AFRICAN MOLE RATS AS MODELS FOR REGRESSIVE EVOLUTION OF THE EYE

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March 2006
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This thesis is dedicated to my grandparents, Yakov Nikitich Nikitin and Taisya Vasilyevna Nikitina.

Эта диссертация посвящается моим дорогим дедушке и бабушке, Якову Никитичу и Таньке Васильевне Никитиным.
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List of Publications and Presentations from this Thesis.

Publications:


Conference presentations:


Abbreviations

Ago – Argonaute
AP – alkaline phosphatase
APTES – 3-(triethoxysilyl)-propylamine
BAC – bacterial artificial chromosome
BCIP – bromochloroindolyl phosphate
BLAST – Basic Local Alignment Search Tool
Bmp – bone morphogenetic protein
BrdU – bromodeoxyuridine
BSA – bovine serum albumin
CB – ciliary body
CHAPS – (3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate)
CMV – cytomegalovirus
DAB – 3,3’-diaminobenzidine tetrahydrochloride
DAPI – diamidino-2-phenylidole
depc – diethyl pyrocarbonate
DMEM – Dulbecco’s modified Eagle’s medium
dNTP – 2’-deoxynucleotide triphosphate
DSE – distal sequence element
dsRNA – double-stranded RNA
E – embryonic day
EDTA – ethylenediaminetetraacetic acid
EGFP – enhanced green fluorescent protein
FCS – fetal calf serum
FGF – fibroblast growth factor
FoxC1 – forkhead boxC1 transcription factor
GFP – green fluorescent protein
HH – Hamburger-Hamilton stage
ICC – immunocytochemistry
IGF – insulin-like growth factor
IRES – internal ribosomal entry site
kDa – kilodalton
Lmx1b – LIM homeobox transcription factor 1 beta
MAB – maleic acid buffer
MABT – maleic acid buffer supplemented with 0.1% Tween-20
MMLV RT – Moloney Murine Leukemia Virus Reverse Transcriptase
Msx1 – msh-like homeobox transcription factor 1
MWM – molecular weight marker
N-cadherin – neural cadherin
N-terminus – amino-terminus
NBT – nitroblue tetrazolium
NCBI – National Centre for Biotechnology Information
OCT – octamer sequence element
Otx1 – orthodenticle homologue 1
P – postnatal day
Pax6 – paired homeobox 6
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDGF – platelet-derived growth factor
PFA - paraformaldehyde
PGK – phosphoglycerate kinase
Prox1 - prospero-related homeobox 1
PSE - proximal sequence element
Ptmb4 - prothymosin β4 (synonym: thymosin β4)
PTW - PBS supplemented with 0.1% Tween-20
RFP - red fluorescent protein
RISC - RNA-induced silencing complex
RNAi - RNA interference
RPE - retinal pigmented epithelium
RT - reverse transcription
RT-PCR - reverse transcription with polymerase chain reaction
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel
SEM - scanning electron microscopy
Shh - sonic hedgehog
shRNA - small hairpin RNA
siRNA - small interfering RNA
Six3 - sine oculis homologue 3
Sox1 - SRY (sex determining region Y)-box containing transcription factor 1
TBS - Tris-buffered saline
TBST - Tris-buffered saline supplemented with 0.1% Tween-20
TEM - transmission electron microscopy
Tgfα/TGFα - transforming growth factor alpha
Tgfβ1/TGFβ1 - transforming growth factor beta 1
Tgfβ1i4 - transforming growth factor beta 1 induced transcript 4
Tgfβ2/TGFβ2 - transforming growth factor beta 2
TM - trabecular meshwork
TUNEL - TdT-mediated dUTP-biotin nick end labelling
UTR - untranslated sequence
UV - ultraviolet
Abstract

African mole rats as models for regressive evolution of the eye

Darkness-adapted mammals with reduced eyes can serve as valuable models for the study of regressive evolution, as well as for research into the genetic and developmental processes underlying the “degeneration” of the eye. The aim of this work was to characterize two African mole rat species (Heterocephalus glaber and Bathyergus suillus) and explore their potential use as novel models for evolutionary developmental eye research. To this end, the histological structure of the adult eye, the development of the eye and the expression of several molecular markers were investigated. The important abnormal features noted were: the abnormal shape and nuclear distribution in the lens, the extremely large ciliary body and delay in the formation of the anterior chamber compared to other ocular structures.

Lens malformations and the consequent abnormal signalling to the anterior segment structures underlie the abnormal development of the ciliary body, the cornea and the iris. Therefore, molecular basis of the abnormal lens development in the mole rats was investigated. In the naked mole rat, BrdU labeling analysis revealed that the presence of nuclei in the centre of the lens was not due to inappropriate proliferation. To find out whether the process of lens differentiation was occurring abnormally, the expression of the crucial lens structural proteins, α and γ-crystallins, was determined by western blots and immunocytochemistry. It was found that γ-crystallin synthesis was terminated prematurely in the naked mole rats, but occurred throughout the life of the Cape dune mole rat, while α-crystallin synthesis occurred normally in both of these animals. In situ hybridization experiments revealed that the cessation of the γ-crystallin synthesis was not due to the termination of transcription, but to the increased degradation of the γ-crystallin mRNA.

The ciliary body in both of the mole rat species is very extensive compared to the mouse. The lens is a source of a diffusible signal that instructs the subterminal optic cup to differentiate as the ciliary body. To explore the hypothesis that the expanded ciliary body zone in the Cape dune mole rat results from the expansion of the prospective ciliary body zone, the expression pattern of two ciliary body-specific genes (Ptmb4 and Tgfβ1i4) that are upregulated by the lens-derived signal in the subterminal optic cup was determined. The Bathyergus homologues of Ptmb4 and Tgfβ1i4 were cloned by RT-PCR. The sequence analysis of these genes revealed high levels of similarity to the coding sequences of the mouse genes. It was found that neither Ptmb4 nor
Tgfβ1i4 were expressed at the tip of the optic cup. These results differ from what is observed in mouse and chick, where Ptmb4 is strongly expressed in the ciliary body.

Therefore, to further explore the possible roles of Ptmb4 in ciliary body morphogenesis, an experimental approach was adopted. To investigate whether Ptmb4 could be secreted during eye morphogenesis, intraocular localization of the Ptmb4 protein and mRNA were compared in the developing chick and mouse eyes. The results of this study revealed that this protein is found in a number of tissues besides those transcribing high levels of Ptmb4, suggesting that it is secreted. In order to understand the role of the secreted Ptmb4 in ciliary body morphogenesis, the full coding sequence of Ptmb4 was cloned into an expression vector, and this construct was used to transfect Cos7 cells. Cells were shown to synthesize high levels of the Ptmb4 protein. The cells expressing Ptmb4 were then introduced into developing chick eye at E2 to E3.5. No alterations in phenotype were observed.

Gain-of-function experiments might not result in an observable phenotype if the gene whose function is investigated has a permissive rather than instructive function. In order to further investigate the role of the Ptmb4 in the optic cup morphogenesis, loss-of-function experiments had to be performed. Chick embryos were chosen for this work because of their accessibility. RNA interference is a novel technique that is very useful for specific silencing of target genes. This method, however, is still not established in chick embryos. To address this, four chick-specific small hairpin RNA expression plasmids were engineered using the chick-specific RNA polymerase III promoters to drive the small hairpin RNA expression, and highly efficient β-actin promoter to drive expression of the marker gene (GFP) in avian cells. The four plasmids were tested for their ability to drive down exogenous red fluorescent protein expression in the chick embryonic neural tube. One of these plasmids was found to efficiently knock down the expression of the red fluorescent protein in the chick embryos in ovo. This novel RNA interference vector will promote the use of the chick model for the analysis of gene function.

This study contributes to the knowledge of the regressive evolution of the eye by providing the first detailed description of the structural, developmental and molecular aspects of the anterior chamber morphogenesis in two microphthalmic subterranean rodents.
1. Introduction

The vertebrate eye is an extremely complex organ, with a structural organization finely tailored to suit the function it must perform. Despite the fact that the eyes of all vertebrates share the same general organization, there is a great evolutionary diversification among different groups. Novel and sometimes very unusual phenotypes arise to meet specific visual needs, and as adaptations to diverse ecological niches occupied by vertebrates on land and in the ocean. For example, the eyes of animals living under conditions of permanent darkness (deep in the ocean, in caves or burrows), where acute vision is of limited or no survival value, often become small, disorganized and dysfunctional.

Examples of evolutionary degeneration of the eye can be found in virtually every vertebrate group, including fishes (the blind cave fish Astyanax), amphibians (Caeciliidae), reptiles (blind snakes Typhlopidae) and mammals (moles, mole rats). (Not surprisingly, no species with degenerate eyes have ever been found among birds, which rely heavily on visual orientation during flight.) These "naturally blind" animals are of immense interest to evolutionary biologists, because investigations into their eye structure and development can provide a better understanding of the evolutionary mechanisms operating during the atrophy of an organ that has become obsolete.

A great evolutionary biologist T. Dobzhansky once said that nothing in biology makes sense except in the light of evolution. This is especially true when a complex structure like the eye is considered. When various ocular phenotypes encountered in naturally blind animals are investigated, new insights into the stability, plasticity and the level of redundancy in the gene regulatory networks governing the eye development will emerge. Thus, the significance of such evolutionary developmental studies extends far beyond advancing the knowledge of vertebrate evolution. Some of the developmental phenotypes observed in the eyes of naturally blind vertebrates challenge the current views of the eye development, and further investigations on these unusual models can add significantly to our current understanding of the process of "normal" eye development.

Despite a number of investigations in recent years (Jeffery, 2001; Yamamoto and Jeffery, 2004; Avivi et al., 2001; Hough et al., 2002; Quilliam, 1966), very little is known about the developmental mechanisms responsible for eye degeneration in darkness-adapted vertebrate species. In fact, only very few recent microanatomical studies on the eye structure of blind mammals exist; most reports available are over 80 years old. To my knowledge, only two studies
of embryonic eye development in mammalian species with reduced eyes have been performed so far, while nothing has been reported on the molecular and genetic aspects of this process. In short, a significant gap in our knowledge of this subject exists, which needs to be urgently addressed. With novel, high-power molecular tools now available, it is now possible to re-examine these important evolutionary models. In this work, I attempted to further the current limited understanding of the degenerate evolution of the ocular structures in mammals by investigating the morphological features, embryonic and postnatal development, and molecular control of eye formation in two species of subterranean rodents, the naked mole rat (*Heterocephalus glaber*) and the Cape dune mole rat (*Bathyergus suillus*). It is hoped that this work will help establish these two animals as novel model organisms for evolutionary developmental biology.

1.1 Structure of the vertebrate eye

The basic organisation of the eye is essentially the same in all sighted vertebrates (reviewed by Ross *et al.*, 2003; Stevens and Lowe, 1997). The eye is a round, fluid-filled organ, whose function is to focus the light reflected from the objects in the environment onto the photoreceptors of the retina, which convert the visual information into a series of electrical impulses and send the signal to the brain via the optic nerve. To successfully perform this function, parts of the eye must be transparent, which places an unusual constraint on the properties of tissues that lie directly in the path of light (cornea, lens, inner retina).

The surface of the eyeball is covered with sclera, which is continuous with the transparent cornea in the front of the eye. The sclera is composed of dense fibrous connective tissue, which protects the eye and helps maintain its shape. The major functions of the cornea are light transmission and refraction of incoming light. The lens, positioned behind the cornea, is a biconvex transparent structure that is responsible for the focussing of incoming light onto the photoreceptors in the retina. The lens separates the two fluid-filled chambers (the anterior and the posterior chamber) in the front of the eye from the bulk of the eye, the vitreous chamber. This latter is filled up with a transparent homogenous gel, the vitreous body, which is responsible for maintaining the shape of the eyeball.

The anterior and posterior chambers of the eye are filled with fluid which provides nutrients to the transparent avascular lens and cornea. This fluid (the aqueous humour) is secreted by the ciliary body, a muscular ring-like structure that is positioned inside the posterior chamber at the level of the lens. The lens is attached to the ciliary body via fibres collectively known as the
zonula fibres; contraction of the ciliary muscle is responsible for the changes in the curvature of the lens (the lens accommodation), which allows the light rays to be focussed on the retina.

