Forkhead Genes in Mammalian Development

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"Nature cannot be tricked or cheated. She will give up to you the object of your struggles only after you have paid her price."
-Napoleon Hill

To my parents

Forkhead Genes in Mammalian Development

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Abstract

This thesis concerns aspects of Forkhead gene biology and it's relation to mammalian development. Genes from three subclasses are discussed, *Foxj3*, *Foxf1* and *f2*, and *Foxe3*.

We have identified and characterized a novel forkhead gene, *FoxJ3*, that is expressed in neuroectoderm, neural crest and mytome, suggesting possible function in the nervous system and muscle. The myotome, which will develop into muscle, along with the mesenchyme lining the intestinal gut, originates from embryonic mesoderm.

Forkhead factors, *Foxf1* and *Foxf2*, are expressed in intestinal mesenchyme derived from splanchnic mesoderm. Foxf function is important for patterning of the gut tube. Removal of Foxf results in a range of intestinal phenotypes, such as agangliosis and megacolon. Both *Foxf1* and *Foxf2* are regulated by hedgehog signaling, Foxf mutants display mesenchymal increase in *Wnt5a* expression, and reduction in *Bmp4* expression. The extracellular matrix is depleted of collagens, and together with altered paracrine factors, this leads to a phenotype where epithelial cells lose polarization and become resistant to apoptosis.

The ocular lens develops from the head ectoderm and a critical factor in its formation is Foxe3. *Foxe3* is, after secondary fiber differentiation starts, expressed exclusively in the lens epithelium. These cells provide the precursors for lens fibers. Fiber cells are elongated, terminally differentiated cells that provide the specialized optical properties of the lens. Ectopic expression of *Foxe3* in the fiber compartment interferes with several aspects of fiber differentiation. The cytoskeletal remodeling and organelle degradation is blocked in transgenic lenses, whereas fiber cell specific expression of crystallins seems to be undisturbed.

Foxe3 is also involved in patterning of the anterior segment of eye. Heterozygous *Foxe3* mutants show defects in differentiation of the cornea, iris and filtration angle. The anterior segment similarities in *Foxe3* and *Pax6* heterozygous mutants provide, along with *Foxe3* expression being dependent on *Pax6* gene dosage, an indication that *Foxe3* is a major contributor to the phenotype of *Pax6* mutants.

Keywords: Foxj3, Foxf1, Foxf2, Foxe3, development, lens, forkhead, transcription, intestine, anterior segment, Bmp, Wnt.

Papers discussed

Paper I

Landgren H, Carlsson P

FoxJ3, a novel mammalian forkhead gene expressed in neuroectoderm, neural crest and myotome.

Dev Dyn. 2004 Oct;231(2):396-401

Paper II

Ormestad M, Astorga J, **Landgren H**, Wang T, Johansson BR, Miura N, Carlsson P

Murine Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extra cellular matrix production.

Development. 2006 Mar;133(5):833-43

Paper III

Blixt Å, **Landgren H**, Johansson BR, Carlsson P

Foxe3 is required for morphogenesis and differentiation of the anterior segment of the eye and is sensitive to Pax6 gene dosage.

Dev Biol. 2007 Feb 1;302(1):218-29

Paper IV

Landgren H, Blixt Å, Carlsson P

Persistent FoxE3 expression block cytoskeletal remodeling and organelle degradation during lens fiber differentiation.

Invest Ophthalmol Vis Sci. 2008 Oct;49(10):4269-77

Paper V

Landgren H, Carlsson P

Foxe3 expression and protein degradation is regulated by growth factor signaling.

Manuscript

Contents

Introduction to the forkhead family

In order to achieve the complexity of an adult human, the fertilized zygote must go through a most stunning development. The human body consists of around 260 different cell types and the behavior and function of these cells are all encoded in our DNA.

Precise regulation of gene expression is crucial for division of labor between tissues in the multicellular organism. Differential transcription of the around 20000 genes in our body is what determines the final appearance and function of all cells and tissues. This is achieved largely through transcription factors, proteins that interact with DNA and contribute to the spatial and temporal control of transcription by binding to proximal (promoter) and distal (enhancer) elements of genes. Transcription factors are involved in all aspects of differentiation decisions and are responsible for controlling which of the 20 000 genes that are active in a given tissue, ultimately giving rise to such different structures such as bone and blood.

One class of transcription factors is the forkhead family (reviewed in Carlsson and Mahlapuu, 2002; Wijchers et al., 2006; Kaufmann and Knöchel, 1996). The name forkhead stems from a Drosophila mutant identified in 1989 that displayed replacement of both fore- and hindgut with ectopic spike-formed structures (Weigel et al., 1989). The protein responsible for that mutant, named Fork Head after the appearance of the phenotype, turned out to be one of many that contains a highly conserved DNA binding domain (Lai et al., 1993). The DNA binding domain, now called forkhead domain or forkhead box, also serves as the basis for classification of this group of proteins, which are present in animals and fungi, but not in plants or protists. Interestingly, the number of forkhead genes in a given species seems to correlate with increased anatomical complexity, starting with four genes in Saccharomyces cereviciae to around 40 in mammals (Katoh and Katoh, 2004). As the forkhead gene family evolved, gene duplication events were followed by divergence through amino acid substitutions (Fetterman et al., 2008). This led to a situation where forkhead genes today show a high degree of homology within their DNA binding domains between subclasses, but display an almost complete lack of homology in the N- or C-terminal domains outside of the forkhead box.

In 1998 a classification system was proposed for this growing family of proteins and the name Fox (Forkhead box), analogous to Hox for homeobox genes, was adopted (Kaestner et al., 2000). The Fox family is divided into subclasses designated by a letter from A to S, and subclass members by number, e.g. FoxE3. The notation follows the standard for each species, e g uppercase for human (FOXE3), all but first lowercase for mouse (Foxe3) and the first and subclass letters uppercase for all other chordates (FoxE3). The classification into subfamilies is based on sequence comparisons of the DNA binding domain and can be found at http://biology.pomona.edu/fox/

The Forkhead domain

The forkhead DNA binding domain consists of 110 amino acids and the structure of a number of proteins have been solved (Clark et al., 1993; Stroud et al., 2006). The forkhead domain fold is a variant of the helix turn helix motif with three α-helixes, three ß-sheets and two loops, or wings, canonically arranged α1,ß1, α2, α3,ß2,w1,ß3,w2, although variants of this organization exist (van Dongen et al., 2000). The two loops resemble the wings of a butterfly; hence, the structure is also called the "winged helix domain" (Clark et al., 1993). However, there are other, evolutionarily unrelated, proteins that share a similar "winged-helix" fold, based on unrelated primary structures (Gajiwala et al., 2000). The fold of the forkhead domain has a striking similarity to that of linker histone H1 and H5. This inspired the hypothesis that part of the forkhead functionality is as a chromatin modifier (Clark et al., 1993)(Cirillo and Zaret, 1999). For example, are FoxA proteins able to open up chromatin structure independently of SWI/SNF and do so most likely through interactions with core histones H3 and H4 (Cirillo et al., 2002).

Forkhead proteins bind DNA primarily through helix 3, the so-called recognition helix (Clark et al., 1993). Helix 3 is also the most highly conserved part of the forkhead domain. Due to the high sequence homology within the recognition helix, most forkhead proteins share a common core target DNA sequence represented by the seven-nucleotide motif RYMAAYA (Overdier et al., 1994; Pierrou et al., 1994). This motif occurs rather frequently in DNA, and additional sequence specificity is determined by amino acids from the carboxy-terminal end of the forkhead domain, which interact with nucleotides flanking the seven-nucleotide core (Overdier et al., 1994; Pierrou et al., 1994). Unlike the majority of transcription factors, forkhead proteins generally bind DNA as monomers and the binding induces a sharp bend in the DNA. Exceptions exist and are members of the FoxP subfamily, which require dimerization for DNA binding to occur (Li et al., 2004). The transactivation properties have been extensively studied and forkhead proteins have been shown to act both as transcriptional repressors and activators (see for example (Freyaldenhoven et al., 1997; Hellqvist et al., 1998)).

