reported in the vertebrate retina, this study represents the first systematic characterization of *Wnt*, *Mfz* and *Sfrp* gene expression in the developing and adult murine retina. I show the localization of transcripts for several Wnt signaling components and canonical Wnt reporter transgene activation in the developing eye. I also show that several members of the Wnt signaling cascade are expressed in the perinatal and adult retina, implicating a post-histogenesis role for this pathway in the eye.

3.2.1 Wnt signaling in the developing neural retina

Based on the ISH analysis, I have localized *Wnt-3*, *-5a*, *-5b* and *-7b* transcripts to the embryonic mouse NR. *Wnt* expression has been reported previously in the chick RPE (*Wnt-3*, *-5a* and *-11*) and NR (*Wnt-11*) (117). I did not detect *Wnt-3* and *-5a* expression in the RPE by ISH in pigmented (C57Bl/6) or albino (CD1) mice (Fig. 2 and data not shown) and I did not detect *Wnt-11* expression in the mouse retina by RT-PCR (Fig. 1). The discrepancy between these results in the mouse and those reported in the chick could be related to species-specific differences in Wnt signaling in eye development. *Wnt-7b* expression has been reported in the mouse optic stalk and dorsal optic vesicle at E9.5 (187), but not at later stages in mouse development.

Wnt signaling via the canonical pathway involving β -catenin stabilization and TCF-dependent gene transcription appears to be activated in NR, CM and other ocular structures, such as the lens. I assessed TCF-dependent transcription in the eye by examining β -gal activity in *TCF/Lef-LacZ* transgenic mice. Reporter gene activity was localized to the outer neuroblast layer of the NR at E13.5, which could reflect the activity of *Wnt-3*, *-5a*, *-5b* and *-7b* that are expressed at low levels in the NR at this stage.

I find *Wnt* gene expression and TCF-dependent reporter gene activation in the neuroblast layer at a time when RPCs are proliferating and making cell fate choices, therefore, it is possible that Wnt signaling may be involved in these processes. Notably, at E13.5 β -gal activity was localized to cells in the neuroblast layer close to the RPE, which correspond to the region where presumptive photoreceptors are localized, raising the possibility that Wnt signaling plays a role in photoreceptor development. *Wnt-3*, *-5a* and *-7b* have been shown to play a role in precursor cell proliferation in a number of tissues including the limb, hair follicle, lung and caudal regions of the embryo (78, 167, 168, 197, 220, 277). *Wnt-5b* is expressed in the developing gut (146), however, the function of *Wnt-5b* in embryonic development is not known. Wnts have also been shown to be downstream targets of other signaling pathways in developing tissues. For example, *Wnt-5a* appears to be a target of Sonic Hedgehog (Shh) signaling in the hair follicle (197). Since Shh signaling has previously been implicated in growth control and organization in the developing retina (116, 145), it will be important to determine if there is a link between Hedgehog and Wnt signaling pathways in retinal development.

The reception of a Wnt signal by a cell will depend in large part on the binding of Wnts to Mfz receptors and the presence of secreted Wnt antagonists, such as Sfrps, in the extracellular milieu that compete for Wnt binding. The expression of several *Mfz* genes in the embryonic chick and mouse eye has been characterized by ISH, and in adult mouse eye by RNase protection on total RNA (19, 67, 258). I have extended those findings by characterizing the localization of four *Mfz* genes at later stages in embryogenesis and in the adult retina in mice. The expression pattern of *Sfrp-2* that I observed in the embryonic eye is in good agreement with previous studies reported by Leimeister et al (143), however, my observation that *Sfrp-1* transcripts localize to the NR, in addition to the RPE, CM and lens, is different from previous reports (143, 196). It is unlikely that this difference in the *Sfrp-1* expression pattern between this study and the previous studies reflects a problem with probe specificity, as I find no evidence that the *Sfrp-1* probe cross hybridizes to *Sfrp-2* (data not shown).

Analyses in this study and those of other groups have revealed a considerable overlap between *Wnt*, *Mfz* and *Sfrp* expression in the NR, as well as in several ocular structures. This overlap in *Mfz* expression suggests the possibility that there are differences in the binding specificities of Wnts for the different Mfz and Sfrp and/or that signaling through different Mfz receptors results in different cellular responses. Based on previous reports showing an interaction between Wnt-5a and Mfz-5 in the *Xenopus* axis duplication assay (111), it is possible that Wnt-5a preferentially signals via Mfz-5, which is also expressed in the NR (19, 258). It is not clear, however, which Mfz the other Wnts present in the NR bind to.

3.2.2 Wnt-13 signaling at the ciliary margin

My findings indicate that Wnt signaling is likely playing an important role in patterning and development of the distal eyecup, the CM. I find that *Wnt-13* is expressed in the cells at the tip of and in the RPE overlying the CM and that β -gal activity in *TCF/Lef-LacZ* reporter mice is upregulated in the CM in cells within and adjacent to the *Wnt-13* expressing cells. These observations are in good agreement with previous reports showing *Wnt-13* expression in this region of the chick eye (66, 113, 135), and with the finding of Dorsky et al, who reported activation of a *Lef1*/ β -catenin-dependent reporter gene in the CM in developing zebrafish eye (53). Although previous studies on chick and on E9.5 mouse embryo have shown that *Wnt-13* expression is restricted to the dorsal half of optic cup (66, 113, 282), I find no evidence for asymmetric expression of *Wnt-13* in the dorsal eyecup at E12.5 and later stages (data not shown), reflecting that Wnt-13 signaling is likely species and developmental stage-dependent in this region. The differences in function of Wnt-13 in chick and mouse will be discussed in more detail in chapter 5.0 in this thesis.

The CM can be subdivided into a proximal and distal region that differentiates as CB and iris, respectively. The pigmented cells in iris are also the source of retinal stem cells found in the adult eye (243). My observation that *Mfz* genes are expressed in non-overlapping subregions within the CM is consistent with the possibility that patterning within this region could be mediated by differences in Wnt signal reception through the different *Mfz* receptors. Wnt-13 signaling might also be kept localized in the CM by the antagonistic action of *Sfrp-2*, which is expressed in the adjacent NR, but not in the CM.

3.2.3 Wnt signaling in the perinatal and adult retina

Wnt signaling may also be playing a role at late stages in retinal development and in the homeostasis and/or function of the adult retina. The expression of *Mfz* and *Sfrp* transcripts in the adult eye has been reported previously (33, 196, 258), and I extend those findings by showing the localization of *Wnts*, *Mfz*-3, -4, -6 and -7, and *Sfrp*-1 and -3 in the mouse NR at perinatal and adult stages. However, *Wnt* genes are expressed at very low levels compared with *Mfz* or *Sfrp* genes. In contrast to Chang et al who showed *Sfrp*-2 expression in the INL of the bovine retina (33), I find that *Sfrp*-2 expression is downregulated in the murine retina after P7. Similarly, Rattner et al (196) reported that *Sfrp*-1 transcripts are restricted to the CM and lens in the adult mouse eye, however, I find that *Sfrp*-1 transcripts are also present in the NR at this stage.

Wnt signaling has been shown to play a role in synapse and axon remodeling and terminal arborization in other regions of the CNS (reviewed by 40), which raises the possibility that Wnt signaling performs a similar function in the perinatal retina. Retinal histogenesis proceeds from the center to the periphery, such that by P7, the cells in the central retina have ceased proliferating and are undergoing differentiation and synaptogenesis (30). The regression of *Sfrp*-2 expression towards the periphery of the retina, together with the onset of *Sfrp*-3 expression in the central retina, is consistent with the possibility that *Sfrp*-2 is involved in early events in histogenesis, whereas *Sfrp*-3 plays a role in later events, such as differentiation and synaptogenesis. The functional significance of Wnt signaling in this context remains to be identified.

3.2.4 Wnt signaling in the developing lens and optic disc

Based on the ISH analysis, it is apparent that Wnt signaling is playing a role in the development of several ocular structures including the lens, cornea and eyelid. I show that *Wnt-3*, *-5b* and *-7b*, at least 4 *Mfz* genes and *Sfrp-1* and *-2* are expressed in the developing lens, which is in good agreement with previous reports that localized *Wnts*, *Mfz* and *Sfrp* gene expression to the lens (19, 67, 117, 143, 196, 232). Jasoni et al reported the expression of *Wnt-13* in the lens epithelium in chick (113), however, I did not detect *Wnt-13* expression in the mouse lens at any stages from E12.5 to E18.5. Some aspects of Wnt signaling in the lens epithelium likely go through the canonical Wnt signaling pathway, as I observed β -gal reporter gene activation in the lens epithelium in E13.5 TCF/Lef-LacZ transgenic embryos, which is in good agreement with a previous study showing activation of a Lef1/ β -catenin-dependent reporter gene in the zebrafish lens (53). Recently, it has been reported that β -catenin, presumably through the canonical Wnt pathway, has dual functions in lens development, being required for lens morphogenesis and suppressing lens fate in periocular ectoderm (225)

Wnt pathway is likely also involved in the development of cells at the optic disc/nerve. The optic disc consists of a cuff of optic stalk-derived glioblasts that initially serve as a conduit for RGC axons into the optic nerve, and they eventually give rise to the astrocytes that are located in the nerve fiber layer on the vitreal side of the NR, which are intimately associated with retinal blood vessels. Hedgehog signaling likely also plays a role in the development of this population (254), however, few other signaling molecules that regulate

this population have been identified. I show that *Mfz-3*, *-7*, *Sfrp-1* and *-2* are highly expressed in the astrocyte population at the optic disc at E14.5, implicating a role for Wnt signaling in the development of the astrocyte lineage of the optic disc.

Based on the data I have shown here, it is reasonable to predict that Wnt-3, -5a, -5b and -7b signaling may be involved in progenitor cell fate determination and proliferation in the developing NR and in homeostasis in the adult retina and that Wnt-13 signaling functions in the patterning and development of distal eyecup structures. Wnt signaling is likely to contribute to the development of the lens, optic nerve, cornea epithelium and eyelid. It is also possible that additional Wnts could play a role in retinal development, as I did not always observe a complete overlap in the expression patterns of the *Wnt*, Wnt receptor and Wnt antagonist genes.

4.0 Mapping Canonical Wnt Signaling in the Developing and Adult Retina

The key to activate canonical Wnt signaling is to stabilize cytosolic β -catenin, which otherwise is phosphorylated by the Gsk3 β /Axin/APC protein complex and degraded through the proteasome system (reviewed by 22, and by 271). Thus introducing activating mutations in β -catenin or inhibiting kinase activity of the Gsk3 β complex, is sufficient in most cases to activate this pathway (reviewed by 22, and by 154). Li⁺ blocks Gsk3 β activity (130), leading to stabilization of β -catenin and activation of this pathway (16, 34, 95, 129, 228), and thus is considered as a Wnt agonist. Stabilized β -catenin enters the nuclei and binds the TCF/Lef transcription factors, transactivating target genes (reviewed by 22, and by 271).

Previous studies have shown that components of Wnt signaling pathway are expressed in a dynamic pattern in the developing and adult retina, indicating a role of Wnt pathway in retinal development and homeostasis (reviewed by 249). Although the non-canonical Wnt pathways are likely operating in the retina, the observation of the activation of the *TCF/Lef-LacZ*, a canonical Wnt reporter transgene, in the CM and the neuroblast layer of the developing retina in mice (148), suggesting this particular pathway possibly plays an essential role during retinal development. Activation of a Lef1/ β -catenin-dependent reporter gene in transgenic fish was also described in the RPE, CM and lens (53). Despite the above studies, the timing of Wnt canonical pathway activation and its relevance to retinal cellular types during retinal development and in the adult retina remains unclear. In the context of

retinal development, expression patterns of *TCF/Lef* transcription factors have been reported in zebrafish, frog (205, 251), and chick retina (135), however, a comprehensive analysis of *TCF/Lef* expression in the mouse retina remains lacking. To gain insight into the function of Wnt signaling through the canonical pathway in the mammalian retina, I characterized the temporal and spatial activation of this signaling pathway in the developing and adult retina of *TCF/Lef-LacZ* reporter mice, and compared the pattern of reporter activation with the expression of *TCF/Lef* transcription factors and cell type specific markers.

4.1 Results

4.1.1 Lithium treatment induces TCF/Lef-LacZ reporter gene activity in retinal explants.

It was showed previously that reporter gene expression in the *TCF/Lef-LacZ* transgenic line could be detected in the developing embryonic eye (148). To confirm that the *TCF/Lef-LacZ* reporter gene expression accurately reflects activation of the canonical Wnt pathway, I treated retinal explants [2.7.1] dissected from the *TCF/Lef-LacZ* reporter embryos with Li^+ , a well-known agonist of the Wnt pathway.

Explants were stained to detect β -gal activity at different time points following Li^+ treatment [2.7.4]. In control explants, β -gal reporter activity was gradually down regulated after one day *in vitro* compared with the intensity of β -gal staining *in vivo*, especially in the

CM area (compare Fig. 12A, C, E with Fig.14D, E). In contrast, Li⁺ treatment led to a significant up regulation of β -gal activity throughout the NR by 15 hours, reaching a maximum level around 24 hours and downregulated by 48 hours (Fig.12). These findings indicate that *TCF/Lef-LacZ* transgene is a faithful reporter of canonical Wnt signaling in the retina and that maintenance of canonical Wnt signaling, especially in the CM, is dependent on signals that are lost upon transfer of the retina to culture.

4.1.2 Activation of the canonical Wnt signaling in the developing retina

Canonical Wnt signaling is activated during retinal development in various model systems (53, 148), however, the timing of canonical Wnt pathway activation at early stages in mammalian retinal development has not been reported. I took advantage of *TCF/Lef-LacZ* reporter mice to explore this issue and characterized the expression of the reporter gene in the retina from the optic vesicle stage E9.5 to P0. Eyecup development in the mouse begins at E8-8.5 as an evagination originating from the neuroepithelium of anterior neural plate. At E9.5, activation of *TCF/Lef-LacZ* reporter gene was observed in a subset of neuroepithelial cells in the dorsal-nasal side of the optic vesicle (Fig. 13A and Table 4). This β -gal⁺ region is contained within the region that will give rise to the CM and peripheral RPE, as defined by the expression of CM/RPE marker *Otx1* (Fig. 13E) (1, 162). At this stage, the onset of the reporter gene expression in the dorsal optic vesicle mirrors the pattern of *Wnt2b* expression (Fig.13C). This asymmetric pattern of *Wnt2b* expression in the dorsal eye has been reported in chick and mouse retina previously (113, 282). At later developmental stages, the *TCF/Lef-LacZ* reporter gene and *Wnt2b* expression are also induced in the ventral eye (148).

Fig. 12. Activation of the *TCF/Lef-LacZ* reporter transgene in response to Li^+ treatment in E14.5 retinal explants.

(A-H) Cross sections of explants in control media (A, C, E and G) or in the presence of Li^+ (B, D, F and H) for 3, 15, 24 or 48 hours were stained with X-gal to detect β -gal activity. The dashed line in F indicates the lens and the triangle in F indicates up-regulation of *TCF/Lef-LacZ* reporter gene activity in the lens equator. Arrows in D and F indicate up-regulation of reporter activity in the NR after 15 or 24 hours treatment, while arrowheads in D, F and H indicate up-regulation of reporter activity in the CM (n (number of explants)=12). CM: ciliary margin; NR: neural retina. Scale bar: 100 μm .

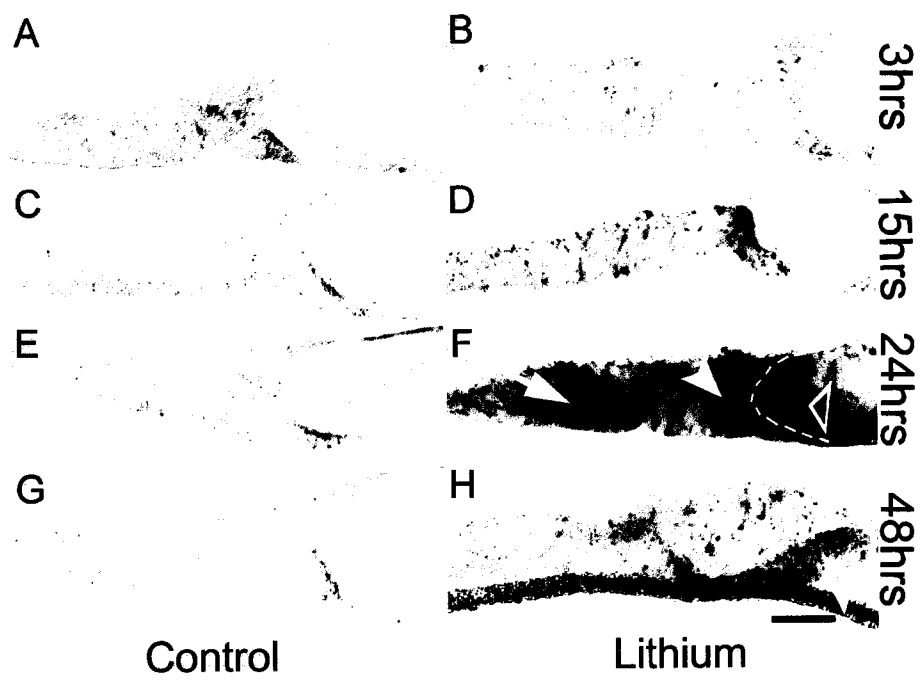


Fig. 13. Differential expression pattern of *TCF/Lef-LacZ* reporter gene, *Wnt2b* and *Otx1* along the dorsal-ventral axis of the optic vesicle.

(A-F) Horizontal sections of the eye at E9.5 were stained with X-gal to detect β -gal activity (A and B), or processed for ISH for *Wnt2b* (C and D) and *Otx1* (E and F). The nasal-temporal axis is indicated as n and t, and the dashed line indicates the rim of forebrain (A). Detection of *TCF/Lef-LacZ* reporter activity at the dorsal-nasal retina (arrowhead in A) corresponds to the location of *Wnt2b* mRNA (arrow in C) and CM/RPE marker *Otx1* in the dorsal (triangle in E) retina. Note that the *TCF/Lef-LacZ* reporter gene and *Wnt2b* is not expressed in the ventral optic vesicle at this stage (filled triangle in B and D). CM: ciliary margin; RPE: retinal pigment epithelium. Scale bar: 50 μ m.

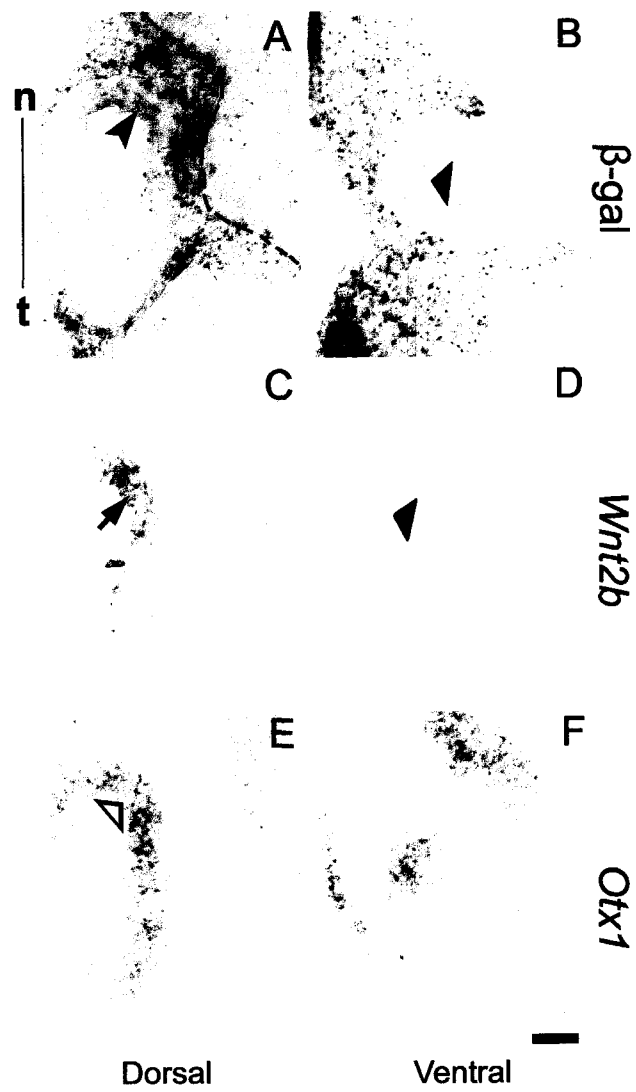


Table 4. Comparison of *TCF/Lef-LacZ* reporter gene activation and expression of *TCF/Lef* transcription factors during ocular development from E9.5 to E14.5*

	OV (E9.5) [#]		CM	RGC		NB	LAE	ONH
	D	V						
Reporter	+	-	+	+ (E12.5 to E14.5) [#]		+	+	-
<i>Tcf1</i>	+	+	+	-		+	+	+
<i>Lef1</i>	+	+	+	+ (E14.5) [#]		+	-	-
<i>Tcf3</i>	+	+	+	-		+	+	+
<i>Tcf4</i>	+	+	-	-		-/+	-	-

* CM: ciliary margin; D/V: dorsal/ventral; LAE: lens anterior epithelium; NB: neuroblast layer; ONH: optic nerve head; OV: optic vesicle; RGC: retinal ganglion cell layer.

[#] Embryonic ages in parentheses indicate that reporter activity or gene expression in this region was restricted to this developmental timepoint.

At E10 the optic vesicle invaginates to form a bi-layered optic cup, with the inner layer developing as NR, the outer layer developing as RPE and the rim of the eyecup the CM. The distal CM gives rise to the iris and the proximal CM gives rise to the two-layered CE, which consists of the outer PCE and the inner nPCE. In the peripheral retina, from E11.5 to E15.5, the *TCF/Lef-LacZ* reporter gene was activated in the CM; from E16.5 onwards, expression of this reporter gene was dramatically upregulated in the prospective nPCE and downregulated in the prospective PCE (Fig.14, Tables 4 and 5).

TCF/Lef-LacZ reporter gene expression was induced in a discrete patch of cells in the NR at E11.5 and by E12.5 onwards, reporter gene expression was predominantly segregated in the inner retina (RGC layer) and the apical side of the neuroblast layer (Fig. 14, Tables 4 and 5). The expression of reporter gene at the apical side of the neuroblast layer was dramatically down regulated after E18.5 (Fig. 14 and Table 5). Activation of the *TCF/Lef-LacZ* reporter gene in the inner neuroblast layer (between the RGC and apical side of the neuroblast layer) was detected from E14.5 until E17.5 and down regulated by birth (Fig.14, Tables 4 and 5). The reporter gene expression was also detected at a high level in the lens anterior epithelium from E11.5 to E14.5; but it was down regulated from E15.5 onwards (Fig.14 and Table 4). The dynamic expression of the *TCF/Lef-LacZ* reporter gene in several distinct regions of the retina during development suggests canonical Wnt signaling plays a role in the development of multiple retinal cell types.

Fig. 14. Expression of *TCF/Lef-LacZ* reporter gene in the developing eye.

(A-I) Horizontal sections of eyes from *TCF/Lef-LacZ* transgenic embryos at E9.5, E11.5, E12.5, E14.5, E15.5, E16.5, E17.5, E18.5 and P0 were stained with X-gal to detect β -gal activity. The nasal-temporal axis is indicated as n and t in A. Insets in panels B, D, E, H and I are enlarged views of the boxed areas. Dashed lines in D and I indicate the boundaries between the prospective PCE (P) and the nPCE (nP) of the CM. Activation of *TCF/Lef-LacZ* reporter gene was detected in the prospective CM (arrowhead in A), the CM and the prospective CE (arrowheads in B-I). It was also detected in the central NR at E11.5 (asterisk in B), the apical side of the NB layer from E12.5 to E17.5 (arrow in E), the inner NB layer from E14.5 to E17.5 (curved arrow in E) and the RGC layer from E14.5 to P0 (triangle in H). Note that activation of the reporter gene in the PCE (P) of the CM is down regulated at P0 (compare enlarged view in D with that in I). CE: ciliary epithelium; CM: ciliary margin; NB: neuroblast; nPCE: non-pigmented ciliary epithelium; NR: neural retina; PCE: pigmented ciliary epithelium; RGC: retinal ganglion cell. Scale bars: 100 μ m for (A), (B and C), (D and E) and (F-I).

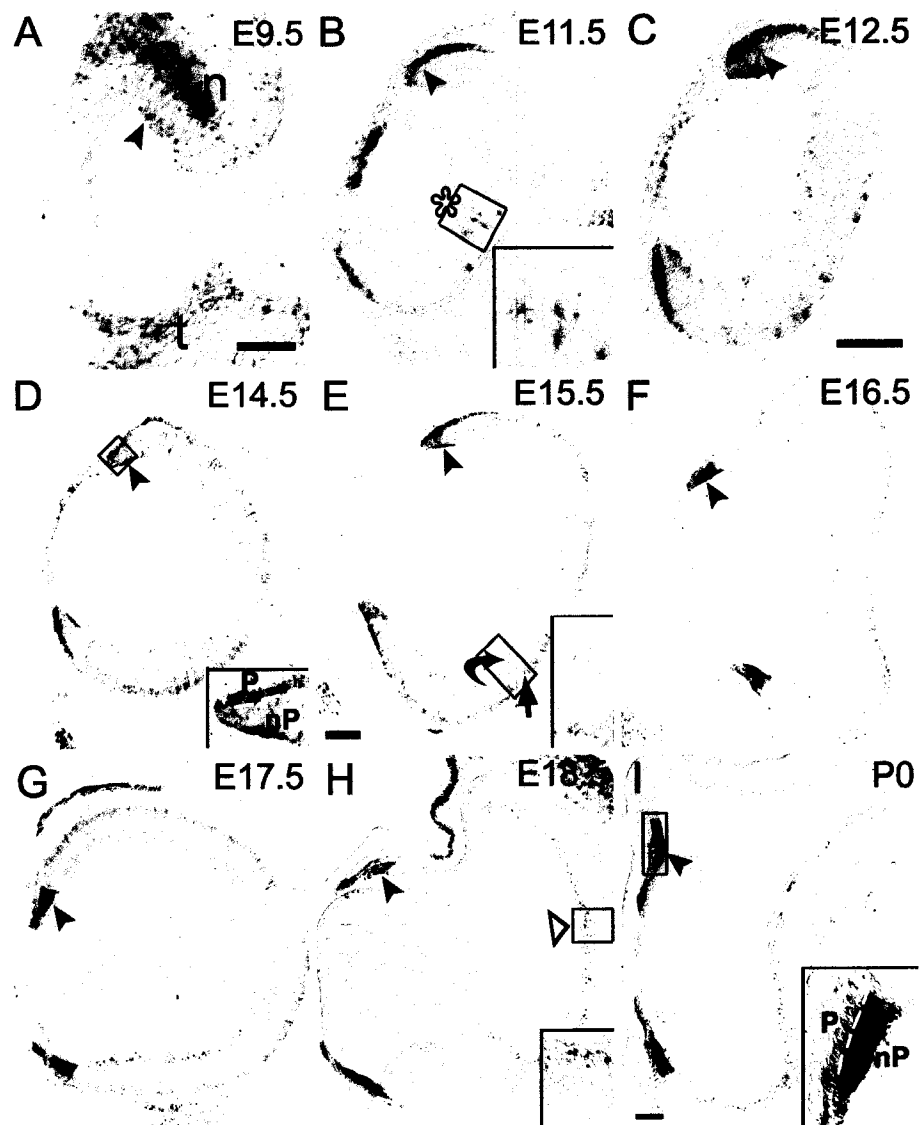


Table 5. Activation of *TCF/Lef-LacZ* reporter gene in the developing retina from E15.5 to P7 and in the adult retina*

CM/CE		RGC	INB/INL	ANB
PCE	nPCE			
+ (- at P7 and adult) [#]	+	+	+ (- from E18.5 to P0)	+ (E15.5 to E18.5)

* ANB: apical neuroblast layer; CM/CE: ciliary margin/ciliary epithelium; INB/INL: inner neuroblast/inner nuclear layer; nPCE: non-pigmented ciliary epithelium; PCE: pigmented ciliary epithelium; RGC: retinal ganglion cell layer.

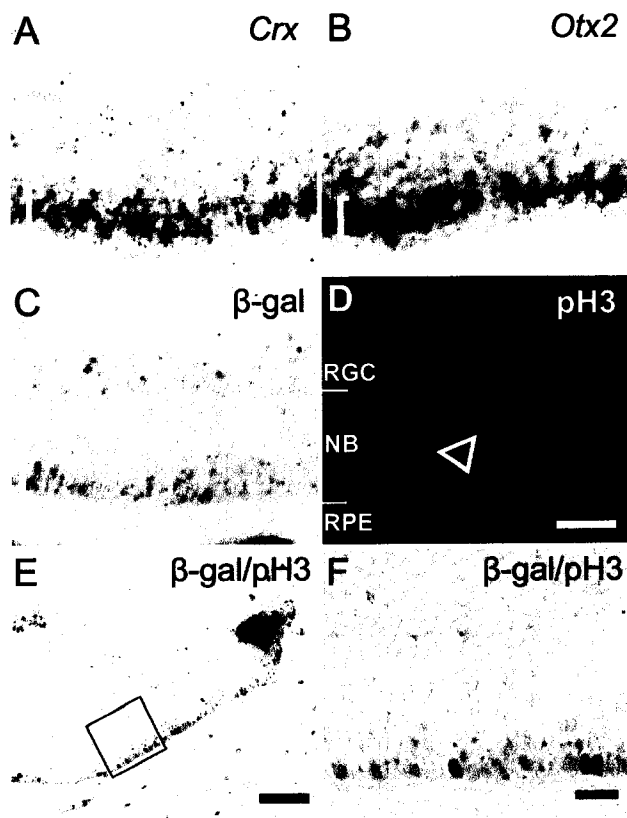
[#] Ages in parentheses indicate that reporter activity in this region was excluded (-) or restricted to this developmental timepoint.