The iris is a highly pigmented contractile diaphragm that extends in front of the lens and forms the pupil. The main function of the iris is the regulation of the amount of light reaching the retina by contracting/expanding. The anterior chamber fluid circulates through the anterior chamber and then is reabsorbed back into the bloodstream via the trabecular meshwork, a structure positioned at the junction between the front surface of the iris and the inner surface of the cornea.

Light perception and preliminary image processing are functions of the retina, a multilayered nerve tissue lining the posterior hemisphere of the eye and composed of light-sensitive photoreceptor cells, interneurons and supporting glial cells. A single cell layer of melanin-containing epithelial cells, the retinal pigmented epithelium (RPE), lines the posterior surface of the retina. One function of the RPE is to absorb scattered and reflected light within the eye, thus preventing glare and ensuring that a crisp, background-free image is formed on the retina.

1.1.1 Structure of the lens

The vertebrate lens is a transparent elliptical organ containing cuboidal lens epithelial cells and long enucleated cells called lens fibres. The entire organ is contained within a fibrous lens capsule, which is secreted mainly by the epithelial cells (Ross et al., 2003). The epithelial cells extend to the equator of the lens where they divide; some of their daughter cells contribute to the maintenance and growth of the lens epithelium, while others elongate, differentiate and move posteriorly to give rise to new lens fibres. The process of new fibre formation continues, albeit at a slow rate, throughout the life of an animal.

Most of the bulk of the lens is composed of the lens fibres, which are long, strap-like or spindle-shaped cells, hexagonal in cross-section, and tightly packed together (Bron et al., 1997). The tips of the fibres join to those of other fibres forming lens sutures; as more fibres are added during the growth of the lens, the suture arrangement becomes more and more complex (Figure 1-2; Kuszac, 1984). The fibre cells are joined to their neighbours by gap junctions to facilitate nutrient exchange within this avascular and largely metabolically
Figure 1-1: Diagram of the cross-section through a human eye (from http://www.medhelp.org/NIHlib/GF-17.html).

Figure 1-2: Drawing of a cutaway view of the adult lens, showing the fibre arrangement and the position of the embryonic lens nucleus (From Kessel and Kardon, 1979).
quiescent structure. Another type of membrane specialization, unique to the lens fibres, are balland-socket junctions, which form when a membrane protrusion from one is encapsulated into an indentation in the membrane of the neighbouring cell (Kessel and Kardon, 1979; Kuszać, 1984). These junctions are thought to allow maintenance of the fibre order, which is essential for lens transparency, while allowing a limited degree of fibre sliding and flexing of the whole lens structure, which is essential for accommodation (Bron et al., 1997). Nuclei and all other organelles of the lens fibre cells are degraded by an apoptotic-like mechanism during development to ensure the transparency of the fibres (Bassnett and Mataic, 1997; Wride and Sanders, 1998). The lens fibre cytoplasm contains up to 30% protein, a large proportion of which is made up of lens specific proteins – crystallins.

1.1.2 Structure of the cornea

Besides letting the light through, cornea also serves to protect the eye surface, therefore this structure must combine transparency with mechanical strength. The cornea consists of three cellular and two acellular layers. The anterior surface of the cornea (facing the outside of the eye) is lined by 5-6 cell layers thick stratified non-keratinized corneal epithelium. This epithelial layer protects the cornea from physical damage, while allowing light to pass through. The replacement for the cells that are sloughed off from the surface of the corneal epithelium is provided by a population of stem cells located in the basal epithelium at the corneoscleral junction (Schlötzer-Schreiber and Kruse, 2005). The basal layer of the corneal epithelium rests on Bowman’s membrane, which separates it from the corneal stroma.

The corneal stroma is the thickest corneal layer. It is composed of specialized mesenchymal cells called keratocytes, and complex fibrous extracellular matrix that is secreted by these cells. The matrix contains collagens type I, V and VI and proteoglycans (keratocan, lumican) (Kao and Liu, 2002); collagen I forms fibres of uniform diameter that are arranged in parallel arrays. This uniformity of the collagen fibre arrangement is essential for the maintenance of the corneal transparency (Meek and Fullwood, 2001; Meek and Boot, 2004). The inner surface of the stroma is bounded by Descemét’s membrane, on which rests the single layered corneal endothelium. This layer is composed of polygonal squamous cells, which are joined to each other by complex arrays of gap, adherens and tight junctions (Joyce, 2003). The corneal endothelium separates the stroma from the aqueous humour of the anterior chamber. The major function of this corneal layer is maintaining of the corneal transparency by regulating the amount of fluid absorbed into the stroma (Joyce, 2003; Bonanno, 2003).
1.1.3 Structure of the ciliary body

The ciliary body extends from the root of the iris and connects to the equatorial region of the lens via zonula fibres. The ciliary body consists of two epithelial layers, the outer of which (facing the sclera) is highly pigmented, while the inner (facing the inside of the eye) is non-pigmented (Ross et al., 2003; Beebe, 1986). In most vertebrates, this structure is folded into radial processes (the ciliary processes), which serve to increase the epithelial surface (Beebe, 1986). The inner epithelium has all the characteristics of a secretory epithelium, including complex intercellular junctions, extensive membrane folding, well-developed endoplasmic reticulum and Golgi complex, and multiple Na/K channels positioned in the lateral plasma membrane (Bishop et al., 2002). The highly vascularized and muscular stroma of the ciliary body is positioned between these two epithelia. The network of capillaries underlies the inner epithelium and extends into the ciliary processes (Ross et al., 2003; Beebe, 1986).

The ciliary body performs several functions crucial for the maintenance of the eye transparency and visual acuity. Its primary function is the secretion of the aqueous humour, which provides nutrients to the avascular lens and cornea. This function is accomplished by the inner ciliary epithelium, which is selectively permeable to small molecules, water, ions and some proteins, but prevents the passage of blood cells from the capillaries in the stroma of the ciliary body. The smooth muscle of the ciliary body allows it to contract or expand, causing the shape changes of the lens involved in accommodation.

1.2 Evolutionary adaptation of the eye to life in constant darkness

Environments such as caves or burrows, where conditions of constant darkness prevail, pose a unique set of challenges to their inhabitants. One of the characteristic features of cave-dwelling or subterranean animals is loss or reduction of ability to perceive light (Poulson and White, 1969; Nevo, 1979). The size of the eye in such animals is often very small compared to their closest sighted relatives, and can be completely covered by skin (Nevo, 1979). Many of the internal ocular structures are malformed, abnormal or absent. Interestingly, the size of the eye and the degree of structural eye degeneration do not necessarily correlate, though most of the really minute eyes also demonstrate severe structural abnormalities.

An additional point is that “degenerate” eyes of the darkness-adapted vertebrates are not all structurally alike. Examination of the reports on histological features of such eyes reveals that at least three general phenotypic categories exist, each with its own unique features. The smallest eyes, completely covered by skin, are seen in Notoryctes (marsupial moles), in the golden moles
Chrysochloris and Eremita (Chrysochloridae; Insectivora) and in the blind cave fish Astyanax mexicanus. The eyes of Notoryctes are vestigial, represented by a mere “hollow ball of pigment”, which is thicker anteriorly and surrounds what appears to be poorly differentiated neural retina; four retinal layers- inner nuclear, inner plexiform, outer nuclear and outer plexiform- can be distinguished (Sweet 1906; Vaughan, 1978). No signs of lens, vitreous body or any anterior chamber structures can be seen in this eye (Sweet, 1906). The eyes of Eremita (Eremita) granti consist of a mass of disorganised cells representing the lens, surrounded by a differentiated retina; no vitreous chamber, cornea, iris or ciliary body can be identified (Gubbay, 1956). The eyes of the cave fish are severely reduced, sunk into the orbit and covered with skin. Histological analysis of the eye structure reveals that the neural retina is present, but it is disorganised and photoreceptors do not differentiate. A small, disorganised lens persists in the eye, but the iris, cornea and ciliary body are absent or rudimentary (Jeffery, 2001). In summary, the eyes of all of these animals are minute and lack vitreous and aqueous chambers, as well as anterior chamber structures such as iris, ciliary body and corneal endothelium.

A unique ocular phenotype is observed in the blind mole rat Spalax ehrenbergi. The eyes of this animal are very small and covered by skin, but unlike the more abnormal eyes discussed above, contain well-differentiated retina and vitreous body. The lens is very small, undifferentiated and seems to undergo cell death, while both the pupil and the anterior chamber of this animal’s eye are completely obliterated by an overgrowth of highly pigmented iris-ciliary body complex (Sanyal et al., 1990).

Finally, some of the subterranean mammals (Bathyergidae, Talpa europaea, Rhyzomyidae) have small eyes that nevertheless are open to the external environment (many have functional eyelids), and exhibit internal architecture more typical of sighted mammalian species, including the iris, ciliary body, anterior and posterior chambers and the retina with clearly distinguishable layers (Quilliam, 1966; Eloff, 1958; Cei, 1946A, B). The lens of the common mole Talpa europaea consists of irregularly shaped nucleated cells, which cannot be called true fibres (Quilliam, 1966), while the lens of Bathyergidae and Rhyzomyidae was reported to have essentially normal structure (Eloff, 1958; Cei, 1946A, B).

The selective driving forces behind the reduction of eyes in animals in subterranean and cave habitats remain poorly understood, despite having been much debated since the time of Charles Darwin. Two theories for the mechanism of this reduction have been proposed (reviewed by Jeffery, 2005). The neutral mutation (or stochastic) hypothesis states that the eye loss results from accumulation of essentially random mutations in the genes important for the eye structure,
as the result of relaxed selective pressure on these genes. An alternative (adaptation) hypothesis, postulates that the loss of the eye can be in some way advantageous to the animals, and thus is selected for. For example, maintaining of the neural network associated with the eye has a high metabolic cost to the animal, so minimizing the size of obsolete neural tissues that are no longer used for visual perception would be advantageous (Nevo, 1998). Alternatively, a single gene may have a function in other tissues/organs besides the eyes; this latter function may offer a survival advantage in the cave/burrow environment and therefore be selected for and amplified at the expense of its role in the eye.

Two prominent researchers in the field of regressive eye evolution, each working with a different animal, seem to agree that eye regression is adaptive; however, different explanations for the adaptive value of the eye loss are offered (Jeffery, 2005; Nevo, 1998). Nevo points out that in the blind mole rat, the neural structures associated with the eye (neural retina, superior colliculus, visual cortex) are all reduced by 90-99%. He argues that the reason for this is metabolic economy, neural tissue being one of the most energetically expensive (Nevo, 1998). On the other hand, Jeffery suggests that conservation of energy is not the main cause for the eye regression in the blind cave fish Astyanax, because the processes of cell division and apoptosis continue simultaneously in this eye well into late larval development, presumably at unnecessary energy cost to the animal (Jeffery 2005; Soares et al., 2004; Strickler et al., 2002). A recent work from the same laboratory has demonstrated that the expression of sonic hedgehog (shh), a midline gene involved in the placement of the two eyefields in the early embryo (see next section for detail), is expanded in Astyanax embryos, causing smaller eye primordia to form (Yamamoto et al., 2004). Shh, like many developmentally important genes, is pleiotropic; that is, it has multiple functions in the embryonic development, also being expressed in the developing teeth, craniofacial structures and tastebuds, which are all enlarged in the cave fish (Jeffery, 2005; Jeffery, 2001). Jeffery proposes that expansion of the head expression domain of shh was selected for because of this gene’s important function in the development of these organs, and the consequent reduction of the eyes was a neutral, non-disadvantageous side effect.

Currently, there is not enough experimental evidence to resolve which of the above hypotheses, or combination thereof, is applicable to the eye degeneration in darkness-adapted animals. Certain constraints on evolutionary changes are clearly imposed by the sequence of gene and tissue interactions operating during development (Gould, 2002; Wolpert, 1998). Therefore, to understand the evolutionary processes responsible for eye degeneration, it is essential to study the embryonic development of the eye and its genetic regulation. Before abnormal development
of the regressive eye can be discussed, the basic process of vertebrate eye development, based on mammalian and chick models, is reviewed.

