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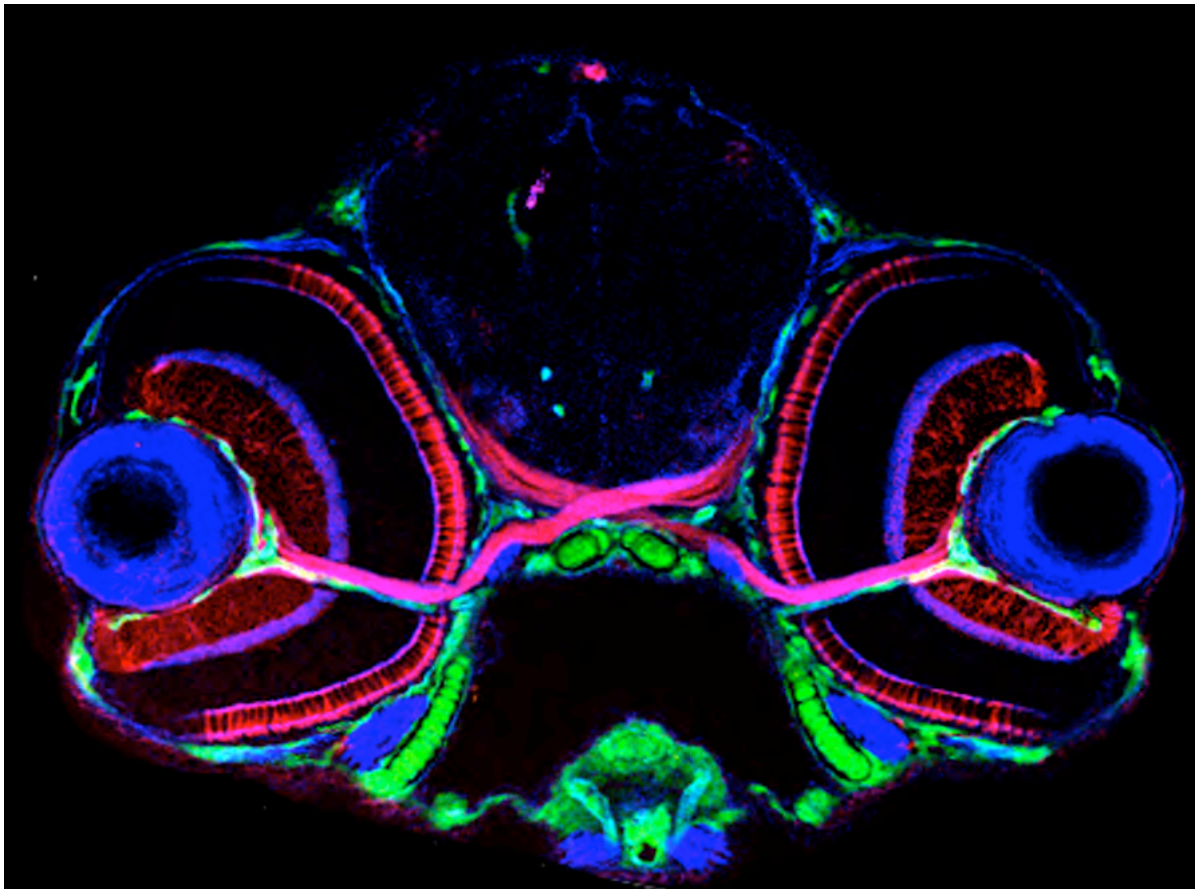
Teleost Retina – A model to study neurogenesis and angiogenesis

Satish Srinivas Kitambi



Stockholm 2009

Teleost Retina – A model to study neurogenesis and angiogenesis

by

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Stockholm 2009

Cover page photo: Lateral cryo section of zebrafish embryo (72hpf) immunostained to show photoreceptors, RGC and optic nerve (in red) and vasculature (in green).

Published by Karolinska Institutet.
PhD Thesis

ISBN number 978-91-7409-365-0

Abstract:

Teleost models, zebrafish and medaka have become popular models to study various aspects of developmental biology and genetics. The rapid embryonic development, transparent embryos and the availability of many mutants for various developmental and molecular pathways contribute to the usefulness of these models. The availability of various biochemical, molecular and genetic techniques applicable on these models facilitate in dissecting developmental processes.

Teleost retina shows very high similarity to that seen in mammalian retina. The arrangement of the six types of neurons and one type of glia is very similar. Zebrafish has been extensively used in gaining insight into the development and functioning of the retina. Medaka, on the other hand has not been so extensively capitalized as zebrafish. The current study characterizes expression of genes mainly from the nuclear receptor family and establishes the role of zebrafish *liver x receptor* in governing the size, patterning and neurogenesis of the retina in zebrafish. We also establish the time line of the retinal patterning of medaka retina. Zebrafish and medaka retina show both similarity and difference in the developmental events governing the patterning of the retina. In zebrafish, retinal neurogenesis follows a fan gradient pattern starting at the ventro-nasal region. In medaka, neurogenesis starts from the central retina. An additional, second domain of neurogenesis is seen with the patterning of photoreceptors in medaka. This observation highlights the possibility of utilizing these two species as comparative models in gaining rapid understanding of retinal development and function.

This study also establishes the time line of vascular development in the zebrafish retina, an important event required for normal function. Similar to neurogenesis, vasculature develops rapidly and this feature was utilized to develop a small molecule-screening assay. The screening resulted in identification of five compounds that produced phenotype ranging from decrease in the number of vessels to loss of vessels specifically in the retina. To gain insight into the mode of action, further analyses of three of the five identified compounds, using either morpholino knockdown or structural similarity search was done. This study highlights the advantage of using zebrafish model to perform medically relevant chemical screen.

List of publications and reports:

The list of papers included in this thesis:

1. **Satish Srinivas Kitambi**, Giselbert Hauptmann (2007). The zebrafish orphan nuclear receptor genes *nr2e1* and *nr2e3* are expressed in the developing eye and forebrain. Gene Expr. Patterns 7, 521-528.
2. **Satish Srinivas Kitambi**, Jarema Malicki (2008). Spatio-temporal Features of Neurogenesis in the Retina of Medaka, *Oryzias latipes*. Developmental Dynamics 237 (12), 3870-81.
3. **Satish Srinivas Kitambi**, Kyle McCulloch, Randall Peterson, Jarema Malicki (2009). Small molecule screen for compounds that affect vascular development in the zebrafish retina. Mechanisms of Development (In press).
4. **Satish Srinivas Kitambi**, Amena Archer, Stefan L Hallgren, Håkan K Olsén, Jan-Åke Gustafsson, Agneta Mode (2009). The role of liver X receptor (*lxr)* in the developing eye. (Manuscript).