4.1.3 Identification of TCF/Lef-LacZ reporter positive cells in the outer neuroblast layer of the developing retina

From E13.5 to E18.5, activation of *TCF/Lef-LacZ* reporter gene was detected in an apical pattern in the outer neuroblast layer, a region that contains differentiating photoreceptors, marked by the expression of homeobox transcription factors *Crx* and *Otx2*, but also mitotic progenitors. *Crx* is expressed soon after a cell is fated to the rod or cone photoreceptor lineage and is essential for terminal differentiation of photoreceptor cells (69, 173). *Otx2* shares a high homology at the amino acid level with *Crx* and is known to compensate for the function of *Crx* in photoreceptor cell fate determination and differentiation (2, 68, 178). To identify the TCF/Lef-LacZ reporter positive cells in this region, I compared β -gal activity with ISH for *Crx*, *Otx2* and IHC [2.5] for phosphoH3, which marks mitotic cells, on serial sections of E16.5 retina. In both the central and peripheral retina, expression of *TCF/Lef-LacZ* reporter gene was detected in a domain that was several cell diameters wide in the outer neuroblast layer, similar to the *Crx* expression pattern (Fig. 15A, 15C). *Otx2* was expressed in a wider area (Fig. 15B), while mitotic cells (anti-phosphoH3⁺) were restricted to a narrow, one-to-two cell wide, stripe of cells adjacent to the RPE (Fig. 15D). Double staining for β -gal and phosphoH3 revealed that the majority of the β -gal positive cells were not mitotic (Fig. 15E, 15F). Therefore, I conclude that the majority of the apically located TCF/Lef-LacZ reporter positive cells at this stage likely correspond to immature photoreceptor precursors.

Fig. 15. Analysis of the TCF/Lef-LacZ reporter positive cells located in the apical neuroblast layer.

(A-F) Horizontal sections of the eye at E16.5 were processed for ISH for *Crx* (A) or *Otx2* (B), stained with X-gal for β -gal activity (blue in C), for IHC with anti-pH3 (red in D) or double stained for β -gal activity (blue in E) and pH3 (brown in E). Hoechst was stained to reveal nuclei (blue in D). A higher magnification view of boxed area in E is shown in F. Brackets indicate the overlapping expression pattern of *Crx* (A), *Otx2* (B) and TCF/Lef-LacZ reporter activity (C) in the apical NB and the triangle (D) indicates a narrow stripe of pH3⁺ cells adjacent to the RPE. A few cells are double labelled for TCF/Lef-LacZ reporter and anti-pH3 (arrowhead in F), whereas majority of the TCF/Lef-LacZ reporter positive cells are not co-labelled with anti-pH3 (arrows in F). NB: neuroblast layer; pH3: phosphoH3; RGC: retinal ganglion cell layer; RPE: retinal pigment epithelium. Scale bars: 50 μ m in (A-D), 100 μ m in (E), 25 μ m in (F).



4.1.4 Dynamic expression of *TCF/Lef* family factors in the developing retina

The expression pattern of the *TCF/Lef* transcription factors in the mammalian retina is largely unexplored. Since the *TCF/Lef-LacZ* reporter gene is regulated by consensus *TCF/Lef* sequences, I compared the expression of *TCF/Lef* factors with *TCF/Lef-LacZ* reporter activity. The expression of the four *TCF/Lef* transcription factors was examined throughout the dorsal/ventral axis of the embryonic retina; no difference was detected in the dorsal versus ventral sections (data not shown). I observed a similar expression pattern of *Tcf1* and *Lef1* in the murine retina, consistent with previous reports showing a high degree of overlap in expression pattern and function of these two genes in fish retina and during murine embryogenesis (185, 251). Both *Tcf1* and *Lef1* mRNA was detected in the optic vesicle at E9.5, with *Lef1* expressed at a higher level than *Tcf1* in the proximal and intermediate optic vesicle that will give rise to CM and RPE (Fig. 16A, 16B and Table 4). From E12.5 to E14.5, both of *Tcf1* and *Lef1* were expressed diffusely in the neuroblast layer and at higher level in both prospective PCE and nPCE of the CM (Fig. 17A-D and Table 4). However, I also detected differences in expression pattern of these two genes. *Tcf1* was expressed in the optic nerve head and lens anterior epithelium (Fig. 17B and Table 4), while *Lef1* expression was detected in the periocular mesenchyme at E12.5 (Fig. 17C). Moreover, *Lef1* expression was observed in an asymmetric pattern in the differentiating RGC layer, with a higher level in the temporal retina at E14.5 (Fig. 17D, 18A and Table 4). Consistent with the possibility that there is an asymmetric distribution of canonical Wnt signaling in the developing retina, I found that the *TCF/Lef-LacZ* reporter gene activity was detected in a

Fig. 16. Expression pattern of *TCF/Lef* transcription factors in the optic vesicle.

(A-D) ISH of horizontal sections of the eye at E9.5 for *Tcf1* (A), *Lef1* (B), *Tcf3* (C) and *Tcf4* (D). The nasal-temporal axis is indicated as n and t, and the dashed line indicates the rim of forebrain (A). The expression of *Lef1* is detected in the nasal and proximal side of the optic vesicle respectively (arrowhead and arrow in B). Note the diffuse expression of *Tcf1* and high level of *Tcf3* throughout the optic vesicle (A and C). Triangle in D indicates low level of *Tcf4* expression in the temporal side of the optic vesicle. Scale bar: 50µm.

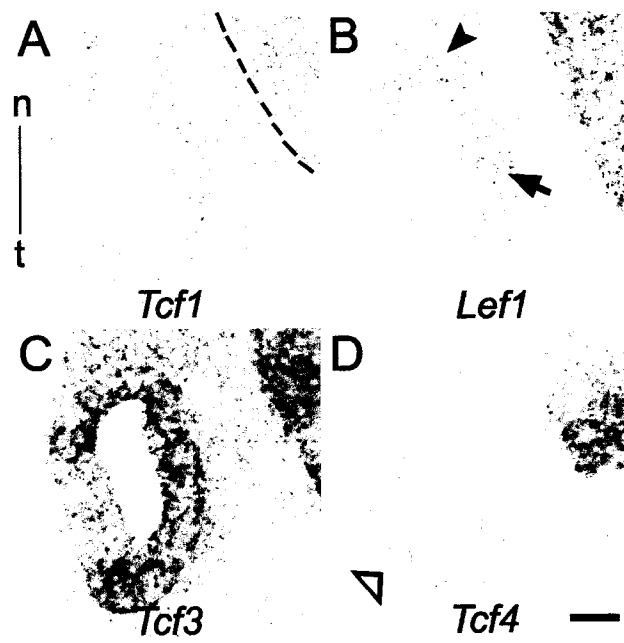


Fig. 17. Expression pattern of *TCF/Lef* transcription factors in the developing eyecup.

(A-H) ISH of horizontal sections of the eye at E12.5 (A, C, E and G) or E14.5 (B, D, F and H) for *Tcf1* (A and B), *Lef1* (C and D), *Tcf3* (E and F) and *Tcf4* (G and H). The nasal-temporal axis is indicated as n and t in A. Inserts are enlarged views corresponding to the boxed areas in B, D and F. Dashed lines in B, D and F indicate the boundaries between the prospective PCE (P) and the nPCE (nP) of the CM. *Tcf1* and *Lef1* are expressed in the CM (arrowheads in A-D). Expression of *Tcf1* and *Tcf3* is also detected in the optic nerve head (triangles in B, E and F). A higher level of *Lef1* expression is detected at the temporal retina at E14.5 (arrow in D). *Tcf3* is expressed in the CM and throughout the NR (E and F); in contrast, expression of *Tcf4* is difficult to detect at E12.5 and E14.5 (G and H). CM: ciliary margin; nPCE: non-pigmented ciliary epithelium; NR: neural retina; PCE: pigmented ciliary epithelium. Scale bars: 100µm in (A, C, E and G) and (B, D, F and H).

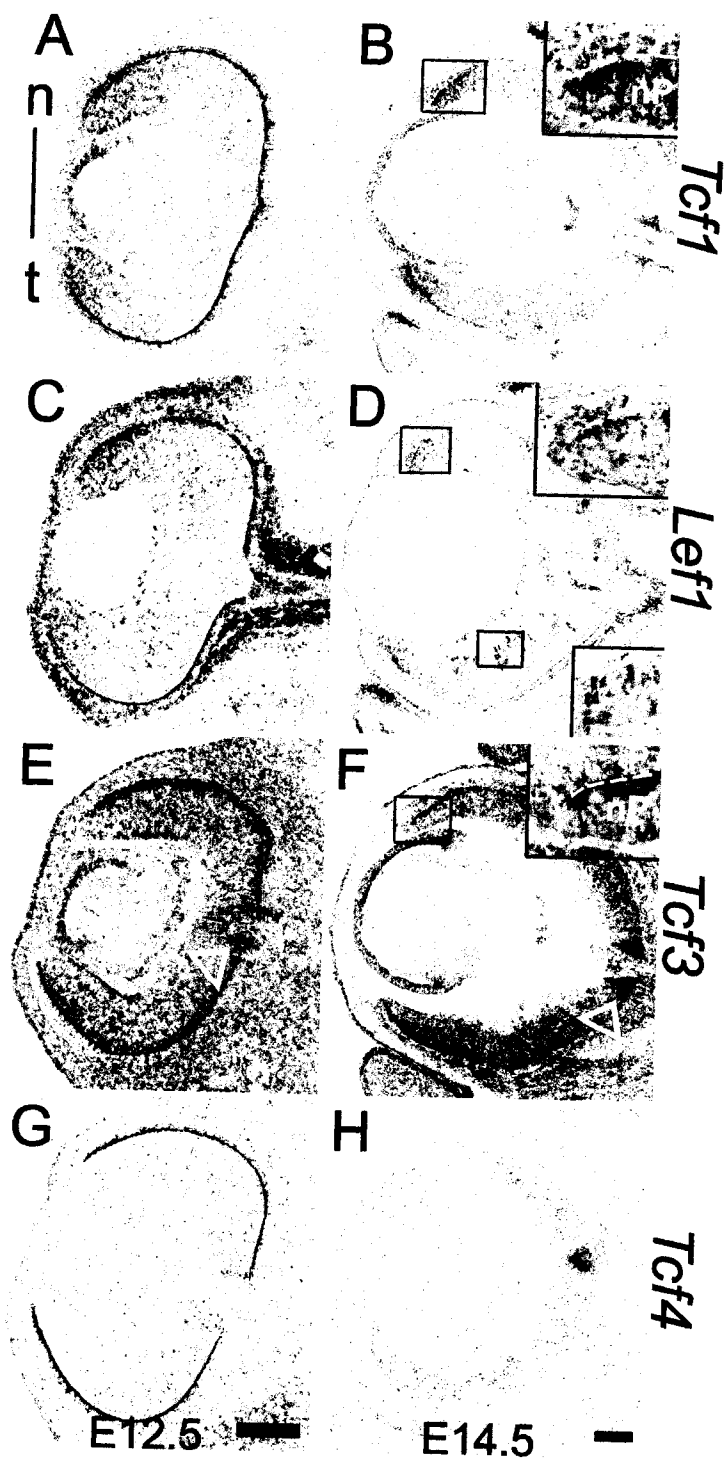
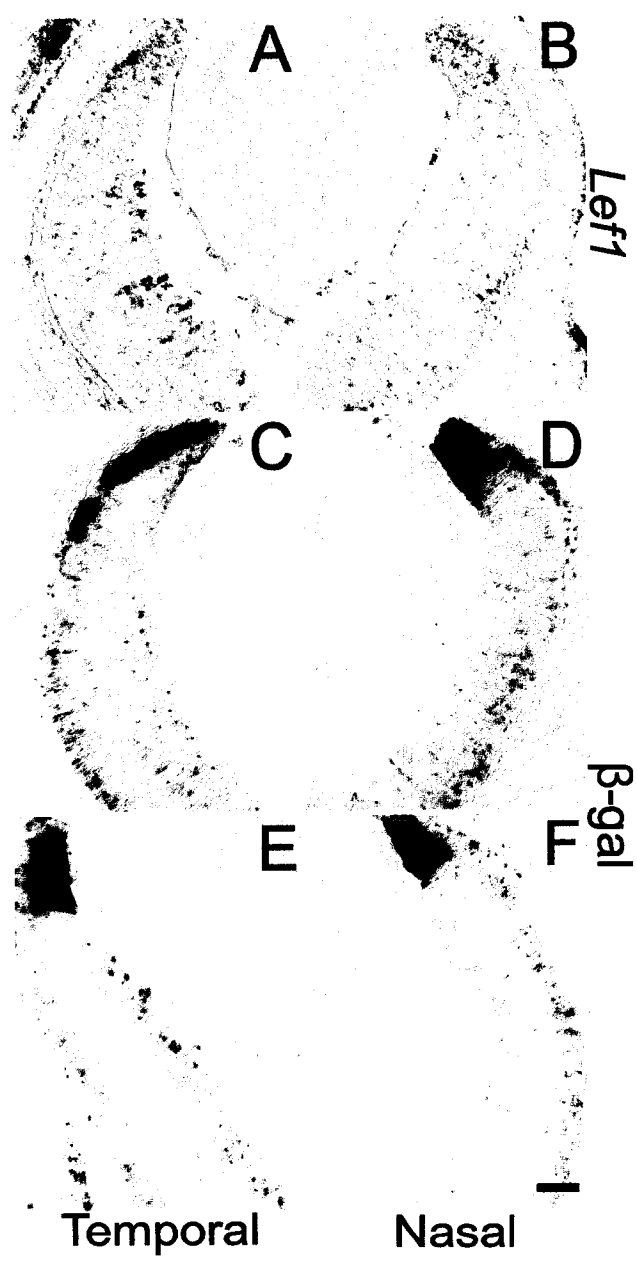


Fig. 18. Asymmetric expression of *Lef1* and *TCF/Lef-LacZ* reporter gene in the temporal retina of the developing eye.

(A-F) Horizontal sections of the eye at E14.5 (A-D) and E17.5 (E and F) were processed for ISH for *Lef1* (A and B) or stained with X-gal (C-F) to detect β -gal activity. Higher level of *Lef1* expression (arrows in A) and *TCF/Lef-LacZ* reporter activity (arrows in C and E) are detected in the temporal retina. Scale bar: 50 μ m.



similar asymmetric pattern in the RGC layer from E14.5 to E17.5 (Fig. 18C-18F).

Tcf3 expression was observed throughout the optic vesicle at E9.5 (Fig. 16C and Table 4), in the entire neuroblast layer and at a higher level in the optic nerve head at E12.5 and E14.5, but was absent from the differentiating RGC layer (Fig. 17E, 17F and Table 4). *Tcf3* transcripts were also detected in the periocular mesenchyme and in the lens anterior epithelium at the developmental stages examined (Fig. 17E, 17F and Table 4). *Tcf4* mRNA was detected at a low level in the optic vesicle at E9.5, and was difficult to detect after E12.5 (Fig. 16D, Fig. 17G, 17H and Table 4). I observed a high level of *Tcf4* expression in the dorsal thalamus at the same stages (data not shown), which was in consistent with previous study (134), proving that the ISH result is reliable.

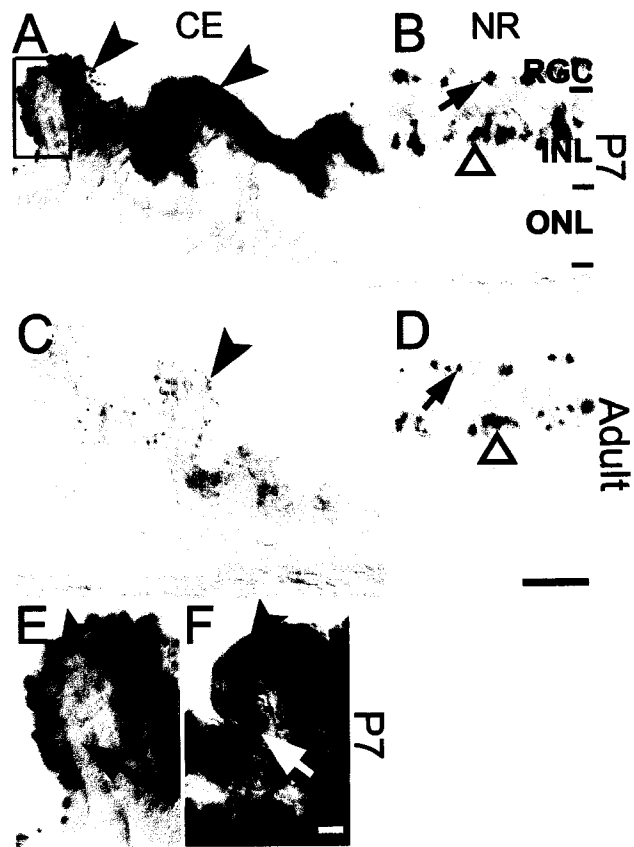
4.1.5 Activation of *TCF/Lef-LacZ* reporter gene in the postnatal and adult retina

Because some Wnts and Wnt signaling components are expressed in the postnatal and adult murine retina (33, 148, 196, 258), I asked whether and how canonical Wnt signaling is activated at these stages.

In the P7 and adult retina TCF/Lef-LacZ reporter activity was detected primarily in the nPCE, the RGC and INL layers (Fig. 19 and Table 5). Note that the TCF/Lef-LacZ reporter gene activity in the adult CE was dramatically down regulated compared with the CE at P7, suggesting that canonical Wnt signaling is attenuated in this region of the adult eye. In the INL layer, the expression pattern of TCF/Lef-LacZ reporter gene was detected at a level much higher than that in the inner neuroblast layer of the late-stage embryos (compare Fig. 19B, 19D with Fig.14F-14I). Moreover, in contrast to my observation that TCF/Lef-LacZ

Fig. 19. Activation of *TCF/Lef-LacZ* reporter gene in the postnatal and adult eye.

Horizontal sections of the eye at P7 (A, B, E and F) and adult (C and D) were stained with X-gal to detect β -gal activity. An enlarged view of boxed area in A is shown in E and the corresponding region from a pigmented retina is shown in F for easier distinction between the PCE and nPCE. Arrowheads in A, C, E and F indicate activation of *TCF/Lef-LacZ* reporter gene in the nPCE, while arrows in E and F indicate that reporter gene expression is not detected in the PCE. *TCF/Lef-LacZ* reporter gene is also activated in the RGC (arrows in B and D) and the INL layers (triangles in B and D) in the central NR. CE: ciliary epithelium; INL: inner nuclear layer; nPCE: non-pigmented ciliary epithelium; NR: neural retina; ONL: outer nuclear layer; PCE: pigmented ciliary epithelium; RGC: retinal ganglion cell layer. Scale bars: 50 μ m in (A-D), 10 μ m in (E, F).

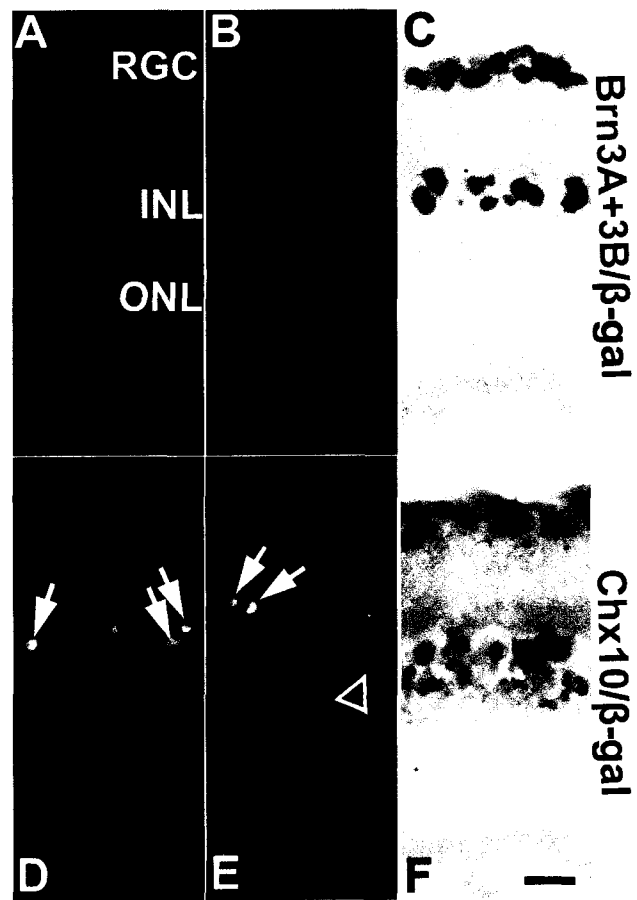


reporter gene activity was gradually downregulated in the CM of retinal explants, its expression was initiated in the INL of the E14.5 explants after 10 days in culture (data not shown), which corresponds to high level TCF/Lef-LacZ reporter gene activation in the postnatal retina. While the source of canonical Wnt signaling in the CM might reside in the RPE, which is removed in the culture, the retinal environment in the explants appears to be permissive for the induction of high levels of TCF/Lef-LacZ reporter gene expression in at least a subset of cells within the explants.

The identity of TCF/Lef-LacZ reporter positive cells was determined by position within the nuclear layers and double staining with β -gal and cell type specific markers. The analyses were performed on P7 and adult retina, with similar results. In the RGC layer, β -gal⁺ cells were identified as subsets of RGC cells (Brn3A⁺ or Brn3B⁺) (273, 274) (Fig. 20). In the INL, β -gal⁺ cells were subsets of amacrine cells (Pax6⁺ and calbindin⁺) (13, 87, 93, 161), but not bipolar (Chx10⁺) (25) or horizontal cells (calbindin⁺) (92, 93) (Fig. 20), implying that canonical Wnt signaling is likely involved in the maintenance or function of RGC and amacrine cells.

Fig. 20. Cell type identification of the TCF/Lef-LacZ reporter positive cells in the adult retina.

(A-F) Horizontal sections of adult eyes were stained with Hoechst to reveal nuclei (A and B), processed for IHC for β -gal (green in D and E) and Pax6 or Calbindin (red in D and E), or double stained for β -gal activity (blue in C and F) and Brn3A+3B (brown in C) or Chx10 (brown in F) with DAB reaction. Arrows in D or E indicate the co-localization of anti-Pax6 or Calbindin with TCF/Lef-LacZ reporter gene expression in the INL, marking amacrine cells. Arrowheads in C indicate the co-localization of anti-Brn3A and Brn3B with TCF/Lef-LacZ reporter gene expression in the RGC layer, marking RGC cells. Note that the horizontal cells (triangle in E) and bipolar cells (Chx10⁺ in F) do not co-express TCF/Lef-LacZ reporter gene. INL: inner nuclear layer; ONL: outer nuclear layer; RGC: retinal ganglion cell layer. Scale bar: 25 μ m.



4.2 Discussion

The expression patterns of the TCF/Lef-LacZ reporter transgene and *TCF/Lef* transcription factors in the developing and adult murine retina was investigated. I show that canonical Wnt pathway activation is dynamic throughout retinal development, suggesting that it plays a role in multiple aspects of retinal development and homeostasis, including the development of the CM and the CE, cell fate specification and/or differentiation, axon guidance, and retinal neuron homeostasis.

4.2.1 Canonical Wnt signaling in the development of the ciliary margin/ciliary epithelium

The CM is a unique region in the developing eye. Although it is neuroepithelial-derived, it is not fated to give rise to retinal neurons; instead the proximal part differentiates as the CE overlying the CB and the distal part differentiates as the iris. A striking feature of my analysis is that the TCF/Lef-LacZ reporter transgene expression was maintained in the CM region of the developing eye from the optic vesicle stage to adulthood. This observation is consistent with a similar reporter analysis in transgenic zebrafish where the expression of a TOPdGFP reporter gene was documented in the CM at the early embryonic stages (53), and suggests a conserved role for TCF/Lef-dependent canonical Wnt signaling in specification and/or formation of this region of the eye. One Wnt gene, *Wnt2b* (formerly known as *Wnt13*), has been reported to be expressed at the tip of the CM and in the RPE cells overlying the CM (135, 148). In several studies on chick retina, cWnt2b was reported to induce accumulation

of β -catenin (135), a feature of canonical Wnt signaling, and to maintain the proliferation of progenitors and inhibit neuronal differentiation of progenitors (136). In the present study *Wnt2b* and *TCF/Lef-LacZ* reporter gene expression is induced in an identical pattern in the dorsal retina at optic vesicle stage, suggesting that *Wnt2b* could be the relevant Wnt signal that is activating the *TCF/Lef-LacZ* reporter gene in the mouse.

4.2.2 A possible role of canonical Wnt signaling in RGC axon guidance

The topographic projection of RGC axons to their major midbrain target, the superior colliculus, is regulated by region-specific differences in RGC responsiveness to graded distribution of guidance cues expressed in the target (reviewed by 165). The differential responsiveness of RGCs to the guidance cues is controlled via graded gene expression along the dorsal-ventral and nasal-temporal axes of the retina (reviewed by 165). The asymmetric expression of A-class Ephrin proteins along the nasal-temporal axis of the retina and the EphA receptor tyrosine kinase along the anterior-posterior axis of the target is part of the mechanism that establishes this topographical map (reviewed by 183). My observation that the *TCF/Lef-LacZ* reporter gene and *Lef1* are asymmetrically expressed in the RGC layer in the temporal retina suggests that canonical Wnt pathway activation plays a role in RGC axon guidance. While the target genes that may be controlled by TCF/Lef-dependent signaling in this context remain speculative, it is interesting to note that *EphB/EphrinB* expression in the intestinal epithelium is regulated by β -catenin/TCF (10, 42) and that graded RGC responsiveness to *Wnt3* has been shown to play a role in retinotectal mapping in the chick visual system (212).

4.2.3 Conserved expression of *TCF/Lef* transcription factors in the vertebrate retina

This study represents the first spatial and temporal expression patterns of *Tcf1*, *Lef1*, *Tcf3* and *Tcf4* in the developing mouse retina. The observation of *Tcf1* expression in the CM of the embryonic mouse retina is consistent with a previous report showing the expression of *Tcf1* in the frog eye (205) and *Tcf7* (the zebrafish orthologue of *Tcf1*) in the retinal margin in fish (251), however, I did not detect dorsal retina-localization of *Tcf1*, as reported in fish. These observations suggest that the expression of *Tcf1* in the retina is highly conserved, but in variable patterns in between species. Expression of *Lef1* in the CM is conserved between mouse and chick (135), whereas the asymmetric distribution of *Lef1* mRNA in the RGC layer has not been reported previously. The detection of high-levels of *Tcf3* expression in the developing retina contradicts previous reports that *Tcf3* is undetectable after E10.5 (134) and is consistent with other studies showing that *Tcf3* is expressed in the embryonic brain of mouse at later stages (71, 219). Moreover, *headless* (the zebrafish orthologue of *Tcf3*) mutant is characterized by a slight reduction in eye size in fish (128), suggesting an important role of *Tcf3* during eye development, however, the roles of *Tcf3* and other TCF/Lef transcription factors in the mouse retina remains to be explored.

The *TCF/Lef-LacZ* transgenic mouse line used in this study is designed to report the TCF/Lef-dependent gene expression. Despite the Li^+ sensitivity of this reporter, will it faithfully recapitulate all canonical Wnt/ β -catenin signaling? It is possible that in some instances the activity of the transgenic reporter gene will not mirror the expression of endogenous Wnt target genes, thereby underestimating the extent and location of canonical

Wnt pathway activation in various tissues. In other situations, activation of this reporter transgene could overestimate the fraction of TCF/Lef activity that is directly downstream of Wnt/ β -catenin signaling. Several non-Wnt ligands have been reported to be able to trigger the canonical Wnt cascade, such as Sfrp1 (244), Dkk2, a member of the Dickkopf family of secreted Wnt antagonists (157), Wise, a secreted molecule (112), and Norrin, the protein product of the Norrie disease gene (275). In particular, Norrin and Wnt receptor, Frizzled-4 function as a ligand-receptor pair, controlling vascular development in the retina and inner ear (275). Nonetheless, the present study systematically mapped the ocular domains and cellular types in which TCF/Lef-dependent gene expression is activated, suggesting multiple roles of this pathway in various developmental processes and homeostasis in the murine retina, which will surely benefit further exploration of Wnt function in the context of mammalian retina and may yield important insights into novel therapeutic approaches to treat or prevent congenital eye diseases.

5.0 Activation of Canonical Wnt Signaling Promotes Ciliary Margin Identity and Controls Neuronal Differentiation in Retinal Explants

Specification of the CM, the periphery of the developing eye, is an active area of research because aberrant function of the CE, which is derived from CM, is associated with eye diseases, such as glaucoma (41, 70), and because this region contains retinal stem cell pools (4, 243). The CM is a unique region in the developing eye. Although it originates from the optic cup, same as the NR, it is not fated to give rise to retinal neurons; instead the proximal part differentiates as the CE overlying the CB and the distal part becomes the epithelia of the iris (11, 201). Previous studies have shown that *Wnt2b* is expressed in the RPE overlying the CM in chick and mouse (66, 113, 135, 148, 149). The expression of several additional components of the Wnt pathway, as well as the activation of TCF/Lef-LacZ, a canonical Wnt reporter transgene, were also detected in overlapping, exclusive or adjacent patterns in this region (149, and reviewed by 249). Based on these pieces of evidence I extended the hypothesis to propose that canonical Wnt signaling, possibly mediated by Wnt2b, plays a role in the development of the CM in mouse.

One prediction of this hypothesis is that the level of canonical Wnt pathway activation would regulate the size of the CM. To test it, I proposed to drive ectopic Wnt signaling in the developing retina and assay for effects on markers of the CM and the NR. Several activators of the Wnt pathway were tested in retinal explants, including Wnt2b,

Wnt3a (a canonical Wnt pathway activator in other system (194, 217, 269)), two isoforms of constitutively active (CA) β -catenin and Li^+ , a well-known activator of canonical Wnt pathway. Several different approaches were used to drive expression of the above Wnt pathway activators, including retrovirus infection, transfection via electroporation and pharmacological treatment in retinal explants, and a transgenic mouse model. The majority of these manipulations were carried out on retinal explants from *TCF/Lef-LacZ* transgenic reporter mice and activation of canonical Wnt signaling was monitored for β -gal activity. To assay for the effects of these treatments on eye patterning, I examined proliferation, cell survival and monitored the expression of several transcription factors that are normally expressed in the retina, specific CM and retinal cellular markers and several genes that have been shown to be Wnt targets in other tissues.

I showed that Li^+ treatment induced canonical Wnt reporter activity, changes in gene expression, nuclear morphology and proliferation index, implicating an induction of CM like characteristic in retinal explants. Li^+ treatment also inhibited expression of neuronal markers. CA β -catenin up regulated Wnt reporter gene expression, albeit weakly, in a cell autonomous fashion; while ectopic expression of Wnt3a led to activation of Wnt reporter gene in a delayed and CM-restricted pattern. Overexpression of Wnt2b failed to induce Wnt reporter activation in the retinal explants. These findings suggest that activation of canonical Wnt pathway leads to a variety of developmental effects, including specification of the CM and control of neuronal differentiation in the murine retina.