1.3 Vertebrate eye development

The basic process of eye development is similar in all vertebrates and has been extensively reviewed (Graw, 1996; Jean et al., 1998; Chow and Lang, 2001; Oliver and Gruss, 1997; Kondoh, 2002). The optic cup forms as a lateral outgrowth of the neuroectoderm of the diencephalon (forebrain), which enlarges to become the optic vesicle. When the optic vesicle contacts the overlying surface ectoderm, an exchange of inductive signals between these tissues is thought to take place, which results in their coordinated invagination to form the lens vesicle and the optic cup (Chow and Lang, 2001). A signal from the distal portion of the optic vesicle induces the part of the overlying epithelium in contact with it to thicken and form the lens placode, which in turn, promotes the invagination of the optic vesicle to form the optic cup (Kondoh, 2002). Continued signalling from the early lens vesicle is also essential for the proper development of the retina, as demonstrated by experiments where the lens was surgically removed (Coulombre and Coulombre, 1964). Invagination of the optic vesicle begins at its ventral side and also involves the stalk that joins the optic cup to the neural tube. The ventrally most part of the vesicle undergoes vigorous proliferation, and this results in the extension of its nasal and temporal halves until they meet along the central midline of the vesicle and form the choroid fissure (Kondoh, 2002). The optic cup at this point consists of two layers of apposing epithelia, the inner one of which will give rise to the neural retina, while the outer layer differentiates to form the RPE. Interactions of the optic cup with the surrounding periocular mesenchyme are essential for proper differentiation of the RPE (Fuhrmann et al., 2000). The optic stalk later differentiates to form the glial cells of the optic nerve.

The lens vesicle formed from the lens placode is spherical and initially hollow, but soon becomes filled by the primary lens fibres, which form by elongation of the epithelial cells located at the posterior of the lens vesicle. Later the tip of the optic cup differentiates to form the ciliary body and iris; both layers of the optic cup are incorporated into these structures. Numerous experimental studies have demonstrated that the lens serves an essential role in the morphogenesis of the anterior eye structures (cornea, ciliary body and iris), which begin to form after the primary lens fibres have elongated. Classical experiments on the embryonic chick eyes, involving surgical removal of the lens (Coulombre and Coulombre, 1957) or implantation of an extra lens (Geniz-Galvez, 1966) demonstrated the crucial importance of this ocular structure in
Figure 1-3: Diagram of mouse eye development (modified from http://dragon.zoo.utoronto.ca/~B03T0801B/eye.html and Cvekl and Tamm, 2004). Abbreviations: LV-lens vesicle; Re-retina; PE-pigmented epithelium; Me-mesenchymal cells; SE-surface ectoderm; EF-choroidal fissure; HA-hyaloid artery; AC-anterior chamber; CEn-corneal endothelium; CS-corneal stroma; CEp-corneal epithelium; SCB-ciliary body stroma; Sir-iris stroma.
the morphogenesis of the pupillary margin. While no details of the histological examination of the ciliary body and iris were reported, the absence of the pupillary margin in the former, and the presence of the supernumerary pupil in the latter case suggests that the lens is an inducer of the iris and the ciliary body formation. When lens cells were ablated during early embryogenesis of the mouse by expressing diphtheria toxin from lens-specific αB or γ-crystallin promoter the lens, ciliary body and the corneal endothelium are either not formed or severely abnormal (Harrington et al., 1991; Klein et al., 1992; Breitman et al., 1989). Recently, Thut and colleagues co-cultured chick and mouse lenses of different ages together with mouse optic cups, and used in situ hybridization for ciliary body-specific markers to demonstrate that lens is the source of a diffusible signal(s) that induces ciliary body specific gene expression at the site of contact between the ectopic lens and the optic cup (Thut et al., 2001).

The three layers of the cornea have different embryological origins: the epithelium is derived from the surface ectoderm, while the stroma and the endothelium are formed by cells of the periocular mesenchyme, which originate in the neural crest and migrate into the space between the lens and the surface ectoderm (Johnston et al., 1979; Cvekl and Tamm, 2004; Gould et al., 2004). Signalling from the lens is also required for the proper morphogenesis of the cornea. In addition to the experiments described in the paragraph above, strong support for this theory comes from experiment by Beebe and Coats (Beebe and Coats, 2000). These authors surgically removed chick lenses from the embryonic eye, and followed the process of corneal differentiation using molecular markers specific for the corneal stroma and endothelium (N-cadherin). The results of these experiments clearly demonstrate that lens epithelium is the source of signal(s) essential for the formation of the corneal endothelium and for the proper differentiation of the corneal stroma (Beebe and Coats, 2000).

1.3.1 Development of the lens

Once the lens vesicle is formed, which in the mouse occurs at E11.0, the process of lens fibre differentiation is initiated (Pei and Rhodin, 1970). The cells in the posterior part of the lens vesicle elongate and fill up the lumen of the vesicle, forming what will later become the embryonic lens nucleus (Francis et al., 1999). In the mouse, elongation of these primary lens fibres is completed by E13.0-E13.5 (Pei and Rhodin, 1970; Theiler et al., 1989). Further growth of the lens is accomplished by proliferation and differentiation of the epithelial cells positioned at the lens equator, the fibres formed in this way are called the secondary lens fibres. New secondary fibre cells form concentric layers around the embryonic nucleus, eventually compacting it, separating it from the lens epithelium and the capsule, and pushing it into the
centre of the lens. These two types of lens fibre cells are virtually identical in their morphology and physiology (Francis et al., 1999; McAvoy et al., 1999; Piatigorsky, 1981).

Differentiation of the lens fibres involves denucleation and loss of organelles, formation of a specialized type of gap junction, and synthesis and accumulation of large number of lens-specific proteins, crystallins. Crystallins (discussed in detail in section 1.5 below) are specialized proteins that form high-order arrangements at the molecular level, making the lens cytoplasm transparent. The transparency of the lens is further ensured by removal of any organelles that could cause light scattering (Appleby and Modak, 1977; Bassnett and Beebe, 1992; Bassnett and Mataic, 1997; Wride and Sanders, 1998). Nuclear degradation in the central lens fibres begins at E15.5 (embryonic day 15.5) in the mouse (Vrensen et al., 1991) and by E8 in the chick (Appleby and Modak, 1977). Fascinatingly, this process of terminal cell differentiation is strongly reminiscent of apoptosis, in fact, at least one member of caspase family is also involved (Ishizaki et al., 1998). The denucleation process is largely completed by P1, so that at birth, no nucleated fibres are found in the centre of the lens (Vrensen et al., 1991). Since removal of mitochondria makes fibre cells metabolically inert, the highly specialized and complex system of gap junctions is established to allow these cells to communicate with the lens epithelium, and aqueous and vitreous humour.

1.3.2 Development of the cornea

As described above, the corneal epithelium is formed from the surface ectoderm overlying the lens, while the corneal stroma and endothelium are essentially neural crest-derived. In the mouse, the ingestion of the periocular mesenchyme into the space between the lens epithelium and the future corneal epithelium begins at E12-E13 (Pei and Rhodin, 1970). It appears that the substrate onto which the cells migrate, the acellular primary stroma, is secreted by the corneal epithelium. Two waves of cell ingestion occur in the chick embryos, while only one has been documented in the mouse (Kidson et al., 1999). Once ingressed, the cells closest to the lens become flattened, contact each other and form adherens and tight junctions. In the mouse, the flattening of the cells is completed by E17.5 (Pei and Rhodin, 1970; Kidson et al., 1999).

Several lines of evidence suggest that formation of the corneal endothelium is essential for the proper morphogenesis of the anterior chamber. Ultrastructural studies of the process of corneal development suggest that as the future corneal endothelial cells are being joined by tight junctions, they withdraw from the surface of the lens, creating a space (Kidson et al., 1999). In
mutant FoxC1-/- and Tgβ2-/- mice and in transgenic mice expressing Tgfα in the lens cells, the corneal endothelium fails to develop, and the fibrous constituents of the corneal stroma appear to adhere to the surface of the lens preventing the formation of the anterior chamber (Kidson et al., 1999; Reneker et al., 2000; Saika et al., 2001). It has been suggested that corneal endothelium serves as a mechanical barrier preventing the sticky corneal stroma from adhering to the surface of the lens (Kidson et al., 1999; Reneker et al., 2000).

1.3.3 Development of the ciliary body

The two epithelial layers of the ciliary body are derived from the two layers of the sub-distal region of the optic cup, while the ciliary muscle and the vascular stroma of the ciliary body (except the vascular endothelium) differentiates from the pericocular mesenchyme, most of which is neural crest-derived (Johnston et al., 1979; Gage et al., 2005). The distal-most ring of tissue at the tip of the optic cup forms the iris. The first morphological indications of the ciliary body development are visible at E14.5 in the mouse (Napier and Kidson, 2005), and by about E4 in the chick. The clear boundary between the neural retina and the nonpigmented ciliary epithelium is established by E15.5, when the differentiation of the retina commences (Napier and Kidson, 2005; Monaghan et al., 1991). By E16.5, the iris and the ciliary body primordia become clearly distinguishable. Morphogenesis of the ciliary folds starts at around birth and is completed by P7 (Napier and Kidson, 2005). It was suggested that the morphogenesis of the ciliary folds is induced by the underlying capillaries (Beebe, 1986; Napier and Kidson, 2005).

1.4 Development of a “degenerate” eye: how much do we understand?

Only a few studies addressing the embryological basis of the eye regression have so far been performed. Histological investigation of the embryonic eye development were conducted on the blind mole rat Spalax ehrenbergi (Sanyal et al., 1990), the cave fish Astyanax mexicanus (reviewed in Jeffery, 2001) and the mole Eremialpa granti (Gubbay, 1956). In all these animals, the initial formation of the optic cup and lens vesicle proceeds normally, even though the optic cup is somewhat smaller in the cave fish compared to the surface-dwelling subspecies. However, the growth of both the optic vesicle and the lens primordium are soon arrested, and the posterior cells of the lens vesicle fail to elongate and to fill the lumen of the vesicle. The lens of all these animals eventually undergoes atrophy, so that only a small rudiment of irregular, necrotic cells represents the lens in the adult. No corneal endothelium, corneal stroma, anterior chamber, iris or ciliary body development is seen in the eye of Astyanax or Eremialpa granti (Jeffery, 2005; Gubbay, 1956). In Spalax, however, a surprisingly different phenotype is observed: the tip of the
optic cup undergoes a spurt of dramatic growth, completely obliterating the anterior chamber and preventing the formation of the corneal endothelium. No ciliary body or iris differentiation is seen in this eye; instead, a highly pigmented, extensively folded structure occupies the entire space between the anterior surface of the lens and the cornea (Sanyal et al., 1990).

What does this sequence of developmental events tell us? As discussed above, several studies have demonstrated that the lens is a crucial regulator of the development of the anterior chamber structures. Therefore, the early apoptosis of the lens is likely to be the reason for the subsequent failure of the development of the anterior chamber structures. The experimental confirmation of this hypothesis comes from the lens transplantation experiments conducted by Yamamoto and Jeffery (2000), whereby the lens of the surface-dwelling Astyanax form with normal eyes was transplanted into the optic cup of the microphthalmic cave form; the subsequent eye development of the cave fish eye was restored to resemble that of the surface fish. Abnormal development of the lens vesicle as the results of alterations in the expression patterns or levels of expression of upstream developmental genes is likely to be the primary cause of the reduced eye phenotype in microphthalmic mammalian species. However, the eye phenotype seen in Spalax apparently defies this kind of explanation, because the tip of the optic cup seems to undergo somewhat ciliary body-like development, despite the profound degeneration of the lens. Unfortunately, no studies have been conducted so far to address this question in Spalax. Indeed, virtually no attention has been devoted to developmental studies of mammalian species with reduced eye.

Since lens abnormalities seem to be the hallmark of the natural eye degeneration, and the lens is known to play such a crucial role in eye morphogenesis, several studies investigated the state of the lens differentiation by examining expression of the major lens fibre markers (crystallins) in adult and embryonic lenses of naturally blind animals. The results and implications of these experiments are discussed in the following section, after a brief review of structure, function and expression of mammalian crystallins.