The biological role of forkhead proteins

Forkhead proteins are involved in many different processes including control of metabolism and developmental patterning and differentiation. This variety will be illustrated by a few examples.

The C. elegans forkhead protein Daf-16 transduce the worm's insulin-like signaling and thereby controls life span, energy storage and dauer formation (Lin et al., 1997; Ogg et al., 1997). This pathway is highly conserved and the mammalian Daf-16 orthologs, the FoxO proteins (FoxO1, FoxO3, FoxO4 and FoxO6), are ubiquitously expressed and at the crossroads of cellular pathways such as insulin/IGF signaling and apoptosis. FoxO proteins can influence the cell cycle by different

mechanisms; deacetylation decreases expression of p27, thereby inhibiting entry into S-phase (Motta et al., 2004; Medema et al., 2000) and FoxO proteins can also promote G2/M transition (Alvarez et al., 2001).

FoxO proteins are involved in mediating responses that protect against oxidative stress, one of the contributors to cellular aging (Kops et al., 2002; Partridge and Brüning, 2008). They regulate metabolism by maintaining gluconeogenic enzymes in the liver and by governing the expression of genes important for stress response (reviewed in (Gross et al., 2008)). Members of the FoxA family influence glucose and insulin homeostasis, apart from their role in early development (reviewed in Friedman and Kaestner, 2006). FoxC2 regulates energy expenditure in adipose tissue; overexpression of *FoxC2* leads to a shift in the balance between brown and white adipose tissue, which results in increased oxygen consumption and reduction in total body fat stores (Xue et al., 2008; Cederberg et al., 2001).

Forkhead gene expression and function is very diverse and there are differences between family members in terms of expression patterns and function. The excellent reviews mentioned in the introduction will provide the reader with extensive in-depth insights into forkhead gene biology.

Early embryology and Foxj3 expression pattern

Introduction

Starting from a fertilized egg cell, it takes millions of cell divisions to arrive at a mature organism. An organism must ensure that the products of these divisions adopt the correct fate. As described in the previous section, this process of development is regulated largely through differences in gene expression profiles between mother and daughter cells put in effect through communication events between cells or groups of cells. All mammalian organs are developed from three early cell populations, called germ layers, which apart from giving rise to the embryo proper also contribute to extraembryonic tissue such as yolk sac. The germ layers and some derived structures are; the ectoderm, that will give rise to the outer epithelium of the body and all neural tissue, such as brain and nerves; the mesoderm, that will develop into bone, muscle, blood, heart etc.; and the endoderm, which will give rise to the primitive gut and it's associated organs, lungs, liver, pancreas etc. Many transcription factors are important for specifying these fates as described in more detail in the following sections of this thesis, and our understanding of these cell and tissue patterning processes often start with acquiring spatial and temporal information of gene expression.

Expression of *Foxj3*

In paper I we describe the expression pattern and genomic description of a hitherto unknown Forkhead gene. It was identified in a whole genome scan in the wake of the genome sequencing projects. The novel forkhead gene, named *FoxJ3*, showed highest sequence similarity to *FoxJ2*. Bioinformatic data mining revealed 13 exons spanning ca 100kb on mouse chromosome 4. Expression analysis using Whole mount in situ hybridization showed that *Foxj3* is expressed at least from embryonic day (E) 8.5 to E12.5. Early expression was found in the neural tube at E8.5 and this expression remains at later stages. Starting at E10.5, *Foxj3* is seen in migrating neural crest cells. Expression persists in neural crest derived structures, such as the eye,mandibular and maxillar components of the first branchial arch, and facio-acoustic and dorsal root ganglia. A segmented pattern with a more ventral localization than dorsal root ganglia appear at E10.5. These expression domains are similar to markers of myotome development and likely represent precursors of myocytes.

Foxj3 show high sequence homology to Foxj2, but share no similarity with Foxj1 outside the forkhead box. Clearly, these genes should constitute separate subclasses. Hence, the functional diversity within FoxJ subclass is large. *FoxJ1* are important for cilia formation and left-right asymmetry (Tamakoshi et al., 2006) while *FoxJ2* are involved in preimplantation processes, and over-expression leads to embryonic arrest with pre-implantation and heart defects (Martín-de-Lara et al., 2008).

The expression pattern of *Foxj3* suggests function in skeletal muscle, and peripheral and central nervous system.

Development of the gastrointestinal tract

Introduction

The gastrointestinal tract is a tube lined by an epithelium specialized in nutrient uptake. It consists of endodermal and mesodermal cells and is shaped during development by extensive paracrine signaling between the two cell populations.

During ontogeny the gut can be divided into three distinct parts, fore- mid- and hindgut. This division follows the different anteroposterior expression domains of Homeobox genes emanating from both endoderm and mesoderm (reviewed in (Beck, 2002)). The foregut will later give rise to esophagus and stomach as well as trachea, lungs, thyroid, liver and pancreas. The mid- and hindgut give rise to small and large intestine respectively. There are also other signaling system important for anteroposterior patterning, for instance does over-activation of Wnt signaling in lung epithelial cells shift them towards an intestinal fate (Okubo and Hogan, 2004).

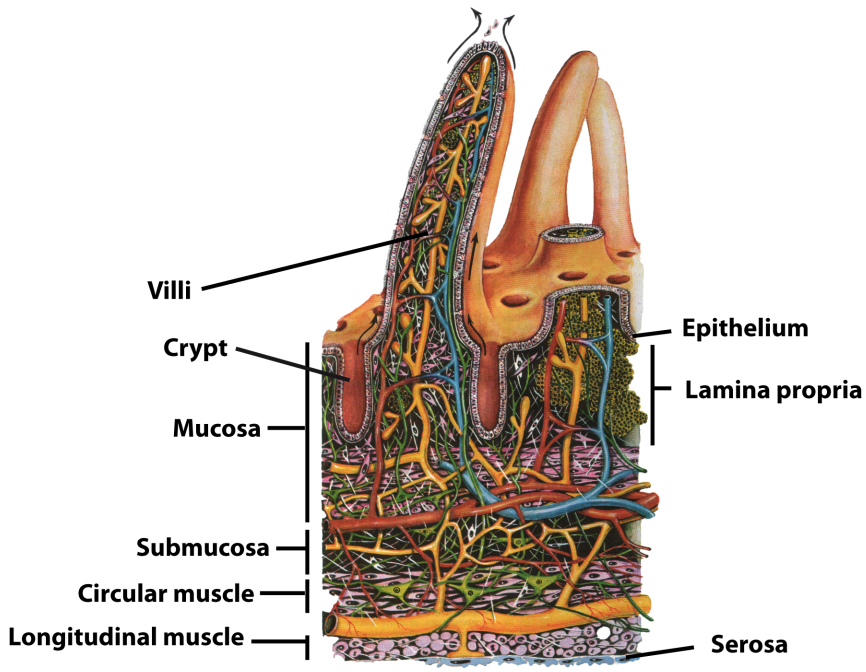

Fig 1. Section of the mature intestinal wall and its many components. Notice the arrows from crypts to the villus top. This is the path of an epithelial cell takes from birth until it is shed into the gut lumen. Image taken from Gray's anatomy: The Anatomical Basis of Medicine & Surgery.

The basic structure of the developing gut consists of endodermally derived epithelium facing the intestinal lumen and mesenchyme derived from the splanchnic part of the lateral plate mesoderm. The mesenchyme will, as development proceeds, become subdivided into the innermost mucosal layer, the lamina propria; the submucosa consisting of connective tissue; two layers of muscle cells of longitudinal and circular organization; and the serosa comprising mesothelial cells and their basal membrane.