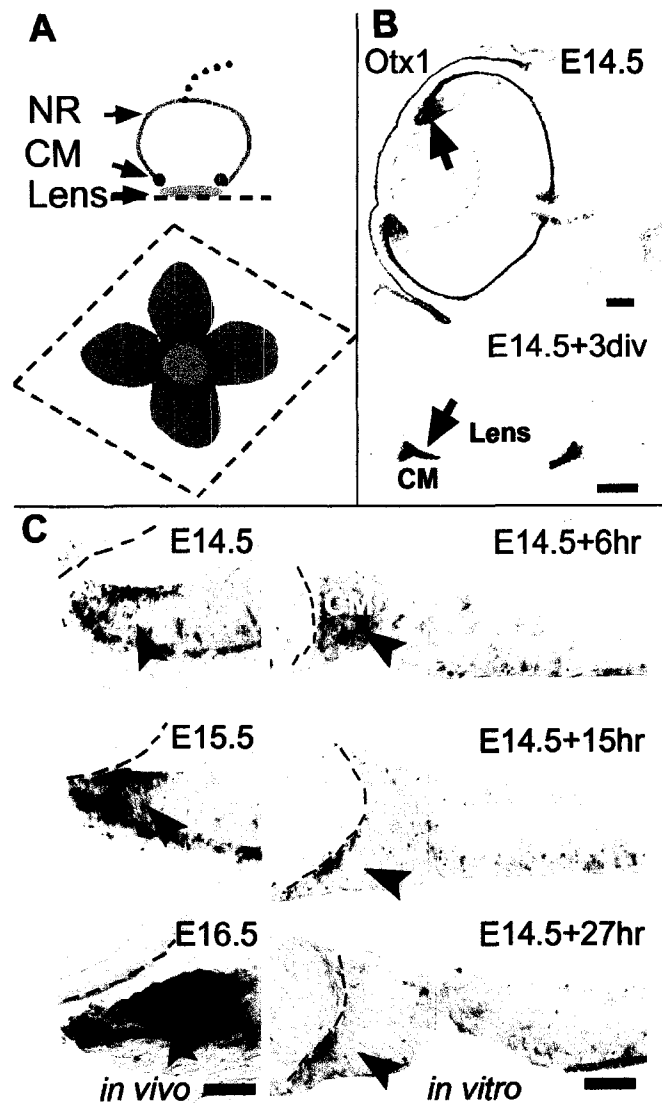
5.1 Results

5.1.1 Explants represent a loss of function model with respect to canonical Wnt signaling in the ciliary margin

Growth of the CM in mice begins around E14.5 and the morphological changes associated with CB development are not apparent until E16.5 (226), however, the partitioning of the eyecup into CM and NR regions is apparent as early as E12.5, as evidenced by the restricted expression of the CM/RPE markers, including *Otx1*, to the distal tip of the eye cup (162). Thus I chose E12-E14 as a suitable stage to monitor effects of Wnt signaling on CM development. Because the lens has been shown to induce the expression of CM genes (240), I established a retinal explant culture system [2.7.1] in which contact between the CM and the lens was maintained (Fig. 21A). In preliminary experiments, I showed that expression of CM marker, such as *Otx1*, as well as several reported Wnt component genes, including *Sfrp2*, *Mfz3*, 4, 6 and 7, are maintained in the explants cultured up to 3 days (Fig. 21B and data not shown) as examined by ISH. However, activation of canonical Wnt reporter gene, as examined by X-gal staining, decays by 15 hours in culture, most notably in the CM, as compared with the equivalent stages *in vivo* (Fig. 21C).

Fig. 21. Retinal explants represent a loss of function with respect of canonical Wnt signaling in the CM.

(A) Diagram shows retinal explant procedure. Eyecups from embryonic mice, dissected free of the RPE and the sclera, were opened at the optic nerve region and flattened onto filters with lens intact and cultured in serum-free explant media. (B) Maintenance of *Otx1* expression, a CM marker, in retinal explants. Cross section from E14.5 explants cultured for 3 days and transverse section from E14.5 embryonic eye were processed for ISH for *Otx1*. Arrows indicate similar expression pattern of *Otx1* in the CM of the explants as that in the embryonic eye. (C) Transverse sections from eyes of *TCF/Lef-LacZ* reporter mice at E14.5, E15.5 or E16.5 (*in vivo*, left panel) and from E14.5 explants cultured for 6hr, 15hr or 27hr (*in vitro*, right panel) were stained with X-gal to detect β -gal reporter activity. Dashed lines indicate the lens. Arrowheads indicate high level of β -gal reporter activity in the CM and it decays by 15 hours in culture (n (number of explants)=5). CM, ciliary margin; NR, neural retina. Scale bars: 100 μ m in (B), 50 μ m in (C).



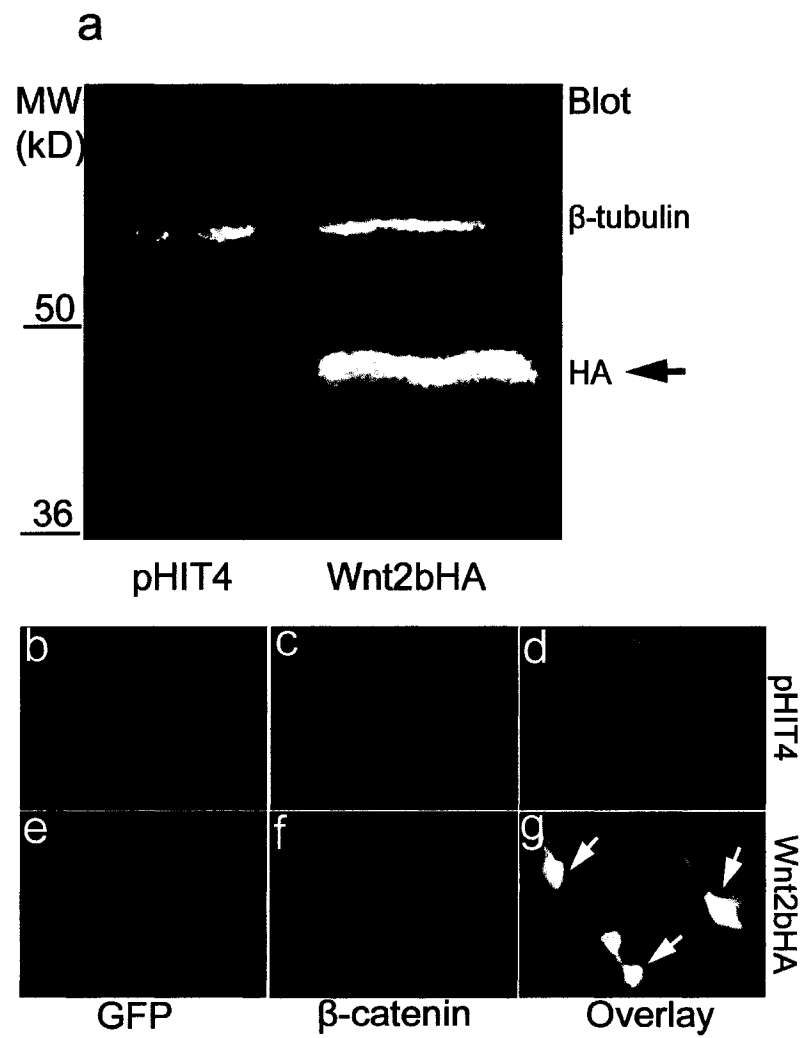
5.1.2 Ectopic expression of *Wnt2b* in the CM fails to activate *TCF/Lef-LacZ* reporter in retinal explants

Wnt2b is expressed in the RPE overlying the CM in the developing retina (135, 148). The RPE was removed when the retinas were dissected for explant culture and *Wnt2b* expression is not detected in the explants (data not shown), which raises the possibility that the failure to maintain high levels of *TCF/Lef-LacZ* reporter gene activation is due to the absence of RPE-derived *Wnt2b*. Chick *Wnt2b* has been shown to activate the canonical Wnt pathway and ectopic *cWnt2b* expression induces Wnt target gene expression, maintains progenitor proliferation and inhibits neural differentiation in the CMZ in embryonic chick retina (135, 136). To determine whether mouse *Wnt2b* behaves in a similar fashion, I constructed a C-terminal epitope-tagged *Wnt2b* expression vector that contains a *GFP* reporter gene (*Wnt2bHA/pHIT4.IRES.hrGFP*, referred to as *Wnt2bHA*) [map in Appendix D] [2.4.4]. Since epitope tags have been reported to inhibit Wnt function in some instances (163), the same expression vector expressing an untagged *Wnt2b*, *Wnt2b/pHIT4.IRES.hrGFP* (referred to as *Wnt2b*) expression vector, was also generated [2.4.4].

In *Wnt2bHA*-transfected 293FT cells, all the *GFP*⁺ cells co-localized with HA protein tag (data not shown) and the Western blot analysis [2.9] with anti-HA antibodies revealed a product of the expected size (approximately 40-43kD) (Fig. 22a), which is in the range of the predicted molecular weight of *Wnt2b* and other Wnt proteins (38-48kD) (218). I observed cytosolic accumulation of β -catenin in the *GFP*⁺ *Wnt2bHA*-transfected cells (Fig. 22g), which is a hallmark of activation of canonical Wnt pathway (reviewed by 270). To further

Fig. 22. Detection of the HA tag and accumulation of β -catenin in Wnt2bHA-transfected cells.

293FT cells were transfected with control plasmid pHIT4 or Wnt2b expressing vector, Wnt2bHA. (a) Whole cell lysates were processed for Western blot for HA tag, and β -tubulin was used as a loading control. Arrow in (a) indicates the position of the HA-tagged Wnt2b fusion protein in lysates from Wnt2bHA-transfected cells. (b-g) IHC to detect GFP reporter (green) and β -catenin (red) in pHIT4 or Wnt2bHA-transfected cells. Hoechst (blue) staining reveals nuclei. Accumulation of β -catenin in the cytoplasm was detected in Wnt2bHA-transfected GFP⁺ cells (arrows in g), while it was not detected in pHIT4-transfected GFP⁺ cells (d).

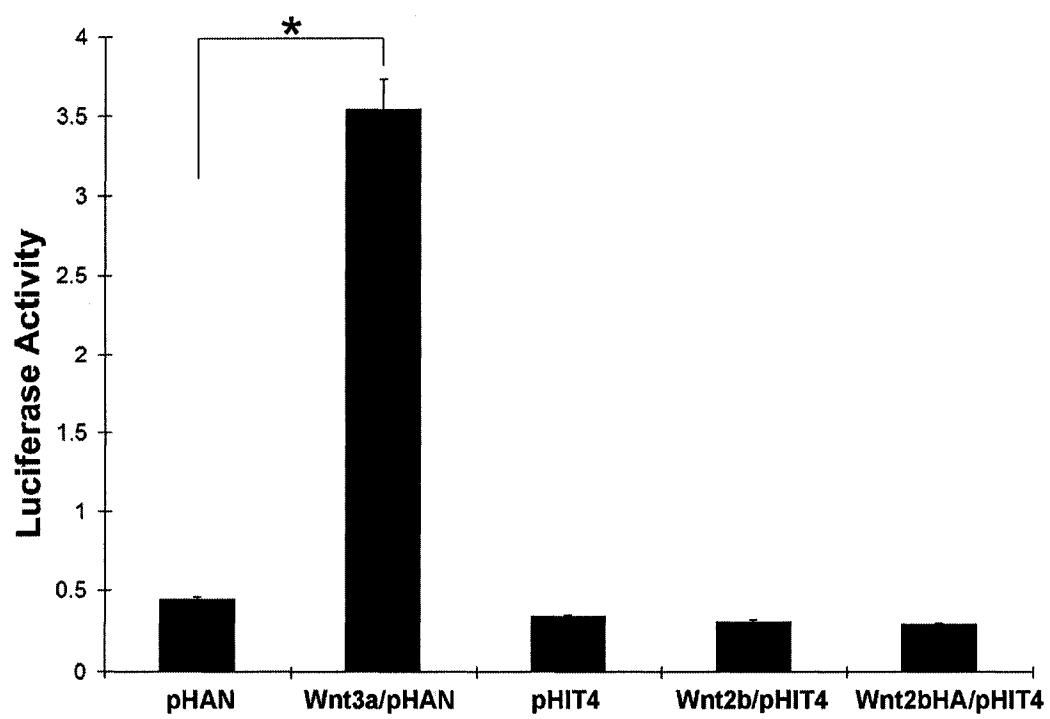


investigate the biological activity of the Wnt2bHA and untagged Wnt2b expression constructs, the TOPFlash luciferase reporter assay [2.3.2] was carried out. This assay has been used extensively to quantitate β -catenin/Tcf-dependent transactivation (133). In this assay the reporter construct, pTOPFlash, contains three copies of the optimal TCF/Lef binding motif (246), upstream of a minimal c-Fos promoter driving luciferase expression. The transactivational property of a test plasmid is valued by measuring luciferase activity after co-transfection with pTOPFlash, while blank expression vectors of the test construct were used as negative controls. A robust response was observed in Wnt3a transfected 293FT cells, however, transfected Wnt2bHA or untagged Wnt2b failed to activate the TOPFlash reporter (Fig. 23).

To investigate the effect of ectopic Wnt2b expression on retinal development, I mis-expressed Wnt2b in E14.5 retinal explants using a retrovirus approach. Wnt2bHA-retrovirus was generated in the pHIT4 system and titered on NIH3T3 cells [2.3.1]. Wnt2bHA expressing retrovirus was used to infect 293 cells at a multiplicity of infection of 10:1. The infection efficiency was higher than 50% as monitored by GFP expression (data not shown). However, this virus infected dissociated retinal cells at low efficiency (< 5%) and did not infect retinal explants (data not shown). I also attempted to target Wnt2b expression to the peripheral retina *in vivo* by generating transgenic mice using Msx1-minimal promoter, which was reported to direct the expression of a LacZ reporter gene as well as other transgenes to the peripheral eyecup (65, 172, 234, 283). A transgene construct, Msx1mp-Wnt2bHA was generated [2.4.4 (c)] and the purified DNA fragment was used for microinjection into

Fig. 23. Wnt2bHA or untagged Wnt2b construct fails to activate the TOPFlash reporter.

293FT cells were co-transfected with test plasmid as indicated, together with pTOPFlash and pRL-PGK, and cultured for 24 hours. Firefly and renilla luciferase activity in the transfected cell extracts were measured. Luciferase activities expressed as arbitrary units were normalized to renilla luciferase activity. All experiments were repeated three times, and results are presented as average of three independent experiments with standard deviations. Statistical significance was evaluated by Student t Test. * $P < E-06$.



pronuclei to generate transgenic mice [2.1.2]. No live born transgenic pups were recovered after two rounds of microinjections [Appendix B]. Examination on one E14.5 founder transgenic embryo derived directly from the founder mouse revealed the ectopic expression of *Wnt2b* in the retina as well as in the brain (data not shown). I presumed that expression of this transgene likely results in early embryonic lethality and thus did not pursue this line of investigation.

I then resorted to an electroporation approach [2.7.3] to induce ectopic expression of *Wnt2b* in the retinal explants. One day after electroporation, GFP⁺ cells co-localized with HA protein tag in *Wnt2bHA*-transfected explants (Fig. 24), indicating that *Wnt2bHA* was expressed in the transfected regions of the explants. To determine whether *Wnt2b* induces canonical Wnt signaling, E14.5 explants from the *TCF/Lef-LacZ* reporter mice were electroporated and the expression of the β -gal reporter was monitored over several days. Transfection of *Wnt2bHA* or untagged *Wnt2b* did not result in an increase of *TCF/Lef-LacZ* reporter gene expression in E14.5 explants cultured for 1, 2, 7 or 10 days, nor did they induce an accumulation of cytosolic β -catenin in E14.5 explants cultured for 2 or 7 days as examined by IHC (data not shown). Untagged *Wnt2b*-transfected explants were also monitored for the expression of a number of Wnt canonical pathway target genes, including *Bmp4*, *C-myc*, *CyclinD1*, *CyclinD2*, *Dkk*, *Dll1*, *Fgf9*, *Lef1*, *Sfrp2*, *Tcf1* (262) by ISH, no change in the expression of these genes was observed with the notable exception of *Bmp4* and *Lef1*, which were up regulated in both of control and *Wnt2b*-transfected explants (Fig. 25). Induced *Bmp4* and *Lef1* expression is restricted to where the plasmid is introduced,

Fig. 24. Detection of the HA protein tag in Wnt2bHA-transfected retinal explants.

E14.5 retinal explants were transfected with control plasmid pHIT4 or Wnt2b expressing vector, Wnt2bHA by electroporation and cultured for 1 day. Cross sections from explants were stained with Hoechst (blue) to reveal nuclei and processed for IHC for GFP reporter (green) and HA tag (red). GFP expression was detected in both of the control and Wnt2bHA-transfected explants (b and f). Arrows in (f-h) indicate that the HA tag is co-localized with GFP expression in the Wnt2bHA-transfected explants (n=3). Scale bar: 100µm.

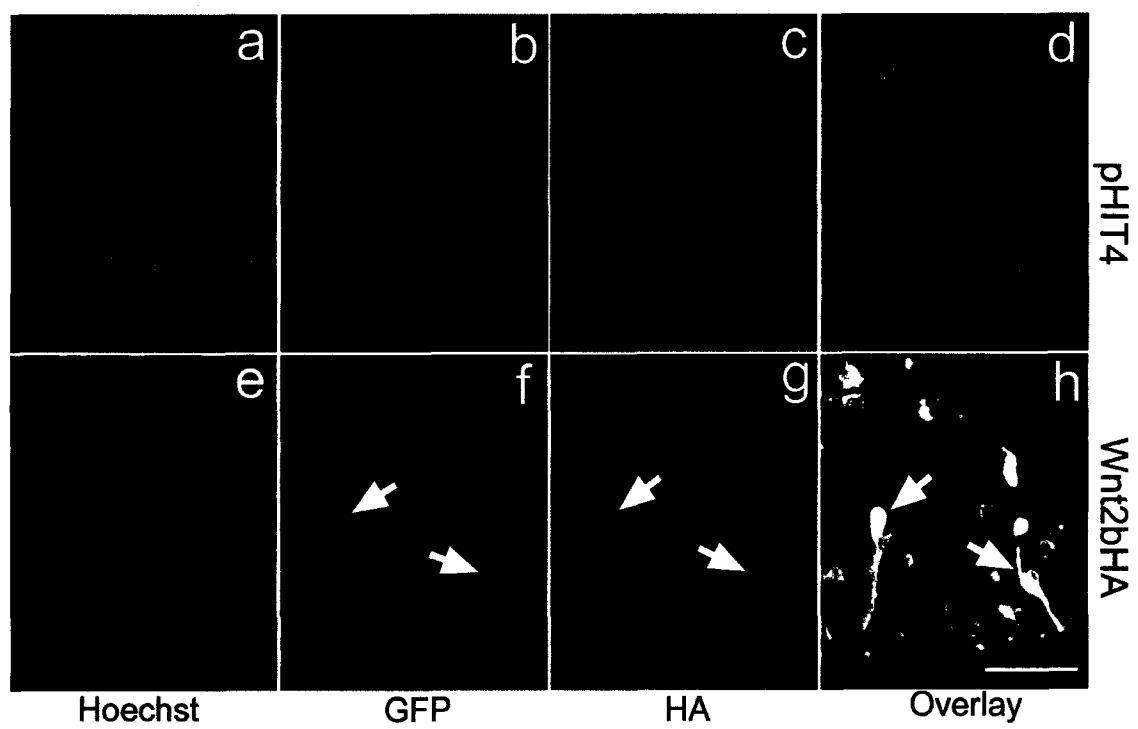
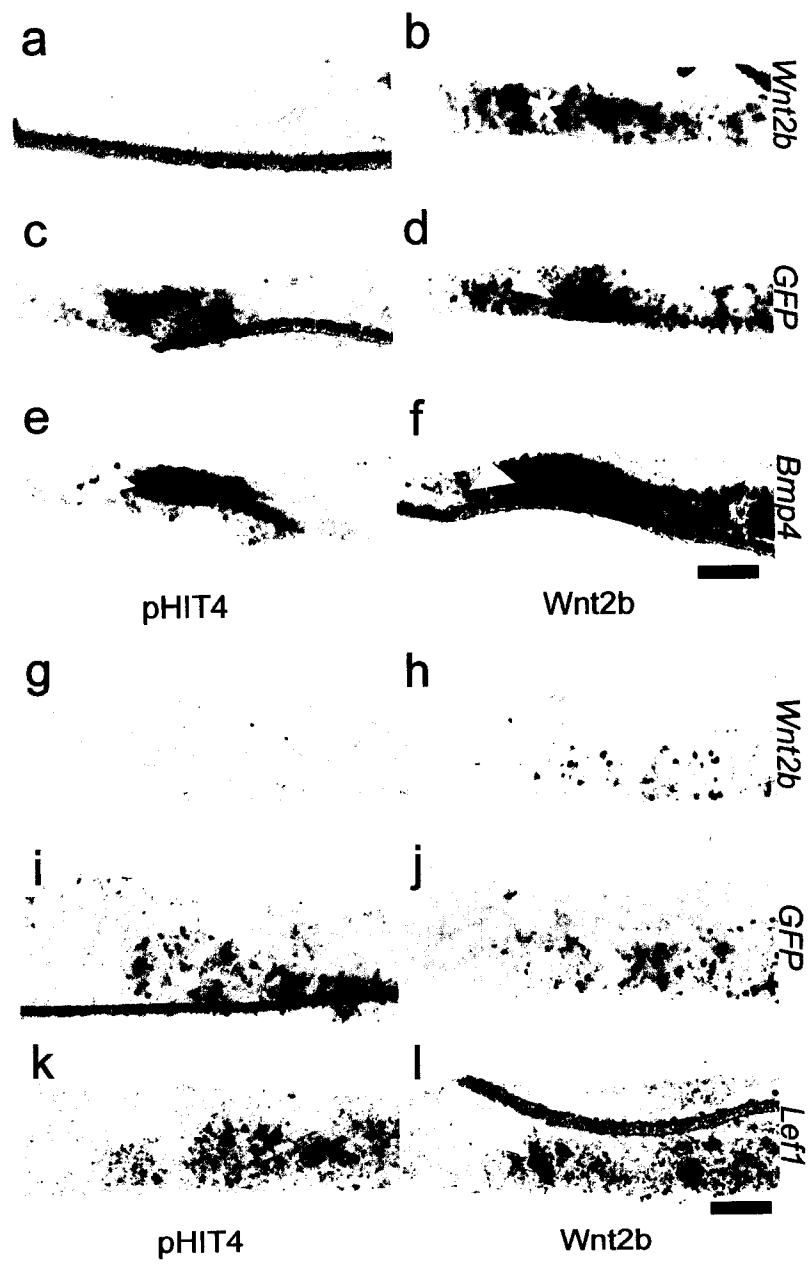


Fig. 25. Up regulation of *Bmp4* and *Lef1* expression induced by electroporation.

Serial sections from E14.5 retinal explants electroporated with untagged Wnt2b or control plasmid pHIT4 and cultured for 2 (a-f) and 10 days (g-l) were processed for ISH for *Wnt2b*, *GFP* and *Bmp4* or *Lef1*. Asterisks (b and h) indicate ectopic expression of *Wnt2b*. Arrows (c, d, i and j) indicate expression of the *GFP* reporter, marking the transfected regions in both control and Wnt2b-electroporated explants, while arrowheads indicate the induced expression of *Bmp4* (e and f) or *Lef1* (k and l) in the corresponding regions (n=5). Scale bar: 100µm.



suggesting that it, or at least part of this effect is an artefact induced by electroporation. To examine the effects of ectopic Wnt2b expression on retinal patterning, Wnt2bHA and untagged Wnt2b-electroporated explants were examined by ISH for a variety of transcription factors that have been shown to play a role in neural progenitor development and eye patterning, and by IHC for the expression of several retinal cellular markers [see a list of examined genes and markers with references in Appendix C] as well as the mitotic cell marker, phospho-Histone H3. No detectable difference on any of these parameters was observed in E14.5 explants transfected with Wnt2b or Wnt2bHA and cultured for 2, 6 or 10 days.

To summarize, ectopic expression of tagged or untagged Wnt2b was achieved in E14.5 retinal explants, but did not induce canonical Wnt pathway activation, as assessed by TCF/Lef-LacZ reporter activity, cytosolic β -catenin accumulation or Wnt target gene expression. Ectopic Wnt2b expression was also not associated with any changes in the expression of developmentally relevant transcription factors and retinal cellular markers. Notably, however, electroporation of plasmid DNA induced the expression of *BMP4* and *Lef1*.

5.1.3 Ectopic Wnt3a activates canonical Wnt signaling in the retinal explants

Because Wnt2b had no discernable effects in my system, I tested the effects of Wnt3a, a well characterized canonical Wnt pathway activator, on retinal development. Wnt3a is one of the best-studied Wnt proteins, in particular in the context of somitogenesis (reviewed by 6, and by 84). Wnt3a acts upstream of Notch signaling in regulating somite formation whereby

Wnt3a signals via Lef1 to up-regulate *Dll1* expression, thus linking Wnt with Notch receptor signaling (7, 72, 83, 102, 108). As opposed to other Wnt proteins, which usually accumulate on the cell surface or extracellular matrix when expressed in cell lines (20, 26, 199), Wnt3a is secreted into culture media from transfected cells (217, 269). Although expression of Wnt3a is not detected in the developing NR, I reasoned that Wnt3a could signal via the endogenous Wnt signaling machinery in the retina and exert effects on retinal development. Therefore, I attempted to induce ectopic Wnt3a expression in retinal explants and assay for effects of canonical Wnt pathway activation on retinal development.

Transfection of a Wnt3a expression construct, Wnt3a/pHAN (referred to as Wnt3a) [2.4.5 (a)] was shown to activate the TOPFlash reporter (Fig. 23), confirming that Wnt3a can activate the canonical Wnt pathway in 293FT cells. E14.5 retinal explants were co-transfected with this Wnt3a construct and a GFP reporter construct, pHIT4.IRES.hrGFP [2.4.2] by electroporation. Similar to the Wnt2b study, *Bmp4* and *Lef1* expression were upregulated in transfected regions of control and Wnt3a-transfected explants (data not shown). In this context, expression of *Bmp4* and *Lef1* served as a convenient readout for locating the electroporated region of the explants. E14.5 retinal explants from *TCF/Lef-LacZ* reporter mice were electroporated with Wnt3a. β -gal reporter activity was examined in explant sections after certain period of culture. Reporter activity in the CM is gradually lost during *in vitro* culture for 2, 6 and 10 days, as addressed earlier. Ectopic Wnt3a expression did not activate the reporter two days after transfection (data not shown), however, extensive activation of TCF/Lef-LacZ reporter activity was detected after 6 and 10 days. This effect

was restricted to the proximal CM, the prospective CE, but not in the prospective iris as assessed by expression of CE/RPE marker, *Otx1* and by the location of β -gal⁺ tissue (Fig. 26 and 27). It was not in the rest of NR even though there was ectopic expression of Wnt3a in the NR (Fig. 26). Thus ectopic Wnt3a activated *TCF/Lef-LacZ* reporter expression in a delayed and tissue-restricted fashion in the CM. Upregulation of *TCF/Lef-LacZ* reporter activity does not co-localize with the ectopic expression of *Wnt3a* (Fig. 26), implying a non-cell autonomous mechanism underlying this effect. Ectopic Wnt3a did not alter expression of *Chx10*, *Crx*, *Notch1*, *Pax6* and *Sfrp2* as examined by ISH, or expression of phospho-H3, or photoreceptor markers recoverin and rhodopsin as examined by IHC (data not shown) [see a list of examined genes and markers with references in Appendix C].

To address the possibility that the limited effect of ectopic Wnt3a was due to limited diffusion from a small number of transfected cells, explants from *TCF/Lef-LacZ* reporter mice were treated with soluble recombinant Wnt3a [2.7.4], which is commercially available and has been reported to induce cytosolic accumulation of β -catenin (127). Based on previous reports showing a short time (2-5 hours) treatment with low concentration (5-100ng/ml) of recombinant Wnt3a is efficient to stabilize β -catenin and long time treatment (over night) leads to morphological changes in cell lines (127, 217, 269), retinal explants were treated with recombinant Wnt3a in doses ranging from 25ng/ml to 250ng/ml for 24 hours and monitored for β -gal reporter activity, the expression pattern of β -catenin by IHC as well as expression of relevant genes by ISH. No detectable difference was observed in Wnt3a treated-explants compared to controls (data not shown).

Fig. 26. Activation of *TCF/Lef-LacZ* reporter gene in the CM in retinal explants electroporated with Wnt3a and cultured for 6 days.

Retinal explants were dissected from E14.5 *TCF/Lef-LacZ* reporter mice and cultured for 6 days following electroporation. Sections from explants electroporated with control plasmid pHAN (a, c and e) or with Wnt3a/pHAN (Wnt3a, b, d and f) were stained with X-gal to detect β -gal reporter activity or processed for ISH for *Wnt3a* or *Otx1*, the CM marker. Insert in (a) shows *Lef1* expression from adjacent section, indicating the electroporated region. Dashed lines indicate the lens. Reporter gene expression was up regulated in the proximal CM (arrow in d), but not in the distal CM, the prospective iris (asterisk in d) as defined by expression of *Otx1* (f), or in the NR (arrowhead in d) in Wnt3a-transfected explants (n=3). Note that up regulation of the reporter gene in the CM does not solely correlate to the location of ectopic *Wnt3a* expression (compare b with d), suggesting a non-cell autonomous mechanism of *TCF/Lef-LacZ* reporter gene induction by Wnt3a. CM: ciliary margin. Scale bar: 50 μ m.