1.5 Lens crystallins: structure, function, regulation and evolutionary diversification

Most of the dry mass of the lens is made up of lens-specific proteins - crystallins. These proteins are extremely abundant in the lens, and constitute about 90% of its total soluble protein (Piatigorsky, 1992). In vertebrates, there are four major crystallin families: α, β, γ and δ; members of the first two families are found throughout the vertebrate taxa, however, γ-crystallins are absent from the lenses of birds and reptiles, they are functionally substituted for by
structurally unrelated δ-crystallins (Piatigorsky, 1984; Treton et al., 1984). Mammalian, fish and amphibian lenses have γ-crystallins as their major structural lens proteins (Platigorsky, 2003; Graw 1997).

Alpha-crystallins are very large oligomers, consisting of around 40 copies of two types of sequence-related subunits (A and B). Each of these two subunits is encoded by a separate gene. The genes are located on different chromosomes (Graw, 1997). Sequence analysis of α-crystallins revealed that they are closely related to the small heat shock proteins (Ingolia and Craig, 1982; van den Heuvel et al., 1985). Both of these types of crystallins are expressed at high levels throughout the lens, in the lens epithelium as well as in the fibres (McAvoy, 1978A, B). αA-crystallin expression is lens-specific, while αB-crystallin is also found in significant amounts in heart, brain, skeletal muscle, lung, thymus and kidney (Dubin et al., 1989). In the lens, α-crystallin functions as a molecular chaperone by binding denatured proteins and keeping them in solution (Horwitz, 1992; Augusteyn, 2004). Because there is no protein turnover in the mature lens, all of the lens proteins have to last for a lifetime. Changes in the protein structure over time can result in protein precipitation; this process is the major cause of age-related cataracts (Brown and Bron, 1996). Therefore, the function of α-crystallins is crucially important for maintaining the transparency of the lens.

β and γ-crystallins are members of a related β/γ-crystallin superfamily, characterized by the presence of the so-called Greek key motifs. The Greek key motifs are stretches of amino acids folded into symmetrical, antiparallel β-sheets, resembling the geometrical pattern associated with ancient Greek art (Blundell et al., 1981; Wistow et al., 1983). Individual β/γ-crystallin polypeptides are composed of two domains, each of which contains two Greek key motifs; the major difference between the two classes of crystallins is the structure of the peptide connecting the domains (reviewed in Lubsen et al., 1988; Graw, 1997). β-crystallins contain a relatively straight connecting peptide, while in γ-crystallins, amino acid composition of the peptide causes it to turn sharply as the protein is folded into the tertiary structure. As the result, all β-crystallins can form intermolecular associations and are therefore found as oligomers, while in γ-crystallins the two domains associate within the same molecule, and so these proteins are found as monomers (Graw, 1997). These two groups of crystallins are immunologically distinct.

Beta- and γ-crystallins are major structural lens proteins. Their ability to form regular molecular arrangements in solution at high protein concentrations minimizes light scattering and ensures uniform passage of light through the lens, making this organ transparent. Six β-crystallin genes are found in mouse, human, rat and chicken (Graw, 1997). These genes are expressed
exclusively in the lens fibres, their expression starts as soon as the lens fibre elongation is initiated (E13.0 in the rat, McAvoy, 1978B). Translated proteins preferentially form heteromeric associations; oligomers composed of two, three, four and eight polypeptide chains are commonly found (Graw, 1997).

There are six individual gamma-crystallin genes in most mammals, arranged in a cluster on the same chromosome, with each gene having its own promoter (Breitman et al., 1984; Lok et al., 1984; Meakin et al., 1987; Peek et al., 1990). In the rat and the mouse, all these genes are transcribed during development, but in human, only four gamma-crystallin genes are transcriptionally active (Meakin et al., 1985; Meakin et al., 1987; Siezen et al., 1988). All six genes are very similar in sequence, as are their protein products, which show 78% to 87% homology in amino acid sequence (Lok et al., 1984; Breitman et al., 1984). Individual γ-crystallins can not be distinguished immunologically. It is very hard to distinguish between individual gamma-crystallins by RNA hybridization, though it can be accomplished if probes corresponding to the most divergent third structural motif of the protein, and stringent hybridization conditions are used (Murer-Orlando et al., 1987).

The γ-crystallin genes can be divided into two groups (crystallins γA/B/C and γD/E/F) based on their exon 2 sequences, which correspond to the third structural motif of the protein (Breitman et al., 1984). These two groups exhibit different physical properties. Crystallins γD/E/F have relatively high phase separation temperatures, which means that they tend to form opaque precipitates at high protein concentrations and low temperatures (Siezen et al., 1988). As many warm-blooded species cannot efficiently regulate their body temperature for a few days after birth, cryoprecipitation of these proteins may account for the formation of reversible "cold cataracts" observed in the neonate rat lenses (Siezen et al., 1988).

Gamma-crystallins are expressed from E13.0 onwards in mouse lens fibre cells, but not in the epithelial cells (van Leen et al., 1987a). A number of transcription factors involved in the regulation of γ-crystallin transcription have been identified. Pax6 and Six3 are known to downregulate γ-crystallin transcription (Chauhan, 2002; Lengler, 2001; Yang, 2004), while Sox1 (Kamachi, 1995; Nishiguchi, 1998), Prox1 (Lengler, 2001) and Maf (Ring et al., 2000; Yang, 2004) serve as transcriptional activators. Retinoic acid responsive elements are also found in the γ-crystallin promoters (Tini et al., 1993; Tini et al., 1995). Combination of insulin and FGF can induce lens fibre differentiation from lens epithelial explants and dramatically stimulates γ-crystallin synthesis, though the exact mechanism by which these factors act on γ-crystallin promoters is still unknown (Chamberlain et al., 1991; Civil et al., 2000; Klok et al., 1998).
Interestingly, studies on rat and mouse lenses demonstrated that there is a dramatic variation in the spatial and temporal distribution of the six γ-crystallin mRNA transcripts and proteins during lens development (Siezen et al., 1988; van Leen et al., 1987a; van Leen et al., 1987b; Aarts et al., 1989; Goring et al., 1992). The three members of the γD/E/F subclass appear to be preferentially synthesized in early development (during embryonic life and up to 60 days postnatally), while γA/B/C crystallins predominate in the mature lens (Siezen et al., 1988). Moreover, the protein levels do not appear to correlate very well with the levels of the corresponding mRNAs, suggesting that gamma-crystallin translation is also differentially regulated (Siezen et al., 1988).

Gamma-crystallins are extremely stable proteins, as they have to last for years, sometimes decades. Low-level degradation of these proteins does occur as part of the normal ageing process, and has been demonstrated in human and rat lenses (Srivastava et al., 1992; Srivastava and Srivastava, 1998). However, increased levels of protein degradation are signs of pathology, and have been shown to be associated with cataract formation (Brown and Bron, 1996).

Synthesis of the γ-crystallins continues throughout the life of an animal in the newly formed fibres at the periphery of the lens. As soon as these fibres mature and lose their nuclei, transcription is no longer possible (Shestopalov and Bassnett, 1999). Continued protein synthesis can still occur, as long as the template mRNA is present. Persistence of gamma-crystallin RNA has been documented in the rat and mouse lenses until 3 month after birth (Goring et al., 1992; van Leen et al., 1987b). It is not clear at present whether γ-crystallin mRNA continues to be detected in the lens because of its extreme stability, or simply because it is a very abundant transcript. In the chick lens, the rate of δ-crystallin mRNA degradation is exactly the same as that of other lens mRNAs (Faulkner-Jones et al., 2003). However, stability of γ-crystallin transcripts can be controlled by a different mechanism. The rate of γ-crystallin mRNA degradation in the mammalian lenses has not been determined. A potent ribonuclease inhibitor was identified in lens fibre cells, and it is thought to contribute to the stability of gamma-crystallin mRNA (Ortwerth and Byrnes, 1971; Ortwerth and Byrnes, 1972).

1.5.1 Crystallin expression in mammals with reduced eyes

Given their almost exclusively lens-specific expression and function, it would be expected that crystallins are no longer found in the degenerate eyes of subterranean mammals. However, the results of the few studies addressing this question reveal that it is not the case. The small, poorly differentiated and nucleated lens cells of the adult common mole Talpa exhibit expression of α-,
β- and γ-crystallins within the appropriate lens compartments, as detected by
immunocytochemistry (Quax-Jeuk en et al., 1985). The lens of the blind mole rat Spalax lacks γ-
crystallin expression (as detected by immunocytochemistry (ICC) using an anti-mouse γ-
crystallin antibody), but still continues to express αA and αB crystallins (Hough et al., 2002;
Avivi et al., 2000; Quax-Jeuk en et al., 1985).

It appears that the lens differentiation can be arrested during the development of regressive eyes
at different time points; therefore one would expect different genetic/developmental mechanisms
to be responsible. Given the crucial importance of the lens in eye morphogenesis and significant
progress that has been made in understanding the transcription regulation of crystallins, it is
surprising that no further investigations were attempted to find out the molecular mechanisms
responsible for the absence of gamma-crystallins in Spalax. Therefore, further molecular
developmental studies are essential to understand the mechanisms controlling lens degeneration
in microphthalmic animals.

1.6 Molecules involved in the ciliary body formation

Compared to the significant advances in our understanding of the molecular regulation of the
lens development, little is known about the molecular mechanisms regulating ciliary body
development. Studies on ciliary body development to date have focussed largely on the
structural and cellular aspects of the process (Coulombre and Coulombre, 1957; Stroeva, 1967;
Bard and Ross, 1982a, b; Reichman and Beebe, 1992; Soules and Link, 2005; Napier and
Kidson, 2005). A handful of transcription factors with demonstrated functions and/or expression
during the development of the ciliary body have been identified, as well as a number of ciliary
body specific potential target genes; these are briefly reviewed below.

Two homeobox genes (Mx1 and Otx1) are known to be expressed during ciliary body
development. Optic cup-specific expression of Mx1 commences at E12.5, in the inner portion of
the optic cup tip. By E13.5 the borders of expression sharpen, and it becomes clear that the
signal is localized to the sub-apical cells of the optic cup, the very cells that form the ciliary
body. Mx1 expression reaches its maximum by E16.5, and then declines slowly until P7
(Monaghan et al., 1991; Zhao et al., 2002). Mx1 knockout mice show no abnormalities in the
ciliary body development; however, it is not clear whether morphogenesis of the ciliary
processes is affected, because these mice die at birth (Satokata and Maas, 1994). Otx1 expression
in the mouse eye is first detectable at E9.5 (Martinez-Morales et al., 2001). This gene is initially
expressed throughout the optic vesicle (Simeone et al., 1993; Martinez-Morales et al., 2001), but
by E12.5 becomes restricted to the pigmented and non-pigmented epithelia of the tip of the optic cup, the RPE, and the ventral optic stalk. As the differentiation of the tissues at the tip of the optic cup proceeds, it becomes clear that both the ciliary body and the iris express this gene (Martinez-Morales et al., 2001). This pattern of expression is maintained until at least P7 (Zhao et al., 2002). The chicken and dogfish homologues of Otx1 also demonstrate this expression pattern. Surprisingly, while the expression is seen in the epithelial layers of the forming ciliary body, it is absent from the mesenchyme that would form the ciliary stroma (Plouhinec et al., 2005). Otx1-deficient knockout mice demonstrate complete absence of the ciliary processes and thinning of the iris (Acampora et al., 1996).

Recently, Zhao et al. (2002) reported that Bmp signalling plays an essential role in ciliary body morphogenesis. Transgenic mice expressing the Bmp inhibitor noggin in a lens-specific fashion exhibited lack of ciliary process formation, abnormal differentiation of the ciliary body epithelia as ganglion cells, and thinning and disorganization of the iris epithelia. Bmp signalling appeared to activate or maintain Msx1 and Otx1 expression in the ciliary epithelia; when Bmp signalling was knocked down in these tissues, Msx1 and Otx1 expression also was downregulated. Bmp7 expression in the tip of the optic cup can be detected as early as E12.5 in the mouse (Hung et al., 2002), while Bmp4 expression is first seen in this region by E14.5 (Chang et al., 2001); Bmp7 is therefore a more likely candidate for the role in regulation of Msx1 and Otx1 during ciliary body (CB) development.