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Abbreviations

CNS	Central nervous system
cv	Choroid vessel
DBD	DNA binding domain
EST	Expressed sequenced tag
GCL	Ganglion cell layer
GD	Gestation days
ha	Hyaloid artery
hv	Hyaloid vessel
hpf	Hours post fertilization
idp	Inner deeper plexus
IHC	Immuno histochemistry
INL	Inner nuclear layer
ISH	In situ hybridization
PNS	Peripheral nervous system
pp	primary plexus
Mb	Mega bases
MYA	Million years ago
NR	Neural retina
NRs	Nuclear receptors
OC	Optic cup
odp	Outer deeper plexus
ONL	Outer nuclear layer
ORF	Open reading frame
OV	Optic vesicle
L	Lens
LBD	Ligand binding domain
RGC	Retinal ganglion cell
RPE	Retinal pigmented epithelium
rv	Retinal vasculature

Databases

http://www.ncbi.nlm.nih.gov/
http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7955
http://www.ensembl.org/index.html
http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg
http://www.grs.nig.ac.jp/medaka/genome/blast.jsp
http://www.sanger.ac.uk/

1. INTRODUCTION:

1.1 The vertebrate eye and its early development

Nature controlled evolutionary processes, that shape complex structures/processes into existence can be readily seen in the visual system. Light has influenced the development of sensory processes in all living organisms. In plants, Chlorophyll is the primary light-sensing moiety and light directs the growth of stem towards it and the roots away from it [1]. In animals, light has been an important selective force in the development and function of eyes. Invertebrate eyes display a wide variety in number, location and function [2,3], and vertebrates have paired chambered eyes, inverted retinal layers and image is formed by refraction of light by the lens and cornea [4].

The eye has evolved, from a primitive organ sensing the intensity and direction of light into a highly specialized sensory structure with different wavelength sensitivity and image contrast [4-7]. The vertebrate eye is an excellent example of the structural and functional complexity of an organ. During vertebrate embryonic development, the eye is specified as the eyefield, a centrally located region in the prospective forebrain [8-10]. The growth of forebrain moves the eyefield forward to form the optic groove. The optic groove induces the formation of lens placode on the ectodermal surface upon contact [10]. The lens placode formed on the surface ectoderm, invaginates and pinches off to form the lens vesicle; this process also creates the optic cup. The 'organizer of the lens' describes this interaction of the optic vesicle and the surface ectoderm [10,11]. The surface ectoderm overlying the lens vesicle forms the cornea and the optic cup gives rise to the neural retina (NR- inner layer of optic cup) and the retinal pigment epithelium (RPE – outer layer of optic cup). The outer lip of the optic cup, where the neural retina and RPE meet, gives rise to iris and ciliary body [10,11].

The vertebrate lens placode formation coincides with the expression of crystalline protein. This protein is preferentially expressed in the lens and is required for maintaining transparency of the lens vesicle [12,13]. The lens vesicle shows polarity, this can be readily seen in the formation of lens fibers. The primary fiber cell mass is formed by the posterior part of the lens, whereas the secondary fibers are formed at the transitional zone to the anterior cells which are proliferating cells [14,15]. Differentiation of the lens fibers is characterized by the onset of crystalline expression and cell elongation with loss of organelles. The coordinated process of invagination of lens placode and optic vesicle establishes the first shape of the eye [14-16]. The

ventral portion of the optic vesicle forms a groove due to the invagination process. This groove runs from the neural retina to the junction of the neural tube and provides a channel for the bloodvessel to enter the retina and the axons to exit the retina. Lateral edges of the groove near the optic vesicle fuse to form the choriod fissure [10,16].

The entire process of eye development is a tightly regulated process (refer figure 1), with genes involved in BMP [17,18] and Wnt signaling [19] pathways, *Pax6* [20-23], play important role in its early development. Loss of function of *Pax6* in mice produces ocular defects [24-26]. In humans, loss of function or missense mutation in *PAX6* is characterized by defects in iris, cornea, lens and hypoplasia of the retina and iris [27-29]. In addition, expression of genes like *RX, SOX2, SIX3, OPTX2* are required for proper patterning of the eye and retina and their loss in humans are characterized by the microophthalmia or anophthalmia and holoprosencephaly [29-32]. The expression of *OTX1*, a homeodomain containing protein, regulates the development of iris and ciliary body and loss of expression results in the absence or malformation of iris and ciliary body [33].

1.2 Early retina exhibits polarity

The developing optic vesicle exhibits a defined axis and specific gene expression pattern. For example, the RPE forms from the dorsal region of the optic vesicle and the retina from the ventral region. As the eye develops, the RPE moves ventrally to cover the entire retina [10,11]. The dorsal region of the optic vesicle in mice exclusively expresses *Mitf*, and loss of function in mice results in the RPE transdifferentiation into the neural retina [34,35]. Expression of *Chx10* is seen only in the presumptive retina and regulates the proliferation of cell in the retina. Mutation of *Chx10* in mice leads to reduction of cell proliferation in the early retina [36].

The early neural retina consists simply of neuro-epithelial cells, however they show a well-defined dorso-ventral and anterior-posterior polarity. The retinoic acid (RA) signaling process plays an important role in specifying the ventral polarity of the retina and closure of the optic fissure. The developmental importance of RA signaling is well documented in loss of function studies of the RA receptors (RAR's) and the retinoid X receptors (RXR's), members of the nuclear receptor family [37,38]. Mice *Rxrα-/-* results in coloboma of the optic nerve and reduction of the ventral retina, and

Rar and *Rxr* double knockout mice show bilateral eversion of the ventral retina and the absence of ventral iris [37,38]. In addition to RA signaling, other genes display restricted expression domains along the dorso-ventral axis. For example *Pax2, Vax2, EphB2, EphB3* are expressed in the early ventral half of the retina and *Xbr-1, Gdf6, Tbx-1, EphrinB2, EphrinB3* are restricted to the dorsal half of the retina. The polarity of the early neural retina is very important for the cell fate determination. Restricted expression of Vax2, Tbx5 and Ephrin signaling has been shown to be necessary for proper retino-tectal patterning of the axons of the retinal ganglion cells. Similar to the anterior-posterior patterning, neural retina also shows dorso-ventral patterning. This is readily seen with the expression of *Foxg1, SOHo1* in the anterior region of the retina and *Foxd1* in the posterior half of the retina [11,39,40].