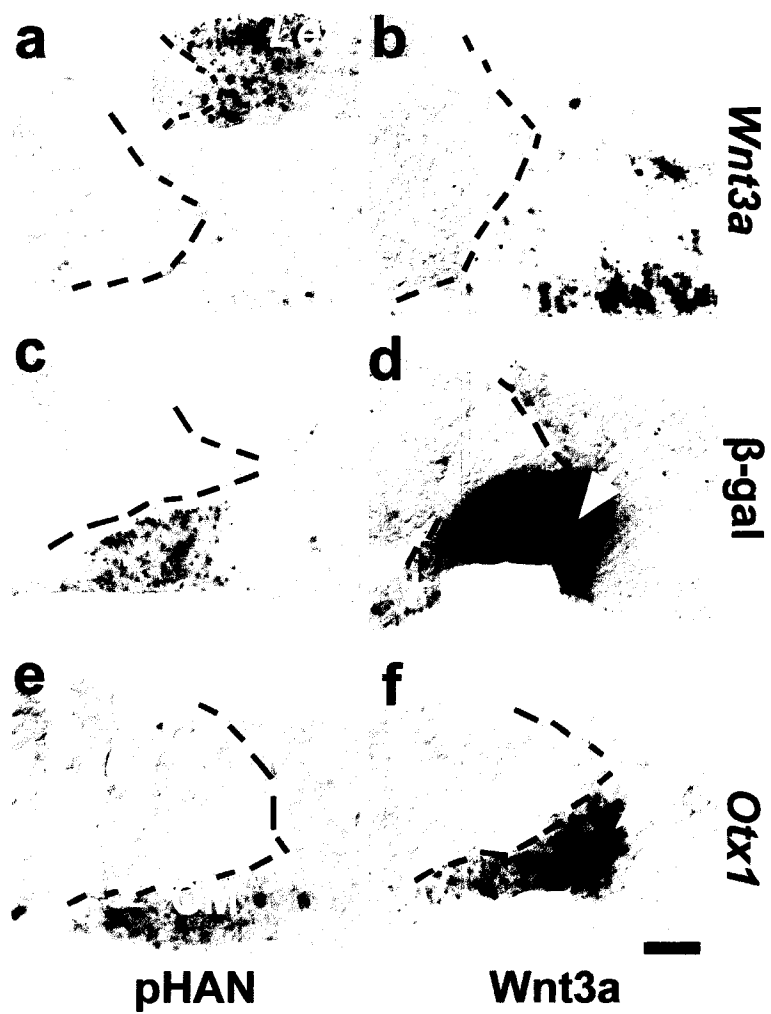
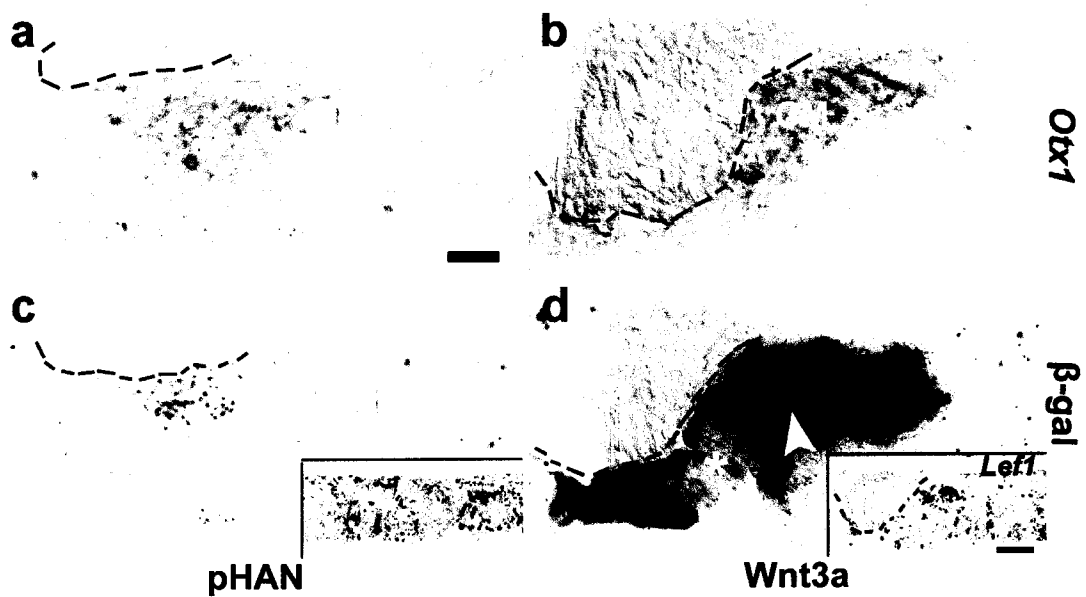


Fig. 27. Activation of *TCF/Lef-LacZ* reporter gene in the CM in retinal explants electroporated with Wnt3a and cultured for 10 days.

Retinal explants were dissected from E14.5 *TCF/Lef-LacZ* reporter mice and cultured for 10 days following electroporation. Sections from explants electroporated with control plasmid pHAN (a and c) or Wnt3a/pHAN (Wnt3a, b and d) were stained with X-gal to detect β -gal reporter activity or processed for ISH for *Otx1*, the CM marker. Inserts show *Lef1* expression from adjacent sections, indicating the electroporated regions. Dashed lines define the lens. Expression of reporter gene is up regulated in the proximal CM (arrowhead in d), but not in the distal CM, the prospective iris (asterisk in d) as defined by expression of *Otx1* (b) in Wnt3a-transfected explants (n=1). CM: ciliary margin. Scale bar: 50 μ m in (a to d), 100 μ m in inserts.



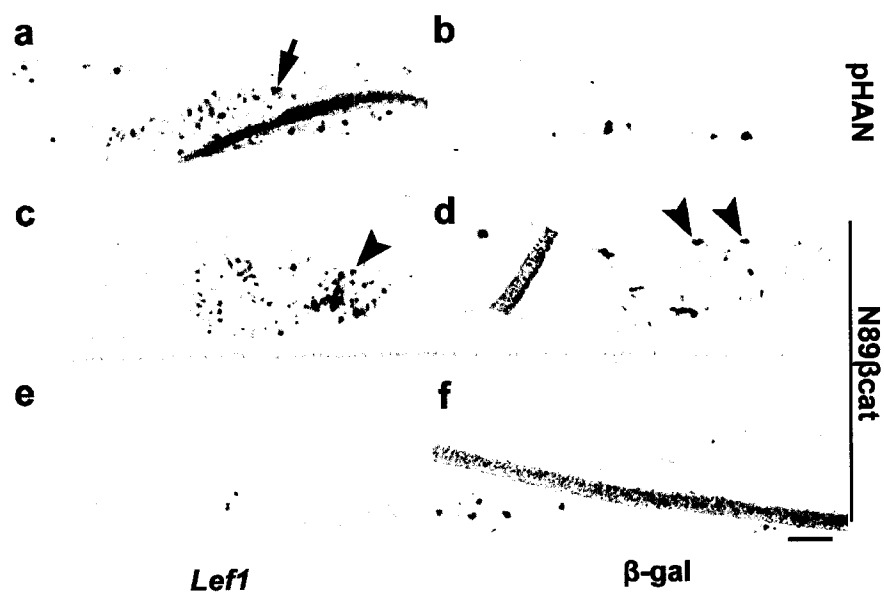
5.1.4 Constitutively active β -catenin up regulated Wnt reporter in the retinal explants

Failure of Wnt2b to activate the TCF/Lef-LacZ reporter and delayed induction of this reporter by Wnt3a could be due to the presence of extracellular Wnt antagonists, or absence of high affinity receptors/co-receptors in the explants, or the induction of a second signal. Therefore, to bypass this problem, I proposed to induce cell-autonomous β -catenin activation with gain of function β -catenin constructs in retinal explants from *TCF/Lef-LacZ* reporter mice.

Two different CA β -catenin constructs were used in this study: N57 β -catenin-HA/pCGN (referred to as N57 β cat) and Myc-N89 β -catenin-IRES-Lef1/pHAN (referred to as N89 β cat) [2.4.3]. Both constructs encode a truncated version of β -catenin lacking 57 or 89 N-terminal amino acids that contains the phosphorylation site for Gsk-3 β , which normally targets β -catenin for phosphorylation and degradation, thus producing a stabilized β -catenin protein that constitutively activates downstream Wnt signaling (44, 174, 209, 237). Ectopic expression of N57 β cat or N89 β cat induced the expression of the *TCF/Lef-LacZ* reporter in E13.5 retinal explants cultured for 1 and 2 days (Fig. 28 and data not shown). The induction of reporter gene expression, albeit weak, was restricted to the transfected region of the explants, indicating that the effect of CA β -catenin on *TCF/Lef-LacZ* reporter gene induction was likely cell-autonomous. Expression of relevant genes, such as reported Wnt target gene *Bmp4*, *C-myc*, *CyclinD1*, *Dkk1*, *Dll1*, *Lef1* (262), CM marker *Otx1*, and retinal progenitor or cellular marker *Crx*, *Notch1* and *Pax6* was also examined by ISH in the transfected explants. No difference was revealed in stabilized β -catenin transfected explants compared to controls,

Fig. 28. Constitutively active β -catenin activates *TCF/Lef-LacZ* reporter gene in retinal explants.

Retinal explants were dissected from E13.5 *TCF/Lef-LacZ* reporter mice and cultured for 2 days following electroporation. Sections from explants electroporated with control plasmid pHAN (a, b) or N89 β cat (c-f) were processed for ISH for *Lef1* as an indication of the electroporated region (a, c, e) or stained with X-gal to detect β -gal activity (b, d, f). Expression of reporter gene was up regulated in isolated cell patches (arrowheads in d), corresponding to the transfected region (arrowhead in c), while the reporter activity (f) in the untransfected area (e) was not affected in N89 β cat-electroporated explants (n=4), suggesting a cell autonomous mechanism. Arrow in (a) indicates induced *Lef1* expression in control explants. Scale bar: 100 μ m.



with the exception of induced expression of *Bmp4* and *Lef1* (Fig. 28 and data not shown), in keeping with observations described earlier.

5.1.5 Effects of lithium-induced canonical Wnt pathway activation in retinal explants

Studies of CA β -catenin in retinal explants provided a proof of concept that cell autonomous expression of a stabilized active component of the canonical Wnt pathway could induce Wnt pathway activation. However, the effect of stabilized β -catenin constructs to induce expression of the *TCF/Lef-LacZ* reporter gene was restricted in a small number of transfected cells, making it difficult to monitor gene expression cell-autonomously; and the artefact of induced expression of *Bmp4* and *Lef1*, which are reported Wnt target genes, became a big concern that transfection in retinal explants via electroporation might be improper for studying Wnt signaling in my explant system. An alternative way to stabilize β -catenin is to inhibit the kinase activity of Gsk-3 β with Li⁺, which prevents phosphorylation of β -catenin and subsequent degradation, resulting in activation of Wnt canonical pathway. In the preliminary experiments, Li⁺ treatment did activate *TCF/Lef-LacZ* reporter gene expression in retinal explants (149); therefore, a pharmacological approach of Li⁺ treatment was used in retinal explants from *TCF/Lef-LacZ* reporter mice to activate canonical Wnt signaling. Activation of the Wnt pathway was monitored for β -gal reporter activity and the effects of this treatment on gene expression, cell division and neuronal differentiation were investigated.

To examine the effects of Li⁺ treatment on gene expression, the expression of reported Wnt target genes was examined by ISH, including *Bmp4*, *CyclinD1*, *Dkk1*, *Dll1*, *Fgf9* and

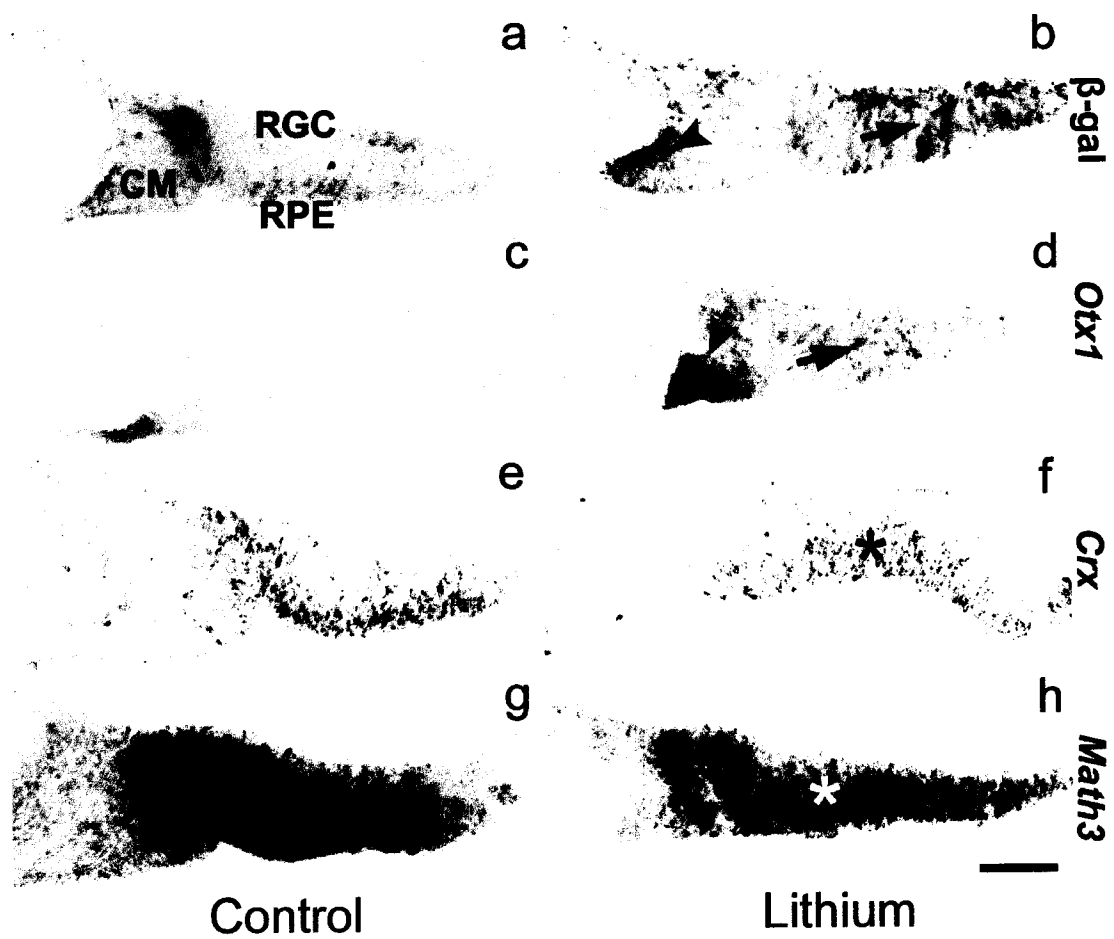
Lef1 (262). The following genes were also analysed: *Chx10* and *Pax6*, to identify progenitors; *Crx* and *Otx2*, to examine development of bipolar cells and photoreceptors as previous data showing that activation of canonical Wnt signaling correlates with *Crx/Otx2* expression in the outer neuroblast layer of the developing retina (149); *Otx1*, to locate CM/CE; *Hes1* and *Hes5*, to monitor Notch signaling; *Math3*, to monitor development of the amacrine cells, as I have shown that canonical Wnt signaling is activated in the amacrine cells in the P7 and adult retina (149); and *Math5*, as this gene was downregulated by ectopic expression of Wnt2b in chick retina (136) [see a list of examined genes with references in Appendix C].

Short-term exposure to Li^+ (9-27 hours) resulted in activation of the TCF/Lef-LacZ reporter and down regulation of *Crx* and *Math3* expression throughout retinal explants (Fig. 29). Li^+ treatment also resulted in an increase in the expression level of *Otx1* in the CM and ectopic *Otx1* expression in the NR (Fig. 29). Change of *Dll1* and *Hes5* expression was also detected in Li^+ -treated explants; however, this effect was less reproducible between samples. No differences were detected for the expression of other genes examined by ISH.

To confirm the Li^+ -induced changes in gene expression, quantitative RT-PCR (qPCR) analysis [2.8.3] was carried out on E14.5 retinal explants cultured for one day after Li^+ treatment. The expression of *Crx*, *CyclinD1*, *Dll1*, *Hes5*, *Math3*, *Otx1* and *Pax6* were examined in 6 independent experiments and in each experiment, RNA samples were from pooled (5-7 explants) controls or Li^+ treated explants. Efficiency of the primer pairs was tested on P0 retinal RNA and used to calculate fold change of gene expression. Fold change of each gene was normalized to *HPRT* and β -actin, and gave similar results. qPCR analysis

Fig. 29. Li^+ treatment up regulates the expression of the *TCF/Lef-LacZ* reporter gene and *Otx1*, and down regulates *Crx* and *Math3*.

Serial sections from E14.5 retinal explants dissected from *TCF/Lef-LacZ* transgenic embryo and cultured in control media (a, c, e and g) or in the presence of Li^+ (b, d, f and h) for one day were stained with X-gal (a and b) to detect β -gal activity or processed for ISH for *Otx1* (c and d), *Crx* (e and f) or *Math3* (g and h). Dashed line in (a) defines the lens. Expression of the *TCF/Lef-LacZ* reporter gene (n=6) and *Otx1* (n=3) is up regulated in the CM (arrowheads in b and d), and in the NR (arrows in b and d) of Li^+ -treated explants. Asterisks in (f and h) indicate the down regulation of *Crx* (n=7) and *Math3* (n=2) expression after Li^+ treatment. CM: ciliary margin; RGC: retinal ganglion cell layer; RPE: retinal pigment epithelium. Scale bar: 100 μm .



confirmed the Li⁺-induced down regulation of *Crx* and *Math3*, and upregulation of *Otx1* (Fig. 30). *Dll1* and *Pax6* were also downregulated, which was not revealed by ISH, in Li⁺-treated explants. However, changes in *CyclinD1* and *Hes5* expression levels were not consistent between samples (data not shown).

To confirm changes in *Pax6* expression, IHC was performed with anti-Pax6 antibodies to identify the cell types that were affected by Li⁺ treatment. Pax6 is expressed in retinal progenitors, as well as in differentiated RGC, amacrine and horizontal cells (101, 195, 255). Neurons can be distinguished from RPCs by the higher intensity of Pax6 expression (64). A remarkable change in nuclear morphology and Pax6 expression pattern was observed after Li⁺ treatment in E14.5 explants cultured for one day. In the control explants, nuclei were mostly round and randomly oriented, and very intense Pax6 immunoreactivity was detected throughout the neuroblast layer and in the majority of cells in the RGC layer, marking the differentiated RGC and amacrine cells (Fig. 31A, C). After Li⁺ treatment, nuclei were more organized and mostly elongated perpendicular to the apical layer of the retina, in a neuroepithelial-sheet like pattern; and fewer intensely-stained Pax6⁺ cells were detected in the RGC layer (Fig. 31B, D), which was in keeping with a recent report showing reduction of Pax6⁺ amacrine cells in chick retina by CA β -catenin (38).

The change in the Pax6 expression pattern implies the effect of Li⁺ treatment on retinal progenitors. Thus I further looked at an additional progenitor marker, Chx10. Chx10 is a HD protein that is also expressed in retinal progenitors (150) as well as in differentiated bipolar cells (13, 25) and a subset of Müller glial cells (207). Retinal

Fig. 30. Quantitative RT-PCR analysis of gene expression in Li⁺-treated retinal explants.

RNA was extracted from E14.5 retinal explants cultured in control media (white bar) or in the presence of Li⁺ (black bar) for one day and processed for quantitative RT-PCR with specific primers for *Otx1*, *Crx*, *Dll1*, *Math3* and *Pax6*. Pooled data from six independent experiments was analysed. Fold change was normalized to housekeeping gene *HPRT*. Up regulation of *Otx1* expression and down regulation of *Crx*, *Dll1*, *Math3* and *Pax6* are significant (n=58). Statistical significance was evaluated by Student t Test. *P<0.001.

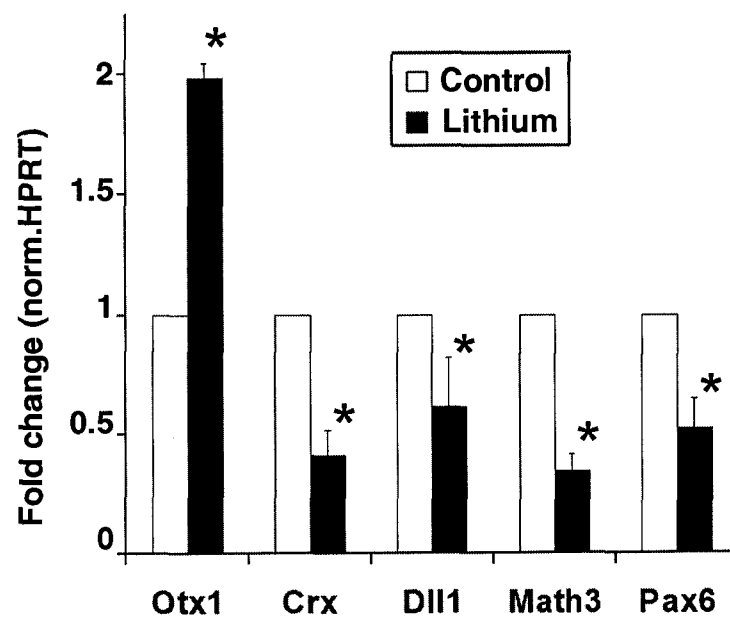
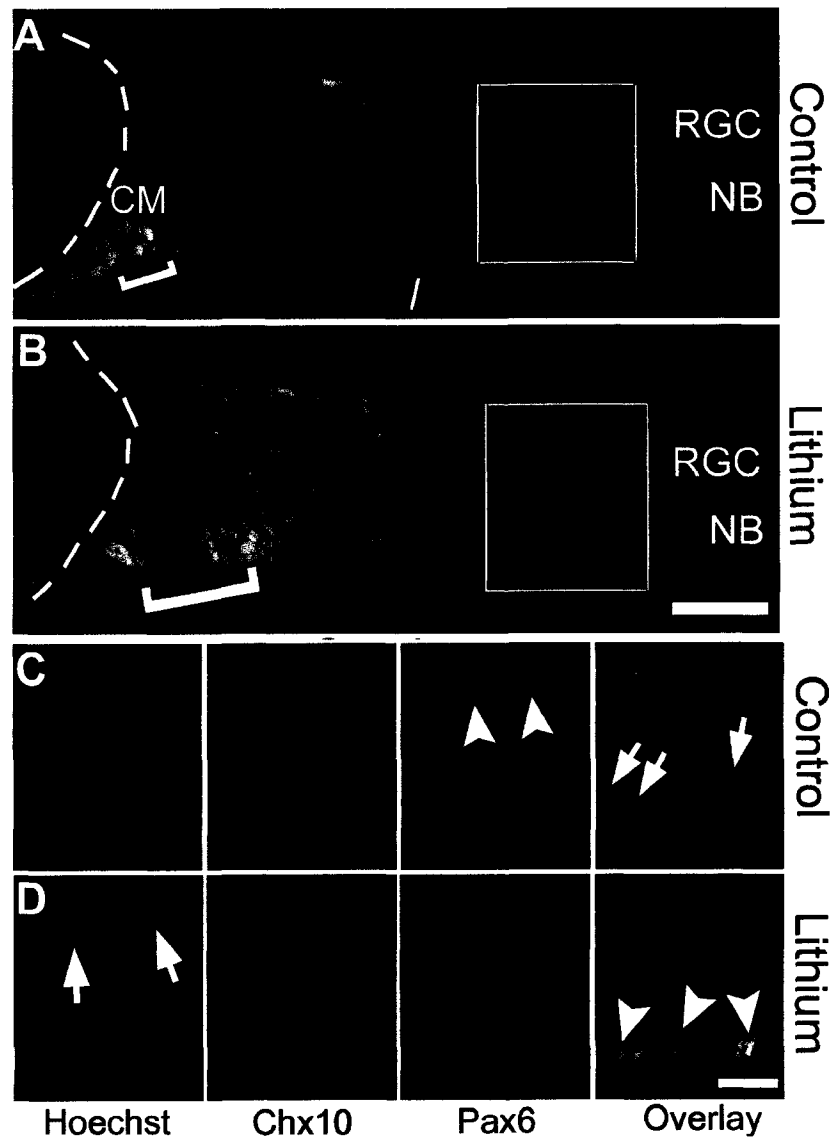


Fig. 31. Short-term Li⁺ treatment alters the expression pattern of Chx10 and Pax6.

Sections from E14.5 retinal explants cultured in control media (panel A) or in the presence of Li⁺ (panel B) for one day were stained with Hoechst (blue) to mark nuclei and processed IHC for Chx10 (green) and Pax6 (red). Dashed lines define the lens. Boxed areas in (panel A and B) are shown in higher magnification in panel C and D, respectively. (A and B): The expansion of Chx10⁺/Pax6⁺ domain of the CM (brackets) is apparent in Li⁺-treated explants compared to the controls (n=3). (C): Arrowheads indicate Pax6⁺ cells in RGC layer and arrows indicate representative round cells in the NB layer with a high level of Pax6 staining in control explants. (D): Arrowheads indicate elongated Pax6⁺Chx10⁺ cells in the NB layer of Li⁺-treated explants (n=3). Arrows indicate small round nuclei with intense Hoechst staining in the RGC layer of Li⁺-treated explants. CM: ciliary margin; RGC: retinal ganglion cell layer; NB: neuroblast layer. Scale bar: 50μm in (A, B), 25μm in (C, D).



progenitors are the only cell type that co-express Chx10 and Pax6, and co-localization of these two proteins was used to identify progenitors in several other studies (13, 63, 64, 175). Of particular relevance to the present study, CMZ cells and nPCE cells of the CB co-express these two proteins in postnatal chick retina (63, 64). Double staining for Chx10 and Pax6 revealed Chx10⁺Pax6⁺ cells in the CM of the control explants (Fig. 31A), whereas in Li⁺-treated explants, Chx10⁺Pax6⁺ CM is expanded (Fig. 31B) and majority of the elongated cells along the apical side of the neuroblast layer are Chx10⁺Pax6⁺ (Fig. 31D).

Significant effects on gene expression in Li⁺-treated explants were revealed, indicating that activation of canonical Wnt signaling rapidly alters gene expression in the developing retina. To determine the functional consequences of activated canonical Wnt pathway in the developing retina, other developmental parameters, such as proliferation and cell differentiation were examined in this context.

Li⁺ treatment induces effects on progenitor gene expression and nuclear morphology, raising the possibility that activation of canonical Wnt signaling affects cell proliferation. To assess cell proliferation, the BrdU-labeling index was measured in E14.5 explants cultured for one day in Li⁺. BrdU is a thymidine analogue that can be incorporated into newly synthesized DNA during S-phase of the cell cycle. The subsequent detection of incorporated BrdU with specific anti-BrdU antibodies is an accurate and comprehensive method to quantitate the degree of DNA-synthesis and estimate the fraction of cells in S-phase. Explants were grown under control conditions or in the presence of Li⁺ for 24 hours and BrdU was added to the culture medium during the last 6 hours of culture. In Li⁺-treated

explants, BrdU⁺-nuclei were less intensely stained and exhibited an elongated morphology (Fig. 32g) similar to morphological change that I observed in Chx10/Pax6 double stained explants. To quantify the BrdU-labeling index, the explants were dissociated into single cells and the percentage of BrdU⁺ nuclei was determined [2.7.2]. The percentage of BrdU⁺ cells was decreased, suggesting a reduction in proliferation, in Li⁺-treated explants (Fig. 32i). This observation also suggested that Li⁺ treatment altered the cell cycle. To investigate this possibility, I examined phosphohistone H3 (pH3), a marker for M-phase cells. The proportion of M-phase cells and pH3⁺ proportion of S-phase cells remained unchanged, implying that M phase as well as G2 to M phase progression was normal in Li⁺ treated explants; however, Li⁺ treatment induced a significant reduction in the proportion of M-phase cells that were BrdU⁺, compared with the controls (Fig. 32i). Therefore, the effect of Li⁺ treatment on cell cycle in explants is complex and likely also involved cell death.

Hoechst staining revealed many round, pyknotic nuclei in E14.5 explants cultured for one day in Li⁺ (Fig. 31D). To determine whether Li⁺ treatment induces cell death, TUNEL assay was carried out. In control explants, apoptosis-mediated cell death was detected in the RGC layer, which has been documented previously in retinal explants (259); whereas, Li⁺-induced TUNEL⁺ cells, in addition to the dead cells usually observed in the RGC layer, were detected in the neuroblast layer as well (Fig. 33). Thus, short term Li⁺ treatment is associated with an increase in cell death, which could contribute to the rapid reduction of Pax6⁺ cells with high intensity in Li⁺-treated explants.

Fig. 32. Effects of Li^+ treatment on nuclear morphology and proliferation.

E14.5 retinal explants were cultured for one day and labelled with BrdU for 6 hours before harvesting. Sections from explants cultured in control media (a-d) or in the presence of Li^+ (e-h) were stained with Hoechst (blue) to reveal nuclei and processed for IHC for phosphoH3 (pH3, green) and BrdU (red). Dashed lines in (c) and (g) indicate the morphology of nuclei. The nuclei were elongated upon Li^+ treatment in comparison to control explants (compare c with g) ($n=4$). Arrows in (d) indicate the $\text{BrdU}^+\text{pH3}^+$ cells in control explants, while arrowheads in (h) indicate the $\text{BrdU}^+\text{pH3}^+$ cells in Li^+ -treated explants. Scale bar: $10\mu\text{m}$. (i) Dissociated cells from control or Li^+ -treated explants were processed for IHC and proportion of pH3^+ cells or BrdU^+ cells in total cells, and BrdU^+ proportion of pH3^+ cells or pH3^+ proportion of BrdU^+ cells were scored. Note that the proportion of BrdU^+ cells in pH3^+ cohort is significantly reduced in Li^+ -treated explants compared to control (N (number of experiments)=2, $n=5$). Statistical significance was evaluated by Student's t test. $*P < 0.05$ and $**P < 0.001$.