There is abundant evidence that the lens induces ciliary body differentiation by means of a functionally conserved diffusible signal, though the molecular identity of this signal is unknown (Coulombre and Coulombre, 1964; Genis-Galvez, 1966; Harrington et al., 1991; Klein et al., 1992; Breitman et al., 1989; Thut et al., 2001). Two downstream targets of this signal in the ciliary epithelium have however been discovered (Ptmb4 and Tgfb1i4). These are relevant to the work presented in this thesis and therefore are discussed in more detail below. Thut et al. (2001) remark that a variety of growth factors were tested for their ability to induce Ptmb4 and Tgfb1i4 transcription in the optic cup, including TGFβ1, TGFβ2, basic FGF, PDGF, TGFα and Bmp7, and none of them individually appeared to mimic the function of the postulated lens-derived signal. It is possible that the lens derived signal involves either combinations of these diffusible molecules, or unknown novel soluble factor(s) (Thut et al., 2001; Mu et al., 2003).

A LIM homeodomain class transcription factor Lmx1b plays a role in the development of the ciliary body and iris stroma. This molecule is expressed in the periocular mesenchyme of the mouse eye from about E10.5, and later - in the ocular tissues derived from these cells (ciliary
body and iris stroma, corneal stroma and endothelium). In transgenic mice lacking \textit{Lmx1b} expression, the prospective iris and ciliary body stromal cells fail to enter these structures and fail to undergo proper differentiation; ciliary body and iris hypoplasia and the absence of the ciliary processes result (Pressman \textit{et al.}, 2000).

Kubota \textit{et al.} (2004) identified a host of other ciliary specific molecules by using subtractive microarrays. Because posthatch chick tissue was used in the experiment, these genes are unlikely to be involved in the initial stages of CB morphogenesis, they are most probably downstream targets of transcription factors expressed earlier (Msx1 or Otx1). Thus while a range of CB-specific molecules and relevant loss-of-function phenotypes have been reported, there is little understanding of the transcriptional regulatory network underlying the morphogenesis of this structure. Clearly, continued efforts in this direction are essential to further our understanding of the molecular aspects of vertebrate eye development.

1.6.1 \textbf{Thymosin beta 4}

One of the genes that is upregulated in the tip of the optic cup by signals from the lens is Ptmb4, which encodes a small (43-44 amino acid) polypeptide thymosin \(\beta 4\) (Thut \textit{et al.}, 2001). The major intracellular function of this protein is binding of monomeric actin and inhibition of spontaneous actin polymerization (Sanders \textit{et al.}, 1992). Ptmb4 constitutes the main actin monomer buffering protein in many tissues (Safer and Nachmias, 1994; Huff \textit{et al.} 2001). It is thought that this cytoskeleton-modifying ability of Ptmb4 is responsible for the multitude of cellular processes that this molecule is known to regulate. For instance, Ptmb4 promotes cell migration during skin and corneal wound closure (Philp \textit{et al.}, 2004), increased cell motility during cancer metastasis (Wang \textit{et al.}, 2003; Cha \textit{et al.}, 2003; Kobayashi \textit{et al.}, 2002), differentiation, migration and adhesion of endothelial cells during angiogenesis (Grant \textit{et al.}, 1999; Malinda \textit{et al.}, 1997) and migration and differentiation of hair stem cells during hair follicle growth (Philp \textit{et al.}, 2004a, b). Ptmb4 interacts with actin monomers through the actin-binding motif LKKTET, which is positioned at the N-terminus of the thymosin polypeptide (Bubb, 2003).

Thymosin \(\beta 4\) also exhibits immunoregulatory (Oates \textit{et al.}, 1989) and anti-inflammatory properties (Goya \textit{et al.}, 1999; Girardi \textit{et al.}, 2003), which cannot be attributed to its actin-sequestering functions, because these effects were observed after the extracellular application of this protein (Bubb, 2003). In a recent report, it was demonstrated for the first time that Ptmb4 can be secreted and taken up by cells in culture (Bock-Marquette \textit{et al.}, 2004). Secreted
thymosin β4 was found in the conditioned medium of Cos1 cells transfected with thymosin β4 expression construct. However, Ptmb4 does not possess a signal sequence for secretion, and is extremely hydrophilic (and thus not likely to cross cellular membranes unassisted); the mechanism of its secretion and intracellular uptake is not understood at present (Al-Nedawi et al., 2004; Bock-Marquette et al., 2004).

Ptmb4 is expressed in a wide range of tissues during development. In the mouse, it is found in blood vessels and heart, consistent with its role in angiogenesis, as well as the central nervous system and peripheral ganglia (Gomez-Marquez et al., 1996; Carpenterio et al., 1995). A very similar pattern was reported in chick embryos, with the expression of this molecule detected in developing blood vessels and neurons, and also in somites, feather buds and in the mesenchyme of the outgrowing limb buds, suggesting its potential role in the morphogenesis of these structures (Dathe and Brand-Saberi, 2004). In zebrafish, Ptmb4 expression is also detected in various neuroepithelial derivatives (trigeminal ganglia, selected areas of hindbrain, Rohan-Beard neurons), in the trunk and tail mesoderm, in the pinched-off somites and later in muscle cells (Roth et al., 1999). In the eye, Ptmb4 was shown to be expressed at high levels in the tip of the optic cup (prospective ciliary body) in mouse (Thut et al., 2001), chick (Kubo et al., 2003; Thut et al., 2001) and zebrafish (Roth et al., 1999). In both chick and mouse, the expression of this gene (as detected by in situ RNA hybridization) is confined to the prospective ciliary zone, not iris or retina (Thut et al., 2001; Kubo et al., 2003). Moreover, only the non-pigmented inner layer of the ciliary epithelium showed Ptmb4 expression in fish and albino mice (Thut et al., 2001; Roth et al., 1999). Ptmb4 transcripts are first detected in the mouse embryonic eye at E12.5, and are maintained until at least P2 (later stages were not investigated) (Thut et al., 2001). In chick embryonic eyes, the temporal dynamics of this gene’s expression have not been reported, but Kubo et al. (2004), reports strong expression of this gene at E5. This very localized pattern of Ptmb4 expression in the eye suggests that this protein has a function in the ciliary body morphogenesis. However, this precise function has not yet been elucidated.

A single functional study has so far been performed to investigate the role of thymosin β4 in embryogenesis. Roth et al. (1999) injected Ptmb4 sense and antisense mRNA into zebrafish embryos at one cell stage. Injection of the sense mRNA did not appear to disturb normal development, apart from a mild effect of increased branching of neuronal tracts. However, knocking out Ptmb4 by injecting antisense RNA resulted in significant abnormalities in the development of the nervous system and somites, including severe defects or complete lack of neuronal tracts and absence of midbrain-hindbrain boundary, as well as irregular somites and abnormal fin and jaw muscles. Because neurons and somite cells undergo extensive cytoskeletal
re-arrangements during pathfinding or, in the case of somites, mesenchymal-epithelial transformation, the developmental defects observed in the absence of Ptmb4 could well be associated with the actin depolymerising function of this protein (Roth et al., 1999). It is of interest that in the above study effects of perturbation in Ptmb4 expression on eye morphogenesis were not reported.

1.6.2 Tgfb1i4

Experiments by Thut et al. (2001) demonstrated that the expression of Tgfb1i4, like that of Ptmb4, is induced in the prospective ciliary body region by a secreted lens-derived factor(s). Transforming growth factor (TGF) β1 induced transcript 4 (Tgfb1i4), also known as TSC-22 (Transforming growth factor β1 stimulated clone 22), is a leucine zipper-containing putative transcription factor that was initially isolated as a gene whose expression was induced by TGFβ1 (Shibanuma et al., 1992). In vitro studies demonstrated that Tgfb1i4 is a potent suppressor of cell proliferation. Downregulation of this gene promotes tumorigenesis (Shostak et al., 2003), while overexpression of Tgfb1i4 can downregulate cell proliferation and induce apoptosis in human breast cancer cells (Hino et al., 2000; Nakashiro et al., 1998) and various other carcinoma cell lines (Kester et al., 1997; Kawamata et al., 1998).

Expression pattern of Tgfb1i4 during chick and mouse embryogenesis has been determined (Dohrman et al., 2002; Dohrman et al., 1999; Kester et al., 2000). Northern blot analysis on human tissues revealed its presence in fetal brain, lung, heart, liver and kidney (Jay et al., 1996). Tgfb1i4 is found in the neural tube and the somitic mesoderm of E8.5 mouse embryo. Later in development, Tgfb1i4 is seen in the dorsal root and facial ganglia, as well as in the mesenchymal compartment of many tissues and organs, including the lung, kidney, intestine, tooth primordia, the limb bud, and in condensing hair follicles, as well as in the prospective cartilage throughout the body and the endothelium of the blood vessels (Dohrman et al., 1999; Kester et al., 2000).

In the chick embryo, Tgfb1i4 expression pattern is essentially analogous to that seen in the mouse (Dohrman et al., 2002). This expression pattern suggests that Tgfb1i4 may be involved in mediating developmentally important epithelial-mesenchymal interactions (Dohrman et al., 1999). During eye development, Tgfb1i4 transcripts can be detected in the tip of the optic cup from E12.5 until at least P2 (Thut et al., 2001). Like Ptmb4, Tgfb1i4 expression is excluded from the tip of the optic cup, which would give rise to the iris, and is confined to the non-pigmented epithelium of the prospective CB (Thut et al., 2001). Developmental expression of this gene in the chick embryonic eye has not been reported.
Very little is known about the function of Tgfb1i4 during vertebrate development, and no developmentally interesting target genes have been identified. Tgfb1i4 overexpression in *Xenopus* leads to inappropriate apoptosis, while morpholino-mediated downregulation of its expression causes inappropriate proliferation and impairs gastrulation movements (Hashiguchi *et al.*, 2004), suggesting that this gene may regulate cellular proliferation and cell death.

### 1.7 African mole rats: a novel model for regressive eye evolution?

Very little is currently known about evolutionary or developmental aspects of natural eye degeneration in subterranean mammals. To bridge this gap, the work described in this thesis was initiated. Of the many existing subterranean mammalian species, African mole rats were the most available. The results of a preliminary histological investigation conducted in this laboratory suggested that while the overall ocular architecture was preserved in these animals, interesting modifications of the anterior chamber structures, in particularly of the lens, were observed (Maughan-Brown, Honours Project, 2001). These preliminary results suggested that African mole rats may provide clues to the genetic processes underlying anterior eye development. Two mole rat species, the Cape dune mole rat and the naked mole rat, were chosen for a detailed histological, embryological, ultrastructural and molecular investigation of their eyes.

The African mole rats belong to the family Bathyergidae, a group of exclusively African burrowing rodents, with every member of the family being adapted to subterranean lifestyle. The family consists of five genera (*Bathyergus* - Cape dune mole rats, *Heterocephalus* - naked mole rats, *Georychus* - Cape mole rats or blesmols, *Heliophobius* - silvery mole rats and *Cryptomys* - common mole rats), which are all endemic to sub-Saharan Africa (Sherman *et al.*, 1991). A wide range of social behaviours is exhibited by the members of this family, from strictly solitary (*Bathyergus*) to what are arguably the most social of mammals (*Heterocephalus*). Among other unique features is the varying degrees of poikilothermy that characterize the various members of this family, which is most pronounced in naked mole rats, whose body temperature fluctuates with ambient temperature in a reptilian fashion (Bennett and Faulkes, 2000).

The genus *Bathyergus* is considered to be the most divergent of the group. Classical phylogenies separate it into a subfamily Bathyerginae. One of the two species comprising the genus, the Cape dune mole rat *Bathyergus suillus*, is the largest member of the family, with adult males reaching up to 1.8kg. *Bathyergus suillus* is a solitary species, endemic to South Africa, inhabiting the
coastal area of the southwestern Cape. *Bathyergus suillus* is a seasonal breeder, with the breeding season lasting from July to October. These animals are extremely aggressive towards conspecifics, and do not reproduce in captivity. Cape dune mole rat is one of the least-studies members of this family. No reports on its ocular structure and visual ability exist (Bennett and Faulkes, 2000).