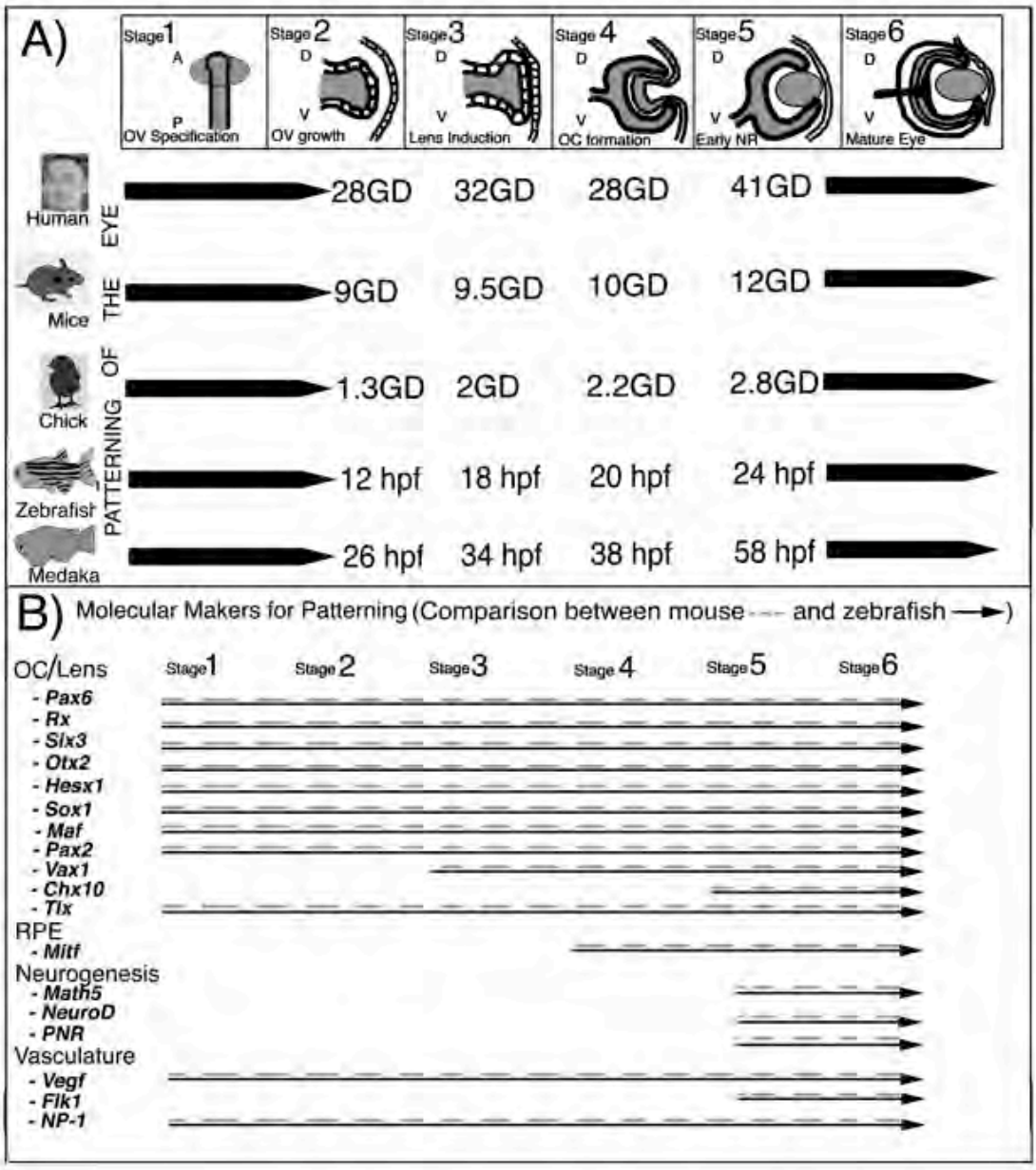

Figure 1. Early eye development in different species. A) Represents early eye development, classified into six stages (Stage 1 to 6). The approximate time required by human, mice and chick to reach the stage are represented by gestation days (GD) and zebrafish, medaka are shown in hours post fertilization (hpf). B) Shows the comparison of the expression time line of various eye markers from zebrafish (black arrow) and mouse (grey broken lines). The following abbreviations are used in the figure; anterior (A), posterior (P), dorsal (D), ventral (V), optic vesicle (OV), optic cup (OC), neural retina (NR), retinal pigment epithelium (RPE).

1.3 Patterning and neurogenesis of retina

Early embryonic retina is simply a neuroepithelial layer made up of progenitor cells. The progenitor cells proliferate and expand in cell number, this event is necessary to achieve the right size and proportion of different cell types [10,41]. The neural epithelial progenitor cells undergo patterning to generate six different types of neurons and one type of glia arranged in three laminar structures. The retinal ganglion cells (RGC) and displaced amacrine cells in the ganglion cell layer (GCL); biopolar, amacrine, horizontal and muller glia cells in the inner nuclear layer (INL); rod and cone photoreceptor cells in the outer nuclear layer (ONL) [42-44]. The cell proliferation, neuronal development and gliogenesis are regulated by multiple transcription factors, mostly from the bHLH factors [44]. For example, in mice, *Tll*, *Hes1* and *Hes5* act to maintain the progenitor cells in undifferentiated state and play an important role in maintaining an adequate supply of retinal progenitors [45-47]. Then, the expression of neural bHLH genes like *Math5, Math3, NeuroD* in mice, regulate retinal cell fate specification and neurogenesis. The retinal cell fate specification and neurogenesis are highly regulated process governing the proper time, position and number of different type of neurons in the retina. In mice, from E10.5 onwards, the retinal progenitors generate neurons, with the retinal ganglion cells (RGC) appearing first and the muller glia cells appearing last. The order of appearance of retinal cell types is conversed across many species with difference in time. The proper patterning of the retina is very important for the normal function of the visual system, defective regulation, as seen in many human abnormalities, at any stage of development produces severe alterations to the development and function of the visual system [13,44,48-50].