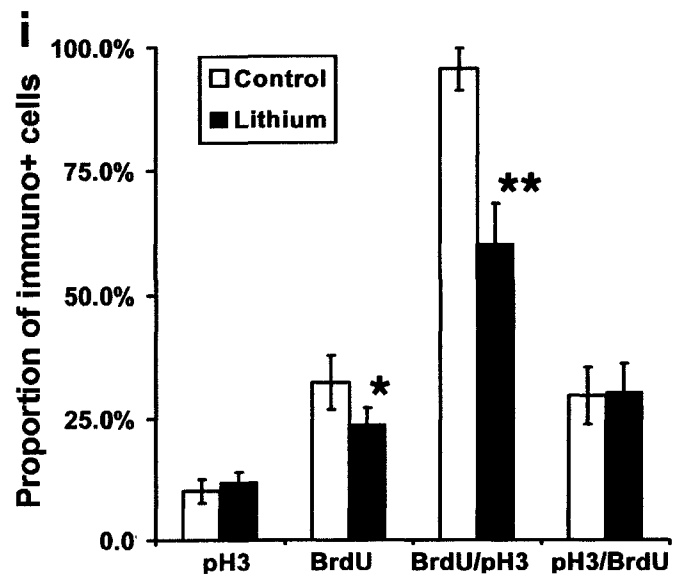
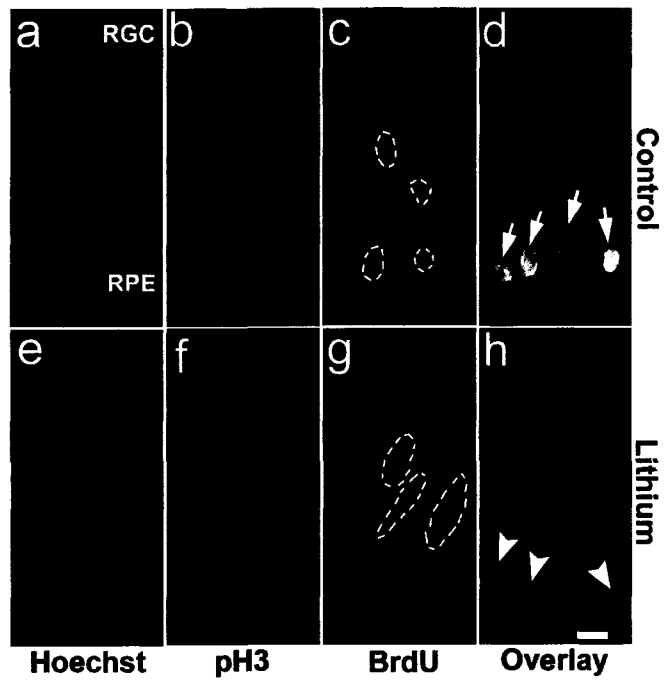
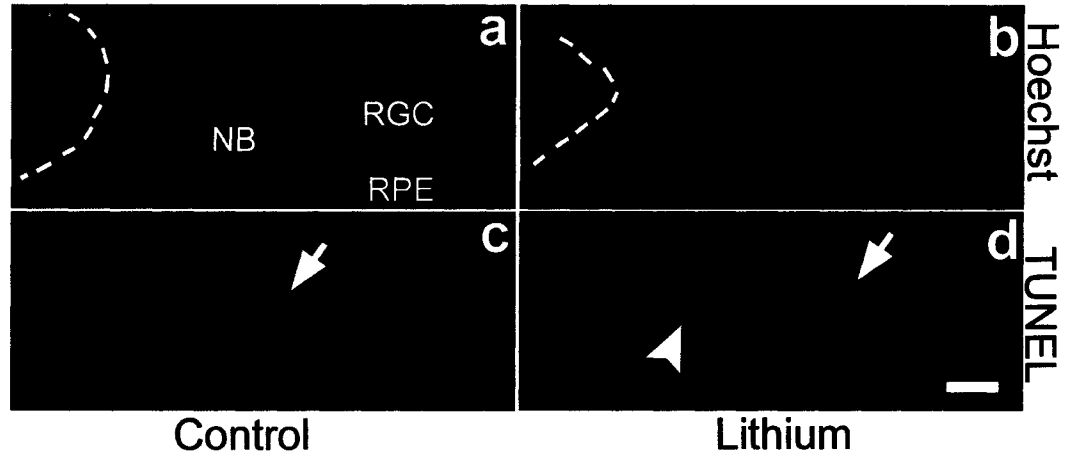


Fig. 33. Li^+ treatment induces cell apoptosis.

Sections from E14.5 retinal explants cultured in control media (a and c) or in the presence of Li^+ (b and d) for one day were stained for Hoechst to mark nuclei and for apoptosis by TUNEL detection. Cell death is usually observed in the RGC layer of retinal explants (arrow in c). There were more apoptotic cells in the RGC layer and the NB after Li^+ treatment compared to controls (arrow and arrowhead in d) (n=2). NB: neuroblast layer; RGC: retinal ganglion cell layer; RPE: retinal pigment epithelium. Scale bar: 50 μm .



To determine the effect of Li^+ on cell differentiation in the retina, explant sections and dissociated cells were stained with cell type-specific markers. In E14.5 explants cultured for one day, equivalent to E15.5 *in vivo*, the RGCs are the most prevalent differentiated cell type. To examine RGCs, IHC was performed for Brn3b (a transcription factor of the POU HD subclass that is expressed exclusively in subpopulation of RGCs (274)) on explant sections and dissociated cells. No significant difference in proportion of Brn3B⁺ cells was detected in Li^+ -treated explants compared to controls (data not shown). To identify cell types that differentiate at later stages, E14.5 explants were cultured for 10 days, equivalent to P5 *in vivo*, a stage when the majority of the retinal neurons have been generated and begun to differentiate (32). TCF/Lef-LacZ reporter activation induced by a 20-hour exposure to Li^+ gradually decays within 5 days in explants (data not shown), however, activation of this reporter by constant exposure to Li^+ peaks around 24 hours and decreases dramatically by 48 hours (149), implying the operation of a negative feedback loop. In an attempt to achieve a medium level of elevated Wnt signaling, I pulsed explants with Li^+ at day 0, day 4-5 and day 9-10 instead of culturing explants with a constant source of Li^+ .

Explant sections were stained for Chx10 and Pax6 to identify bipolar and amacrine cells (RGCs, which can also be identified with Pax6 staining, gradually die in explants cultured longer than 24 hours (259)). The distribution of cells in the control explants recapitulates what is observed *in vivo* with Pax6⁺ amacrine cells located in the RGC layer and inner region of the INL, and Chx10⁺ bipolar cells in outer region of the INL (Fig. 34b). After Li^+ treatment, the boundaries between RGC, INL and ONL layers were poorly defined

(compare Fig. 34a and c). Pax6⁺ or Chx10⁺ cells were not localized to the RGC and INL; instead these cells were scattered in the outer layer of the explants. Double staining of Chx10 and Pax6 also revealed Chx10⁺Pax6⁺ cells, possibly progenitors (Fig.34d). Consistent with this possibility, BrdU⁺ cells were detected in the outer layer of Li⁺ treated explants after 10 days (Fig.35d), indicating cell division persisted in Li⁺-treated explants compared with controls. Moreover, Li⁺ treatment appears to suppress rosetting in the ONL, a laminar abnormality usually observed in retinal explants due to loss of RGC-derived Shh signaling (259) (compare Fig. 34a and c). A reduction of rhodopsin⁺ photoreceptors and syntaxin⁺ amacrine cells was also observed in Li⁺-treated explants cultured for 10 days as assessed by IHC on explants sections and quantified in dissociated explants (Fig. 35). Thus I concluded that Li⁺ treatment reduces cell differentiation in the retina.

Fig. 34. Long-term Li^+ treatment in retinal explants alters the expression pattern of Chx10 and Pax6.

E14.5 retinal explants were cultured for 10 days in control media or pulsed with Li^+ at day 0, day 4-5 and day 9-10. Sections from control (a and b) or Li^+ -treated explants (c and d) were stained with Hoechst (blue) to reveal nuclei and processed for IHC for Chx10 (green) and Pax6 (red). Control explants have well defined RGC, INL and ONL (dashed lines in a), whereas Li^+ -treated explants do not exhibit a clear distinction between the RGC and the INL (c), however, the developing ONL (dONL) exhibits less rosetting than in the controls (compare asterisk in a and c). Distinct layers of Chx10^+ cells and Pax6^+ cells are detected in the INL of control explants (arrows and arrowheads in b), while the Pax6^+ cells and Chx10^+ cells are randomly distributed in the outer layer of retina after Li^+ treatment (arrows and arrowheads in d) (n=2). The > symbol in (d) indicates a $\text{Pax6}^+\text{Chx10}^+$ cell in Li^+ -treated explants. INL: inner nuclear layer; NB: neuroblast layer; ONL outer nuclear layer; RGC: retinal ganglion cell layer. Scale bar: 10 μm .

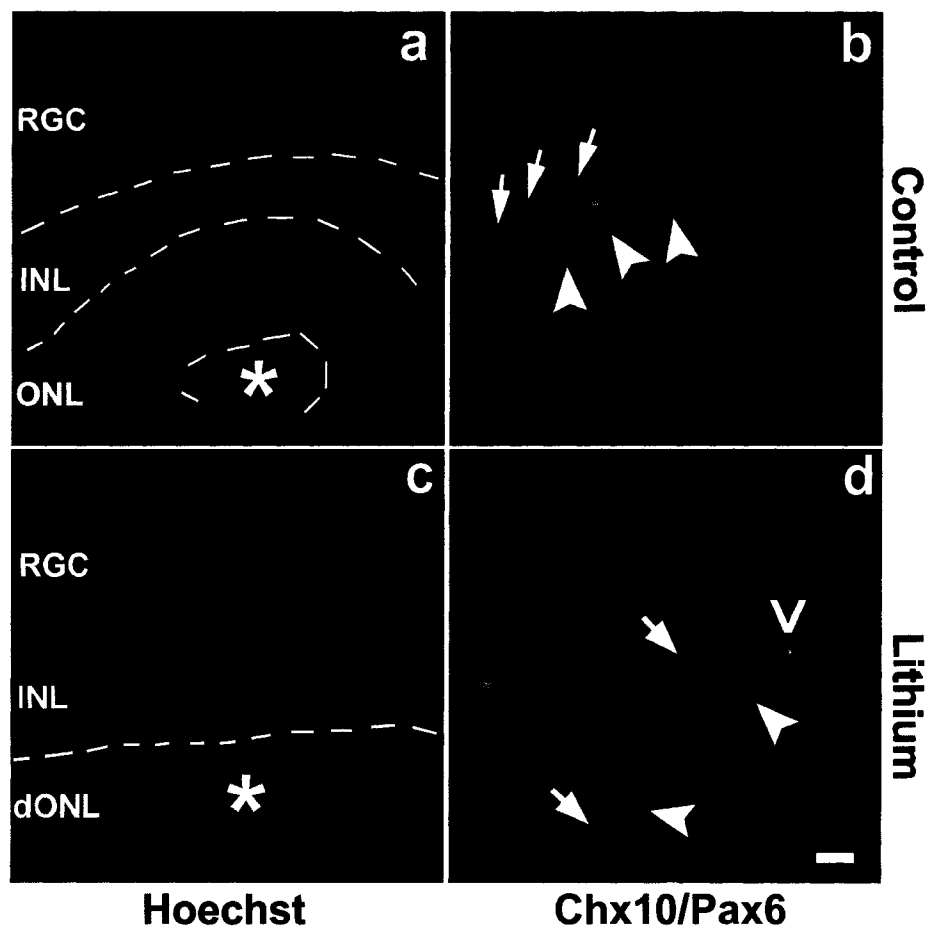
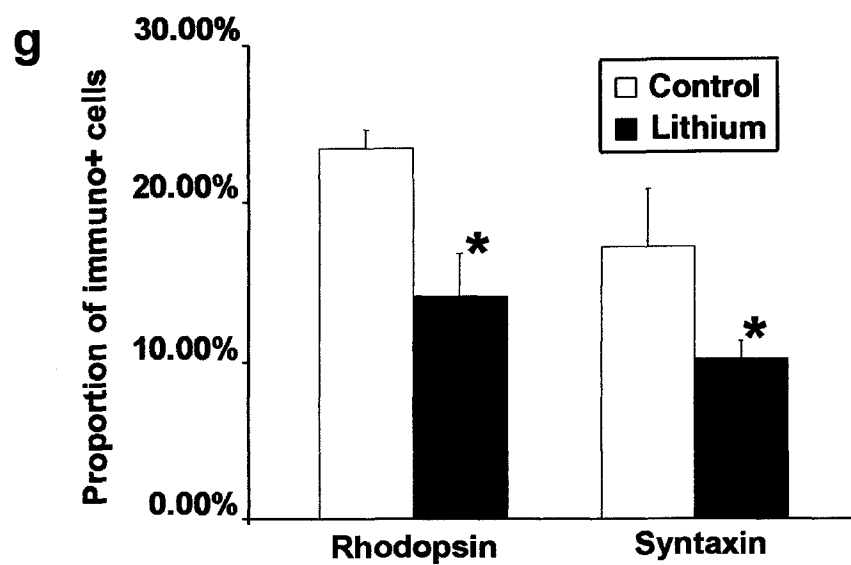
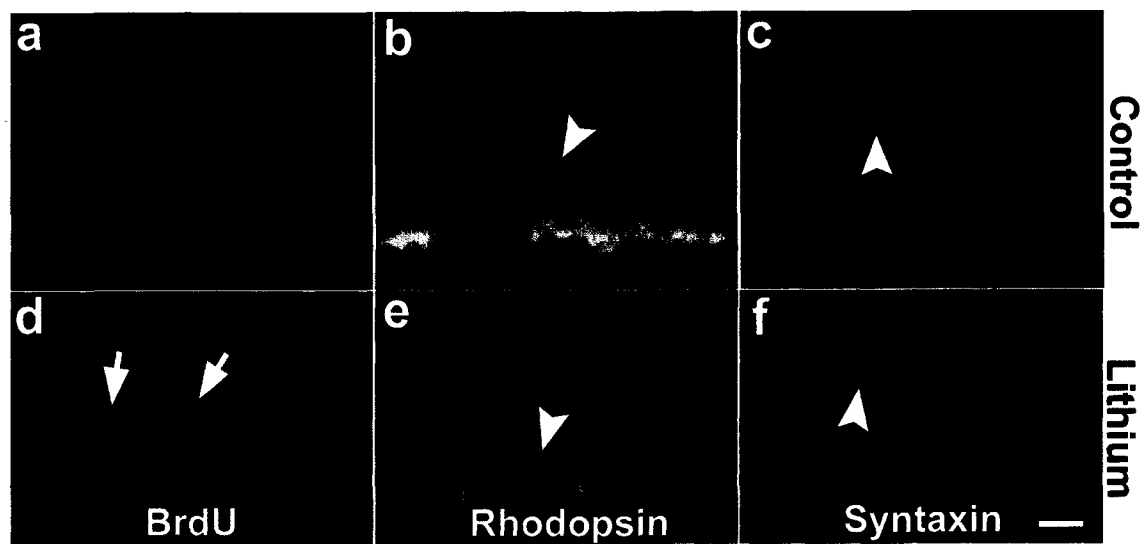


Fig. 35. Persistence of S-phase cells and reduced formation of photoreceptor and amacrine cells in long-term Li^+ treated explants.

E14.5 retinal explants were cultured for 10 days in control media or pulsed with Li^+ at day 0, day 4-5 and day 9-10. BrdU was added to the culture media for the last 6 hours. Serial sections from control (a-c) or Li^+ -treated explants (d-f) were stained with Hoechst (blue) to reveal nuclei and processed for IHC for BrdU (a and d), rhodospin (b and e) or syntaxin (c and f). Arrows in (d) indicate BrdU^+ cells, marking S-phase cells in Li^+ -treated explants. Arrowheads indicate that rhodopsin $^+$ photoreceptor cells (b and e) and syntaxin $^+$ amacrine cells (c and f) are reduced after Li^+ treatment. Scale bar: 25 μm . (g) Control (white bars) or Li^+ -treated explants (black bars) were dissociated and processed for IHC for rhodospin or syntaxin and proportion of specific cell types were scored (N=2, n=6). Statistical significance was evaluated by Student t Test. * $P < 0.05$.



5.2 Discussion

Previous findings from our group and others have raised the possibility that canonical Wnt signaling is involved in the development of the CM. To test this idea, retinal explants dissected from the developing retina were used as a model system. Canonical Wnt reporter activity was gradually downregulated in explants from *TCF/Lef-LacZ* transgenic mice. Thus, similar to loss of function for Shh signaling in retinal explants (259), this system represents a loss of function model for canonical Wnt pathway activation, which could be rescued by ectopic expression of Wnt3a. Li^+ treatment, which stabilizes β -catenin, activated canonical Wnt signaling, leading to ectopic CM gene expression and reduction of differentiation in the explants. Thus, canonical Wnt signaling plays multiple roles in a variety of developmental events in the mammalian retina, including specification of CM, neuronal differentiation, cell cycle progression and cell survival.

5.2.1 Canonical Wnt signaling promotes CM character in the murine retina

Based on five major criteria, I have proposed that canonical Wnt signaling promotes CM character in the murine retina. First, activation of Wnt signaling induces ectopic expression of a CM marker and expands the CM. Second, Li^+ -induced reduction in BrdU intensity and slowing of the cell cycle is similar to what has been observed in the CM (reviewed by 11). Third, $\text{Chx10}^+\text{Pax6}^+$ CM domain is expanded in Li^+ -treated explants. Fourth, the shape of the nuclei in Li^+ -treated explants is reminiscent to elongated nuclei observed in the CM. Fifth, persistence of progenitors and dividing cells, and reduced

differentiation in the explants is similar to what is observed in the fish and chick CM, the least mature and latest differentiated region of the vertebrate retina (89, 105, 141, 164).

Ectopic Wnt3a up regulated the expression of *TCF/Lef-LacZ* reporter in the CM, however this effect was delayed (for 6 days) compared with the rapid effect of Li^+ . The delay could reflect the requirement to accumulate sufficient amounts of protein to overcome extracellular inhibitors, such as Sfrp2 (73, 143, 148). However, since treatment with high concentrations of recombinant Wnt3a did not induce a rapid response in explants, this is less likely. Another possibility is that the competence of the cells to respond is lacking, however, Li^+ could induce a rapid CM gene expression. Finally, another plausible explanation for the delay is that Wnt3a exerts its effect through the production of a secondary signal.

I observed that electroporation induced non-specific expression of two Wnt target genes, *Bmp4* and *Lef1*, in the retinal explants, however, I did not investigate whether this change was also reflected at the level of the protein. Bmp signaling was previously shown to be required for CB morphogenesis at late stages of eye development (283), however, the CM/CB development was not affected in the presence of upregulated *Bmp4* expression in electroporated control explants, at least in terms of the pattern of gene expression that I monitored. Thus, it is unlikely that this artefact caused a functional effect. Nonetheless, this finding remind me that I should be cautious by using electroporation, in particular, in the context of a Wnt study.

5.2.2 Proliferation and cell cycle effect of Wnt canonical pathway in the vertebrate retina

There are several conflicting reports regarding the effect of Wnt pathway activation on proliferation in the vertebrate retina. Kubo et al have shown that Wnt2b promotes RPC proliferation in dissociated and reaggregated cells and leads to continuous growth in explants of chick retina (135, 136). Expression of stabilized β -catenin was shown to increase cell proliferation in the central retina in zebrafish (276) and activation of the canonical Wnt pathway through Frizzled5 increases proliferation in the *Xenopus* retina (248). However, other investigators have shown that expression of stabilized β -catenin or Wnt2b inhibits cell proliferation in the chick retina (38).

I observed that short exposure to Li^+ had a rapid effect on the cell cycle: it reduced the intensity of BrdU staining and the proportion of BrdU⁺ cells, suggesting a reduced cell proliferation. A study carried out in chick has shown that onset of CE gene expression is followed by the reduction in proliferation that results in the thinning at the tip of optic cup and the formation of epithelial monolayer (138). My finding of inhibited cell proliferation by Wnt pathway activation provides a clue in dissecting the mechanism underlying the morphogenesis of the peripheral retina. Although repeated exposure to Li^+ in long term cultured explants resulted in ectopic BrdU⁺ cells. I interpret this as a persistence of progenitors secondary to the inhibition of neuronal differentiation that I observed. Therefore, observations from my study argue that activated Wnt signaling leads to more slowly dividing immature cells, but does not necessarily promote cell proliferation or retinal growth. Reasons underlying the discrepancies among the above noted studies in different species could be that effects of Wnt/ β -catenin signaling are cellular context and developmental stage dependent, as

suggested previously (99, 248). The possibility that there are significant species differences with respect to the role of this pathway during retinal development also remains to be investigated.

I also observed a complex effect on cell phase progression after Li^+ treatment. Canonical Wnt signaling has been suggested for a role in regulating cell cycle progression, but varying in effects in different cellular contexts. It has been shown that activated β -catenin induces G2 arrest in epidermal keratinocyte cells (184), however, inhibition of this pathway also has the same effect in colorectal tumor cells (214). It was reported that CyclinD1 is upregulated by the canonical Wnt signaling at the mRNA and protein level and inactivation of this pathway inhibits expression of *Cyclin D1*, resulting in G1 phase arrest in the colon carcinoma cells (236). Recently, Bjorklund et al performed a large scale RNAi screen for factors regulating cell-cycle progression in *Drosophila* (18). They identified Wnt/ β -catenin signaling, among the classical regulatory factors and other pathways, as a controller of G1 phase (18). Therefore, while the Wnt canonical pathway appears involved in cell cycle control, the exact function remains a matter of further investigation in different cellular contexts, such as in the retina. Extensive analyses should be carried out to understand the proliferation kinetics of CM and CE development.

5.2.3 A role of the canonical Wnt signaling in neurogenesis suppression

The rapid downregulation of *Pax6* and *Math3* expression, and reduction of syntaxin⁺ amacrine cells induced by Li^+ suggest that Li^+ likely acts as a general suppressor of neurogenesis via inhibition of HD and bHLH transcription factor expression. The bHLH

gene *Math3* and *NeuroD* are functionally redundant for amacrine cell development (109), however, misexpression of each gene alone does not increase amacrine neurons (90), while the combination of *Pax6* and *Math3* or *Pax6* and *NeuroD* is sufficient to promote amacrine cell fate (109), suggesting a fine regulation of HD and bHLH genes on amacrine cell fate specification. Now it has been generally accepted that HD and bHLH networks co-operate to specify cell fate in the retina (reviewed by 31, and by 160). However, the larger question is what regulates the amounts and activities of these transcription factors during this process. My findings support a role of canonical Wnt signaling in inhibiting neurogenesis by controlling the expression levels of HD and bHLH factors. Consistent with this interpretation, cWnt2b was reported to inhibit RPC differentiation by downregulating proneural bHLH genes expression in chick retina (136).

5.2.4 Differences of Wnt2b expression and function in the retina in chick and mouse

Overlapping and adjacent expression domains of *Wnt2b* with the canonical Wnt reporter gene in the peripheral retina of the *TCF/Lef-LacZ* transgenic mice and lack of *Wnt2b* expression in explants correlating with the decay of the Wnt reporter activity (149 and this study, reviewed by 249) have raised the possibility that Wnt2b is likely the signal that induces the Wnt reporter expression in this region. However, I found no evidence that murine Wnt2b activates this pathway in retinal explants. This finding contrasts with studies of cWnt2b in chick retina showing that cWnt2b activates canonical Wnt pathway as determined by the up regulation of *Lef1* and cytosolic accumulation of β -catenin (135), and that ectopic expression of *cWnt2b* or stabilized β -catenin develops similar phenotypes (38).

There are several explanations for the discrepancies between my results and the findings in chick. Studies in chick retina were carried out in dissociated retinal cells or *in ovo* or with different gene delivery methods from my study. These differences could have, for instance, resulted in higher concentrations or longer duration of expression of *cWnt2b*. The effect of Wnt2b may require additional factors, possibly from the RPE, that are not present in my explants. It is also possible that I might have been outside a critical developmental time window for observing an effect of Wnt2b in explants. The activity of extracellular antagonists, such as Sfrp2 (143, 148), also likely inhibits Wnt signaling in the explants, but may not in dissociated cells. Finally, it is possible that murine Wnt2b simply functions differentially compared to *cWnt2b* in the eye. The expression pattern of *Wnt2b* is remarkably different in chick and mouse eye. Although both of them are detected in the peripheral RPE, *cWnt2b* is also expressed in the lens epithelium, while murine *Wnt2b* is not (38, 149, reviewed by 249). Due to the crucial role of lens in CM/CB development (reviewed by 107), it is reasonable to ask whether the lens-derived *cWnt2b* signals to the CM to regulate its development, and the RPE-derived *cWnt2b* has other functions as yet unknown. If such were the case, Wnt2b could not be the signal to activate the Wnt reporter in the CM/CE in mouse. Then the next question is what and where the ligand is in the periphery of the murine eye.

6.0 Canonical Wnt signaling drives ciliary margin specification in the murine retina

The high levels of canonical Wnt reporter activation in the CM, together with the expression of *Wnt2b* and *Frizzled* receptors in overlapping and adjacent domains in this region of the eyecup suggests a function of the Wnt canonical pathway in the development of the CM. I hypothesized that Wnt signaling, through the β -catenin dependent canonical pathway, plays a role in CM development and the formation of its derivatives. As shown in Chapter 5.0, activation of this signaling pathway by ligand, Wnt3a or by agonist, Li^+ induced Wnt reporter activity and Li^+ treatment led to changes in gene expression, proliferation index and differentiation that were similar to CM, supporting my hypothesis that canonical Wnt signaling specifies CM identity in the developing murine retina.

To further dissect the role of this pathway in anterior eye development, we investigated the effects of modulating canonical Wnt signaling in the peripheral eye in transgenic mice. α -*Cre-GFP* (referred to as α -*Cre*) transgenic mice, where Cre activity is primarily restricted to progenitors in the retina at embryonic stages (122, 161, 256), were crossed with β -catenin transgenic mice (*Catnb*^{+lox(ex3)}), in which exon3 of β -catenin is flanked by loxP sites (88). Cre expression was monitored by IHC with anti-GFP antibodies. In compound transgenic mice (α -*Cre*; *Catnb*^{+lox(ex3)}), referred to as *Catnb* ^{Δ ex3}, Cre-mediated excision of floxed exon3 removes the Gsk3 β phosphorylation site in β -catenin, producing a stabilized β -catenin, which constitutively activates target genes. Activated Wnt/ β -catenin

signaling in the retina was monitored by crossing in the *TCF/Lef-LacZ* canonical Wnt signaling reporter transgene (171) to generate the compound transgenic mice with Wnt reporter, *Catnb* ^{$\Delta ex3$} ; *TCF/Lef-LacZ* (referred to as *Catnb* ^{$\Delta ex3$} ; *LacZ*) [2.1]. We found that the peripheral retina in the β -catenin stabilized eyes was disorganized. The strict correlation of *Cre* expression with the activation of *TCF/Lef-LacZ* reporter and Wnt target gene expression indicated that the canonical Wnt pathway is activated in the *Cre*⁺ region in a cell-autonomous fashion. The *Cre*⁺ β -gal⁺ region exhibited expression of a number of CM markers, but lost NR identity, suggesting a conversion of NR into CM. In postnatal eyes, the peripheral eye exhibited a profile of gene expression similar to the CE, while the pigmented tissue expanded and extended into NR, implicating a cell fate conversion of NR and expansion of the CB in this region, but the morphological appearance of the CB is disturbed.

The α -*Cre* transgene is also expressed in subsets of differentiated RGC and amacrine cells in the late developmental stages (122, 161, 256). We found that expression of cellular markers for these retinal neurons, as well as several other retinal cell types, were extensively downregulated in the β -catenin compound transgenic mice.

6.1 Results

6.1.1 Expression of stabilized β -catenin results in expansion of the CM

At embryonic stage E14.5, *Cre* expression, as monitored by anti-GFP staining, is localized to the CM and adjacent peripheral retina in *Catnb*⁺; *LacZ* littermate controls (α -*Cre*; *Catnb*^{+/+}; *TCF/Lef-LacZ*) (Fig. 36b), consistent with previous reports (122, 161). We also observed that *Cre* expression is detected in the inner layer of the CM, the prospective nPCE and the inner layer of IPE (iIPE), but not in the overlying RPE layer, the prospective PCE and the outer layer of IPE (oIPE) (Fig. 36b insert). This observation contrasts with a previous study (122), but is consistent with Davis-Silberman et al who found no evidence for *Cre* activity in the pigmented layer in the periphery (48).

Examination of E14.5 tissue in *Catnb* ^{Δ ex3}; *LacZ* mice revealed that there was a dramatic increase in TCF/Lef-*LacZ* reporter activity and expression of Wnt target genes, *Lef1* and *BMP4*, that was restricted to *Cre*⁺ region of the retina (Fig. 36g, 36h and Fig. 37g). Thus, α -*Cre* targeted expression of stabilized β -catenin induces cell-autonomous activation of canonical Wnt signaling in the peripheral retina. The peripheral retina of the *Catnb* ^{Δ ex3}; *LacZ* mice was severely disorganized, contained cellular rosettes and there was no clear distinction between the RGC and NB layers, compared with retinas from controls (Fig. 36e). The lack of RGC differentiation in the *Cre*⁺ region of the *Catnb* ^{Δ ex3} retina was confirmed by IHC with RGC-specific antibodies (Fig. 39). All of the *Cre*⁺ cells in the *Catnb* ^{Δ ex3}; *LacZ* retina were located in this disorganized peripheral region of the eye cup, compared with *Catnb*⁺; *LacZ* transgenic mice where the *Cre*⁺ cells were distributed between

Fig. 36. Activation of canonical Wnt signaling in the peripheral retina of E14.5 β -catenin compound transgenic mice.

Serial retinal sections from E14.5 control littermates, *Catnb*⁺;*LacZ* (a-d) and β -catenin compound transgenic mice, *Catnb*^{*Δex3*};*LacZ* (e-h) were stained with Hoechst (a, e) to reveal nuclei and processed for IHC for GFP (b, f) to identify Cre expression, or stained with X-gal for β -gal reporter activity (c, g) and ISH for *Lef1* expression (d, h). Insert in (b) shows a large view of the CM. Dashed lines in (e) depict the invaded tissue from the periocular mesenchyme. *Cre* expression is detected in the nPCE and iIPE in the control littermates (insert in b). The eye and vitreal compartment (# in a and e) are smaller and the CM is extensively disorganized, with abnormal rosettes (arrow and arrowhead in e) in the *Cre* expressing regions (arrow and arrowhead in f) in the *Catnb*^{*Δex3*};*LacZ* mice. The TCF/Lef-*LacZ* reporter is activated (compare arrows and arrowheads in c and g) and *Lef1* expression is up regulated (compare arrows and arrowheads in d and h) in an identical pattern as *Cre* expression (n=4), implying a cell-autonomous mechanism. Note anterior region of the invaded mesenchymal tissue is mixed with *Cre*⁺ β -gal⁺*Lef1*⁺ cells (outlines in f-h), but the posterior region is not (asterisks in g and h). CM, ciliary margin; iIPE/oIPE, prospective inner and outer iris pigment epithelium; NR, neural retina; PCE/nPCE, prospective pigment and non pigmented ciliary epithelium. Scale bar: 100 μ m.