The naked mole-rat (*Heterocephalus glaber*) is found in the hot, arid regions of Kenya, Somalia and Ethiopia. Large colonies, usually composed of 75 to 80 related individuals, live in extensive burrow systems that can be up to three kilometres long and occupy an area greater than 100 000 square metres (Sherman et al., 1991). Naked mole-rats are eusocial, exhibiting a "truly social" structure with a reproductive division of labour, cooperative care of young and an overlap of generations (Jarvis, 1981). Since most naked mole-rats within a colony do not reproduce, the definition of adulthood as reproductive maturity is difficult to apply. The youngest captive mole-rats reported to be reproductively active were 8-12 months old (Jarvis, 1991). Therefore, for the purposes of this study, "adults" were designated as being over 12 months old, regardless of their reproductive status. The peculiar social system of the naked mole-rats and their tendency to establish new colonies by fission results in extremely high levels of inbreeding both within a single colony and between the colonies of a particular geographic region. The inbreeding coefficient of these animals is the highest recorded among wild mammals, and is similar to that for the inbred strains of laboratory mice (Reeve et al., 1990). This genetic homogeneity provides an additional advantage for the use of this animal in developmental studies, because there would be less developmental variation due to genetic background.

When this study was initiated, very little was known about the Bathyergid eye. In recent years, these unusual animals have attracted attention of the scientific community; however, recent investigations were concerned mainly with the retinal architecture and the remaining function of the visual pathways in the eye of these animals (Cernuda-Cernuda et al., 2003; Peichl et al.; 2004; Hetling et al., 2005). No developmental studies have been recently conducted on this animal’s eye outside this laboratory.

At the beginning of this project, there was little information on the structure of the mole rat eye, and that information was old and somewhat contradictory (Cei, 1946b; Hill et al., 1957). For instance, Cei (1946b) described the mole-rat lens as poorly differentiated with primitive characteristics, while Hill et al. (1957) stated that the lens is differentiated; similarly, there was a disagreement between these two authors concerning the structure of the iris. Preliminary investigation in this laboratory reported a number of abnormalities in the lens, cornea and
anterior chamber, characteristic of these animals (Maughan-Brown, 2001). However, due to variability among the mole rats and the small number of specimens examined, uncertainties remained. Maughan-Brown observed the absence of anterior chamber in several juvenile but not adult mole rat specimens, but did not investigate when and how this structure developed. Structural abnormalities at the posterior of the lens, appearing before P30 were reported, but the cellular mechanisms responsible for their formation were not investigated. Retinal degeneration was observed in one out of the three specimens studied.

1.8 Aims of this study

Investigations of eye structure and development in naturally blind animals can provide important insights into the molecular and developmental mechanisms of evolutionary processes. Unfortunately, most of the studies conducted on naturally blind mammals are several decades old, and therefore are mainly descriptive. Another limitation is that often only the adult eye phenotype is considered, because the earlier developmental stages are not easily available. It is therefore essential to search for novel model animals with naturally reduced eyes suitable for developmental studies. With the advent of modern molecular biology and identification of a range of molecular markers specific for various eye tissues, it is now possible to investigate the molecular mechanisms underlying eye degeneration in these animals.

The broad aim of this work was to explore two species of African mole rats as possible novel models for study of natural eye regression, and also to investigate the molecular and genetic mechanisms responsible for the formation of the microphthalmic eye in these animals. To this end, it was necessary to provide a detailed description of the histology and ultrastructural features of the eye at different developmental stages. When this work was under way, it became clear that in both of these animals, the most abnormal structures are those of the anterior chamber, i.e. the lens, the ciliary body and the cornea.

The specific aims of this study were:

1) To confirm and expand the preliminary investigation of the naked mole rat eye morphology (Maughan-Brown, Honours thesis, 2001) using a larger sample size that would adequately represent all age groups and postnatal developmental stages.

2) To describe the histological structure and embryonic development of the microphthalmic eye of the Cape dune mole rat.
3) To determine the possible molecular causes of the abnormal lens fibre morphogenesis in the mole rats by investigating the expression of $\alpha$- and $\gamma$-crystallins and the levels of cell proliferation at different developmental stages;

4) To investigate the role of the lens in the ciliary body development in the Cape dune mole rats by examining the expression pattern of several candidate ciliary body-specific genes that are induced by signalling from the lens ($Ptmb4$ and $Tgfb1i4$);

5) To describe the dynamics of the anterior chamber and corneal endothelium formation in the naked mole rats using transmission electron microscopy.

As this study proceeded, it was discovered that one of the ciliary body specific markers, $Ptmb4$, was absent from the ciliary zone of the Cape dune mole rats. In order to understand the functional significance of this finding, it was deemed necessary to investigate the function of this molecule in eye development. The next aim therefore, was

6) To investigate the function of thymosin beta 4 in the development of the ciliary body using the chick model.

During the execution of the penultimate portion of this work, it became clear that the best way to investigate the role of $Ptmb4$ in ciliary body morphogenesis is by downregulation of its expression in an experimentally accessible model such as the chick embryo. However, techniques for gene knockdown that could be suitable for this experiment were not established in the chick model. Therefore, the final aim of this project was to design and construct an RNA interference vector suitable for use in the chick embryos. The experimental approaches used and the results of this part of the project are discussed separately in Chapters 5 to 7 of this thesis.
2. Materials and Methods

2.1 Animals

Juvenile and adult naked mole-rats (Heterocephalus glaber) were obtained from the Department of Zoology, University of Cape Town, where a number of successfully breeding colonies have been established (Jarvis, 1981). Adult naked mole-rats were sacrificed with chloroform or halothane and pups by decapitation. Eyes were harvested from mole-rats at postnatal days 0, 1, 5, 14, 21, 32 and 34 and from ten adults of various ages.

Cape dune mole rats (Bathyergus suillus) were obtained from the Cape Town International airport, where these animals are captured and culled as part of the airport’s pest control program. Animals were sacrificed with chloroform, because halothane and a number of other anaesthetics were found to be ineffective. Embryos were removed and staged according to Theiler system for mouse embryonic development (Theiler, 1989). Despite the differences in the length of gestation between these animals (mouse – 19 days, Bathyergus – 60 days, J. Jarvis, personal communication), their early developmental stages are virtually identical. Increased length of intra-uterine development in the Cape dune mole rats results in some of the later embryonic stages being equivalent to the postnatal stages of the mouse. All studies were carried out in compliance with the guidelines of the Animal Ethics Committee of the University of Cape Town.

2.2 Histology and eye measurements

The eyes of 19 mole-rats, aged one day to 12 years, were analysed using standard histological techniques. After dissection, the eye globe was placed in ice-cold, fresh 4% paraformaldehyde (PFA) in phosphate buffered saline. A sharpened tungsten needle was used to pierce the posterior hemisphere of the eyeball, allowing a rapid penetration of fixative into the eye. Eyes were fixed in PFA overnight, and then dehydrated through a series of increasing ethanol concentrations and embedded in paraffin wax. The eyes were embedded so that sectioning would be through the vertical meridian of the eye. Sections 4 or 5 μm thick were stained with haematoxylin and eosin using standard procedures. Images were captured using a Carl Zeiss AxioCamHR digital camera mounted on an Axioskop 2 microscope, or Kodak DC290 digital camera and Nikon Microphot-FX microscope. The measurements of the diameter of the eye, lens, ciliary body, iris and cornea were made using the Axiovision 2.4 software package. The lengths of the Bathyergus retina and ciliary body were determined using Photoshop 6 software package. To ensure consistency in the results, measurements of various structures within the eye
were taken of histological sections through the centre of the pupil. The diameters of the whole eye and the lens were separately measured along the anteroposterior axis (at the level of ora serrata in the mole-rat) and the dorsoventral axis (along the line extending from the cornea to the retina and passing through the centre of the eye). The average values of these two measurements, for each eye and lens diameter, were then calculated. In order to circumvent errors that could be introduced into the measurements of ciliary body and retina length by retinal detachment from the RPE and shrinking of the retina, the length of the RPE and the pigmented ciliary epithelium were measured.

2.3 Transmission electron microscopy (TEM)

Eyes were fixed overnight in Karnovsky’s fixative (1.5% paraformaldehyde, 0.5% glutaraldehyde in 0.1M Sorenson’s phosphate buffer) and then cut in half equatorially and the anterior segment returned to fixative for another hour. Samples were washed in 0.1M Sorenson’s phosphate buffer (0.087M Na₂HPO₄, 0.013M NaH₂PO₄ in distilled water, pH 7.6), postfixed for 90 minutes in 1% osmium tetroxide solution in distilled water, stained for 30 minutes in 2% uranyl acetate, dehydrated and embedded in epon-araldite or Spurr’s resin. Sections of 1μm thick were stained for 5 seconds with 1% toluidine blue in 1% borax. Ultra-thin sections were stained in 8% saturated uranyl acetate and Reynold’s lead citrate, and then viewed and photographed using a JEM109 transmission electron microscope at 120kV.

2.4 Immunohistochemistry

2.4.1 Paraffin wax sections and cryosections

For BrdU incorporation assays, a 14-day old mole-rat was injected with 300 μg/g body weight BrdU (Boehringer-Mannheim) for two days, and two adult mole-rats, four and 12 years old, with 15 μg/g body weight at weekly intervals over a period of 14 weeks. All animals were sacrificed 24 hrs after the last injection. Eyes were removed, fixed in 4% PFA and processed to paraffin wax as described above. Paraffin sections, 5μm thick, were dewaxed and rehydrated, washed in tris-buffered saline (TBS), treated briefly with proteinase K (Roche), and denatured with 1.5M HCl for 30 minutes at 37°C. Sections were blocked with 0.5% bovine serum albumin (BSA) in TBS for 30 minutes, and incubated with a mouse monoclonal anti-BrdU antibody (Roche), diluted 1/50 in blocking solution, overnight at 4°C. Sections were washed extensively in TBST (TBS + 0.1% Tween 20) and incubated with Alexa 488-conjugated goat anti-mouse secondary antibody (1/1000 dilution in blocking solution; Molecular probes, Eugene, Oregon, USA) for 1 hr in the dark at room temperature. Sections were washed extensively in buffer, counterstained
for 10 minutes with DAPI (1:100 in TBS) at room temperature, and then mounted in Mowiol. The slides were viewed and analysed as described above.

For anti-α-crystallin and anti-γ-crystallin immunocytochemistry, 5 μm paraffin wax sections, cut through the centre of the pupil, were placed on APTES-coated slides, dewaxed and rehydrated, rinsed with TBS and incubated with 0.3% H₂O₂. Sections were blocked in 3% BSA in TBS for 1-1.5 hrs at room temperature prior to incubation with 1/200 dilution of rabbit anti-αA/B-crystallin polyclonal antiserum (a gift from Professor H Kondoh, Osaka university, Japan) or 1/500 dilution of rabbit anti-γ-crystallin antiserum (generously provided by Dr Linlin Ding, Joseph Horwitz Laboratory, Jules Stein Eye Institute, University of California, Los Angeles) overnight at 4°C. The next day, the sections were washed extensively in TBST, and incubated with peroxidase-conjugated swine anti-rabbit antiserum (DAKO) for 2 hrs. Antigen-antibody complexes were detected by 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma) colour reaction. Sections were lightly counterstained with hematoxylin, dehydrated through ethanol series, cleared in xylol and mounted in Entellan (Merck).

For determination of Ptmb4 localization in the eye, 10 μm cryosections of P0 mouse eye were placed on SuperFrost slides (Menzel GmbH & Co, Germany), rinsed with TBS and incubated with 0.3% H₂O₂ in methanol to inhibit endogenous peroxidase activity. Sections were blocked in 3% BSA in TBS for 2 hrs at room temperature, and then incubated overnight at 4°C in 1/200 dilution of rabbit polyclonal antibody against four N-terminal amino acids of Ptmb4 (Immunodiagnostik, Germany). The next day, the sections were washed extensively in TBST, and incubated with peroxidase-conjugated swine anti-rabbit antiserum (DAKO) for 2 hrs. Antigen-antibody complexes were detected by 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma) colour reaction. Sections were lightly counterstained with hematoxylin, dehydrated through ethanol series, cleared in xylol and mounted in Entellan (Merck).