In order to achieve this organization, extensive signaling between different cell populations is required, particularly epithelial-mesenchymal crosstalk. Many different paracrine factors and their signaling network components are present in the gut. Hedgehog proteins are morphogens involved in many developmental processes (reviewed in Ingham and McMahon, 2001). Mouse has three hedgehog proteins Desert (DHH), Sonic (SHH) and Indian hedgehog (IHH). They transduce their signal through Patched and Smoothened trans-membrane proteins and Gli family transcription factors. Two hedgehogs, *SHH* and *IHH*, are expressed in the gut endoderm and are important for patterning along the anteroposterior and radial axes. *SHH* and *IHH* mutants have reduction in intestinal smooth muscle, gut malrotation, abnormal innervation similar to Hirschsprung's disease and imperforate anus (Ramalho-Santos et al., 2000). Ectopic expression of *SHH* in the hindgut altered expression of *BMP4* and *Hox* genes (Roberts et al., 1998), adding further evidence for the notion that hedgehog signaling is important for gut patterning. Wnt signaling is important for control of proliferation in the intestinal epithelium. The Wnt pathway maintains stem/progenitor cells by promoting cell cycle progression and inhibiting differentiation. BMP signals, on the other hand, have an opposite effect by controlling stem cell number in the crypts of the intestinal villi (Wnt and BMP signaling is reviewed in Scoville et al., 2008).

The mesenchymal and epithelial cells together produce the extracellular matrix (ECM) that make up the basement membrane for the epithelial cells. It is rich in collagens, laminins and proteoglycans such as perlecan and entactin/nidogen. The basement membrane not only provides physical support, induces polarization and acts as a survival signal for the epithelium, but also influences the efficiency of paracrine signaling, for example by binding growth factors and paracrine signal molecules (Wang et al., 2008b).

FoxF genes and gut development

Foxf1 and *Foxf2* are two closely related forkhead genes expressed in various tissues during embryonic development (Ormestad et al., 2004), e g the splanchnic mesoderm that surrounds the embryonic gastrointestinal tract. *Foxf1* is important for development of organs derived from the foregut, such as lungs, trachea and esophagus (Mahlapuu et al., 2001). *Foxf2* null mutants are born with cleft palate and die at birth (Wang et al., 2003).

In paper II we show that both *Foxf1* and *Foxf2* are important for the development and patterning of the embryonic gut tube. *Foxf2* $^{-/-}$ animals have a dilated thin-walled distal colon that occasionally ends in a blind sac (intestinal atresia). Compound heterozygotes, *Foxf1*$^{-/+}$;*Foxf2*$^{-/+}$, display a gut phenotype similar to that of *Foxf2*$^{-/-}$ animals. Hence, they will collectively be referred to as *Foxf* mutants. Human congenital megacolon is caused by a lack of innervation of the distal colon (Carrasquillo et al., 2002). *Foxf* mutants have a similar loss of enteric neurons in the colon. The smooth muscle cells in the thin, disorganized intestinal wall were poorly differentiated. This phenotype has similarities with the hedgehog mutants (Ramalho-Santos et al., 2000). *Foxf* genes are mesenchymal targets of epithelial hedgehog signaling in the foregut (Mahlapuu et al., 2001) and in the lateral mesoderm of early embryos (Astorga and Carlsson, 2007) and inhibition of *Foxf* expression by the smoothened inhibitor cyclopamine in embryonic intestinal explants, verified that *Foxf1* and *-f2* are downstream of hedgehog also in the gut.

Hedgehog has been shown to activate expression of *BMP4* in the intestine (Roberts et al., 1995) and *BMP4* is a target for *Foxf1* in the early extraembryonic and lateral mesoderm (Astorga and Carlsson, 2007). We therefore investigated *BMP4* expression in *Foxf* mutants and found a significant reduction of *BMP4* expression in the intestine, whereas tissues where *Foxf* proteins are not expressed did not show any changes in *BMP4* levels. Intestinal smooth muscle differentiation is inhibited by BMP4 (De Santa Barbara et al. 2005; Sukegawa et al., 2000) and in *Foxf* mutants expression of smooth muscle actin expanded into the villus core.

Foxf mutants had poor adhesion between cell layers throughout the intestine, due to a deficiency in ECM production. The ECM is rich in collagens, laminins and heparan sulphate proteoglycans. Immunostaining for collagen I and IV showed a dramatic reduction in all parts of the gut. Furthermore, a dominant-negative Foxf2-GFP fusion protein completely abrogated collagen production and secretion in both primary E18.5 gut fibroblasts and 3T3 cells, which indicates that the ECM deficiency is due to a cell autonomous requirement for normal *Foxf* gene dosage in the mesenchymal cells.

Anchorage to the ECM of the basement membrane triggers integrin signaling in the epithelial cells, which is necessary for survival and induces cell polarity. When epithelial cells lose contact with the ECM they undergo apoptosis (called anoikis when epithelial cells die as a result of losing contact with their substrate) and this happens normally when epithelial cells born in the crypts reach the top of the villus at the end of their migration. Due to ECM deficiency, intestinal epithelial cells become depolarized, with a rounded shape and mis-localization to apical and basal membranes of the adherens junction components, E-cadherin and ß-catenin. The apical exposure of junctional proteins led to adhesion between villi, and in the most severe cases a complete occlusion of the gut lumen. In spite of the overt depolarization, the epithelial cells failed to induce anoikis, which indicated that they were partly resistant to apoptosis.

The epithelium of the gut is continuously renewed through proliferation of progenitor cells in the villus crypts. At E18.5, staining with a proliferation marker such as PCNA showed intense proliferation in the intervillus pockets, which are the embryonic predecessors of the crypts, and only post-mitotic cells in the villi. In *Foxf* mutants, this distinct border had dissolved and actively cycling cells were present along the entire villus axis. Ectopic proliferation and resistance to apoptosis suggested abnormal activation of the Wnt pathway. Elevated Wnt signaling was confirmed by showing that ß-catenin, a Wnt signal transducer, was localized in the nuclei of epithelial cells throughout the villus of *Foxf* mutants. The source of the increased Wnt signaling was identified as overexpression of *Wnt5a*. Bmp4 was shown to inhibit *Wnt5a* expression, thereby establishing a link from SHH/IHH (from epithelium), via Foxf to BMP4 and Wnt5a (all in the mesenchyme), to control of degradation of ß-catenin (in the epithelium).

A prediction from this model is that inactivation of *Foxf* alleles due to mutations would increase the frequency with which intestinal adenomas are formed, which should in turn lead to an elevated risk of developing intestinal carcinoma. This is in line with a larger body of evidence showing the importance of stromal cells for tumor initiation and progression (illustrated by Yauch et al., 2008).

Foxe3 and lens development

Introduction

The lens is the organ that, together with the cornea, gathers and focuses light onto the retina. The retina converts the light to nerve impulses which, when interpreted by the brain, results in vision. The main focus of this chapter is the development of the anterior segment of the eye in general and the lens in particular. The lens is a highly specialized organ that has some characteristics that separates it from other tissue. It is simple in its organization with an anterior epithelial layer that is proliferative and provides progenitors for fiber cells. Mature fiber cells are the end-points of differentiation and are elongated, rich in lens-specific proteins, and devoid of cellular organelles. The lens is avascular and relies on oxygen and nutrients from the aqueous humour that bathes it's anterior side. A common cause of impaired vision is lens opacity, presenting clinically as cataract.

Lens formation

The first event of eye development, at E8.0, is outgrowth from the forebrain of the optic vesicle (OV). The lens develops from a thickening in the head surface ectoderm, the lens placode, at E9.0-E9.5 (the steps of lens induction is thoroughly reviewed in (Donner et al., 2006)). The thickening occurs soon after contact is made with the optic vesicle (OV). The lens placode invaginates together with the outer part of the optic vesicle to form the lens pit and the optic cup respectively. This process occurs differently in different species. In mice lens specification precedes the contact of OV with the lens competent surface ectoderm. There is a specific need for the OV until the 23-somite stage, and in culture, lens formation occurs without OV after this stage (Furuta and Hogan, 1998). The optic cup will later form the neural retina and pigmented retinal epithelium.