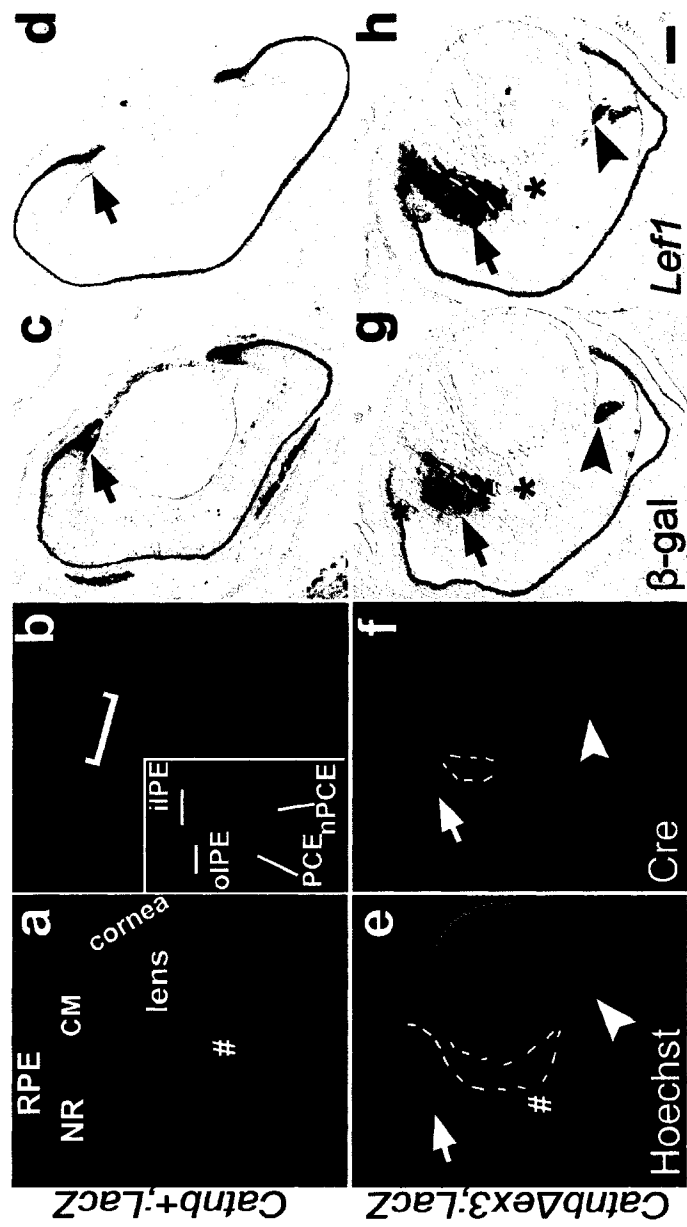
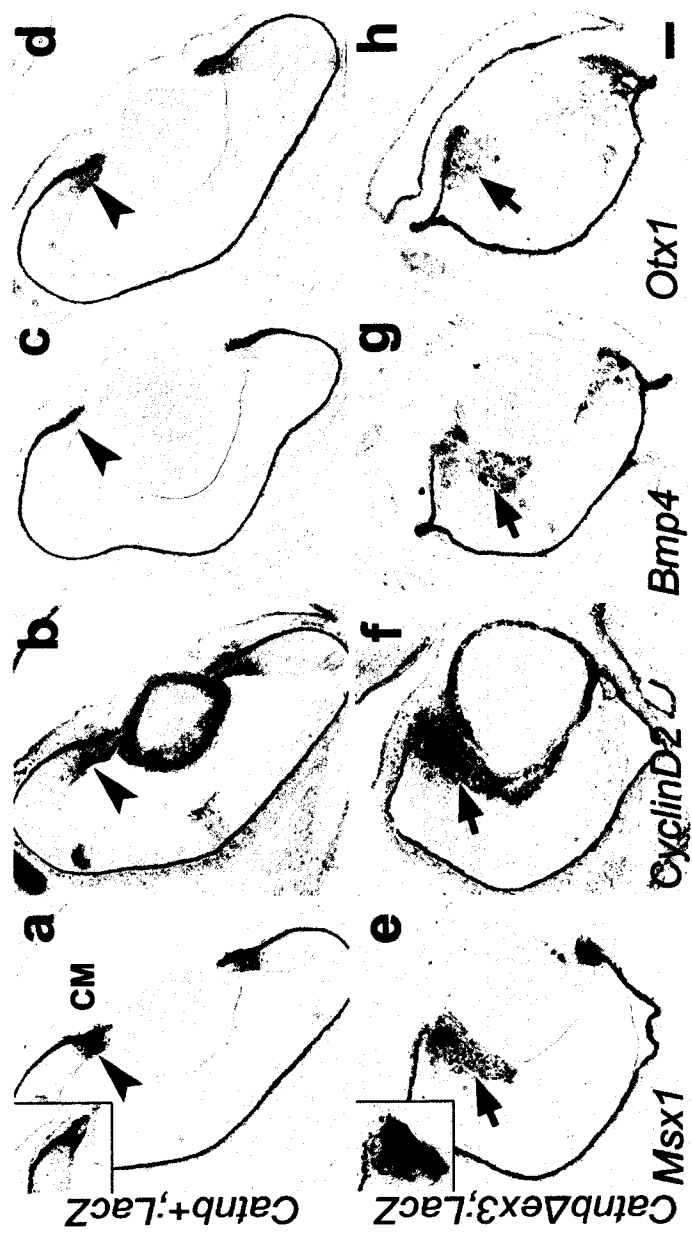


Fig. 37. Expansion of CM marker expression in the β -catenin-stabilized peripheral retina at E14.5.

Serial retinal sections from E14.5 control littermates, *Catnb*⁺;*LacZ* (a-d) and β -catenin compound transgenic mice, *Catnb*^{*Δex3*};*LacZ* (e-h) were processed for ISH for CM markers, *Msx1*, *CyclinD2*, *Bmp4* and *Otx1*. Inserts show β -gal activity (a, e) in adjacent sections, revealing an increase in the domain of activation of Wnt signaling in the *Catnb*^{*Δex3*};*LacZ* mice. An expansion in the expression domains of all the examined CM genes was apparent in *Catnb*^{*Δex3*};*LacZ* mice (compare arrows with arrowheads) (n=4). CM, ciliary margin. Scale bars: 100 μ m.



the CM and the NR (Fig. 36b and 36f). *Catnb* ^{Δ ex3};LacZ eyes also exhibited an accumulation of mesenchymal cells posterior to the lens, indicating that the invasion of mesoderm and neural crest derivatives into the eye was abnormal (Fig. 36e and 39).

To identify the aberrant peripheral tissue, the expression of CM and NR markers was examined by ISH with specific riboprobes. The region of expression of CM markers, such as *Msx1*, *CyclinD2*, *Bmp4* and *Otx1*, was markedly increased in the *Catnb* ^{Δ ex3};LacZ retina compared with controls, and was restricted to the β -catenin-stabilized region (Fig.37). In contrast, NR markers, including *CyclinD1*, *Crx*, *Math3* and *Sfrp2*, were excluded from the region with expanded CM marker expression in the *Catnb* ^{Δ ex3};LacZ mice (Fig. 38). Comparison of *Cre* expression with Wnt reporter staining confirmed that these changes in gene expression were autonomous to the cells expressing stabilized β -catenin (Fig.36-38). We concluded that expression of stabilized β -catenin drives CM specification in a cell autonomous fashion.

As the eyes in the *Catnb* ^{Δ ex3} transgenic mice were smaller than control littermates (Fig. 37, 38), we asked whether this might be due to defects in vasculature formation leading to nutrition deficiency and growth retardation. IHC with anti-collagen IV, marking endothelial cells, and with smooth muscle actin (SMA), marking pericytes, showed that hyaloid vasculature overlaid the basal retina and the lens capillary in controls (Fig. 39b and 39c). The hyaloid system was formed in the eyes of E11.5 and E14.5 *Catnb* ^{Δ ex3} embryos, but the morphology appeared abnormal as collagen IV⁺ or SMA⁺ cells were intermixed with invaded mesenchymal cells (Fig. 39e, f and data not shown).

Fig.38. Loss of NR marker expression in the β -catenin-stabilized peripheral retina at E14.5.

Serial retinal sections from E14.5 control littermates, *Catnb*⁺;*LacZ* (a-d) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3};*LacZ* (e-h) were processed for ISH for NR markers, *CyclinD1*, *Crx*, *Math3* and *Sfrp2*. Inserts show *Lef1* expression (a, e) or β -gal activity (d, h) in adjacent sections, revealing an increase in the domain of activation of Wnt signaling in the *Catnb* ^{Δ ex3};*LacZ* mice. These NR genes are not expressed in the CM in the control littermates (arrowheads in a-d) and their expression is excluded in the β -catenin activated region, an expanded region compared to the controls, in the peripheral retina of *Catnb* ^{Δ ex3};*LacZ* mice (arrows in e-h) (n=2). Scale bars: 100 μ m.

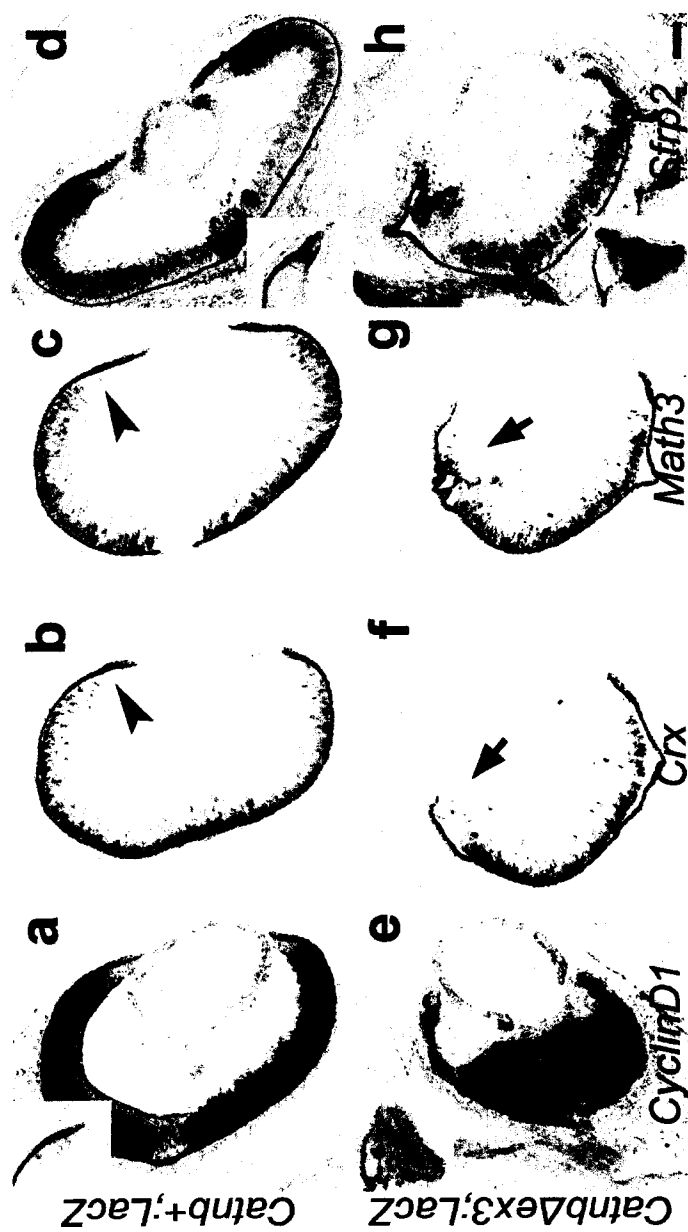
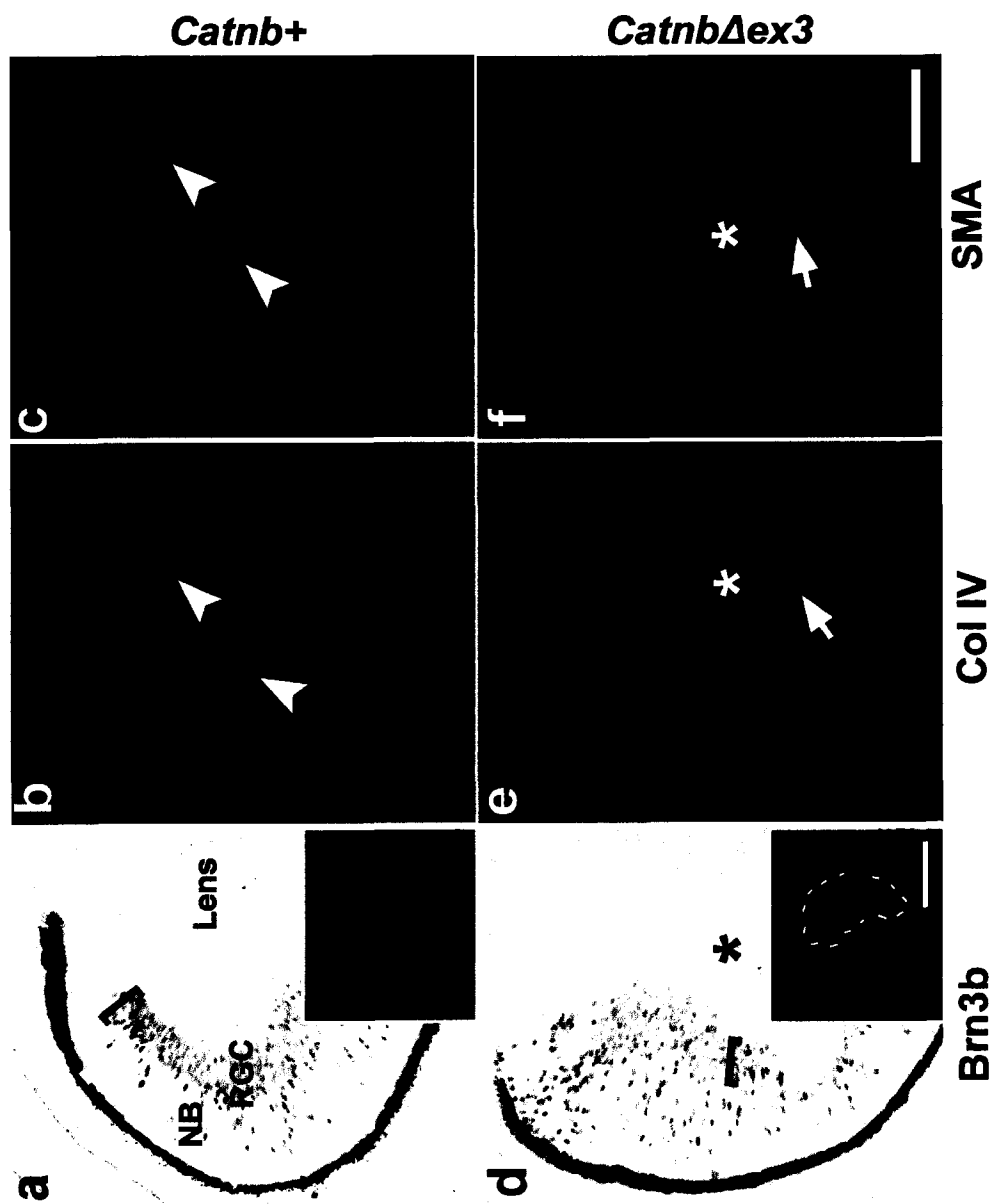


Fig. 39. Changes in the generation of RGCs and formation of the hyaloid vasculature in the eyes expressing stabilized β -catenin.

Serial retinal sections from E14.5 control littermates, *Catnb*⁺ (a-c) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (d-f) were processed for IHC for Brn3b (a and d), Collagen IV (Col IV, b and e) or smooth muscle actin (SMA, c and f). Hoechst staining (blue) reveals nuclei. Inserts in (a and d) show the domains of *Cre* expression in adjacent sections. Dashed lines (d) indicate the region corresponding to the *Cre*⁺ region in the eye of *Catnb* ^{Δ ex3} mice. Asterisks (d-f) indicate the invaded mesenchymal tissue. RGC and NB layers are well defined (black and white brackets in a) in the retina in controls. RGCs are excluded in the *Cre*⁺ region (dashed lines in d) and disorganized in adjacent region (arrow in d), but maintained in the unaffected central retina (black and white brackets in d) in *Catnb* ^{Δ ex3} mice (n=1). Arrowheads indicate Collagen IV⁺ endothelial cells or smooth muscle actin⁺ pericytes in the hyaloid vessels in controls, while the hyaloid system is disorganized and intermixed with invaded mesenchymal cells in the *Catnb* ^{Δ ex3} mice (arrows in e and f) (n=2). Scale bars: 100 μ m in (a-f) and for inserts.



6.1.2 Phenotypes in the earlier eyecup suggest transdifferentiation of NR to CM fated tissue

At E14.5, all of the Cre⁺ cells in the *Catnb* ^{Δ ex3};LacZ retina expressed CM markers (Fig. 37), raising the possibility that expression of stabilized β -catenin induced the transdifferentiation of cells from NR to CM. If such were the case, we would expect to see a normal distribution of Cre⁺ cells at earlier stages in eyecup development soon after expression of the α -Cre transgene. Eye size and the distribution of Cre⁺ cells were comparable in *Catnb* ^{Δ ex3};LacZ and *Catnb*⁺;LacZ mice at E11.5 (Fig. 40). However, even at this stage, we detected robust Wnt reporter activity, expression of Wnt target genes and the initiation of the CM gene expression profile in Cre⁺ cells in *Catnb* ^{Δ ex3};LacZ mice (Fig. 40g, 40h and Fig. 41). Thus, we could detect the initiation of CM development at a time when Cre⁺ cells were still localized to the prospective NR region of the eyecup. We did not observe a difference in TUNEL staining in the *Catnb* ^{Δ ex3} mice compared with *Catnb*⁺ mice at E11.5 (data not shown). Thus, the absence of Cre⁺ cells outside the expanded CM in the *Catnb* ^{Δ ex3} eye at later stages is unlikely to be secondary to death of a subset of Cre⁺ cells that failed to participate in the aberrant CM. The simplest interpretation of these findings is that ectopic canonical Wnt signaling induces the transdifferentiation of NR to a CM fate.

6.1.3 No evidence of selective proliferation in the β -catenin stabilized region of the compound transgenic mice

To address whether the increase in the CM was secondary to a selective outgrowth of cells expressing stabilized β -catenin, we examined Ki67, a proliferation marker by IHC.

Fig. 40. Activation of canonical Wnt signaling in the β -catenin stabilized retina at E11.5.

Serial retinal sections from E11.5 control littermates, *Catnb*⁺;*LacZ* (a-d) and β -catenin compound transgenic mice, *Catnb*^{*Δex3*};*LacZ* (e-h) were stained with Hoechst (a, e) to reveal nuclei and processed for IHC for GFP (b, f) to identify *Cre* expression, or stained with X-gal for β -gal reporter activity (c, g) and ISH for Wnt target, *Lef1* expression (d, h). Insert in (b) shows a large view of the CM, revealing *Cre* expression in the NR but not in the RPE at the CM region in controls. There are rosettes (arrowhead in e) in the *Cre*⁺ region (f) of β -catenin stabilized eye. The domains with up regulation of *TCF/Lef-LacZ* reporter (g) and *Lef1* expression (h) strictly overlap with the *Cre*⁺ region (f) (n=2). Asterisks (g and h) denote the CM and brackets (g and h) denote the NR. Note the general structure and the size of the eyecup look normal in the *Catnb*^{*Δex3*};*LacZ* mice. CM, ciliary margin; NR, neural retina; RPE, retinal pigment epithelium. Scale bars: 100 μ m in (a-h), 25 μ m in insert.

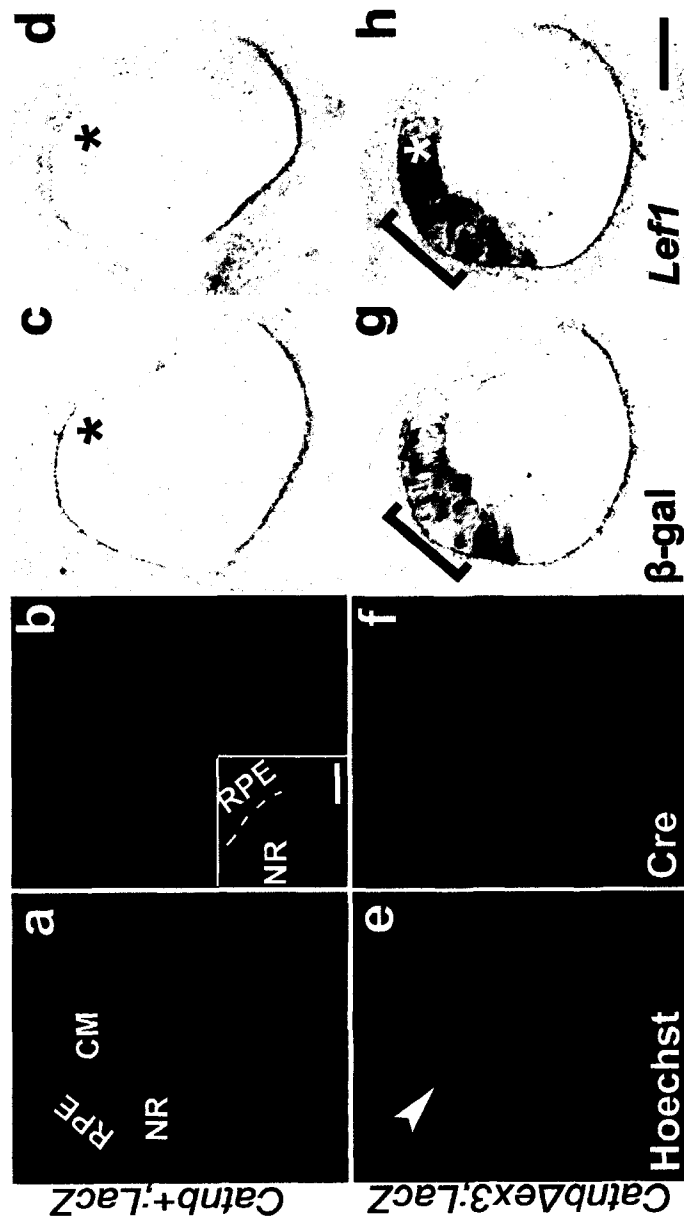
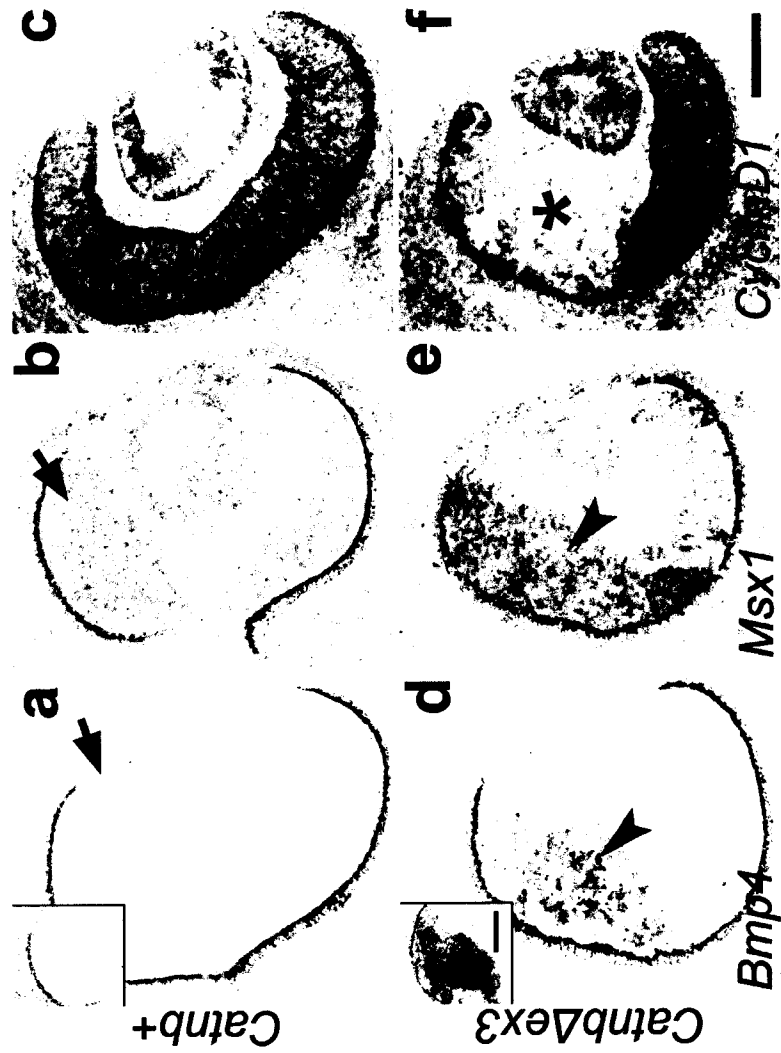


Fig. 41. Stabilized β -catenin specifies a CM gene expression profile in the peripheral retina at E11.5.

Serial retinal sections from E11.5 control littermates, *Catnb*⁺ (a-c) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (d-f) were processed for ISH for CM marker genes, *Bmp4*, *Msx1* and NR marker *CyclinD1*. Inserts show *Lef1* expression (a and d) in adjacent sections, revealing activation of Wnt signaling in the *Catnb* ^{Δ ex3} mice. Expression of *Bmp4* and *Msx1* is dramatically up regulated (compare arrows in a and b with arrowheads in d and e), while *CyclinD1* is markedly down regulated (asterisk in f) in the β -catenin activated region in the *Catnb* ^{Δ ex3} mice (n=2). Scale bars: 100 μ m in (a-f) and for inserts.



At E11.5, Ki67⁺ nuclei were mostly spindly shaped and the density of Ki67 staining showed a central high to periphery low gradient in the *Catnb*⁺ controls, consistent with previously observed gradient rate of proliferation at this stage (Fig. 42b). The gradient pattern of Ki67 staining in the eye of the *Catnb* ^{Δ ex3} mice was comparable to the control littermates; however, the Ki67⁺ nuclei in the β -catenin stabilized region had a more rounded appearance (Fig. 42f). By E14.5, Ki67⁺ nuclei were highly compacted throughout the NR, while the CM contained fewer and less intensely stained Ki67⁺ cells compared to the central retina in the control littermates (Fig. 42d). The cells were disorganized in the Cre⁺ region of the eye in the *Catnb* ^{Δ ex3} mice, however, both Ki67⁺ and Ki67⁻ nuclei presented in this region, a pattern comparable to that in the CM in the controls (Fig. 42h). We thus conclude that selective proliferation of Cre⁺ cells is unlikely to account for the expansion of the CM region in the *Catnb* ^{Δ ex3} transgenic mice. However, quantification for Ki67 or other proliferation marker should be carried out to completely rule in or out the possibility of selective outgrowth.

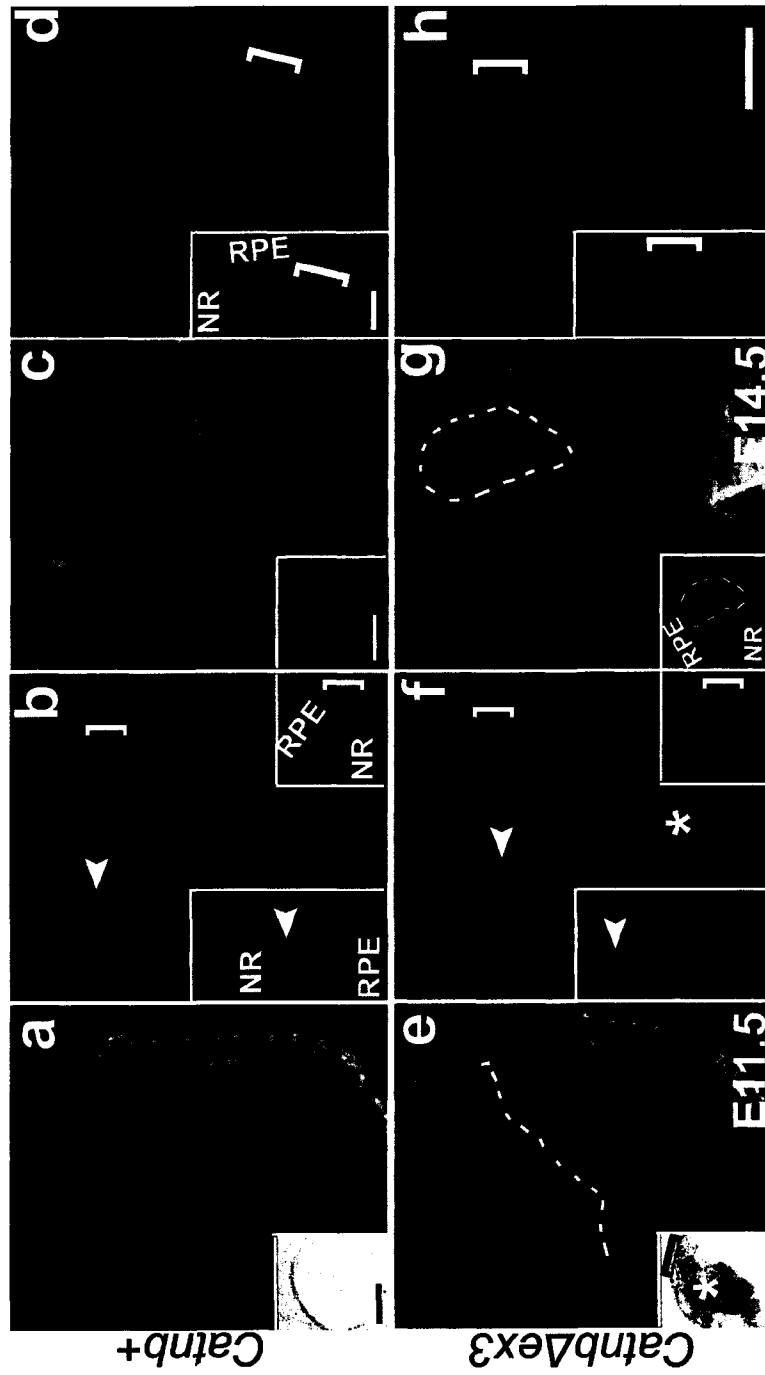
6.1.4 Expression of stabilized β -catenin down regulates retinal progenitor gene

Chx10* and *Pax6

Rowan et al have reported a small eye phenotype and expanded CB in the peripheral retina in the *Chx10* null mutants (*or^J* mice) (207). An aberrant peripheral retina was also reported in mice with conditional inactivation of *Pax6* gene in the peripheral retina (161). *Chx10* and *Pax6* are paired-like HD transcription factors and are expressed in embryonic retinal progenitors, including the CM. Loss of *Chx10* is associated with optic nerve hypoplasia and failure of bipolar cell development (25). Some aspects of this phenotype have

Fig.42. Stabilized β -catenin alters nuclei morphology with no effect on cell proliferation in the developing eye.

Serial retinal sections from E11.5 or E14.5 control littermates, *Catnb*⁺ (a-d) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (e-h) were stained with Hoechst (blue) to reveal nuclei and processed for IHC for a proliferation marker, Ki67 (b, d, f, h). Inserts in (a and e) show *Lef1* expression in the adjacent sections, revealing activation of Wnt signaling in the CM (bracket) and NR (asterisk) in *Catnb* ^{Δ ex3} mice. Inserts in (c and g) show α -Cre expression in the adjacent sections. Inserts in (b, d, f and h) are large views of Ki67 staining in the peripheral (brackets) or central (arrowheads) retina. Dashed lines (e and g) define the β -catenin stabilized regions. At E11.5, the intensity of Ki67 staining in the β -catenin stabilized region is comparable to that in the control littermates (compare arrowheads in b with f). Arrowheads in (b and f) indicate spindle shape of nuclei in the controls versus round in the region expressing stabilized β -catenin in the *Catnb* ^{Δ ex3} mice (n=2). The nuclei in the β -catenin unstabilized region looks normal (asterisk in f) in the compound transgenic mice. At E14.5, the CM contains both Ki67⁺ and Ki67⁻ cells in the controls (brackets in d), and similar pattern of Ki67 staining was detected in the Cre⁺ region in the *Catnb* ^{Δ ex3} mice (brackets in h) (n=2). NR, neural retina; RPE, retinal pigment epithelium. Scale bars: 100 μ m in (a-h), for inserts in (a, e) and in (c, g), 25 μ m for insert in (b, d, f and h).