2.4.2 Cultured cells

Cos7 cells were transfected with pCIG and pCIG+Ptmb4 (see Section 2.6.2 below). Twenty four hrs after the transfection, the cells were fixed in MEMFA (1xMEM (0.1M MOPS, 2mM EGTA, 1mM magnesium sulphate, pH 7.4), 3.7% formalin) for 10 minutes at room temperature, then rinsed three times for 5 minutes in PBS, permeabilized in PBSTx (PBS containing 0.1% Triton-X) for 5 minutes and rinsed in PBS. The cells were then blocked in 1% BSA in PBS for 1-2 hrs at room temperature, and incubated in 1/200 dilution of anti-Ptmb4 antibody (Immunodiagnostik, Germany) in blocking solution overnight at 4°C. The next day, the cells
were washed extensively in PBS (five-six 5-minute changes), and incubated in 1/2000 dilution of anti-rabbit Cy3-conjugated secondary antibody (Jackson Immuno-Research laboratories). The cells were viewed using Zeiss Axiovert 200M fluorescence microscope.

2.5 Gel electrophoresis and western blot analysis

2.5.1 Proteins extracted from the lens

Lenses were dissected from four two-day-old naked mole-rat pups and one 6-year old adult naked mole rat, as well as from one 2-year old and one 8-months old Cape dune mole rat. The lenses were homogenized in 20μl extraction buffer (0.1M Tris-HCl, 1% Nonidet P40, 0.01% SDS, 1 μg/ml aprotinin and 0.1mM phenylmethylsulfonyl fluoride) at pH 7.2 to extract the water-soluble proteins, and centrifuged at 12 000g for 15 minutes. The water-insoluble proteins from the pellet were resuspended in 5 μl of 4x sample dye (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125M Tris; 0.03% bromophenol blue) and then diluted to 10 μl with extraction buffer. The supernatant (10 μl) was boiled to denature proteins, electrophoresed on a 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), along with 5 and 1 μg samples of 2-day old mouse lens protein extract, and transferred to a nitrocellulose membrane (Hybond-C, Amersham Life Science) at 8V, in 25 mM Tris-HCl, 5% methanol, overnight. The membrane was blocked with 10% fat-free milk solution for 1.5 hours, and γ-crystallins were detected with 1/1000 dilution of rabbit anti-γ-crystallin antiserum (provided by Dr Linlin Ding). Primary antibodies were detected with horseradish- peroxidase-conjugated swine anti-rabbit polyclonal antibody, using ECL fluorescent detection system (Amersham Life Science). After transfer, the gel was fixed in 20% methanol, 7% acetic acid solution overnight with constant agitation, washed three times in distilled water, and silver-stained for 15 minutes with 0.05 M solution of AgNO₃. The protein bands were visualised after addition of the developer (0.005% citric acid, 0.02% formaldehyde). The reaction was stopped with 1% acetic acid solution, and the gel was photographed using Chemilmager.

2.5.2 Proteins secreted from cultured Cos7 cells

Cos7 cells transfected with pCIG and pCIG+Ptmb4 were incubated for 24hrs to allow the plasmid-encoded genes to be expressed. The next day, the culture medium was replaced with serum-free DMEM, and the cells were incubated for another 24hrs. Then, the medium was aspirated and the secreted proteins precipitated with 3x volume of acetone at -20°C overnight. The protein pellet was collected by centrifugation at 500g, washed twice with 70% ethanol, and
air-dried for 1 hr at room temperature. The protein pellet was resuspended in 1 ml of extraction buffer.

Transfected Cos7 cells were washed twice in PBS, scraped from the bottom of the 10-mm dish, and resuspended in 150 μm extraction buffer. The cells were vortexed and trituated to release the proteins. Protein extracted from the cells was used as the positive control. The protein concentration was determined using BioRad assay as recommended by the manufacturers, and 30 μg of each protein sample were loaded on 15% acrylamide gel. The rest of the western blot procedure was exactly as described in Section 2.5.1, except that rabbit anti-Ptmb4 antibody (Immunodiagnostik, Germany) was used in 1/200 and 1/2000 dilution.

2.6 Cloning

2.6.1 Probe templates

Bathyergus-specific probes for Ptmb4 and Tgfb1i4 were obtained by RT-PCR (reverse transcription with polymerase chain reaction) on total RNA isolated either from embryonic heads or eyes. RNA was prepared using TriPure Isolation Reagent (Roche, Germany), according to the manufacturer's instructions. Degenerate primers were designed by aligning the coding sequences of the mammalian and chick Ptmb4 and Tgfb1i4, and selecting the most conserved regions. Primers containing degenerate nucleotides at no more then three positions were designed to minimize non-specific amplification. The primer sequences were:

5'-Ptmb4 degenerate: 5'-GGCTGAGATC/TGAGAAA/GTTGG -3'

3'-Ptmb4 degenerate: 5'-AAATAAGAAA/GGCAATGCTC/TGT -3'

5'-Tgfb1i4 degenerate: 5'-GCATGAAT/AT/GCCCAATGT/GT-3'

3'-Tgfb1i4 degenerate: 5'-TTACACTAC/AATACACAAATATACA-3'

The RT mix was assembled as follows: 0.5-1 μg template RNA; 3 μl of 20 μM dT15; 4 μl of 5 mM dNTPs; 0.5 μl of RNasin Ribonuclease inhibitor (Promega); 1 μl of M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase, Promega), 4 μl of M-MLV RT 5 x reaction buffer (Promega). The total volume of each reaction was 20 μl. First strand cDNA synthesis was carried out at 42°C for 2 hrs. To control for DNA contamination, a reaction with no M-MLV RT was included; any product obtained from a PCR reaction carried out on this control would indicate a presence of contaminating genomic DNA in the RNA preparation.
The PCR conditions were as follows: denaturation - 94°C for 2 minutes; annealing and amplification – 25 cycles of 94°C for 1 minute, 52°C for 2 minutes, 72°C for 1-2 minutes; final extension - 72°C for 5 minutes. A hybrid PCR Sprint machine was used for all PCR amplifications.

The amplified fragments of the expected size (171bp for Ptmb4 and 1.1kb for Tgfb1i4) were column-purified, cloned into pGem-T Easy Vector System (Promega, Madison, USA) according to the manufacturer’s instructions. The individual clones were sequenced by Inqaba Biotech (Pretoria, South Africa).

Crystallin γ D (crygD) clone was obtained in a plasmid (pME18S-FL3) that did not contain any of the viral RNA polymerase promoters, and so it was not possible to use it for probe synthesis directly. In order to obtain a suitable probe template, the crystallin coding sequence was excised using XhoI, and ligated into XhoI site of pCDNA3 (Invitrogen), after treating the vector with shrimp alkaline phosphatase (USB Corporation, Cleveland, USA) to prevent self-ligation.

2.6.2 Ptmb4 expression construct

The full coding sequence of the chick Ptmb4 was amplified from a clone isolated from chick neural crest cDNA library (Lee, unpublished) using the following primers:

5'-Gg Ptmb4 XhoI: 5'-GATCTCGAGATGTCCGACAAACCCGA-3'

3'-Gg Ptmb4 EcoRI: 5'-GATGAAATTCTCATTTGCAGTCACCCGC-3'

Expand High Fidelity PLUS PCR System (Roche, Germany) was used to ensure the highest accuracy of amplification. The PCR product was column-purified (QIAGEN), and directionally ligated into the chick-specific expression vector pCIG (Megason and McMahon, 2002). The insert-containing clones were identified by colony PCR using pCIG sequencing primers:

pCIG seqFor: 5’-TACAGCTCCTGGGCAACGTG-3’

pCIG seqRev: 5’-GCTTCGGCCAGTAACGGTAG-3’

The final construct was sequenced in both directions to confirm that there were no amplification errors.
2.7 In situ hybridization

Table 2-1 below summarizes the probe templates that were used in this work.

Table 2-1: Probe templates used for in situ hybridization.

<table>
<thead>
<tr>
<th>Name of the template</th>
<th>Organism</th>
<th>Length of the insert</th>
<th>To make sense probe</th>
<th>To make antisense probe</th>
<th>Source of the clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptmb4</td>
<td>mouse</td>
<td>508bp</td>
<td>NolI; T7</td>
<td>EcoRI; T3</td>
<td>IMAGE (Invitrogen)</td>
</tr>
<tr>
<td>cPtmb4</td>
<td>chick</td>
<td>500bp</td>
<td>NolI; SP6</td>
<td>SalI; T7</td>
<td>Vivian Lee (Caltech)</td>
</tr>
<tr>
<td>BsPtmb4</td>
<td>Bathyergus suillus</td>
<td>170bp</td>
<td>SalI; T7</td>
<td>SalI or Spel; T7</td>
<td>this work</td>
</tr>
<tr>
<td>Tgfb1i4</td>
<td>mouse</td>
<td>1.6kb</td>
<td>NolI; SP6</td>
<td>SalI; T7</td>
<td>IMAGE (Invitrogen)</td>
</tr>
<tr>
<td>BsTgfb1i4</td>
<td>Bathyergus suillus</td>
<td>1.1kb</td>
<td>NolI; SP6</td>
<td>SalI; T7</td>
<td>this work</td>
</tr>
<tr>
<td>Msx1</td>
<td>mouse</td>
<td>500bp</td>
<td>NolI; T7</td>
<td>EcoRI; T3</td>
<td>IMAGE (Invitrogen)</td>
</tr>
<tr>
<td>Otx1</td>
<td>mouse</td>
<td>527bp</td>
<td>NolI; T7</td>
<td>EcoRI; T3</td>
<td>IMAGE (Invitrogen)</td>
</tr>
<tr>
<td>Lmx1b</td>
<td>mouse</td>
<td>1.3kb</td>
<td>BamHI; T7</td>
<td>HindIII; T3</td>
<td>Brigit Hogan (Duke)</td>
</tr>
<tr>
<td>CrygD</td>
<td>mouse</td>
<td>1.0kb</td>
<td>EcoRI or HindIII; SP6</td>
<td>Xbal; T7</td>
<td>IMAGE (Invitrogen) and this work</td>
</tr>
</tbody>
</table>

2.7.1 Probe preparation

To synthesize digoxigenin(dig)-labelled RNA probes, the template clone was linearized with the appropriate enzyme, cleaned up by phenol-chloroform method or column-purified, and in vitro transcription reaction (1μg linearized DNA, 1mM each rGTP, rATP and rCTP, 0.35mM dигrUTP, 0.65mM rUTP, 1 x transcription buffer, 10mM DTT, 1μl RNase inhibitor and 2μl RNA polymerase) was carried out for 2 hrs at 37°C. The amount and the integrity of the RNA probe synthesized were then assessed on 1% agarose gel, and the probe precipitated with 4M LiCl and 100% ethanol. The RNA probe was then resuspended in the probe mix to the final concentration of 1-10ng/μl and stored at -20°C.

2.7.2 In situ hybridization on sections

In situ hybridizations on mouse and mole rat eye and embryonic head sections were performed as described by Etchevers et al. (2001). All aqueous solutions used in probe preparation and tissue treatment prior to the hybridization step were treated with 100μl/L depc (diethyl
pyrocarbonate, Sigma) overnight at 37°C, then autoclaved to inactivate depc. Similarly, all the
glassware used in slide preparation was rinsed in depc-treated water and baked at 180°C for at
least two hours to destroy RNase activity.