As the lens pit deepens the connection with the surface ectoderm narrows, forming the lens stalk. The lens stalk degenerates, possibly through apoptosis, around E11.5 (Ozeki et al., 2001), and the lens vesicle, a hollow sphere, is thereby pinched off from the surface ectoderm. The posterior half of the lens vesicle thickens and the

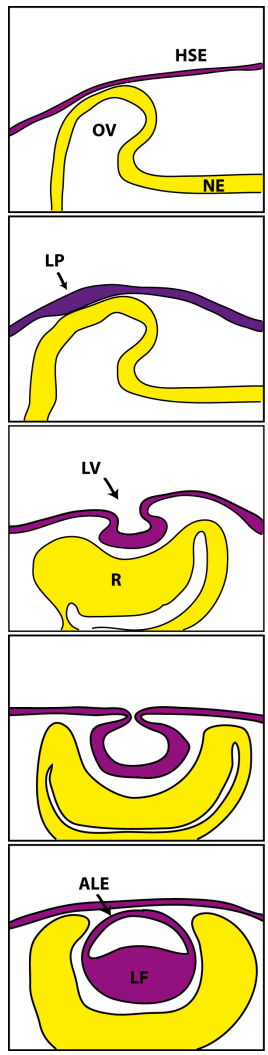

Fig 2. Schematic figure of early lens development. OV optic vesicle. NE neuroectoderm. HSE Head surface ectoderm. LP Lens placode. ALE Anterior lens epithelium. LF Lens fibers.

epithelial cells elongate to fill the entire cavity. This primary fiber differentiation is completed at E13.5. The anterior cells of the lens vesicle remain proliferative and persist as the lens epithelium. Thereby, the basic organization of the mature lens is established: a proliferative epithelium covering the anterior hemisphere of a spherical body of differentiated fiber cells.

Epithelial cells above the lens equator proliferate and migrate posteriorly, into the transition zone, where they initiate the differentiation to fibers. The consequence of this is that from E14.5 there is a continual addition of secondary lens fibers with no appreciable loss from the lens center. The rate of lens growth gradually diminishes until the final, adult size is reached. From this point, the production of new secondary lens fibers is balanced by loss of old fibers and no net growth occurs.

Many genes and signaling pathways are engaged in controlling the timing and execution of the lens development program. Some are involved in both early events, such as placode formation, as well as late events, such as secondary fiber differentiation or lens homeostasis. FoxE3, which is the main topic of this thesis, is such an example.

Molecular events of early lens development

Of particular importance for lens development is the *Pax6* gene. The absence of *Pax6* causes complete lack of eyes (Hill et al., 1991) and seems to be at the apex of the eye development pathway. The mammalian Pax6 protein can induce formation of ectopic eyes in both vertebrates and flies (Chow et al., 1999; Halder et al., 1995). Expression of *Pax6* is found in several tissues that contribute to eye development, such as the anterior neural plate and the outgrowing optic vesicle. It is also expressed in a broad segment of head ectoderm, but becomes restricted to the lens placode after the ectoderm has made contact with the optic cup (Grindley et al., 1995). Ectodermal expression of *Pax6* is crucial for lens formation, since *Pax6* deficient head ectoderm transplanted onto wild type optic vesicle does not form a lens (Fujiwara et al., 1994). Head ectoderm was found to be more sensitive to alterations in *Pax6* gene dosage than the optic cup, when a *Pax6* allelic series was analyzed (Favor et al., 2008).

The regulation of *Pax6* expression has been extensively studied and several cis-acting elements important for its regulation have been identified. Examples include the ectodermal enhancer, about 3kb upstream, the SIMO element some 160kb downstream of the transcription start site, and the DRR region further downstream from SIMO (Kammandel et al., 1999; Kleinjan et al., 2006; Kleinjan et al., 2001; Williams et al., 1998). Deletion of the ectodermal enhancer leads to decreased *Pax6* expression, preferentially on the nasal side, and results in a phenotype similar to that found in heterozygous *Pax6* (Small eye, *Sey*) animals, with a persistent stalk between the lens and cornea (Dimanlig et al., 2001).

Two molecules of the BMP family are produced by the OV at the time of lens placode formation. Both BMP4 and BMP7 are important for lens induction and development; both act upstream of placodal *Pax6* and induces lens placode formation (Furuta and Hogan, 1998; Wawersik et al., 1999). BMP4 has been shown to induce expression of *Sox2* in the presumptive placode, the expression of which is tightly linked to lens specification and, hence, no lens is formed in *BMP4* null mutants (Furuta and Hogan, 1998).

FGF signaling is another major player in lens development, which will be further discussed below. FGF receptor inhibition reduces levels of *Pax6* in lens placode ectoderm (Faber et al., 2001). There is no single obvious candidate for the critical FGF ligand, but in the case of induction of the olfactory placode, FGF8 has been shown to promote olfactory fate at the early placode state (Bailey et al., 2006).

From results of a large body of work (see for example Wigle et al., 1999;Blixt et al., 2000; Yamada et al., 2003; reviewed in Lang, 2004), a transcription network model for lens formation can be outlined. In this model, Pax6 controls lens development by regulating *Mab21l1*, *Foxe3*, *Sox2*, *Six3*, and *Prox1*. Furthermore, these transcription factors interact genetically with each other as exemplified by the relationship between *Foxe3* and *Prox1* (Blixt et al., 2000; Paper IV).

Lens fiber differentiation

During development, the lens acquires a thick basal lamina, called lens capsule, which encloses the lens and serves as the basement membrane for the epithelial cells. It is rich in ECM proteins such as collagen IV, laminins, heparan-sulfate proteoglycan (HSPGs) and fibronectin. These proteins can, apart from their structural role influence growth factor signaling by binding ligands such as FGF and BMP (Wederell and de Iongh, 2006; Schulz et al., 1997; Wang et al., 2008b). As a basement membrane, the capsule induces integrin-mediated signaling in epithelial cells, which is important for differentiation and survival (reviewed by Walker and Menko, 2008).

Changes in cellular organization

The process of lens epithelial to fiber cell terminal differentiation requires extensive remodeling of cellular function and appearance. Most striking is the dramatic increase in cell length, a process that obviously requires extensive changes in the cytoskeleton. During differentiation the fiber cells elongate and migrate towards the center of the lens; their ends following the lens capsule posteriorly and the fiber-epithelial interface anteriorly towards the optical axis. Eventually, they lose contact with the substratum and make contact with fiber cells migrating from the opposite side in structures called sutures (Kuszak et al., 2004). The migration of fiber cells utilize the same molecular machinery used for fibroblast cytoskeletal dynamics including Rho family small GTPases. These proteins modulate lens cell movement, and inhibitors of Rho GTPase activity causes cataract (Rao et al., 2002; Zelenka, 2004).

During the final stages of fiber differentiation cells lose all membrane-bound organelles (Bassnett, 2008). This process shares many similarities with apoptosis, even though caspases, key players in the apoptotic cascade have yet to be shown to be involved in fiber differentiation (Zandy et al., 2005). The cues that trigger organelle degradation are not fully understood. Hypotheses include signaling by TNF-α related molecules (Wride and Sanders, 1998), and a passive process based on gradients of metabolites (discussed in Bassnett, 2002). Due to the loss of organelles, including nuclei, fiber cells are not transcriptionally active and rely on metabolites from cortical fibers and epithelial cells. This transport is facilitated by an elaborate system of gap junctions formed by connexin46 and 50. The importance of the metabolic coupling enabled by the gap junction network for homeostasis, is illustrated by cataract formation in *connexin* mutants (Xia et al., 2006).

Growth factor regulation of lens development

The lens is not an isolated tissue, but is located in an environment rich in growth factors, and where reciprocal signaling between tissues is common (lens growth factor regulation is reviewed in Lovicu and McAvoy, 2005). The organization of the lens is maintained throughout life with an actively proliferating anterior epithelium and a posterior compartment of fiber cells, that make up the bulk of the lens and gives it its optic properties. Classical experiments done in the 1960s illustrated the influence of the ocular environment on lens polarity, shape and growth. When chicken lenses were surgically inverted so that the epithelium faced the retina, posterior epithelial cells differentiated into fibers, whereas cells from the lens equator proliferated and colonized the anterior hemisphere to create a new epithelium (COULOMBRE and COULOMBRE, 1963). The lens influenced the surrounding ocular tissue as well (COULOMBRE and COULOMBRE, 1964), demonstrating the role of the lens for growth and differentiation of other ocular tissues.