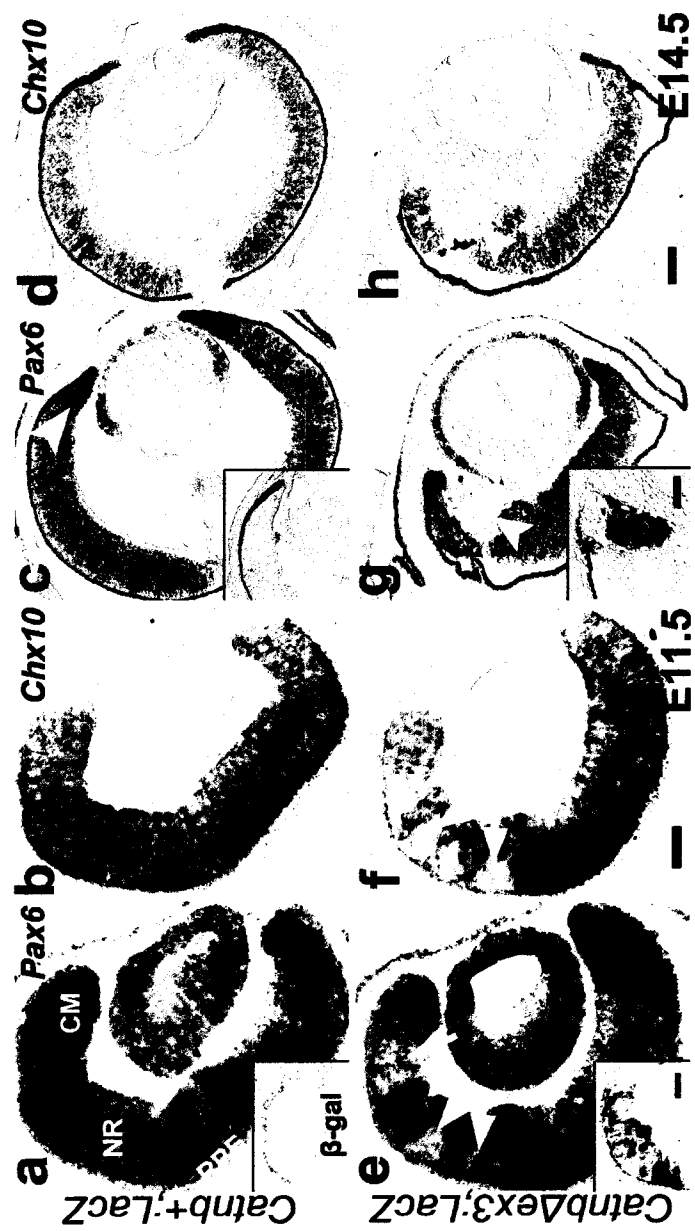
been attributed to a role for *Chx10* in proliferation (reviewed by 144), although this is controversial (152). *Pax6* is required for the multipotent state of progenitors and its inactivation led to the change of neural potential of the RPCs (160). The *Catnb*^{*Δex3*} mice also exhibited small eyes and expansion and disorganization of the CM, thus we asked whether expression of these two genes is affected in these transgenic mice. Examination in E11.5 and E14.5 tissue revealed down regulation in expression of both genes in the region expressing stabilized β -catenin (Fig.43). The identical pattern of reporter activity and loss of gene expression strongly suggests a cell-autonomous mechanism underlying this effect (Fig.43). Moreover, at E14.5, the β -catenin stabilized region showed a solid pattern of reporter activation (Fig. 43g insert) and completely excluded the expression of *Chx10* and *Pax6* (Fig. 43g, 43h); whereas at E11.5, activation of Wnt reporter and lowered level of *Chx10/Pax6* expression located in cell patches distributing within 1/3-1/2 region of the eyecup (Fig. 43e, 43f). Thus activation of Wnt canonical signaling was associated with *Chx10/Pax6* atypical reduction in the β -catenin activated retina.

6.1.5 Maintained CE/CB identity with abnormal neurogenesis in the peripheral eye of postnatal mice expressing stabilized β -catenin

Given the patterning changes in the peripheral eye of embryos expressing stabilized β -catenin, we looked at later stages to characterize the development of the non-neural derivative of the CM. It should be noted that, at later stages, *α -Cre* is also active in differentiated RGC and amacrine cells. Thus we generally evaluated the eye size and morphology of the *Catnb*^{*Δex3*} mice at P7. Further analysis was done in two parts, the

Fig. 43. Loss of *Chx10* and *Pax6* expression in the β -catenin stabilized region of the embryonic eye.

Serial retinal sections from E11.5 (a, b, e and f) or E14.5 (c, d, g and h) control littermates, *Catnb*⁺; *LacZ* (a-d) and β -catenin compound transgenic mice, *Catnb*^{*Δex3*}; *LacZ* (e-h) at were processed for ISH for *Pax6* or *Chx10*. Inserts show β -gal activity in adjacent sections, revealing activation of Wnt signaling in the eye expressing stabilized β -catenin. Dashed lines in (g and h) define the Wnt activated tissue. Loss of *Chx10/Pax6* expression is in an identical pattern as that of reporter activation at E11.5 (arrows in e and f) and E14.5 (arrowheads in g and h) (n=2). Note that *Pax6* is normally highly expressed in the CM at E14.5 in the controls (arrowhead in c). CM, ciliary margin; NR, neural retina; RPE, retinal pigment epithelium. Scale bars: 50 μ m in (a, b, e and f) and for the inserts in (a, e), 100 μ m in (c, d, g and h) and for the inserts in (c, g).



development of the peripheral and the central NR.

At P7, the eyes of the *Catnb^{Δex3}* mice were smaller, poorly shaped with severely disorganized periphery (Fig. 44e). The vitreous was packed with cells, likely derived from the periocular mesenchyme (Fig. 44e, 44g). The lens was larger compared with the control littermates, and appeared closely associated with the NR (Fig. 44e).

In the eye of control littermates at this stage, the peripheral region contained clearly defined ciliary processes, with *Cre* expression in subset of the cells in the nPCE, but not in the PCE (Fig. 44c), in keeping with a previous report (48). The iris was extended from the root of the CB and also expressed *Cre* transgene (Fig. 44c), also consistent with Davis-Silberman et al (48). In the eyes of mice expressing stabilized β-catenin, *Cre* expression was barely detected in the region that normally forms the CB (Fig. 44g) and the few GFP⁺ cells that were detected likely represented RGC or amacrine cells from the NR, based on morphology of the cells. The peripheral retina of compound transgenic mice contained rosettes (Fig. 44e), with the pigmented cells invaded deep into the peripheral NR and mixed with the non-pigmented tissue, forming ciliary process-like structures (Fig. 45, 46). We could not see the iris in the *Catnb^{Δex3};LacZ* mice in the region that normally forms iris (Fig. 45b, 45d and data not shown). Expression of *TCF/Lef-LacZ* reporter and Wnt target gene *Lef1* indicated the activation of Wnt canonical pathway in the disorganized peripheral region of the eye in the *Catnb^{Δex3};LacZ* mice (Fig. 45b, 45d). CM/CE marker was detected in this region (Fig. 45f), suggesting a CM/CE identity. Thus we conclude that the CE/CB is

Fig. 44. Expression of stabilized β -catenin results in morphological changes in the eye of the postnatal mice.

Retinal sections from P7 control littermates, *Catnb*⁺ (a, b) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (e, f) were stained with Hoechst (a, e) to reveal nuclei and processed for IHC for GFP to identify *Cre* expression (b, f). High magnification views of peripheral eye (asterisks in a, e) and central retina (open arrowhead in a and e) are shown in c, g, d and h, respectively. Dashed lines define the lens (a and e) and the invasion of mesenchymal tissue (g). Vitreous is apparent in the controls (# in a), but lost in the *Catnb* ^{Δ ex3} mice (e). *Cre* expression was detected in the nPCE (arrowheads in c) and iris (insert in c), but not in PCE (arrows in c) in the peripheral eye of control littermates. The peripheral eye of *Catnb* ^{Δ ex3} mice is disorganized with *Cre* expressed in a few cells in this region (g) (n=3). Arrowheads and arrows in (d and h) indicate *Cre*⁺ cells in RGC layer and INL respectively. Note that both IPL (compare brackets in d and h) and OPL (compare open arrowheads in d and h) in the eye of *Catnb* ^{Δ ex3} mice are thinner than the controls (n=3). INL, inner nuclear layer; NR, neural retina; PCE/nPCE, pigmented and non-pigmented ciliary epithelium; RGC, retinal ganglion cell layer; ONL, outer nuclear layer. Scale bars: 100 μ m in (a, b, e and f), 50 μ m in g and (c, d and h), 25 μ m for insert.

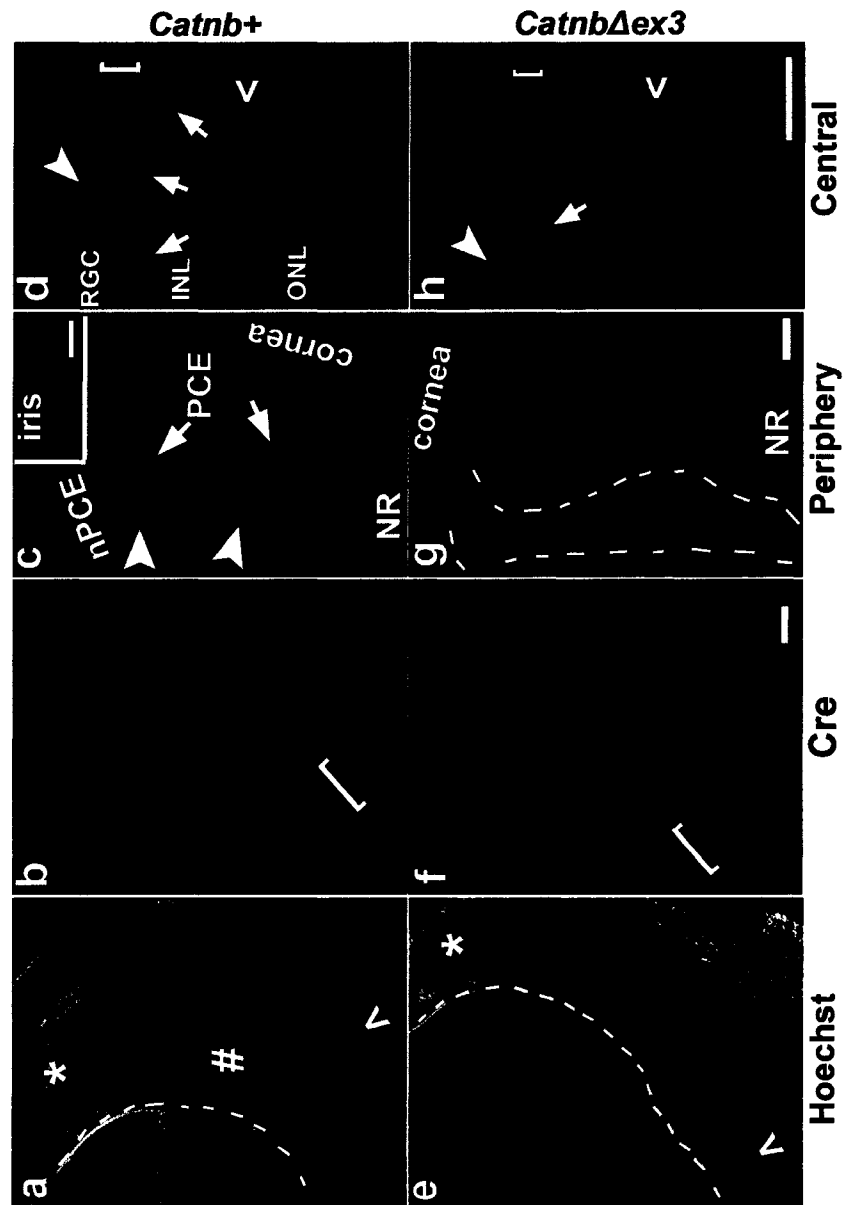
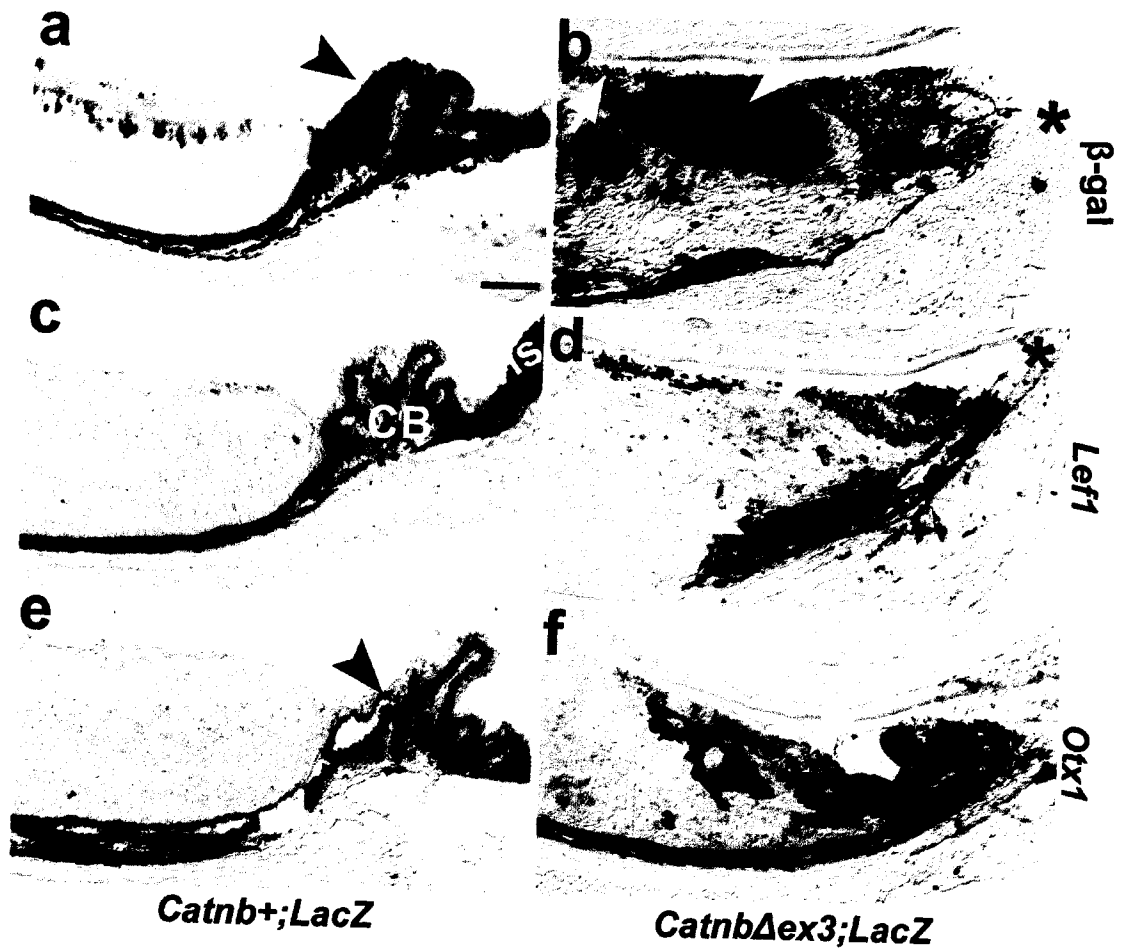


Fig. 45. Activation of Wnt signaling induces CM/CE gene expression in the peripheral retina of the postnatal mice.

Serial retinal sections from P7 control littermates, *Catnb*⁺; *LacZ* (a, c, e) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3}; *LacZ* (b, d, f) were stained with X-gal for β -gal reporter activity (a, b) and ISH for Wnt target *Lef1* (c, d) and CM marker *Otx1* (e, f) expression. Insert in (a) is a high magnification view of iris, revealing reporter gene expression in a subset of cells. Arrowheads in (a and e) indicate normal activation of *TCF/Lef-LacZ* reporter and expression of *Otx1* in the CE of control mice. Arrows in (b, d, f) indicate the pigmented tissue in the NR region of *Catnb* ^{Δ ex3}; *LacZ* mice. In the peripheral retina of the mice expressing stabilized β -catenin, activation of *TCF/Lef-LacZ* reporter (arrowhead in b) overlaps with the induction of *Lef1* (arrowhead in d) and *Otx1* (arrowhead in f) expression, implying a cell autonomous mechanism (n=2). CB, ciliary body; CE, ciliary epithelium; NR, neural retina. Scale bars: 100 μ m in (a-f), 25 μ m for insert.



maintained in the periphery, where stabilized β -catenin activates canonical Wnt pathway, but CE/CB does not undergo proper morphogenesis.

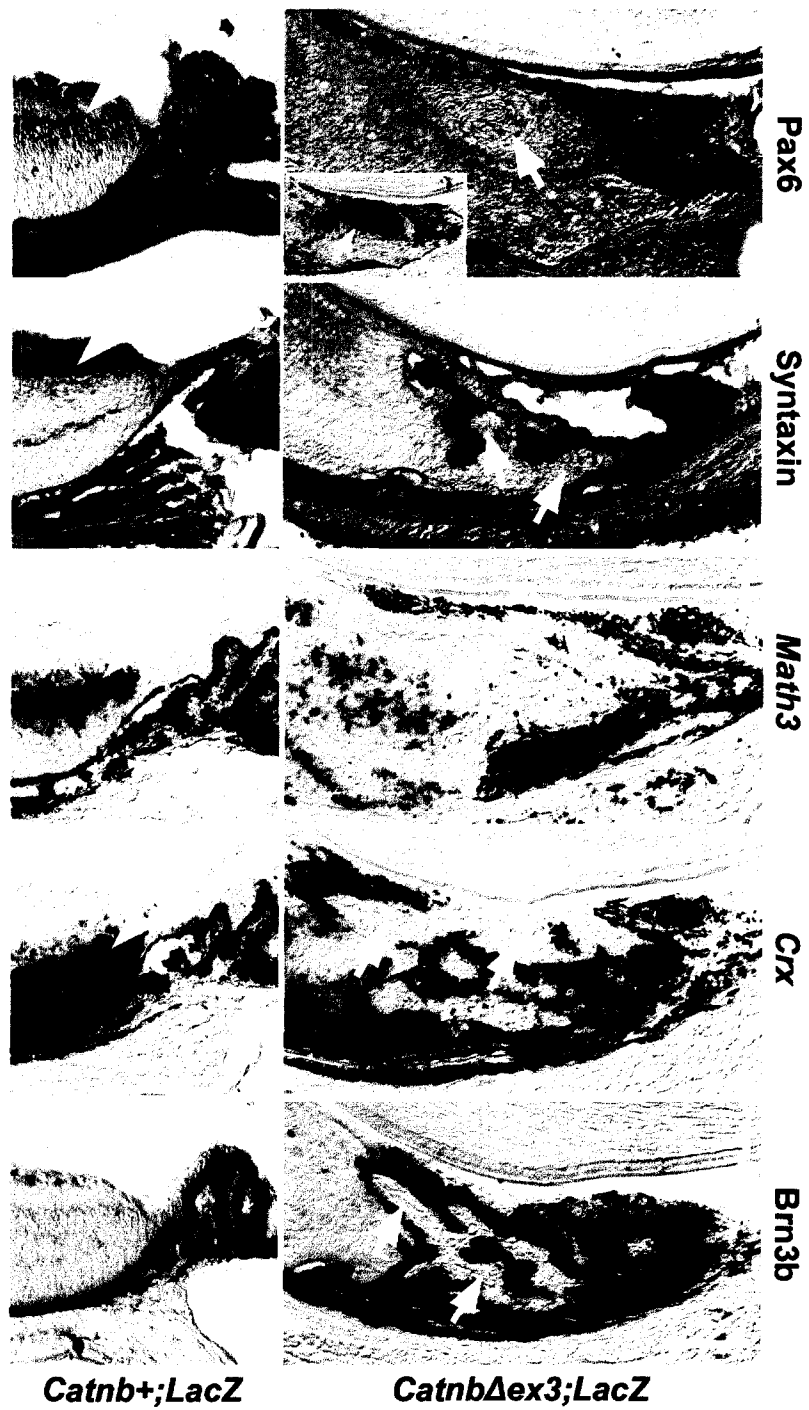
The phenotype of an aberrant peripheral retina was also reported in the mice with a conditional inactivation of the *Pax6* gene in the eyes (161). Marquardt et al showed exclusive expression of amacrine cell markers in the peripheral eyes of the *Pax6* deficient mice. We thus examined markers for differentiating and differentiated neurons and asked whether they were presented or not in the peripheral eye expressing stabilized β -catenin. In the region that Wnt reporter was activated, no *Pax6*⁺ or syntaxin⁺ amacrine cells, or Brn3b⁺ RGCs were detected (Fig. 46b, d, j). Loss of *Math3* expression, a marker for amacrine cells confirmed problem of amacrine cell differentiation (Fig. 46e, f). Expression of *Crx*, a marker for photoreceptors was also excluded from this region, suggesting loss of photoreceptors (Fig. 46g, h). Thus there was a general reduction in neurogenesis, including amacrine cells, in the peripheral eye expressing stabilized β -catenin.

6.1.6 Stabilization of β -catenin results in a reduction of mature neurons in the central retina of the postnatal eye

As noted above, there is a second wave of α -*Cre* expression, which is in differentiated RGC and amacrine cells at late stages (122, 161, 256). Thus we asked what the effect of stabilized β -catenin was on the differentiation of retinal neurons by examining specific markers in the postnatal NR. We also examined expression of *TCF/Lef-LacZ* reporter and BrdU incorporation to monitor activation of Wnt/ β -catenin signaling and cell proliferation, and markers for other cellular types to ask if there were indirect effects on those cells.

Fig. 46. Inhibition of neurogenesis in the peripheral retina of postnatal mice expressing stabilized β -catenin.

Serial retinal sections from P7 control littermates, *Catnb*⁺ ;*LacZ* (a, c, e, g and i) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} ;*LacZ* (b, d, f, h and j) were processed for IHC for Pax6 or syntaxin to detect amacrine cells, Brn3b to detect RGCs, or ISH for *Math3* and *Crx* expression. Insert in (b) shows β -gal reporter activity in adjacent section, indicating activation of canonical Wnt pathway in the peripheral region (arrow). Expression of all the examined markers or genes was excluded from the β -gal⁺ region (compare arrows with arrowheads) (n=2). Scale bars: 100 μ m in (a-j) and for insert.



Because development of the peripheral eye was perturbed, we monitored cell types in the central retina. *Cre* expression was detected, but in fewer cells, in the central retina of the *Catnb^{Δex3}* mice compared to the control littermates (compare Fig. 44b with 44f, 44d with 44h). The IPL and OPL layers were thinner; whereas the thickness of INL and ONL in the *Catnb^{Δex3};LacZ* mice appeared similar to the controls (Fig. 44d, 44h). The *TCF/Lef-LacZ* reporter was activated in fewer cells, but in similar pattern as in control littermates (Fig. 47d). We also detected BrdU⁺ cells in the INL of the *Catnb^{Δex3};LacZ* mice (Fig. 47f).

Staining with cell type specific markers [see a list of retinal cellular markers and references in Appendix C] revealed changes in a number of cell types in the *Catnb^{Δex3}* mice: RGCs, amacrine, bipolar and a subset of horizontal cells were reduced in the central retina of the mice expressing stabilized β-catenin compared to controls (Fig. 48, 49). *Cre* expression was not detected in the region containing bipolar or horizontal cells (Fig. 49 inserts), suggesting a non cell-autonomous effect on these cell types. Notably, staining for rhodopsin and cralbp did not reveal any difference of mature rod photoreceptors and Müller glial cells in the central retina of the *Catnb^{Δex3}* mice compared to the control littermates (data not shown).

Fig.47. Changes in Wnt responding cells and persistence of dividing cells in the central retina of postnatal mice expressing stabilized β -catenin.

Serial retinal sections from P7 control littermates, *Catnb*⁺; *LacZ* (a-c) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3}; *LacZ* (d-f) were stained with X-gal to detect β -gal activity (a, d) and processed for IHC for BrdU (c, f). Hoechst staining (b, e) reveals nuclei. Inserts in (a and d) show *Cre* expression in adjacent sections. There are fewer β -gal⁺ reporter cells in the RGC (compare arrows in a and d) and INL (compare arrowheads in a and d) layers in the eye expressing stabilized β -catenin compared to control littermates (n=3). Activated β -catenin resulted in an increase of BrdU⁺ cells (arrowhead in f) in the central retina (n=2). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell layer. Scale bars: 50 μ m in (a-f) and inserts.

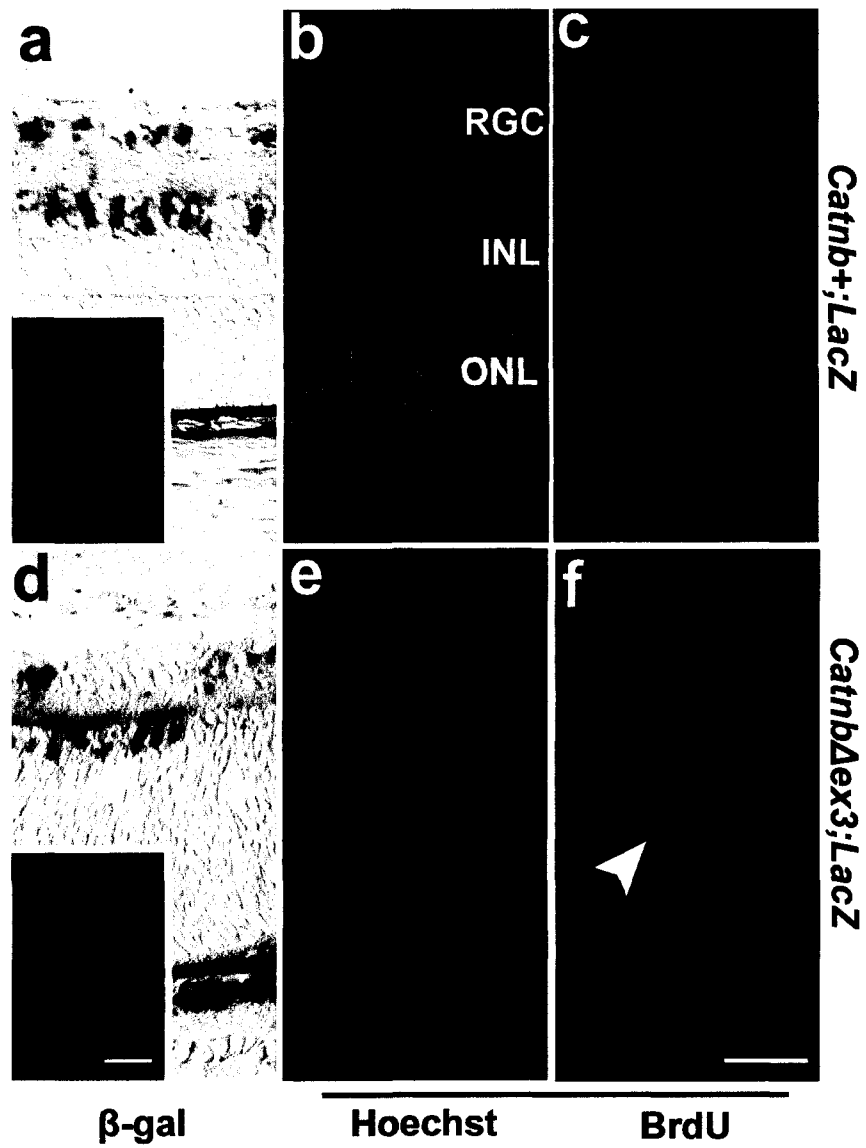


Fig. 48. Stabilized β -catenin reduces mature RGCs and amacrine cells in the central retina of the postnatal mice

Retinal sections from P7 control littermates, *Catnb*⁺ (a-d) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (e-h) were processed for IHC for Brn3b (a, e), Pax6 (b, f), syntaxin (c, g) or calretinin (d, h). Hoechst staining reveals nuclei. Inserts in (a, e) show *Cre* expression in serial sections. In the RGC layer, Brn3b⁺ (arrowheads in a and e), Pax6⁺ (arrowheads in b and f) and calretinin⁺ (arrowheads in d and h) cells, marking RGCs and amacrine cells, are remarkably reduced in the *Catnb* ^{Δ ex3} mice compared to controls (n=3). In the INL layer, Pax6⁺ amacrine cells (arrows in b) are lost and calretinin⁺ amacrine cells (compare arrows in d and h) are reduced in the *Catnb* ^{Δ ex3} mice (n=3). Anti-syntaxin staining revealed disorganized IPL (compare brackets in c and g), confirming problem of amacrine cells, while syntaxin⁺ cells in the INL (compare arrows in c and g) appear normal in the *Catnb* ^{Δ ex3} mice. Note that the IPL shows high levels of abnormal immunoreactivity to Pax6 staining (bracket in f) in the mice expressing stabilized β -catenin. INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; RGC, retinal ganglion cell layer. Scale bar: 25 μ m.