1.4 Vasculature of the eye

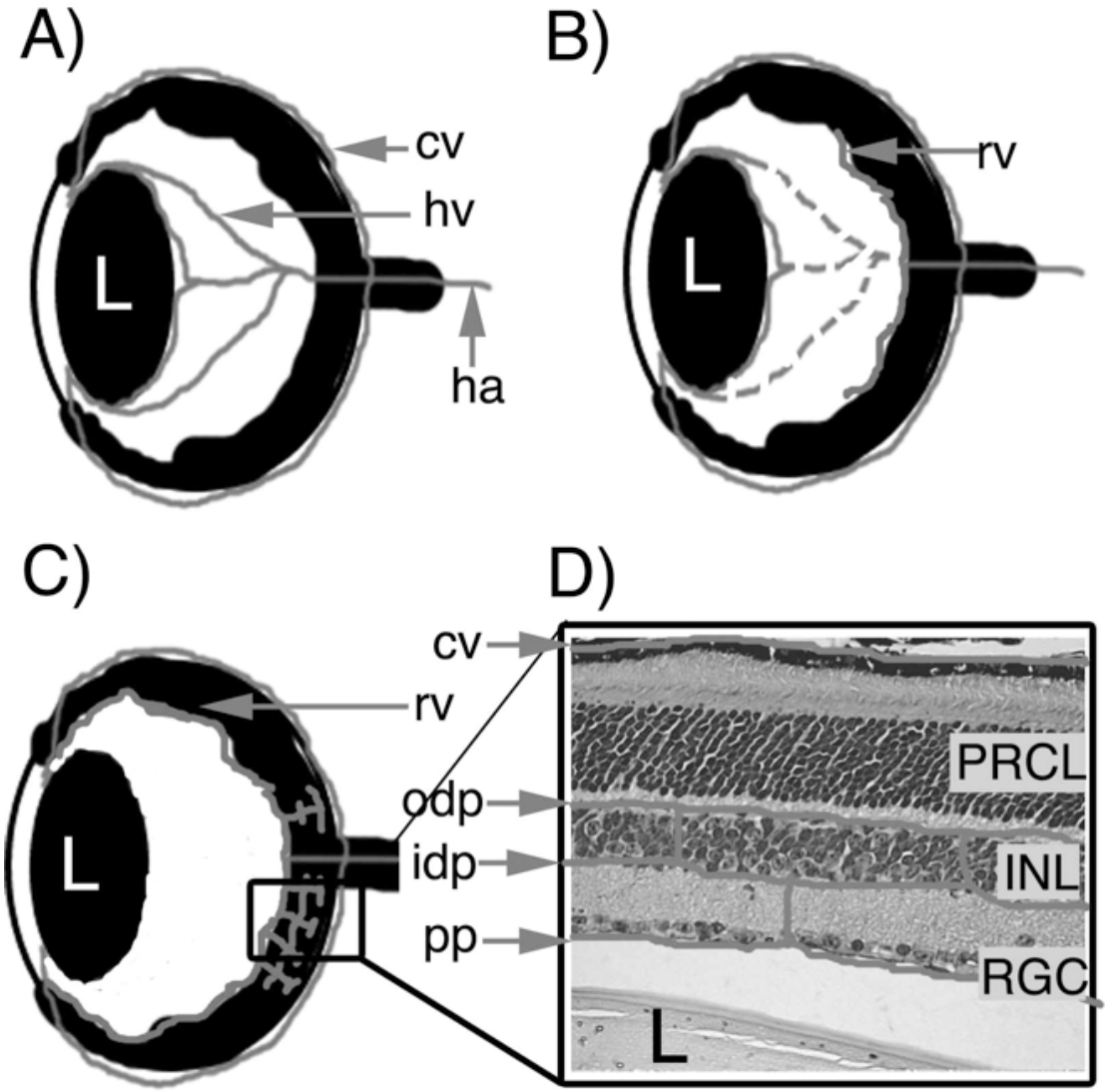

Figure 2: Development and remodeling of the vasculature of the eye. A) The hyaloid (hv) and choroids vasculature (cv) are the initial vasculature systems seen in the developing eye. Blood comes into the eye via hyaloid artery (ha). B) Hyaloid vasculature regresses and is replaced by retinal vasculature (rv). C) The retinal vasculature (rv) completely replaces the hyaloid vessels, and form deeper plexus inside the retinal layers. D) The primary plexus (pp) of the retinal vasculature forms the inner (idp) and outer (odp) deep plexus. Lens is abbreviated as L. The figure is adapted and modified from the article, Angiogenesis (2007) 10:77-88.

Embryonic vascular development occurs basically via two processes, vasculogenesis and angiogenesis. The process in which the embryonic haemangioblast cells cluster to form a tube like structure is termed vasculogenesis. Endodermal derived tissues like lungs, pancreas, heart, and dorsal arota are vascularized via this process. Aniogenesis is a process where new vessels sprout from preexisting vessels. This type of process is seen in structures like brain, retina, and kidney [51,52]. Angiogenesis also plays a role

in different pathological conditions like proliferative diabetic retinopathy and wound healing. Vasculature is one of the earliest organs formed and plays a major role in supplying oxygen and nutrient during development [51,52]. Many signaling pathways, and Vegf in particular play an important role in vasculature development. The primitive vascular network in the developing embryo is actively remodeled. This remodeling involves growth of new vessels, regulation of the vessel diameter and regression of vessels. Remodeling of the vasculature results in the formation of mature network. Proper formation and regulation of vasculature is very important for the survival of the embryo as defects can cause lethality. For example, mice deficient in tie2 show severe defects in vasculature and is lethal, and loss of angiopoietin2 in mice cause defects in vessel sprouting and regression [51-54].

Similar to other tissues of the body, oxygen and nutrients to the mammalian eye are supplied via three different vasculature systems (refer Figure 2). They are hyaloid, choroid and retinal vasculature. While choroid and retinal vasculatures are the main systems in the adult retina, the developing retina initially receives oxygen and nutrients via hyaloid vasculature. The hyaloid vasculature is a transient vasculature system and is later replaced by the mature retinal vasculature [51-53,55].

The hyaloid artery enters the optic vesicle through the choroids fissure and reaches the posterior lens. It then forms a network around the lens, called the tunica vasculosa lentis (TVL). The TVL network grows and reaches the anterior part of the lens and forms the pupillary membrane (PM). This completes the development of the hyaloid vascular system (HVS). This system is made up of only arteries and the HVS system joins the choriodal system to drain blood. The HVS system supplies oxygen and nutrients to the early retina and once formed completely, it undergoes a process called regression. By the process of regression, the HVS vessels are removed and are replaced by retinal vasculature. Failure of the HVS to regress is associated with cataract, retinal detachment and retinal hemorrhage in humans. The failures of HVS regression in humans are collectively called persistent fetal vasculature [51,54]. In mice, knockout of Lrp5, Frizzled-4, Arf, Ang2 or BMP-4 results in persistent fetal vessels [51].

Retinal vasculature system supplies oxygen and nutrient to the adult retina. It is divided into two components, the superficial primary and the deep secondary vessel system. The primary vessels penetrate into the retina and branch along the inner and outer surface of the inner nuclear layer of the retina. Retinal pathologies, like

proliferative diabetic retinopathy, venous occlusion and retinopathy of prematurity (ROP) are characterized by rapid vessel proliferation, thereby severely affecting proper visual function [51-53,55].

The mesoderm surrounding the early optic vesicle gives rise to the choroidal vasculature (CV). This vascular network surrounds the outer part of the eye. The development, expansion around the eye and density closely follows the spreading of pigmentation of the retinal pigment epithelium. As the CV grows to cover the eye, the anterior part of the network joins to form a circular annular vessel. The growth of CV depends on inductive signals from the differentiated RPE cells. Inductive signals like FGF and VEGF from the RPE layer play an important role in the formation of CV. In human coloboma conditions with defects in RPE, CV is severely affected. In mice over expression of dominant negative FGF or absence of VEGF also causes defects in the formation of CV. In humans, defective CV or choroidal neovascularization is commonly seen in pathological conditions like age-related macular degeneration (AMD) and diabetes leading to progressive loss of vision [56].

The three vascular systems play an important role in proper development, survival and functioning of the retina. Abnormal functioning of one or several vascular systems is seen in various human pathological conditions, indicating its functional importance [51-53,55].

1.5 Nuclear receptors

Nuclear receptors (NRs) constitute a metazoan specific family of transcription factors that regulate gene expression in response to various extracellular or intracellular signals [57-60]. Upon binding of ligands, the NRs undergo a conformational change, which enables binding to various cofactors and *cis*-regulatory sequences called nuclear receptor response elements (NRE's) thereby modulating gene expression [57,58]. These factors play an important role in development, reproduction, metabolism and homeostasis and are characterized by the presence of highly conserved structural domains. The domain structure includes, from N-terminus to C-terminus, the A/B modulatory domain, the DNA-binding domain (DBD), hinge domain region D, the ligand binding domain (LBD) and a highly variable C-terminal F-binding domain [58,59,61]. The conservation of the domains and the sequence similarity has led to classification of this superfamily into seven sub-families

(numbered from 0 – 6) [58,59]. The evolutionary diversity of this family can be readily seen in the number of receptors seen in different species. Over 270 NRs have been identified in the nematode worm *Caenorhabditis elegans* [62], but only 48 NRs in human, 49 in *Mus musculus* and 21 NRs in *Drosophila melanogaster* have been reported so far [63,64]. Apart from the presence of multiple subfamilies and the evolutionary diversity, the NRs also show diversity in preference for ligands, which may range from endogenous ligands such as steroid hormones, thyroid hormones, leukotrienes, postaglandins, fatty acids and exogenous compounds like drugs, environmental pollutants. Some of the NRs donot have identified ligands and are therefore collectively called 'nuclear orphan receptors' [57-59]. The regulation of various important biological processes by NRs has made them preferred candidates for small molecule drug design to control functions associated with major diseases [65-67].