Over the years, much work has been invested into identifying the factors responsible for lens cell behavior. The use of lens epithelial explants has identified many molecules important for lens epithelial differentiation, starting with insulin (Piatigorsky, 1973) in 1973. Important advances in our understanding of the origin of these lens stimulatory factors came even earlier, with studies that demonstrated the importance of the neural retina for lens growth (COULOMBRE, 1965). Later, it was shown in rat lens epithelial explants that the cells could be induced to proliferate and differentiate in co-culture with neural retina (McAvoy, 1980). The molecules secreted from the retina, originally termed "fiber differentiation factor", we today know as members of the fibroblast growth factor family (FGF). It is now clear that there are differences in the composition of the milieu of the posterior and anterior lens faces, with the posterior (vitreous humour) promoting differentiation and the anterior (aqueous humour) epithelial cell maintenance.

FGF signaling

The mammalian FGF family of growth factors consists of 22 members, 13 of which are expressed in the lens (reviewed in Robinson, 2006). The FGF ligands signal through one of four receptor tyrosine kinases FGFR1-4. Upon ligand binding, receptors dimerize which leads to tyrosine auto-phosphorylation and activation of one of several intracellular signaling cascades. *FGFR* genes are alternatively spliced, which increases the number of variants far beyond four, and alternative splicing influences ligand specificity and affinity (Zhang et al., 2006). Detailed studies of *FGFR1* showed an increased expression at the onset of fiber differentiation, and higher expression in the germinative and transition zones, compared to central anterior epithelial cells (de Iongh et al., 1996). The importance of FGF signaling during lens development, and redundant functions among receptors, was recently shown by generation of mouse *FGFR* triple mutants with severe lens defects (Zhao et al., 2008).

Important insights into the role of FGFs in lens biology, came from rat lens epithelial explant studies, where epithelial cells were grown in culture with the lens capsule intact, thereby maintaining them on their original basement membrane. McAvoy and Chamberlain found that FGF1 and FGF2 could influence lens epithelial explants in a dose dependent manner, where low concentrations induced proliferation, intermediate migration, and high concentration differentiation (McAvoy and Chamberlain, 1989). These experiments, together with results showing higher concentrations of FGF in vitreous compared to aqueous humour (Schulz et al.,

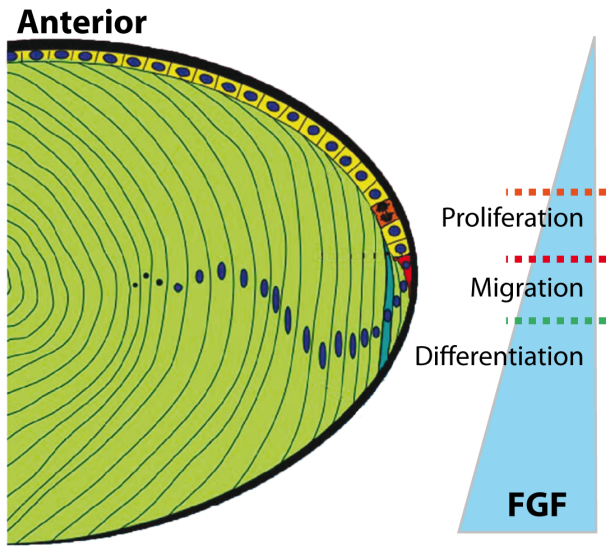

Fig 3. Schematic diagram of the mature lens with epithelial cell overlying elongated terminally differentiated fiber cells. To the right is illustration of the FGF gradient postulated in the eye and the different threshold concentrations that invoke differential lens cell response.

1993) form the basis of the theory that an FGF gradient exists within the eye, and that this gradient determines the location of the transition zone (see Lovicu and McAvoy, 2005). Results from transgenic mice in which the FGF pathway has been disturbed generally support this model. Overexpression of *FGF* leads to elongation and cell cycle withdrawal of central epithelial cells (Robinson et al., 1995; Lovicu and Overbeek, 1998; Robinson et al., 1998), whereas mice expressing a secreted form of FGFR3 that binds FGF display the reverse phenotype, compared to ligand overexpression (Govindarajan and Overbeek, 2001). To date, FGFs remain the only growth factors known to induce fiber differentiation in the mammalian lens.

TGF-ß signaling and fiber differentiation

TGF-ß related proteins comprise a large family of signaling molecules whose signaling is normally transduced by Smad proteins (Massagué, 1998). Multiple receptors are expressed in the lens (de Iongh et al., 2004) and both Smad1 (downstream of the BMP receptor) and Smad2 (TGFß receptor) are phosphorylated in elongating fiber cells, which is a telltale sign of active signaling (Belecky-Adams et al., 2002; Beebe et al., 2004). Inhibition of BMP signaling with Noggin, or deletion of receptors, interferes with fiber cell differentiation (Belecky-Adams et al., 2002; Faber et al., 2002).

Over-expression of a kinase deficient TGFßR leads to defects in terminal differentiation of lens fiber cells (de Iongh et al., 2001). However, Beebe et al reported persistent Smad2 phosphorylation and normal lens formation in *TGFßRII* knock out mice, which would argue that TGFß is dispensable for lens development and that phosphorylation of Smad2 can occur independently of TGFßRII (Beebe et al., 2004). In a pathological situation, anterior subcapsular cataract, which can occur after injury to the lens epithelium — for instance, as a result of cataract surgery — TGFß and Smad3 are involved by inducing epithelial-mesenchymal transition (EMT) (Saika et al., 2004).

The Wnt pathways

The first evidence for Wnt involvement in lens development came from studies of the Frizzled co-receptor *Lrp6* null mutant displaying persistent connection between lens and cornea (Stump et al., 2003). Many Wnt signaling components are expressed in the lens (Stump et al., 2003) and inactivation of ß-catenin specifically in this tissue leads to disruption of epithelial and fiber cell differentiation (Cain et al., 2008). ß-catenin stabilization and nuclear translocation is the end point of the canonical Wnt pathway and many of the components of this pathway are expressed in the lens. However, there are also reports of non-canonical (e.g. Ca2+-, PI3-K/AKT -mediated) Wnt signaling in the lens (Chen et al., 2006).

Juxtacrine signaling by Notch

During lens induction in Xenopus, Notch signaling has been shown to directly activate *Foxe3* in the surface ectoderm in response to the Notch ligand Delta2 in the optic vesicle (Ogino et al., 2008). In the developing lens of mouse embryos, Notch target genes *Hes1* and *Herp2* are expressed specifically in the epithelium, whereas the Notch ligand Jagged1 (Jag1) is present in the subepithelial fiber cells (Jia et al., 2007; Rowan et al., 2008). Genetic abrogation of Notch signaling leads to expansion of the expression of the Cdk-inhibitor p57 and a reduction in lens size (Jia et al., 2007).

Lens cell proliferation

Lens epithelial cells in the germinative zone, immediately anterior of the transition zone, proliferate much more rapidly than anterior cells. While it was initially postulated that only germinative zone cells where capable of responding to mitogenic signals it has later been shown that all epithelial cells maintain proliferative potential (Beebe, 1992; Zhou et al., 2006). Many growth factors have been shown to act as mitogens for lens cells; in epithelial explant cultures FGF, IGF, PDGF-A, PDGF-D, EGF, and HGF will stimulate proliferation (Iyengar et al., 2006; Choi et al., 2004; Ray et al., 2005). A number of growth factor transgenic mice have been generated that alter proliferation in the lens. Overexpression of *IGF1* displaces the transition zone posteriorly and changes the size and location of the germinative zone, all with a small change in proliferation (Shirke et al., 2001). PDGF-A also stimulates epithelial cell proliferation, but at the same time increases the expression of certain fiber differentiation markers (Reneker and Overbeek, 1996). However, the *PDGFR-α* knock out has only a slight reduction in lens size (Soriano, 1997), which shows that PDGF receptor signaling is not essential for proliferation, nor for lens development in general.