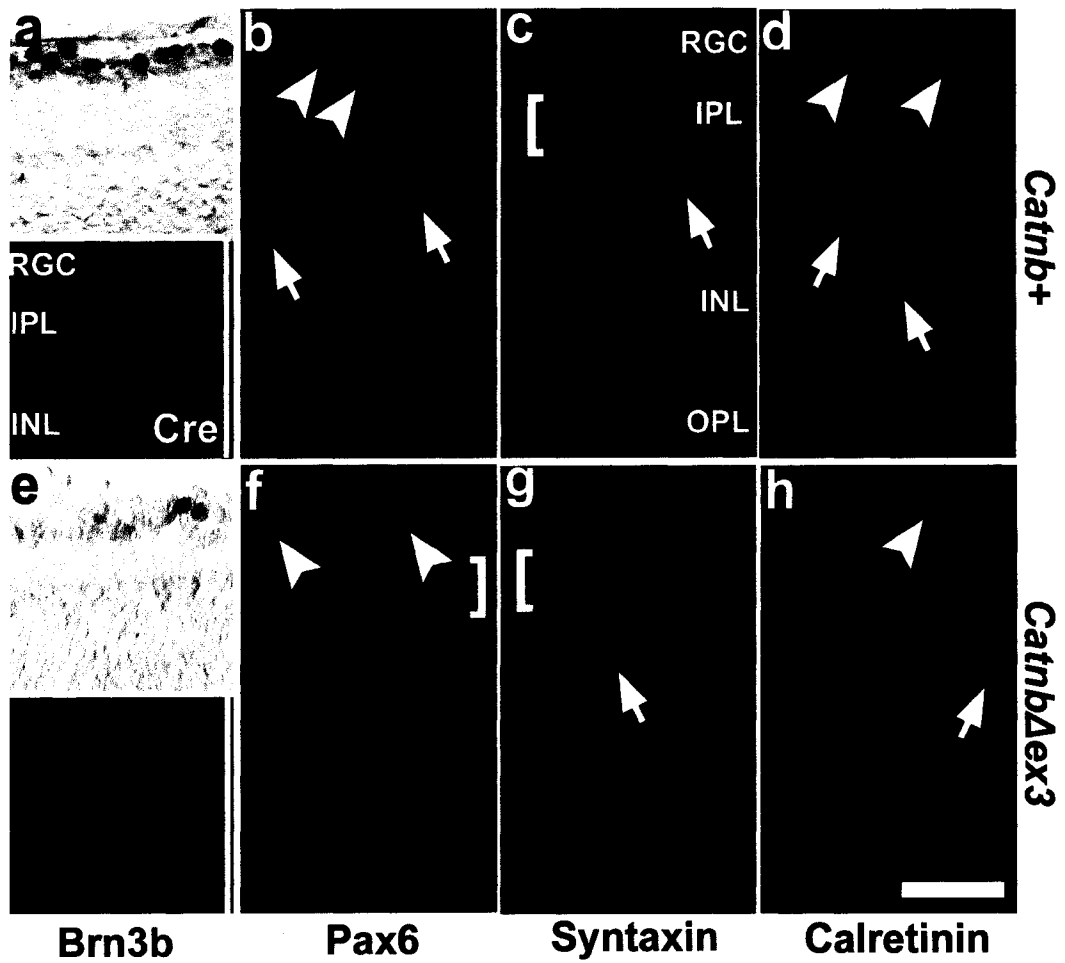


Fig.49. Stabilization of β -catenin in the central retina resulted in non cell autonomous changes in bipolar and horizontal cells in the postnatal eye.

Retinal sections from P7 control littermates, *Catnb*⁺ (a-c) and β -catenin compound transgenic mice, *Catnb* ^{Δ ex3} (d-f) were processed for IHC for Chx10 (a, d), Prox1 (b, e) or calbindin (c, f). Hoechst staining (blue) reveals nuclei. Inserts in (a, d) show *Cre* expression from serial sections. Asterisks in (a, d and inserts) denote the OPL. Highly stained Chx10⁺ bipolar cells locate apically in the INL (bracket in a) in controls, but in the retina expressing stabilized β -catenin, Chx10⁺ cells are less intensely stained and shift to the basal region of the INL (bracket in d) (n=2). Prox1⁺ bipolar cells (brackets in b and e) are markedly reduced in the *Catnb* ^{Δ ex3} mice. Calbindin⁺ horizontal cells (open arrowhead in c) are lost in the retina expressing activated β -catenin (f), while Prox1⁺ horizontal cells appear unaffected (compare open arrows in b and e). Note that Prox1⁺ amacrine cells (arrows in b and e) and calbindin⁺ amacrine cells or RGCs (arrows and arrowheads in c and f) are reduced in the eye of *Catnb* ^{Δ ex3} mice compared to control littermates. INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell layer. Scale bar: 50 μ m in (a-f), 25 μ m for inserts.

6.2 Discussion

6.2.1 Activation of canonical Wnt signaling converses the NR into CM

In the *in vivo* model of conditional expression of stabilized β -catenin we observed activation of the canonical Wnt reporter, induction of a CM-like gene expression profile and an expanded CB structure at late stages, all of which were restricted to Cre⁺ cells. Thus, expression of stabilized β -catenin in the embryonic eye is sufficient to expand the CM/CB. We did not observe an increase in proliferation or an increase in cell death in the eyes of the *Catnb*^{*Δex3*} mice, arguing that the phenotype in the *Catnb*^{*Δex3*} mice is not due to selective cell survival or increased proliferation. Thus our interpretation of the *Catnb*^{*Δex3*} phenotype is a transdifferentiation of the NR into CM/CB. A converting status of *Chx10/Pax6* expression observed at E11.5 supports this model. Consistent with our findings, a recent study on chick retina revealed that activation of canonical Wnt pathway, via stabilized β -catenin or ectopic expression of *cWnt2b*, inhibits *Chx10* expression and induces CB/iris identity (38), suggesting that this function of Wnt signaling in the establishment of peripheral eye fates is evolutionally conserved. Further investigations need to be carried out to prove this model in mice, such that by doing fate mapping with a genetic reporter to show that the cells are actually derived from the NR.

6.2.2 Elevated levels of β -catenin results in phenotype different from that caused by loss of *Chx10* or *Pax6*

Stabilized β -catenin leads to downregulation of *Chx10* and *Pax6*, but the phenotype in the *Catnb* ^{Δ ex3} mice is different from *or*^J mice, which are null for *Chx10* or transgenic mice with conditional inactivation of *Pax6*. The *or*^J mice develop a progressive hyperpigmentation in the peripheral retina such that most of the retina appears pigmented (103, 207). In the *Catnb* ^{Δ ex3} mice, the CB-like peripheral region is expanded with a clear distinction between the pigmented and non-pigmented tissue and the developing CM is much more disorganized compared with the *or*^J mice. However, we cannot rule out the possibility that the pigmentation effect of loss of *Chx10* in retinal cells (207) may contribute, in part to the expansion of the pigmented tissue in the peripheral eye of the *Catnb* ^{Δ ex3} mice. Knockout of *Pax6* in the peripheral retina results in exclusive development of amacrine cells in the affected region (161); in contrast, although *Pax6* expression was downregulated in the peripheral retina of *Catnb* ^{Δ ex3} mice, amacrine neurons did not develop in this region, demonstrating that induction of stabilized β -catenin signaling does not phenocopy knockout of *Pax6* in this region of the retina. We conclude that the phenotype presented here is unique to the *Catnb* ^{Δ ex3} transgenic mice, suggesting a specific role of Wnt/ β -catenin signaling in directing development of the CM and its non-neural derivatives.

The CB pigmented layer in the adult retina contains cells with stem cell identity (4, 62, 243). Despite the expanded CM/CB in the mice expressing stabilized β -catenin, there were fewer RSC spheres received from the peripheral pigmented tissue in postnatal and adult *Catnb* ^{Δ ex3} mice compared with control littermates (unpublished data from YP Wang in Wallace lab). This is unexpected given the fact that the PCE of the *Chx10* null retina

produces more retinal stem cells (43, 243) and *Chx10* expression is dramatically down regulated in the *Catnb^{Δex3}* mice. It is possible that improperly formed CB does not provide a perfect niche for the stem cells pools, or that loss of *Pax6* expression affects the production of neural spheres. There could also be technical problems with dissociating that tissue from these malformed eyes. All the possible molecular bases remain as a matter for further investigation.

6.2.3 Possible mechanism underlying the small eye phenotype

There are several factors that could account for the microphthalmia that we observed in the *Catnb^{Δex3}* transgenic mice. The CM has a lower rate of proliferation compared to the central retina and thus, conversion of a major part of the NR into a slower growing CM could markedly reduce eyecup growth. Invasion of mesenchyme to the vitreous likely affects blood supply to the eye and negatively affects growth. Finally, it is possible that the CB function is affected in these mice and this could alter development and growth of the eyecup. It has been suggested that the secretion function of the CE starts prior to the morphological maturation of the CB (reviewed by 107). The aberrant CM in the developing eye could partially lose its ability to produce components that are important in coordinating the growth and morphogenesis of the optic cup (reviewed by 11).

6.2.4 Reduction of neurogenesis in the postnatal eye likely due to improper differentiation or cell death

There are severe abnormalities in the RGC layer and INL of the *Catnb^{Δex3}* mice, including a reduction of several retinal cell types and in the thickness of plexiform layers, the

latter of which suggests reduction in cell processes. Thus the NR in the eye expressing stabilized β -catenin is not well developed. Fewer RGCs are likely due to cell death because their development in the central regions looks normal at embryonic stages. There is a reduction in the amacrine cells; but our analysis does not allow us to distinguish whether this is secondary to cell death, failure to specify these cells or downregulation of specific markers. Changes in other cell types other than RGCs and amacrine cells are possibly secondary to growth defects in the eye. To differentiate between these possibilities, further analysis, including dissociated cell counting and analysis at several developmental stages, is needed to address whether there is cell death at any stages, whether the total cell number remains the same and what the cells are in the central region of the postnatal eyes expressing stabilized β -catenin. It is also worthwhile to target activated Wnt signaling to subset of specific cell types in the central retina and identify Wnt function in these retinal cells.

7.0 Conclusions and Remarks

Based on the pattern of gene expression and Wnt reporter activation reported by our group and others (149, and reviewed by 249), I hypothesized that canonical Wnt signaling plays a role in retinal development, in particular, the specification and formation of the CM and its derivatives. *In vitro* and *in vivo* studies presented here demonstrate that activation of this pathway induces a CM like identity in the NR and expands the CM/CB in the periphery of the eye. Thus, increased Wnt canonical signaling is sufficient to promote CM specification in the mammalian eye. Findings in this study are in partial agreement with and have extended the observations from other parallel studies carried out in chick retina (38, 135, 136).

It is also found in this study that Wnt/ β -catenin signaling activation reduces retinal differentiation, however, it is difficult to conclude whether this is a direct or indirect consequence of the activated Wnt pathway. In long term cultured retinal explants, reduced neuronal differentiation may be the result of a general suppression effect of Li^+ on neurogenesis, but I cannot rule out the possibility that other indirect factors are also involved during the long course of the culture. In perinatal β -catenin compound transgenic mice, stabilized β -catenin in the central retina is restricted by α -Cre activity, to amacrine neurons and RGCs, yet multiple cell types are affected. It could be that these indirect effects in the central retina of transgenic mice are also secondary to disruption in CB development. CB is

critical to the maintenance of the functional eye (reviewed by 11) and retinogenesis, as many components of the retinal inner limiting membrane, which is important for normal retinal development (85), are synthesized in the CB (86, 96). However, at least two types of retinal cells are formed normally in the central region of the NR, implying that the aberrant function of CB might not be a major reason that causes disruption of neuronal differentiation. Alternatively, it could be that increased level of β -catenin in amacrine cells and RGCs induces regional changes in the differentiation of specific neighbouring cells.

The studies presented in this thesis have raised a number of additional points that are worthy to pursue experimentally.

1. The gain of function study provides evidence that ectopic Wnt signaling is sufficient to promote CM development and the formation of the non-neural derivatives in the eye. But is this pathway also necessary for the formation of CM/CB? Disruption of canonical Wnt signaling downregulates peripheral gene expression and leads to iris hypoplasia in chick retina (38), and I showed decay of reporter activity in retinal explants that are missing *Wnt2b* expression, suggesting that this pathway is likely required for peripheral eye development. To address this possibility, I could reduce Wnt/ β -catenin signaling in the peripheral retina and monitor CM/CB development. In preliminary experiments it has been shown that inactivation of β -catenin in the peripheral retina reduces CM gene expression and the size of the CM (unpublished data from YP Wang in Wallace lab). In addition, an important question is whether *Wnt2b* is the ligand that signals to the CM/CB in mouse. To identify *Wnt2b* function in this region of the eye, I could knock down *Wnt2b* expression using a conditional

approach and look at the effects on the development of the peripheral eye. It would also be worth looking for other possible ligand candidates. Proteins other than Wnts can trigger the canonical Wnt cascade, such as Norrin, the protein product of the NDP (reviewed by 177). It has been known that Norrin and Frizzled-4 receptor function as a ligand-receptor pair, controlling vascular development in the retina (275). However, no CB problems have been reported in either loss of NDP in patients and mouse model (155, 275), or gain of function of NDP in transgenic mice (182) .

2. Dual functions of β -catenin in Wnt signaling and cell-cell adhesion have been well recognized. What is less clear, however, is whether and how activated Wnt/ β -catenin signaling affects the pool of β -catenin employed in cell-cell adhesion (reviewed by 22, and by 176). Transgenic expression of stabilized β -catenin in neural precursors results in mice with enlarged brains due to a substantial increase in precursor proliferation (37). But to what extent is this effect on proliferation due to direct activation of canonical Wnt pathway targets versus alterations in cell fate due to cadherin-dependent changes in asymmetric cell division (reviewed by 14, 36, 253)? At this point in time, efficient methods or reagents to distinguish whether a particular phenotype is mediated by transcriptional activation or cell adhesion function are lacking. Thus, one has to bear in mind that the phenotype associated with stabilized β -catenin in the retina could be due to perturbation in both functions of β -catenin.

3. It has been shown in this study that Wnt/ β -catenin canonical signaling drives CM specification. But what is unknown is the downstream Wnt target gene that specifies CM cell fate. A previous study has implicated BMP signaling in CB development (283) and

utilization of transgenic mice through activated Wnt signaling increased *Bmp4* expression, which likely contributed to the expanded CM. I showed that electroporation induces high level ectopic expression of *Bmp4* in retinal explants; but it has no effect on CM gene expression. It is also worth noting that the increase in *Bmp4* expression level in the Cre⁺ cells does not recruit non-Cre⁺ cells to participate in the aberrant CM in *Catnb*^{Δex3} mice. Thus I believe that ectopic *Bmp4* might contribute to the CM expansion, but is not sufficient to drive expression of CM gene profile. The CM marker gene *Otx1*, which is indispensable for CB formation, as the *Otx1* null mice completely lose CB (1), is also a good candidate for a target of Wnt signaling. Requirement of *Otx1* function in the context of Wnt signaling during the peripheral eye development could be investigated using gain and loss of function approaches, such that targeting ectopic *Otx1* to the CM and asking whether this produces a similar phenotype as *Catnb*^{Δex3} mice. The expression pattern of *Msx1*, another CM marker, suggests a role for this HD transcription factor in patterning the peripheral region of the developing eye (172). Moreover, *Msx1* is a reported Wnt target in carcinoma cells (268). Thus *Msx1* could also be a good candidate responsible for the expansion of the CM in the β-catenin compound transgenic mice. *Msx1* deficient mice have been reported and survive till birth (211), however, an eye phenotype has not been reported. Examination for CM in those *Msx1*^{-/-} mice could provide evidence whether *Msx1* is required for the normal formation of the CM. It would also be helpful if ectopic *Msx1* is targeted to the peripheral retina and look at the effects on CM and CB. Notably, *Msx2*, which is also a Wnt target gene (268), has an identical expression pattern with *Msx1*, and forced expression of *Msx2* results in

microphthalmia and the spreading of RPE/CM marker expression to the entire NR (272), implicating these *Msx* genes in this context are worthy of further investigation.

In conclusion, the comprehensive analysis of Wnt signaling component gene expression and activation of canonical Wnt pathway presented here have implicated multiple roles of Wnt signaling in the context of mammalian retinal development and homeostasis. Functional studies carried out *in vitro* and *in vivo* reveal that, similar to other areas of the CNS, Wnt signaling functions in a variety of developmental processes and cellular contexts in the mammalian retina, particularly in the specification of the CM and its non-neural derivatives. Findings from this study will benefit further exploration of Wnt function in the mammalian eye and shed light on the general field of CNS development.

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9.0 Contributions of Collaborators

List in the alphabetical order of the surname of collaborators:

Yves De Repentigny (Technician, Kothary lab, OHRI and University of Ottawa, Canada) performed the pronuclear microinjections, from receiving the *Msx1mpWnt2bHA* DNA fragment up to generation of transgenic mice described in Chapter 5.0.

Kazuki Kuroda (Postdoctoral fellow, Rudnicki lab, OHRI and University of Ottawa, Canada) performed the TOPFlash assay, from receiving the testing plasmids up to Excel analysis of the results described in Chapter 5.0.

Chantal Mazerolle (Technician, Wallace lab, OHRI and University of Ottawa, Canada):
I have been collaborating with Chantal to accomplish the study in Chapter 6.0. Basically, I designed every experiment and finished microscopic imaging and data analysis. Chantal performed the experiments, including *in situ* hybridisation, immunohistochemistry and majority of the X-gal staining. Both of us were involved in tissue preparation.

Othman Mohamed (Ph.D student, Dufort lab, McGill University, Canada) prepared the embryonic tissue from *TCF/Lef-LacZ* transgenic mice analysed in Chapter 3.0 and prepared the adult tissue analysed for IHC in Chapter 4.0. For both cases, Othman contributed from setting mouse timemate up to tissue embedding.

10.0 Appendices

Appendix A. Oligonucleotides^a

Gene	Sequence (5'---3')	PCR condition		Reference (Genbank No.)
		AT (°C)	Mg ²⁺ (mM)	
<i>α-Cre</i>	F atgcttctgtccgtttgccg R cctgttttgacaggttcagcg	57	1.5	^b
<i>β-actin</i> ^c	F gtacttgcgctcaggaggag R ttgctgacaggatgcagaag	58~60	1.5	NM_007393
<i>Catnb</i>	F gacaccgctgcgtggacaatg R gtggctgacagcagcttttctg	65	1.5	^d
<i>Crx</i>	365F cag■■gtctgggtcaagaatcg 460R acgagccttggtctgtgc	58	1.5	NM_007770.2
<i>CyclinD1</i> ^c	F aggaagcgggccaggtagtt R agtgcgtgcagaaggagatt	58~60	1.5	NM_007631
<i>Dll</i>	1007F cactgacc■■caatctgtctgc 1119R gatgcactcatcgcagtagc	58	1.5	NM_007865.2
<i>Hes5</i>	280F tgaaacacagcaaag■■ccttc 418R agcttcatctgcgtgtcg	58	1.5	NM_010419.2
<i>mHPRT</i> (set 1)	9F ctgctttccggagcggtagc 740R caacttgcgctcatcttagg	56	1.6	J00423.1
<i>mHPRT</i> (set 2) ^c	F ctttgctgacctgctggatt R ctttgctgacctgctggatt	58~60	1.5	NM_013556
<i>Math3</i>	58F accccgggaaagag■■aatc 163R tgagagctcagaccttgtcc	58	1.5	NM_007501.2
<i>Msx1mp</i>	1115F acgctgcagaccgatgccacctgactt 1415R agaaggccaactcgagcga	66	1.0	S73812

Appendix A. Oligonucleotides^a (Cont'd)

Gene	Sequence (5'---3')	PCR condition		Reference (Genbank No.)
		AT (°C)	Mg ²⁺ (mM)	
<i>Otx1</i>	423F tcacagctggacgtgctc	58	1.5	NM_011023.3
	540R tgaaccaaacctggactctg			
<i>Pax6</i>	605F cggcagaagatcgtagagc	58	1.5	NM_013627.2
	719R tggatacgttttcattgtccag			
<i>TCF/Lef-LacZ</i>	F cagtggcgtctggcggaaaacctc	62	1.5	e
	R aaacaggcggcagtaaggcggtcgg			
<i>Wnt-1</i>	1201F aaatcgcccaacttctgca	53.2	1.5	BC005449
	1799R aatacccaaagaggtcacagc			
<i>Wnt-2</i>	323F cggcctttgtttacgccatc	55.3	2.25	NM_023653
	836R tgaatacagtagtctggagaa			
<i>Wnt-2b</i> (set 1)	844F tgtactctgcgcacctgct	60	2.25	AF070988.1
	1162R tgcactcacactgggtgac			
<i>Wnt-2b</i> (set 2)	115F aaccaagcttatgctgaagctgcagggtga	61	2.0	AF070988.1
	1284R gacggaattctcaggtctggtccagccact.			
<i>Wnt-2b</i> (set 3)	1033F tgtgtcttgacaaggctgc	51~65	1.5	AF070988.1
	1281R aatgtcgacggtctggtccagccactct.			
<i>Wnt-3</i>	529F gccgacttcggggtgctggt	61.4	2.0	NM_009521
	846R cttgaagagcgcgtacttag			
<i>Wnt-3a</i>	603F attgaatttgagggaatggt	57.5	1.5	NM_009522
	920R cttgaagtacgtgtaacgtg			
<i>Wnt-4</i>	122F tgtacctggccaagctgtcat	60	2.25	NM_009523
	466R tccggtcacagccacactt			
<i>Wnt-5a</i>	1175F tcctatgagagcgcacgcat	60	2.25	NM_009524
	1399R cagcttgccccggctgttga			

Appendix A. Oligonucleotides^a (Cont'd)

Gene	Sequence (5'---3')	PCR condition		Reference (Genbank No.)
		AT (°C)	Mg ²⁺ (mM)	
<i>Wnt5b</i>	604F tcggaggagcagggccgagc	64.8	2.25	M89799
	829R cagcttgccctggcgggtga			
<i>Wnt6</i>	556F atggatgcgagcagcacaagcg	59.4	2.25	M89800
	798R tttgccgtcgttggtgccca			
<i>Wnt7a</i>	412F caaggccagtaccactggga	56.5	1.75	M89801
	719R ggctccacgtggacggcctc			
<i>Wnt-7b</i>	271F accgtcttcgggcaagaact	63.9	2.25	M89802
	531R cctggcgttcttttgatct			
<i>Wnt8b</i>	534F aacgtgggcttcggagaggc	62.9	1.5	NM_011720
	806R gcccgcgccttgacagcaggt			
<i>Wnt10a</i>	1005F aaagtcccctacgagagccc	52.3	1.0	NM_009518
	1184R cagcttcgacggaagctt			
<i>Wnt10b</i>	370F cggctgccgcaccacagcgc	59	1.0	U61970
	549R cagcttggtcttaagccggt			
<i>Wnt11</i>	496F gccatgaaggcctgccgtag	62.9	2.0	X70800
	648R gatggtgtgactgatgtgg			

^a Primer sequences for *Wnt-2*, *-3*, *-3a*, *-5a*, *-5b*, *-6*, *-7a*, *-8b*, *-10a*, *-10b* and *-11* were the same as those used by Lako et al (140). *mHPRT* (set 1) and (set 2) were used for *Wnt* gene expression analysis in chapter 3.0 and qPCR in chapter 5.0 respectively; *Wnt2b* (set1) was used for *Wnt* gene expression analysis in chapter 3.0; *Wnt2b* (set2) and (set3) were used for construction of *Wnt2b* constructs in chapter 5.0. Number prior to the sequence indicates location of the 1st nucleotide of the primer within a referred cDNA; underline indicates the restriction enzyme site; ■ indicates the boundaries of two adjacent exons. AT: annealing temperature; F: forward primer; Mg²⁺: magnesium; R: reverse primer; Reference: Genbank accession number or literature; ^b (161); ^c Designed by Dr. M. Furimsky, OHRI; ^d (88); ^e (171).

Appendix B. Number of embryos and pups delivered after pronuclear injection of the *Msx1mp-Wnt2bHA* construct^a

Transfer	Age of tissue	Live	Rabs/Dead pups	TG
1	E12.5	8	4	0/8
2	E14.5	4	2	0/4
3	P21	4	n/a	0/4
4	P21	4	n/a	0/4
5	E14.5	4	6	1/4 ^b
6	P2	2	1	0/2
7	P2	1	1	0/1

^a Genotyping was performed by PCR on live embryos and pups only. E: embryonic; n/a: not available; P: postnatal; Rabs: reabsorptions; TG: transgenic embryos or pups.

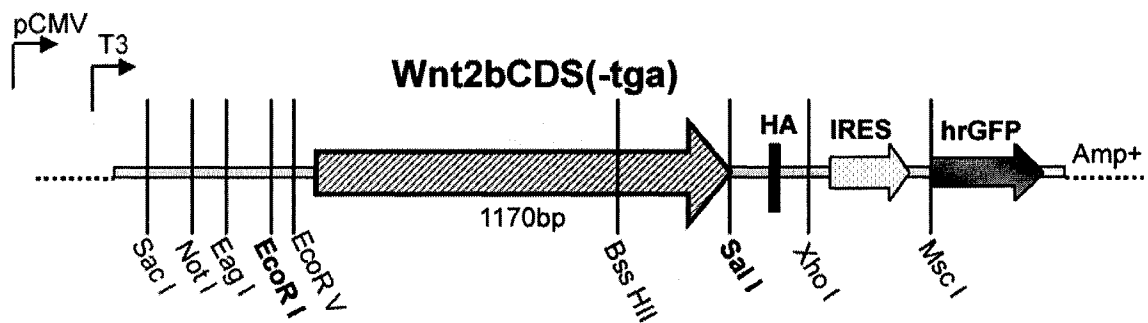
^b confirmed by Southern blot.

Appendix C. Summary of transcription factors and cellular markers examined to evaluate the effects of ectopic Wnt signaling on retinal patterning^a.

Gene name	Type	Expression	Reference
Brn3b	Pou domain TF	RGC	^h
Calbindin	Ca ²⁺ sensor protein	horizontal/amacrine/RGC	ⁱ
Calretinin	Ca ²⁺ sensor protein	amacrine cells/RGC	ⁱ
Chx10	HD TF	RPC/bipolar/ Müller glial cells	^{b c d}
Cralbp ⁺	retinoid binding protein	Müller glial cells	^t
Crx	HD TF	photoreceptor cells	^e
Hes1	bHLH repressor	retinal progenitors	^l
Hes5	bHLH repressor	retinal progenitors	^m
Math3	bHLH activator	bipolar/ differentiating amacrine cells	^{n q}
Notch1	transmembrane receptors	developing neural retina	^o
Otx1	HD TF	CM/ CE/iris epithelium/RPE	^f
Otx2	HD TF	photoreceptor cells	^p
Pax6	HD TF	RPC/ RGC/amacrine/horizontal	^g
PKC α	Kinase	rod bipolar cells	^{h, i}
		Subset of amacrine/photoreceptors	ⁱ
Prox1	HD TF	bipolar/amacrine/horizontal cells	^{r, c, s}
recoverin	Ca ²⁺ sensor protein	bipolar cells	^h
		rod and cone photoreceptors	ⁱ
rhodopsin	GPCR	rod outer segments	^j
syntaxin	synaptic protein	amacrine/horizontal	^k

^a bHLH, basic helix loop helix; CM, ciliary margin; GPCR, G-protein coupled receptor; HDTF, homeodomain transcription factor; RGC, retinal ganglion cell. ^b (150); ^c (13, 25); ^d (207); ^e (35, 68, 173); ^f (1, 162, 283); ^g (101, 195, 255); ^h (227); ⁱ (93); ^j (74, 213); ^k (5, 100, 233); ^l (91); ^m (91); ⁿ (91); ^o (9, 51, 147); ^p (178); ^q (241); ^r (3); ^s (60); ^t (24)

Appendix D. Plasmid map for Wnt2bHA/pHIT4 (6708bp)



TGA-removed full length Wnt2b cDNA is subcloned at the EcoR I & Sal I sites in frame with HA tag in pHIT4/IRES.hrGFP. Shown are known unique restriction enzyme sites. Vector is incomplete. CDS, coding sequence.

11.0 CURRICULUM VITAE

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EDUCATION

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Molecular cloning, *In situ* hybridization, Immunohistochemistry, Tissue culture,
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- 2001-2007 Graduate student (M.Sc. and Ph.D.), University of Ottawa
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- 2000-2001 Associate professor, Poultry science, Shandong Academy of Agricultural
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Molecular characterization of infectious bursal disease virus (IBDV) and
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- 1999-2000 Visiting scientist, Microbiology and Immunology, University of Ottawa
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- 1993-2000 Assistant professor, Poultry science, SAAS, P.R.China

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- 1995-1998 Graduate student (M.Sc.), Shandong University, P.R.China
Isolation and identification of IBDV field strains in Shandong province and the analysis of RE sites on VP2 gene
- 1988-1993 Junior researcher, Poultry science, SAAS, P.R.China
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HONOURS AND AWARDS

- 2005--Present Admission scholarship in science (Ph.D.), University of Ottawa
- 2003-2005 Excellence scholarship in science (Ph.D.), University of Ottawa
- 2003-2005 Graduate student award (Ph.D.), Foundation Fighting Blindness Canada
- 2004 First prize for presentation on the Annual Research Day, University of Ottawa Eye Institute
- 2003 Admission scholarship in science (Ph.D.), University of Ottawa
- 2001 Excellent research award, principal investigator, SAAS, P.R.China
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- 1999-2000 Visiting scientist fellowship, China Scholarship Council, P.R.China
- 1995 Science and technology prize 3rd level, researcher, SAAS, P.R.China
- 1995 Excellent paper of the year, Shandong Association of Husbandry and Veterinary Medicine, P.R.China

PUBLICATIONS

Peer-reviewed Papers

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Chapters in books

Wang SY, Wang BW, Chen KW, Ning ZH, Yang JM, Si JL, Li TS, Li BM, **Liu H** (1998)

Chinese Poultry Science. *Shandong Sci Tech Press*. ISBN7-5331-2150-3.

POSTER PRESENTATIONS AT MEETINGS

- 04/2002 **Liu H** and Wallace VA. Wnt gene expression in the murine retina. *Ottawa Health Research Institute 1st Anniversary Retreat Day Celebration Scientific Symposium*. Ottawa, ON.
- 05/2002 **Liu H** and Wallace VA. Wnt gene expression in the murine retina. *University of Ottawa Eye Institute 16th Annual Research Day*. Ottawa, ON.
- 03/2003 **Liu H**, Mohamed O, Dufort D and Wallace VA. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *23rd Great Lake Mammalian Development Meeting*. Toronto, ON.
- 11/2003 **Liu H**, Mohamed O, Dufort D and Wallace VA. The role of Wnt signaling in mammalian retinal development. *Ottawa Health Research Institute 3rd Annual Research Day*. Ottawa, ON.
- 11/2004 **Liu H**, Mohamed O, Dufort D and Wallace VA. The Wnt signaling and retinal development. *Ottawa Health Research Institute 4th Annual Research Day*. Ottawa, ON.
- 05/2005 **Liu H**, Mohamed O, Dufort D and Wallace VA. Wnt signaling is involved in retinal development. *University of Ottawa Eye Institute 19th Annual Research Day*. Ottawa, ON.
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- 05/2005 **Liu H**, Mohamed O, Dufort D and Wallace VA. The role of Wnt signaling in retinal development. *Association for research in vision and ophthalmology annual meeting*. Fort Lauderdale, FL.