1.6 LXRs and cholesterol regulation

Cholesterol and its derivatives, such as steroids and metabolites, act as ligands for a numbers of NRs. These NRs includes receptors for fatty acids (PPARs), bile acids (CAR, FXR, PXR) and oxysterols (LXRs) and play important role in cellular and whole body cholesterol homeostasis [68]. Oxysterols are formed from oxidized cholesterol and, their amounts are proportional to the cholesterol content in a cell. Oxysterols are ligands of Liver X receptors (LXRs) and therefore LXRs act as important cellular cholesterol sensors [69,70]. LXRs form permissive heterodimers with retinoid X receptors and, upon ligand binding stimutate the expression of target genes that regulate cholesterol homeostasis. LXRs operate either by: promoting the conversion of biliary cholesterol into bile acid excretion, or inhibiting the intestinal cholesterol absorption, or activating the excretion of biliary cholesterol or bile acid, or promoting reverse cholesterol transport [69-71]. The main role of LXRs is the regulation of cholesterol metabolism, however it also plays a role in inflammation, autoimmune reaction, insulin sensitivity and artherosclerosis. Many currently used drugs like thazolidinedione derivatives, fibrates and statin also modulate the function of LXRs and, changes in its expression levels are also seen in many diseases [72-76].

1.7 Teleost models

In recent years teleost models, zebrafish and medaka have become popular models to study various aspects of developmental biology and genetics. The rapid external development, transparency of embryos, housing many fish together and the possibility of obtaining large number of offsprings are advantages readily offered by these models [77-80].

The zebrafish, *Danio rerio*, has been more extensively used than medaka. Zebrafish shows a high degree of anatomical and physiological homology to that of other high order vertebrates and has highly similar cellular structure, signaling processes, and cognitive behavior. Sensory systems like vision, olfaction, taste, touch, balance and hearing are amongst the homologies [81,82].

The medaka, *Oryzias latipes* is very similar to zebrafish in its anatomy and physiology. Medaka and zebrafish evolutionary lineages have been separated for around 110MYA, this separation offers a very good opportunity to uncover conserved and divergent pathways guiding various developmental and metabolic processes [77-80]. The collection of various mutant lines and development of various biochemical, molecular and genetic techniques facilitates studying developmental biology, wound healing, metabolism and physiology. Teleost models are also gaining popularity for toxicity and efficacy screening of chemicals, pharmaceuticals and pesticides which can be correlated in terms of human health risks [78,83-86].

Retina of the zebrafish and medaka displays very high similarity to that of mammalian retina. Similar to other vertebrates, the teleost retina is made up of six different types of neurons and one type of glia cells. The retinal ganglion cells (RGC) are the first neurons to be generated followed by other classes of neurons. The similarity to other vertebrates, rapid development of the retina and the availability of many mutants showing defects in development and functioning of the retina make these models valuable for elucidating underlying genetic pathways in retina development [77,78,87-89].

2. AIM:

The study design of this thesis work was to primarily increase the understanding of the process of retinal neurogenesis in two different teleost models, zebrafish and medaka and to establish small molecule screening assay tool using zebrafish retina.

The specific aims of this study were –

a. To understand the pattern of neurogenesis in zebrafish retina by characterizing the expression of nuclear receptor genes (zebrafish *tll, pnr, lxr*).
b. To characterize the retinal lamination and neurogenesis in medaka.
c. To understand the role of zebrafish *liver x receptor* in retinal development.
d. To establish the pattern and timeline of retinal angiogenesis in zebrafish.
e. To develop small molecule screening procedures using zebrafish retina.
f. To identify and characterize small molecules that show specific effect on retinal vasculature.

3. METHODS:

Certain methodological techniques are central to this thesis work, and hence a short description of these techniques may enable a better understanding of the described work.

In situ hybridization (ISH) was used extensively to characterize the spatio-temporal expression pattern of various genes during this study (**Paper I, II, III**). This is a widely used and an efficient technique to detect specific DNA or RNA sequences in whole embryo or tissue section, by using labeled complementary DNA or RNA. The complementary DNA or RNA can be labeled with either fluorescent or non-fluorescent markers. In this study we used digoxygenin labeled complementary RNA (simply called probes), which was in turn detected using anti-digoxygenin antibody linked with alkaline phosphatase. This alkaline phosphatase on the antibody facilitates the detection of the antigen (digoxygenin) and antibody (anti-digoxygenin) complex by using substrate like BM purple or BCIP-NBT or fast red to produce a color reaction. Multiple transcripts can be detected using this procedure (**Paper I**). Already established protocols were used to characterize the expression pattern of various genes in the whole embryo. We have developed a modified procedure for ISH on sections (**Paper II**).

Immunohistochemistry (IHC) is a frequently used technique to localize proteins in a cell of a tissue section or whole embryo. This procedure explores the principle of using antibody to detect a protein, to which it specifically binds. The detection of the complex can be done using fluorescent or non-fluorescent methods. IHC with antibodies specifically targeting different retinal neurons were used in this study (**Paper II, III**).

Small molecule screening tool. Small molecules (either drug candidates, plant extracts, metabolites, xenobiotic, toxic compounds or pollutants) can be easily evaluated using the zebrafish model. The rapid external development, small size and early transparent development and the possibility to get few hundreds of embryos from a single pair have greatly added to the use of zebrafish as a vertebrate screening tool. The availability of transgenic fish also contributes to the usefulness of this model. We have utilized the fli1:EGFP transgenic line, which express EGFP in all endothelial cells, to identify chemicals that specifically effect the retinal vasculature (**Paper IV**). The procedure involved adding the chemical to the fish water and

administering it to the developing embryos kept in 96 well plate. The EGFP signal provides an efficient readout, which was observed using a fluorescent microscope. Although such procedures have been used before to screen for chemicals affecting the trunk vasculature and cardio vasculature development, the procedure for screening chemicals affecting retinal vasculature was first described in this study.

Morpholino Knockdown (MO) is a very efficient technique employed to produce a transient knockdown of the gene of interest in zebrafish. Morpholinos are nucleases resistant modified oligo nucleotides, which can be designed to block splicing of nuclear RNA into mRNA or to block initiation of transcription of the gene towards which the morpholino is directed. The efficiency of knockdown can be analyzed by PCR or western blotting techniques aimed at detecting the mRNA or protein of the gene of interest. Using this technique, we have knocked down the developmental expression of zebrafish *lxr* (**Paper IV**) and *ace* (**Paper III**). As the embryo grows, the concentration of morpholinos decreases thereby the translation of the gene of interest is gradually restored. This is a good method to produce knockdown and study the role of the gene during embryonic development. In our experiments with zebrafish *lxr* (**Paper IV**), we used morphlinos and blocked the Lxr protein synthesis, the specificity of the knockdown was demonstrated by the possibility of rescuing the phenotype by injection of synthetic *lxr* mRNA. We also used this technique to test the structure function relationship of Enalapril maleate, a chemical compound identified during the small molecule screening process (**Paper III**). Enalapril maleate is a FDA approved drug that causes dilation of blood vessel by inhibiting *angiotensin-converting enzyme (ace)*. To further investigate the mode of action of this compound, we performed a morpholino knockdown of *ace*, which was verified using PCR (**Paper III**).