Cell cycle regulation of lens cells

For fiber differentiation to occur, lens cells must exit the cell cycle. A key event in the cell cycle withdrawal is the regulation of Retinoblastoma protein (Rb) by cyclin dependent kinases (CDKs). Lens cells of *Rb* mutants failed to exit the cell cycle and had a higher propensity for apoptosis (Morgenbesser et al., 1994). Upstream regulators of Rb are the CKIs, p27 and p57, which function redundantly to inhibit CDKs, thereby keeping Rb active and forcing cells out of the cell cycle. Double mutants for these CKIs have hyperproliferation of the lens epithelium (Zhang et al., 1998). A similar phenotype was observed when Rb was inhibited by transgenic expression of E6 and E7 oncogenes from human papillomavirus in the lens epithelium (Nguyen et al., 2002). Expression of the CDK inhibitors *p27* and *p57* is activated by the transcription factor Prox1, a protein present in the equatorial region of the lens (Wigle et al., 1999).

The anterior segment of the eye

The anterior segment of the eye comprises the cornea, ocular drainage structures (trabecular meshwork and Schlemm's canal), iris, ciliary body and lens. These structures develop from four embryonic sources; surface ectoderm, neural ectoderm, head mesoderm and neural crest (anterior segment development is reviewed in (Gould et al., 2004)). At E12.5, the prospective cornea consists of one to two layers of cells derived from the surface ectoderm. The following day or two sees migration of periocular mesenchyme, which consists of cranial paraxial mesoderm and neural crest cells. By E15, the corneal endothelium and a small anterior chamber can be identified. The anterior rim of the optic cup forms the epithelium of the ciliary body and iris and serves as a substrate for periocular mesenchyme migration, beginning around E15. Later, the ocular drainage structures differentiate from the mesenchyme at the root of iris and cornea. The development of the murine anterior segment, in particular the drainage structures and iris, continues after birth and are not fully mature until after three weeks of age (Smith et al., 2001).

The tissues of the anterior segment are actively producing and responding to growth factors. The lens is important for development of the cornea, as demonstrated by lens ablation experiments in chicken (Zinn, 1970). Recently, Zhang and colleagues performed essentially the same experiment in mice using transgenesis. Expression of diphtheria toxin in the lens resulted in its complete ablation by P8, with defects in

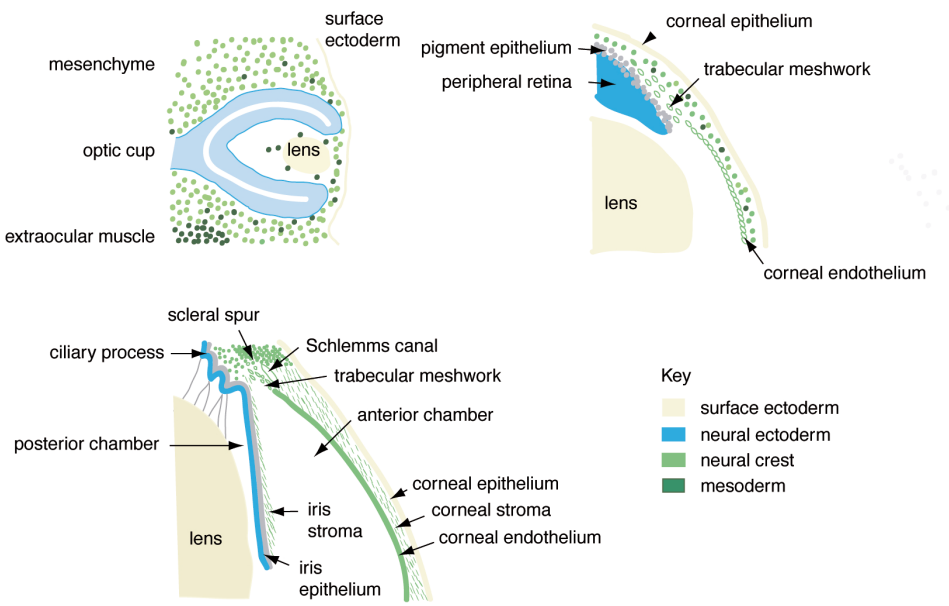

Fig 4. The basic structures, and development of the anterior segment of the eye. Anterior is to the right. Modified from Sowden 2007.

corneal endothelium and iris as secondary effects (Zhang et al., 2007). The lens expresses *TGFß2*, which induces expression of *Pitx2* and *Foxc1* in periocular mesenchyme, thereby directing development towards corneal stroma, endothelium and trabecular meshwork (Ittner et al., 2005; Saika et al., 2001; Evans and Gage, 2005). Other TGFß superfamily members involved are BMP4 and -7. BMPs are important for correct differentiation of the ciliary body, as demonstrated by overexpression of *Noggin* — a BMP antagonist — in the lens (Zhao et al., 2002). Heterozygous *BMP4* mice have anterior segment defects, although with variable penetrance, in most cases associated with high intraocular pressure (IOP) (Chang et al., 2001). In humans, elevated levels of TGFß are found in patients with primary open-angle glaucoma and high IOP (Ochiai and Ochiai, 2002). A TGFß-associated increase in ECM-production impedes trabecular meshwork permeability and increases outflow resistance, with rising IOP. This effect of TGFß can be antagonized by BMP4 and BMP7 (Fuchshofer et al., 2007; Wordinger et al., 2007).

Summary of Foxe3 results

Targeted mutation and anterior segment

Here, we verify *dyl* as a null allele of *Foxe3* by creating a targeted deletion of the DNA binding domain of *Foxe3*. The targeted mutant is indistinguishable from the *dyl* mutant, with loss of the lens epithelium and a small vacuolated lens.

Anterior segment defects in Foxe3 heterozygotes

Earlier studies have demonstrated requirement of the lens for development of the anterior segment (Beebe and Coats, 200; Zinn, 1970; Zhang et al., 2007). Since *Foxe3* mutants display severe lens defects, a detailed study of the anterior segment was conducted. *Foxe3* mutants have improper differentiation of cornea, iris, ciliary body and trabecular meshwork. The cornea in *Foxe3* $^{-/-}$ animals have a lax structure that lacks proper stratification as illustrated by the uniform distribution of ZO-1, a component of tight junctions. The iris and ciliary body are replaced by a structure that resembles a rudimentary iris but is lacking most histological hallmarks of both iris and ciliary body. Furthermore, the iris/ciliary body structure adheres to the abnormal cornea endothelium, effectively obliterating the filtration angle. Misplaced localization of the cell adhesion protein N-cadherin to all mesenchymal tissue facing the anterior chamber may contribute to the observed adherences.

Examination of heterozygous mutants showed similar defects, with an undifferentiated more compact iris compared to wild type. The iris defects are accompanied by an edematic and vascularized corneal stroma, indicative of a dysfunctional iris. Similar defects were seen in Pax6 heterozygotes, analyzed in parallel.

Ectopic *Foxe3*

In paper IV we investigated the effect of *Foxe3* mis-expression on the mouse lens. Using a modified aA-crystallin promoter, a mouse was created that expresses *Foxe3* in the fiber compartment of the lens. The lenses of these mice form normally, but perinatal defects are obvious. Transgenic lenses are cataractic and contain large vacuoles at birth. The fiber structure is severely perturbed, with the normally very highly ordered fibers being replaced by fibers that are loosely packed and very non-uniform in appearance, as judged by electron microscopy. The Tg(Cryaa-Foxe3) lens has a gap in Foxe3 protein localization just posterior to where the endogenous gene is turned off, despite presence of mRNA from the transgenic construct. Microarray analysis comparing wildtype and transgene lenses of two days old mice confirms the impression from histology. Genes important for terminal differentiation, such as *DLAD*, a DNAse responsible for nuclear breakdown, are reduced as well as genes important for morphological change. All in all, the impression from transcript

profiling is that ectopic expression of *Foxe3* in fiber cells results in an epithelialization of the fiber cell gene profile.

Foxe3 ex-vivo

In Paper V we analysed Foxe3 regulation utilizing an explant system, where whole lenses were cultured ex-vivo. We found that *Foxe3* expression is dependent on signaling between lens fiber and epithelial cells. Earlier work have suggested that Foxe3 is necessary for lens epithelial cell proliferation (Blixt et al., 2000), we now show that presence new data on the relationship between Foxe3 and proliferation. Foxe3 is abruptly removed when fiber differentiation starts (paper IV) and we show that signaling events is likely to cause this removal through a pathway that targets Foxe3 for destruction. We further found evidence for Map kinase involvement in *Foxe3* turnover. The targets of Foxe3 are unknown and to that end we identified transcriptional targets of Foxe3.

Discussion

Foxe3 is expressed from E9.5 in the lens placode and later becomes confined to the lens vesicle. Initially, expression is evenly distributed, but is later lost in the posterior half as these cells differentiate to fiber cells. After E13.5, *Foxe3* is exclusively expressed in the anterior epithelium of the lens (Blixt et al., 2000). However, prior to E13.5, at E9-11 there is a transient expression domain of *Foxe3* in the diencephalon (Blixt et al., 2000). What function this has is unclear, as well as the fate of the expressing structures.

Dysgenetic lens is a spontaneous mouse mutant identified in 1979 (Sanyal and Hawkins, 1979). Previous work with cloning and sequencing of *Foxe3* indicated that mutations in *Foxe3* are responsible for the *dyl* phenotype, characterized by a small and vacuolated lens that fails to separate from the surface ectoderm (Blixt et al., 2000). It was also shown that two mutations in the Forkhead box of *Foxe3* were present in the *dyl* allele and that these mutations abolished DNA binding (Blixt et al., 2000; Ormestad et al., 2002). The variable phenotype of *Foxe3* $^{dyl/+}$ mice, a persistent connection between lens and cornea, is very similar to the human congenital disorder Peters' anomaly (Ormestad et al., 2002; Stone et al., 1976) and mutations in the forkhead domain of *Foxe3* have been found in human patients (Ormestad et al., 2002; Semina et al., 2001). Taken together, earlier work indicated a role for *Foxe3* in proliferation and survival of the lens epithelium, inhibition of fiber differentiation, and development of the anterior segment of the eye.

Foxe3 and anterior segment differentiation

The anterior segment of the eye comprises the cornea, anterior chamber angle (made up of the trabecular meshwork and Schlemm's canal), iris, ciliary body and lens. The view of the lens as an organizer for the anterior segment development is supported by lens ablation experiments (Beebe and Coats, 2000; Zhang et al., 2007; Zinn, 1970).

In *Foxe3* heterozygotes, the lens epithelium is phenotypically normal and defects in the anterior segment are therefore related directly to *Foxe3* deficiency, and not the lack of lens epithelium. The identity of the factors responsible for anterior segment dysgenesis in *Foxe3* $^{-/+}$ animals are currently unknown, but must involve factors produced by the lens epithelium and secreted into the anterior chamber fluid.

The cornea of the *Foxe3* mutants is loosely organized and the endothelium is underdeveloped. The phenotype is more severe in the homozygous mutant, where the endothelium is altered beyond recognition. Descemet's membrane is fairly normal even in *Foxe3* null mutants indicating that some endothelial identity persists. The heterozygote endothelium seems to maintain a partial barrier function, but the appearance of fluid-filled vacuoles between endothelial cells led to the hypothesis that alterations mediated by *Foxe3* results in misplacement of ion pumps that normally keep the cornea dehydrated. A predicted consequence of this would be the edematous corneal stroma observed in the *Foxe3* heterozygotes. Another feature of the mutant

cornea is vascularization, suggesting hypoxia within the stroma, a tissue normally lacking any vessels. This indicates that the defects seen in iris differentiation have functional consequences, since the iris is the primary source of oxygen, nutrients, and metabolites for the avascular tissues of the anterior segment, cornea and lens. The iris is more severely affected in homozygous mutants, where both iris and ciliary body are replaced with a tissue sharing resemblance of both. This rudimentary structure display adherence to both lens and cornea. The trabecular meshwork and Schlemm's canal are absent from the *Foxe3* $^{-/-}$ mutant and while they do form in the *Foxe3*$^{-/+}$ mutant, the chamber angle is sometimes closed by adherences between iris and cornea. The function of the trabecular meshwork and Schlemm's canal is to drain the aqueous humour, and defects in this process can cause an increase in intraocular pressure. This in turn leads to glaucoma, the leading cause of blindness in the world (Quigley, 1996). This suggests that mutations in *FOXE3* might be a cause for congenital glaucoma in humans.

Regulation of Foxe3 expression

Several lines of evidence points towards *Pax6* being upstream of *Foxe3*. Reduction of *Pax6* gene dosage led to lowered *Foxe3* expression at E10.5 and E11.5 (paper III); *Pax6* mutants can not activate a LacZ reporter driven by the *Foxe3* promoter (Brownell et al., 2000); Pax6 can activate the Zebrafish foxE3 ortholog (Kenyon et al., 1999). Furthermore, the phenotypes in the anterior segment of *Foxe3* and *Pax6* heterozygous mutants are very similar (paper III; Baulmann et al., 2002) and both *FOXE3* and *PAX6* mutations have been shown to cause Peters' anomaly in humans (Hanson et al., 1994; Ormestad et al., 2002). Taken together, these data provides a strong case for *Pax6* being upstream of *Foxe3* and suggest that anterior segment defects in both mutants are due to a common mechanism.

There are a number of reports detailing other transcription factors that can influence *Foxe3* expression. A possible link between *Pax6* and *Foxe3* might be *Mab21l1*, the mutants of which, lack *Foxe3* expression during early lens development (Yamada et al., 2003). Two reports suggest other direct upstream activators of *Foxe3*. One is *Sip1*, a Smad interacting protein first expressed in the lens placode and later maintained in the lens epithelium (Yoshimoto et al., 2005). In vitro assays suggest that Sip1, synergistically with Smad8, binds and activates the *Foxe3* promoter. In Xenopus, the Notch pathway is upstream of *Foxe3* and the Notch mediator Su(H) (Rbpj in mammals) was shown to interact with a conserved enhancer sequence in the *Foxe3* promoter (Ogino et al., 2008). Furthermore, the conserved enhancer contained binding site for Otx2, and Smad1, suggesting that Smad and possibly the TGFß family of growth factors might be involved in regulation of *Foxe3*.

Foxe3 and lens differentiation

The Tg(Cryaa-Foxe3) mouse has ectopic expression of *Foxe3* in the fiber compartment. This leads to incomplete fiber differentiation, and histological comparison of Tg(Cryaa-Foxe3) and wild type lenses revealed a slightly smaller, vacuolated lens with highly disordered and loosely packed fiber cells (paper IV). Ectopic *Foxe3* also abrogated organelle degradation. Part of the reason for defective organelle breakdown became evident when gene expression profiles of Tg(cryaa-Foxe3) and wild type lenses were compared. The transgenic lens had reduction in transcripts for *DLAD,* a lens-specific DNAse responsible for DNA degradation (Nishimoto et al., 2003). Interestingly, *Foxe3* null mutants also have reduction in *DLAD* mRNA (Medina-Martinez et al., 2005). This may seem contradictory, but it any mutant with general delays/defects in fiber differentiation is likely to have reduced expression of fiber cell markers. One factor implicated in regulating organelle degradation is TNF-α (Wride and Sanders, 1998). Many genes in the TNF-α pathway have altered expression in the Tg(cryaa-Foxe3) lens (paper IV), loosly supporting the idea that organelle degradation is a regulated process.

The general interpretation when histology and gene expression profiles are put together is that ectopic expression of *Foxe3* leads to a partial epithelialization of the fiber cells. Support for this notion comes from comparison with a different microarray data-set, comparing the expression profiles of epithelial cells vs. cortical fibers; Transcripts upregulated in Tg(cryaa-Foxe3) are normally present at a higher level in epithelial cells (Nakahara et al., 2007; Paper IV).