4. RESULTS AND DISCUSSION:

To understand the process of neurogenesis and vasculature in the retina, we used two teleost models, zebrafish and medaka. Both models are widely used to understand various developmental/genetic and metabolic processes.

Paper I characterizes the spatio-temporal expression pattern of two orphan nuclear receptors in zebrafish, the orphan nuclear receptor family genes *nr2e1* and *nr2e3*, which are highly related to each other. Using bioinformatics tools, the zebrafish genome database was searched to identify and assemble zebrafish *nr2e1* and *nr2e3* gene sequence. The sequence information was used to scan the EST database to identify possible hits corresponding to *nr2e1* and *nr2e3*. The EST database scan resulted in identification of one EST corresponding to *nr2e1* and two ESTs corresponding to *nr2e3*. The full sequence of *nr2e1* EST showed and open reading frame of 1191 nucleotides (nt), 417nt 5'UTR and 149nt 3'UTR. A full sequence analysis of nr2e3 ESTs revealed that the open reading frame was made up of 1260nt. The putative protein products obtained by translation of *nr2e1* and *nr2e3* genes showed high similarity in the DBD and LBD region with orthologs from different species. Whole-mount *insitu* hybridization and RT-PCR were used to characterize the expression profile of these two genes.

Zebrafish *nr2e1* was expressed from bud stage of development as a transverse stripe in the rostral brain rudiment. The expression lies anterior to the posterior midbrain as shown with markers like *pax2.1* and *eng2*. Around 10 somite stage the expression was seen in the optic primordium and the forebrain region. The zebrafish optic primordium appears around 7 somite stage and the optic cup is formed around 15 somite stage. The expression of *nr2e1* increased in the optic primordium by 15 and 20 somite stage. The presumptive neural retina at this stage is made up of mitotically active progenitor cells. At 20 somite stage the expression was found in the anterior brain and in olfactory placodes. Expression in olfactory placode was also seen at 24hpf and was weakly detected at 36hpf. Strong expression was seen in the tectum, telencephalon, hypothalamus, dorsal thalamus and pretechtum at 1dpf. Expression was seen through out the retina at this stage, which was decreased and seen to be concentrated as a ring of cells around the lens by 48hpf. This region corresponds to the mitotically active germinal zone of the retina.

Expression profiling by RT-PCR indicated that there was no maternal or gastrulation stage expression of *nr2e1*. Expression was detected in the 5 somite stage sample and continued to be expressed till 5dpf. In the adult zebrafish tissue the expression was detected in brain and eye. The expression pattern of the zebrafish *nr2e1* corresponded to the area of expression of *nr2e1* in other species.

Zebrafish *nr2e3* expression was first detected at 7-somite stage embryo in the epiphysis region. The expression in the epiphysis preceded the retinal expression. The development and differentiation of the epiphysis is completed before the retinal neurogenesis, this was also seen with the expression pattern of the photoreceptor specific nuclear receptor (*pnr* or *nr2e3*). The epiphysis primarily consists of two types of neurons, the photoreceptors and projection neurons. The epiphysial expression of *nr2e3* was seen in the region corresponding to the photoreceptor cells by 48hpf. In the retina, expression of *nr2e3* was seen at the ventronasal region in 25hpf old zebrafish embryos. The expression followed the retinal neurogenic wave of expression, where the early retina progenitors exit mitosis at the ventro nasal region first, then spreading from the ventronasal region to dorso-temporal regions. The expression of *nr2e3* precedes the appearance of photoreceptors in the retina. This is similar to that seen in other species. By 48hpf the expression was restricted to the entire photoreceptor cell layer of the retina.

The expression pattern of both *nr2e1* and *nr2e3* seem to be similar to that seen in other species, indicating a conserved role of these two receptors through evolution.

Paper II describes the pattern of lamination and neurogenesis in the retina of the medaka fish. The availability of closely related species provides us with an interesting opportunity to look into the conservation of the process of neurogenesis and patterning of the retina. Medaka and zebrafish have around 110MYA (million years ago) of evolutionary separation and the entire teleost species have undergone whole genome duplication around 400MYA. Loss or redundancy of expression or gain of function of duplicated genes follows this genome duplication. By genome database search for POU class genes and nuclear receptors, we have earlier demonstrated that the loss of genes is not always similar in zebrafish and medaka (refer licentiate thesis). We have demonstrated differential expression of duplicated genes in zebrafish (refer licentiate thesis work). As a first step to exploit the two closely related species in elucidating the molecular pathways/processes, we decided to study the retinal

patterning of medaka. Through this work we highlight the benefits of using two teleost models in parallel to understand the developmental processes governing retinal patterning.

Medaka retina shows similarity to zebrafish retina in the arrangement of neurons in the retinal lamina and many antibodies directed against zebrafish proteins cross-react with corresponding medaka proteins. However several interesting differences are also apparent, the most obvious is the duration of development. Medaka eye development and development in general takes a longer time than in zebrafish. The longer development process can be advantageous, as it would allows proper characterization of the onset of gene expression or mutant phenotype. This is very useful when characterizing developmental pathways. The longer duration of development was obvious when the development of photoreceptors was studied using Zpr1 antibody and opsin RNA probe. In addition to the longer duration of development, medaka also exhibited differences in photoreceptor neurogenesis pattern. Unlike zebrafish, where neurogenesis starts in the ventro nasal region, medaka photoreceptor neurogenesis was found to start at two independent locations in the retina. The first domain of expression was seen in the central retina which moved dorsally first and then ventrally. The second domain, independent of the first domain of expression, was seen in the ventral retina. The pattern of the second domain of expression is very similar to that seen in zebrafish and goldfish and may be evolutionary reminiscence. In addition to the presence two domains of origin, medaka retina also showed a different arrangement of photoreceptors in the retina than that seen in zebrafish. The photoreceptor cells in medaka, as shown by IHC and electron microscopy are arranged in a square mosaic pattern. This is different to zebrafish, where the photoreceptors are arranged in a row mosaic pattern. The data presented in this study paves a way for further analysis of retinal development using both the teleost models in parallel. The similarity and differences in retinal development and the availability of many retinal mutants will be very useful in understanding the eye (retina) development from evolutionary, molecular and functional contexts.

Paper III characterizes the development of retinal vasculature in zebrafish and uses small molecule screening methodology to identify and characterize chemicals that affect retinal vasculature. Similar to other vertebrates, zebrafish retina is vascularized via angiogenesis, however the early events of the development of retinal vasculature

have not been described. Through this work we show that around 24hpf, the endothelial cells enter the retina through the choroids fissure and move below the posterior region of the lens. The endothelial vessels formed an extensive vascular network around the lens by 48hpf and within the next 24hrs, moved from the posterior part to the anterior part of the lens to covering it entirely. The network of vessels around the lens was termed as intraocular vasculature; the anterior region of this intraocular vasculature forms a ring vessel that opens into the surface vasculature. During the period of the formation of the intraocular vasculature, two other vasculature systems of the eye also develop, the surface and the choriod vasculature. At 28hpf, a single vessel was seen to develop opposite to the choriod fissure, this was the beginning of the surface vasculature. By 48hpf, two additional vessels form on the surface of the eye and they were named as, nasal, dorsal and ventral vessels. The three vessels fuse at the posterior end to form another ring vessel. Blood enters through the nasal and exits via the dorsal and ventral vessel. The choroid vasculature was not extensively followed, but by 9dpf, endothelial vessels formed polygonal structures and covered the entire eye. The intraocular vasculature undergoes dynamic remodeling and by 30dpf, completely detaches from the lens and falls on top of the retina.

The vascular patterning of the retina is a dynamic process and depends on the close coordination of various other process and signaling events involved in the patterning of the retinal neuroepithelial sheet. Mutations affecting the neuronal patterning and/or polarity also affect the vascular development of the retina as shown in Figure 3. For example, defects in sonic hedgehog signaling are known to affect the neurogenesis and neuronal patterning of the retina. A defect of this signaling event also has an effect on the vasculature development, as seen in smoothend mutant (*smo*) [90]. In the smoothend mutant, endothelial cells enter the retina via the choroids fissure and spread around the lens, but proper vessel formation was absent. The endothelial cells instead aggregated and spread around the lens. In oko meduzy mutation [89], which affects the polarity and neuronal patterning of the neuroepithelium, the endothelial cells failed to form vessels and also failed to cover the lens. In some cases, the endothelial cells invade the retina. Another retinal patterning mutant, *out of sight* [89] showed a very loose formation of intraocular vessels around the lens.

The rapid development of retinal vasculature and the small size of the embryo offer a good opportunity to characterize the vascular development using small molecule

screen or to screen for medically relevant chemicals. We developed a pharmacological assay methodology to manipulate vasculature and identify medically relevant compounds. Through this assay procedure, we identified five compounds that produced retinal vasculature specific phenotypes. Out of the five compounds, only one produced visible side effect on the retinal lamina. The compounds, Enalapril maleate, Pyrogallin, Albendazole, Mebendazole and Zearalenone produced phenotype ranging from widening of vessels to loss of vessels in the retina. Enalapril maleate is an ACE (angiotensing converting enzyme) inhibitor, to elucidate whether the effect produced by enalapril maleate was via ACE inhibition, morpholino knockdown against ACE was done. The knockdown did not produce the same effect indicating either independent mechanism of action of enalapril maleate or insufficient inhibition. Albendazole and mebendazole show structural similarity, to characterize the structure-function relationship, a database search was performed and five more compounds structurally similar to albendazole and mebendazole were identified. These structurally similar compounds showed a similar phenotype in exposed embryos to that produced by albendazole and mebendazole indicating a conserved structure-function relationship.

Through this work we have established zebrafish retina as a model to perform pharmacological screen to identify and characterize medically relevant compounds.

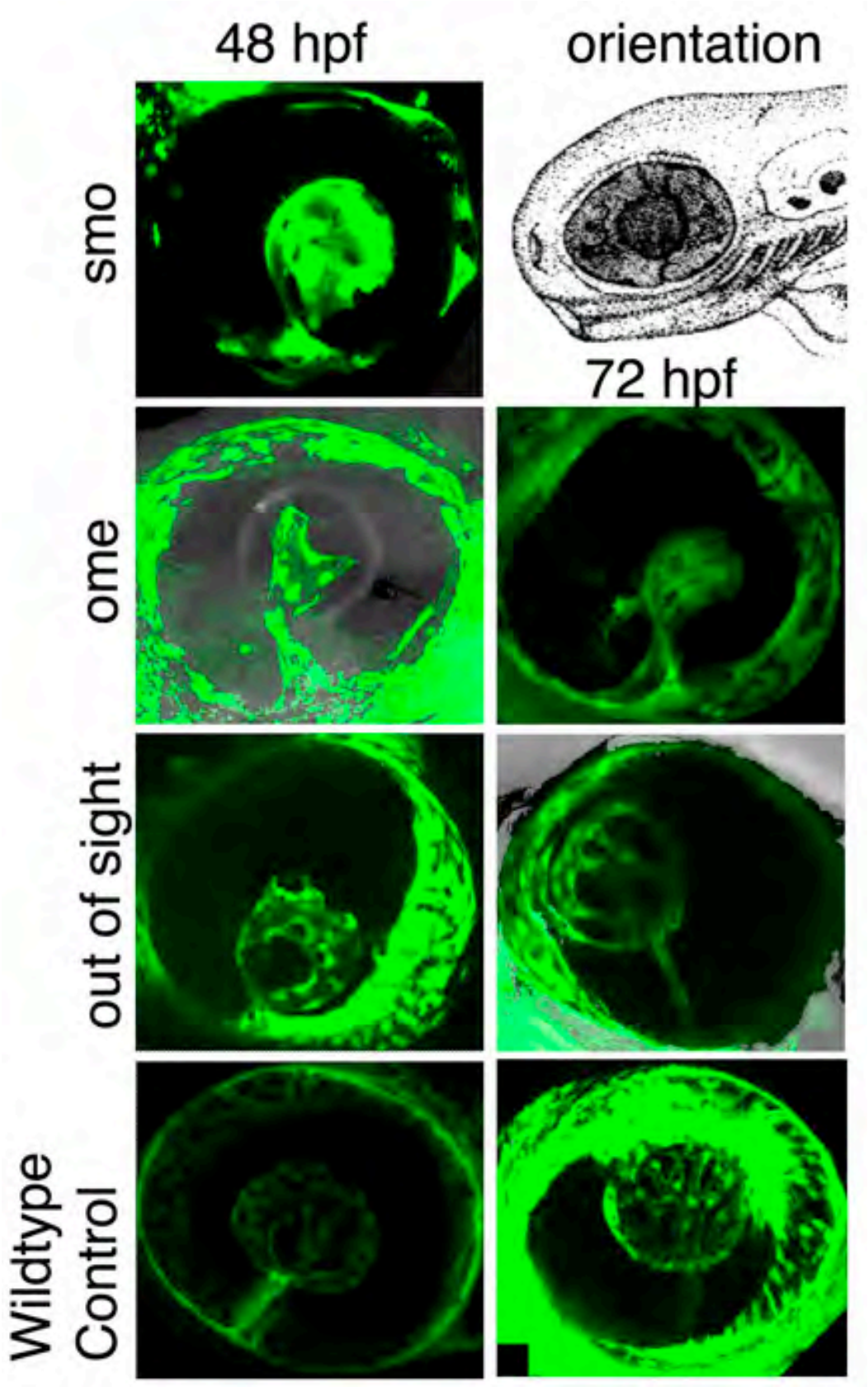

Figure 3: Retinal vasculature of different mutant zebrafish. Embryos are oriented laterally to view the eye and vessels behind the lens are imaged. Images panels shows the wildtype control vasculature (bottom panel) and zebrafish mutant lines, *smoothend (smo)*, *oko meduzy (ome)* and *out of sight* at 48 hpf and 72 hpf stage of development.