Foxe3 and initiation of fiber differentiation

The model for lens fiber differentiation, proposed in paper V, involves growth factor induced degradation of Foxe3 at the lens equator. Removal of Foxe3 seems to be required for differentiation to initiate and proceed properly (Blixt et al., 2000; Rowan et al., 2008); paper IV). Growth factor signaling have been shown to influence proliferation, migration, and differentiation in lens epithelial explants (McAvoy and Chamberlain, 1989). Most, if not all, of these responses are mediated through ERK and PI3-K/Akt kinases, and the outcome is determined by the concentration of ligand, which in turn determines the amount and duration of ERK and PI-3K phosphorylation (Iyengar et al., 2007; Lovicu and McAvoy, 2001; Wang et al., 2008a). There is some notable division of labour between these two kinases. The accumulation of beta and gamma-crystallins can be blocked by inhibition of Akt but not ERK phosphorylation (Wang et al., 2008a). This leads to the hypothesis that different cellular cascades influence different parts of fiber differentiation. Furthermore, it increases the possible number of upstream agonists. The bipartite nature of fiber differentiation is also evident from Tg(Cryaa-Foxe3) lenses, where the morphology of fiber cells is dramatically affected, but crystallin gene expression, along with some of the other most highly expressed lens genes, are not altered (paper IV).

It is clear that FGF2 alone initiates fiber differentiation. However, the response of lens epithelial explants exposed to vitreous is stronger, suggesting that other growth factors can augment the response of FGF2. Signaling through the PI3-K, Cdc42 and JNK axis can be triggered by Wnt ligands and this cascade can influence cytoskeletal behavior (Schambony and Wedlich, 2007).

In support of non-canonical Wnt signaling being the mode of ligand transduction in the lens is a report by Kreslova et al showing that, while ß-catenin conditional knockout do have a lens phenotype, it is likely not due to ß-catenin-mediated signaling since no activity of a canonical lacZ reporter was found in the lens (Kreslova et al., 2007).

Changes in cytoskeletal modification have been proposed as a trigger for fiber differentiation, and PI3-K activation is necessary for this cytoskeletal reorganization to occur (Weber and Menko, 2006). The transcript profiling of Tg(Cryaa-Foxe3) showed altered expression of many genes modulatory to Map kinase pathways (paper IV). The reported phosphorylation patterns differ, p-P38 is found in epithelial cells and p-JNK is found in both epithelial and fiber cells (Chen et al., 2006; Lovicu and McAvoy, 2001; Li et al., 2003).

In light of higher concentrations of FGFs being present in vitreous compared to aqueous (Schulz et al., 1993), FGF are likely to control where the initiation of fiber differentiation takes place.

Foxe3 and control of differentiation competence

Many reports have shown consequences for lens development when BMP pathway components are altered. The BMP pathway has been shown to be a positive regulator of fiber differentiation (Belecky-Adams et al., 2002) and the gene expression profile of Tg(Cryaa-Foxe3) fits with BMP signaling being a fiber-promoting pathway (paper IV). The overall phenotype of *BMP* receptor and *Smad* null mutants suggest that BMP signaling have a role in fiber differentiation rather then epithelial survival and maintenance (Beebe et al., 2004; Rajagopal et al., 2008). Of course, the possibility remains that BMP4 signal in the epithelium is mediated through a non-BMP receptor using a non-Smad dependent intracellular pathway Another hypothesis is that BMP signaling influences juxtacrine factors in fibers cells, such as Jagged or any other factor capable of triggering a response in epithelial cells.

Foxe3 and maintenance of lens epithelium

Recently, three papers details Notch signaling in lens development (Jia et al., 2007; Rowan et al., 2008; Ogino et al., 2008). The lens of *Rbpj* conditional mutants is smaller than normal with an anteriorly displaced transition zone (Jia et al., 2007; Rowan et al., 2008). The reverse seems to be the case for a constitutively active Notch mutant (Rowan et al., 2008). All changes in position of the transition zone are accompanied by movement of the boundary between Foxe3 positive and negative cells. The third paper, by Ogino et al, describes the identification of a conserved upstream enhancer sequence in the *FoxE3* promoter that bind Su(H), the Xenopus homolog of Rbpj, making Notch a candidate for direct activation of *FoxE3* expression (Ogino et al., 2008).

Jagged is, as mentioned earlier, expressed in fiber cells, and is down-regulated in response to deletion of Notch signaling in *Rbpj* mutants (Jia et al., 2007; Rowan et al., 2008). This is expected based on what is known about Notch signaling in other systems. One of the developmental functions of Notch signaling is cell fate determination and ligand and receptor are never present in the same cell for any length of time (Lai, 2004). A proposed function for Notch is epithelial survival and control of lens size (Jia et al., 2007; Rowan et al., 2008). Foxe3 is lost in posterior, but retained in anterior lens vesicle cells in *Rbpj* conditional null mutants (Rowan et al., 2008). It is clear from the *Rbpj* mutant that epithelial cells do differentiate prematurely, as seen in *Foxe3* mutants (Blixt et al., 2000; Jia et al., 2007).

Foxe3 and proliferation

One of the previously defined roles of Foxe3 was to promote proliferation. To date, no lens mutant or in-vivo situation in the mouse have been reported where normal non-fiber differentiated lens epithelial cells do not express *Foxe3*. In zebrafish however, *Foxe3* knock down with morpholino results in increased proliferation and a multilayering of the epithelia (Shi et al., 2006) arguing that at least in Zebrafish proliferation is not dependent on *Foxe3* at the developmental stages investigated.

To investigate the presence of stem cells in the lens epithelium, Zhou et al performed long term chasing experiments and found long-term label retaining cells in the central part of the epithelium. Lighter labelled cells were present in the germinative zone arguing that the site of the most stemcell-like cells in the lens is in the most anterior part of the epithelium. However, after perturbation both slow and faster cycling cells can be induced to rapid proliferation showing that all lens epithelial cells maintain proliferative capacities (Zhou et al., 2006).

The proliferation rate of the lens epithelium seems to diminish with age, and rat lens explant experiments indicate that there is a mechanism that negatively regulates proliferation as the lens grows in size (McAvoy and McDonald, 1984). There is no appreciable loss of cells in the adult lens and the accepted consensus is that the lens is a tumor-free organ. Together this suggests that there must exist strong control over lens growth and size.

The highest proliferative activity is in the germinative zone just anterior to the transition zone (Shirke et al., 2001). If FGF concentrations within the eye are perturbed, the transition zone moves in either direction. It seems from FGF trangenesis that the germinative zone is altered correspondingly (Govindarajan and Overbeek, 2001; Robinson et al., 1995).

Summary and future directions

Lens epithelial cells are faced with the decision between proliferation/survival or differentiation and these decisions seems to be at least partly controlled by instructive signals from the fiber mass. Our model predicts a role for Foxe3 in maintaining the lens epithelium, inhibiting differentiation, as well as controlling the production of secreted factor influencing other tissues in the eye.

Acknowledgements

The every day version of scientific inquiry can be described in the words of TH Huxley, “the slaying of a beautiful hypothesis by an ugly fact”. Now, this might sound discouraging, and it is. Nobody likes ugly facts, especially not if they are killing your beautifully thought-out hypothesis. Nevertheless, it happens more often than not. During these times, it is nice to have good people around you and that has certainly been true for me, both scientifically and personally. The place and time has come for me to show my gratitude to the people that have made my experience over that last years truly great, and have inspired me to keep going despite of all the “ugly facts”.

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Everything cannot be about science (even though, it would be sweet if it was). Hence, my life outside of the lab have been just as important for this thesis to come true, and by that I would like thank to all my family and friends on the outside.

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“Every honest researcher I know admits he's just a professional amateur. He's doing whatever he's doing for the first time. That makes him an amateur. He has sense enough to know that he's going to have a lot of trouble, so that makes him a professional.”

Charles Franklin Kettering (1876-1958) U. S. Engineer and Inventor.

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