Paper IV represents the study of the expression of zebrafish *liver X receptor* during zebrafish development and characterization its functional implications using morpholino knockdown. The *liver X receptors* act as cholesterol sensors and have established role as regulators of lipid metabolism and reverse cholesterol transport. There are two liver X receptors in mammals, (LXRα and LXRβ), in zebrafish, only one *lxr* has been identified. The expression pattern of this receptor was characterized by ISH. The zebrafish *lxr* is maternally expressed and was detected in the entire optic promordium. As the embryo develops, around 55hpf, the expression became more localized in different layers of the retina, mainly to the RGC layer and optic nerve. By 72hpf the expression was seen to be exclusively in the RGC layer and the RPE layer of the retina and was continuously detected in these regions till 120hpf (last stage analyzed). Similar expression dynamics was seen in the developing brain, the expression was seen in the entire brain primordium and as the development proceeded, got localized to clusters of cell in the brain. The expression pattern indicates an early developmental role for zebrafish *lxr*. To further understand the role of *lxr*, we performed a morpholino (MO) knockdown. The MO-injected embryos (morphants) did develop normally, the optic promordium, lens and the early neural retina formed normally. However neurogenesis of the retina was not initiated. This absence of neurogenesis was evident with absence of *nr2e3* transcript from the retina and the failure of detection of different retinal neurons using antibodies such as Zpr1, Zn8, GABA. In addition to the absence of neurogenesis, the size of the retina and the brain were dramatically reduced. The embryo was also characterized by increase in cell death and decrease in lipid content. The absence of neurogenesis was retina specific as neurogenesis were seen in the brain. The MO knockdown of zebrafish *lxr* indicated its involvement in the regulation of proper eye/brain size, neurogenesis and laminar patterning of the retina.

5. CONCLUSIONS AND FUTURE DIRECTION:

This study has shown both similarities and differences in patterning of the retina in zebrafish and medaka fish. Neurogenesis in zebrafish follows a fan gradient of patterning that begins at the ventronasal region of the retina and proceeds dorso-temporally to cover the entire retina. In medaka, this process starts at the central retina, proceeds dorsally first and then ventrally to cover the entire retina. In the case of photoreceptors neurogenesis in medaka, in addition to the central to peripheral pattern, a second pattern appears at the ventral retina. This second domain is likely to be a reminiscent of the pattern seen in zebrafish.

In addition to the difference in patterning, there is also difference in the time taken for the development of the eye and retina, which takes longer time in Medaka than in zebrafish. This study highlights the advantage of using these two closely related species as comparative models in elucidating various developmental and disease processes. The availability to the genome sequence from zebrafish, medaka and another closely related species fugu, provides the possibility of generating genomic models that can be compared to understand gene function and regulation.

The rapid embryonic development and the availability of very similar molecular, biochemical and genetic techniques offer a very good possibility to quickly characterize the function of a gene in development. For example, in this study we have performed morpholino knockdown of nuclear receptor *liver x receptor (lxr)* and analyzed the phenotype produced (Paper IV). The zebrafish *lxr* plays an important role in retinal patterning and neurogenesis and its absence is characterized by absences of retinal neurons and lack of patterning of the retina. However the molecular and biochemical pathways that are affected should be further studied.

The rapid development of zebrafish retina was also capitalized in characterizing the retinal angiogenesis. Zebrafish retinal vasculature shows both similarities and differences to the mammalian vasculature. The hyaloid regression is incomplete and there is no intra retinal vasculature. The availability of many mutants showing retinal vasculature defects (figure 3) allows a rapid elucidation of the molecular processes involved in proper vascular patterning.

In this study we have also shown the possibility of using the rapid embryonic development as a tool to perform small molecule screens on the retina. Further studies

have to be performed to characterize their function and medical relevance of the molecule identified in this study.

Small molecule/chemical screening using zebrafish is getting very popular. However there is a need to develop assay/screening strategies. The identified small molecules coupled with biochemical and molecular methods in zebrafish would provide a platform to address various developmental and disease processes.

6. ACKNOWLEDGEMENTS:

The doctorate study at KI has been a very rigorous experience for me. Through this process I have realized two very important things, first is that, I enjoy science, and the second most important thing is that I really cannot control events, but I can choose how to respond to them.

I would like to express my sincere gratitude to Dr. Lotta Hambraeus and Södertörns Högskola for their support. I am very impressed with Lotta's hard working nature and leadership style and I hope to interact with leaders like her in future.

My co-supervisor, Dr. Jarema Malicki, Harvard Medical School, for allowing me to explore and enjoy science under his aegis. I have seen very few people who are so intelligent, dedicated and hard working like him. Working with him has taught me to enjoy science.

Professors Agneta Mode, my supervisor and Jan-Åke Gustafsson, Karolinska Institutet, for helping me complete my graduate studies on time. They offer lot of intellectual freedom and I had a very productive and constructive interaction with them.

My co-supervisor, Dr. Per Kylsten, Södertörns Högskola for his timely help and support. My progress would have failed if he had not stepped in at the right time.

My co-supervisor and mentor, Drs. Thomas Burglin and Arindam Majumdar, Karolinska Institutet for support and constructive advice during my graduate studies.

Drs. Lennart Nilsson, Karolinska Institutet and Catarina Ludwig, Prefect of Södertörns Högskola, Maria Lundin, and Anna Lehmusto, Kerstin Beckenius, Silsa Hellborn and Ann Fredriksson for their timely help during my studies.

Drs. Randall Peterson, Michaud Norman, William Sewell, Zhiqiang Chen, Francesca Pignoni at Harvard/MGH for support and help with my experiments. I am very amazed with Norm's knowledge, experience and patience in teaching and handling students.

My buddies Abraham Behre, Temesgen Fafu and Magnus Crona for their constant support and encouragement.

My colleagues at Harvard Med/MEEI. – Drs. Yoshihiro Omori, Chengtian Zhao, Xiaotang Jing, Yan Jiang, Tianyi, Zhiyan, Qingziang, Xiao-Hong Wen and buddies Janisha Biyanwila, Kristina Bellanger, Kevin Kotredes, Andria Schiler, Andreas Warkentine, Sam Kumar for their constant support and encouragement.

My buddies Alia, Stefan Hällgren, Marianna, Mickael Durand-Dubief, Isobel, Ekatherine, Carolina, Abe, Helmi, Marianna, Yuntao, Monica, Julian, Fredrick, Anthony (chounan), Anna, Rebecca, Rachel, Azadeh, Heidi, Indranil, Petra, Emma, Martin, Prasad, Ida, Ingela, Parthena, Oskar, Wessam, Asim, Micheal, Maria, Andreas, Inderpreet, Ramesh, Elancheziyan for their support and creating a good work environment.

Dr. Monika Andersson Lendahl, Kent Ivarssen, Lilijana Obernovic from the Novum Zebrafish Facility for their help and support.

Administration of Södertörns, Marie Bollmark, Marie Granroth, Sam, Johan, Todd, Anders, Asim for their participation in helping me perform my duties.

JAG/AMO group members, and Leu, Wei, Maria Bondesson and Administration of BioNut, Ingwar Lennerfors, Kristina Bergholm, Monica Ahlberg, Marie Frazen, Rikard, Lena Magnell.

Mr. Joseph Chen and Cori Obrien administration of Harvard Med/MEEI for their prompt help.

To my family, especially my Mom and Gayathri for constantly supporting me throughout this process.

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