Tissues were dissected in PBS, fixed in freshly prepared 4% PFA in phosphate buffered saline
(PBS), dehydrated through ethanol series and embedded in paraffin wax. Sections were cut at
7μm, placed on SuperFrost slides (Menzel GmbH & Co, Germany) and allowed to dry overnight
at 42°C. Then sections were dewaxed in two changes of xylol, re-hydrated through ethanol series
of decreasing concentration, rinsed in depc-treated water and PBS, and treated with 20μg/ml
proteinase K solution in depc-treated PTW (PBS+0.1% Tween-20) at room temperature. The
duration of the proteinase K digestion varied with the age of the specimen; 7-minute treatment
was optimal for E11.5 to E14.5 embryonic specimens, for E16.5 to P0 10-minute treatment was
used. Afterwards, the proteinase K activity was quenched with 2mg/ml of glycine in PTW for 5
minutes, slides were washed twice with PTW, post-fixed with 4% PFA for 20 minutes at room
temperature and rinsed with PTW and 2xSSC. Hybridization solution (0.2M NaCl, 0.01M Tris,
0.01M PO₄, 0.005M EDTA, 50% deionized formamide, 10% dextran sulphate, 1 mg/ml yeast
tRNA, 1xDenhardt’s, 0.1% CHAPS, 0.1% Tween-20) containing 1-10ng/μl dig-labelled RNA
probe was then added to the slides, and they were incubated at 60-65°C overnight under
siliconized coverslips.

The next day, the slides were decoversipped by soaking them in wash solution (50% formamide,
1xSSC, 0.1% Tween 20) at room temperature, and then washed extensively to ensure the
complete removal of the unbound probe. Two 30-minute high stringency washes (65°C, in 1 x
SSC, 50% formamide, 0.1% Tween-20), and six 30 to 45 minute MABT (0.1M maleic acid,
0.15M NaCl, 0.2M NaOH, 0.1% Tween-20, pH 7.5) washes at room temperature were
performed. The slides were then blocked in 2% Boehringer blocking reagent (Roche, Germany)
+ 20% heat inactivated sheep or goat serum in MABT for 1.5 hrs at room temperature. Next,
1/2000 dilution of anti-DIG-AP antibodies (Roche, Germany) was applied to the slides overnight
at room temperature. Afterwards, extensive washes (MABT 5-6 times 30-45 munites each wash
at room temperature) were performed to remove the unbound antibody, the slides were rinsed
twice with 0.1M Tris pH9.5, equilibrated in NTMT (0.1M NaCl, 0.1M Tris-HCl, 50mM MgCl₂,
0.1% Tween 20), and treated with the colouration solution (4.5μl/ml NBT, 3.5μl/ml BCIP in
NTMT). This last step was done with constant monitoring, every hour, until sufficient colour
development was observed. Then the reaction was stopped by rinsing the slides several times in
distilled water, the colour product fixed into the sections by treatment with 4% PFA for 20
minutes at room temperature, and the slides coverslipped in Mowiol and viewed under the Nikon Microphot-FX microscope.

2.7.3 Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using the method of Sauka-Spengler et al. (2001). The principle behind this procedure is essentially the same as for the section hybridization, except that whole chick embryos or embryonic heads were used instead of the sections. The probe templates used are listed in Table 2-1. Probe preparation was done exactly as described above (Section 2.7.1). All aqueous solutions used in probe preparation and tissue treatment prior to the hybridization step were treated with 100 μL/L depc (diethyl pyrocarbonate, Sigma) overnight at 37°C, then autoclaved to inactivate depc. Similarly, all the glassware used in slide preparation was rinsed in depc-treated water and baked at 180°C for at least two hours to destroy RNase activity.

Embryos were removed from the egg, washed in PBS, and fixed overnight in 4% PFA in PBS. Embryos were then dehydrated through methanol series (25%, 50%, 75% and 100%) and stored in 100% methanol until needed. Then, they were re-hydrated, washed twice with depc PBS and digested with 10 μg/ml proteinase K to aid the penetration of the probe. Hamburger-Hamilton stage 16 (HH16) embryos were treated for 25 minutes, HH20-HH22 embryos – for 35 minutes. Thereafter, proteinase K activity was quenched with 2mg/ml glycine, embryos were post-fixed in 4% PFA, rinsed 4 times in depc PBT, and washed in 50% and 100% hybridization mix (50% deionized formamide, 1.3xSSC, 5mM EDTA, 200μg/ml tRNA, 0.2% Tween-20, 0.5% CHAPS, 100μg/ml heparin). To minimize background, tissues were pre-hybridized in the hybridization mix at 70°C for at least 3 hrs. Then pre-warmed hybridization mix with added dig-labelled RNA probe (1-10ng/μl) was added, and samples incubated at 70°C overnight with constant rotation.

Next day, the embryos were washed six times with hybridization solution at 70°C for 30-45 minutes each wash, then twice with 50% hybridization solution in MABT at 70°C, and 4-5 times with MABT at room temperature. The tissues were blocked in blocking solution (see section 2.7.2) for 3-4 hrs at room temperature, and incubated in 1/2000 anti-dig-AP antibody solution in block overnight at 4°C on the nutator. The unbound antibody was removed by extensive washing in MABT (6-8 times for 1 hour each). After equilibrating the embryos in NTMT, the signal was revealed with 4.5 μl/ml NBT, 3.5 μl/ml BCIP.
After the colour developed to the desired level, the embryos were washed in PBS, post-fixed in 4% PFA and dehydrated to 100% methanol to remove purple background. The embryos were re-hydrated to PBS prior to photographing. In order to visualize the internal staining pattern, embryos were cryoprotected in 5% and 15% sucrose in PBS, embedded in OCT, frozen and cryosectioned at 20 μm.

2.8 In ovo electroporation

Fertile chicken (Gallus gallus domesticus) eggs were obtained from Atlantis farms (Cape Town, South Africa). The eggs were incubated horizontally at 38°C and 50% humidity until the embryos reached the desired developmental stage. To expose the embryos, 2-3ml of albumen was removed from the egg and elliptical windows were made in the eggshell directly above the embryo. A one in 25 dilution of India ink in chick Ringers (120mM NaCl, 1.5mM CaCl₂, 5.0mM KCl, 0.81mM Na₃H₂PO₄, 0.15mM KH₂PO₄, pH7.4) was injected under the vitelline membrane to visualize the embryo. The plasmid pCIG was used in concentration 2 μg/μl. To make it easier to monitor the progress of injection, 1 μl of non-toxic blue food colourant (CI 42090 and CI 14720) was added to the DNA solution. The plasmid-dye mixture was injected into the eye of HH20 and HH25 embryos using a pulled glass needle. Two platinum wire electrodes, about 0.5mm in diameter, were then positioned on the opposite sides of the embryo’s head, with the anode placed next to the injected eye. Chick Ringers was used to wet the embryo and the electrodes. Five pulses of 25.0V (100ms in length with an interval of 900ms) were delivered to the embryo using a square-pulse electroporator (B.E.S. 2004, Caltech). The eggs were sealed with thick plastic tape, and re-incubated for a further 24hrs. The embryos were then dissected out, fixed in 4% PFA for 5-12 hrs, washed extensively in PBS and viewed under Zeiss Axiovert 200M inverted fluorescence microscope.

2.9 Cell culture and transfection

Green African monkey kidney cell line COS7 was grown in standard DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% heat inactivated fetal calf serum (FCS), 1% penicillin and streptomycin, in an atmosphere of 5% CO₂, at 37°C. Transfections with the pCIG+Ptmb4 and empty pCIG as a control were performed with FuGENE transfection reagent according to the manufacturer’s instructions. To obtain adequate transfection efficiency (50%), 10μg of the plasmid were used per 35mm dish.
2.10 Intraocular cell injection

Fertilized chicken eggs were obtained from commercial sources (Atlantis farms, Cape Town). The eggs were incubated horizontally at 38°C and about 50% humidity until the embryos reached Hamburger-Hamilton stage 12 (48hrs of incubation) or 20 (HH20, 72 hrs incubation). To expose the embryos, 2 to 3 ml of albumen were removed from the egg and elliptical windows were made in the eggshell directly above the embryo. In the case of HH12 embryos, a small volume of 5% solution of India ink in chick Ringers was injected under the embryo to enhance its visibility. The vitelline membrane was carefully removed from the area around the right eye. The cells transfected the day before, were lifted from the plate with trypsin, washed twice with FCS-containing medium and allowed to recover in fresh DMEM+FCS on ice for 10-15 minutes. The cells were then washed with serum-free medium, and resuspended in 20μl of fresh DMEM. The cells were mixed with 1 μl of non-toxic blue food colourant to make it easier to monitor the success of the injection. The cell suspension was then injected into the right optic vesicle (HH12 embryos) or next to the RPE of the right eye (HH20 embryos) using a pulled glass needle. A few milliliters of chick Ringers solution were added to prevent the embryos from drying out, then the eggs were re-sealed with wide plastic tape and re-incubated for a further 24 to 72hrs. After the embryos were allowed to develop to the desired stage, they were removed from the egg and viewed under fluorescent microscope. Only the specimens that were successfully injected, as determined by the presence of green fluorescence within the eye or in the eye area, were processed further. The embryos were fixed in 4% PFA for 5-12 hrs, washed in PBS, and cryoprotected with increasing concentrations of sucrose in PBS (5% and 15% for at least 3 hrs each). The next day, the embryos were frozen in OCT compound and sectioned at 16 μm on the cryostat. The sections were viewed under inverted fluorescent microscope and photographed using a Nikon camera.
Figure 2-1: Diagram of the cell injection procedure.
3. Results

3.1 Naked mole-rats are microphthalmic

In this study, the eye histology of 10 adult (aged from 1 year to 12 years) and 7 juvenile (P1 to P34) naked mole-rats was investigated in detail. The eyes from mice of corresponding ages were used for morphological and developmental comparisons. The mouse was chosen for this comparison, because it belongs to the same order as the mole-rat (Rodentia), has a comparable body size, good visual acuity and well-studied eye structure. At birth, the mole-rat pups are morphologically very similar to the mouse pups in body size and general appearance (Fig. 3-1A). The eyelids of both species are closed at birth and open two weeks later in the mouse (Graw, 1996) and between 21 and 30 days after birth in the naked mole-rat (O’Riain, 1996). The eyes of the adult naked mole-rat are deeply sunk into the head, and the eyelids are thickened and generally kept closed unless the animal is alarmed (Fig. 3-1B; Sherman et al., 1991). In comparison to the mouse, adult naked mole-rats have significantly smaller eyes. In order to compare the differences in size of the eye and intraocular structures, the diameter of both the unfixed whole eyes and the histological eye sections were measured as described in Materials and Methods. It was found that, on average, the eye of the adult mole-rat is two times smaller in diameter, and therefore eight times smaller in volume than the mouse eye (n=9; Table 3-1). Thus the naked mole-rat eye can justifiably be termed “microphthalmic”.

The naked mole-rat eye is typically round in shape, but lacks the turgidity characteristic of the mouse eye. This feature was particularly evident after dissection: the eyeball could be easily pinched or indented with forceps (much like squashing a football without air).

The internal organisation of adult mole-rat eyes is similar to that of the mouse, with the main structures – cornea, lens, retina, anterior chamber, iris and ciliary body being discernible. However, a number of significant differences, related to the structure and cellular features of the cornea, iris, ciliary body, lens and retina were found.

3.2 The naked mole-rat cornea

The mouse cornea is made up of a distinct corneal epithelium, stroma and endothelium (Fig. 3-3A). It has been reported (preliminary work that formed a part of this study) that the adult mole-rat cornea has an epithelial layer, a proportionately thinner stromal layer, and, in normal histological sections there appears to be no identifiable corneal endothelium present (Nikitina et al., 2004; see Appendix B). However, scanning electron microscopic (SEM) examination of the mole-rat corneas revealed that in the adult and in 21-day old mole-rat, an endothelial layer
appears to be present, even though it does not exhibit the regular cobblestone pattern characteristic of the mouse endothelium (Nikitina et al., 2004; see Appendix C). In order to verify that what was seen was indeed endothelium, transmission electron microscopic (TEM) examination of the adult (4-year-old) mole-rat eye was conducted. Transmission electron microscopic examination of a cross-section through a four-year old mole-rat cornea revealed the presence of large rounded vacuoles, enclosed between two neighbouring cells, which were flattened and joined by tight junctions (Fig. 3-5A). Because such transcellular transport vacuoles and tight junctions are typical features of mouse corneal endothelium, it was concluded that true corneal endothelial cells are lining the anterior chamber of the naked mole-rat eye.

3.3 The naked mole-rat retina and optic nerve

When the mole-rat eyes were bisected, no structured gel-like vitreous body was observed. Instead, the eyeball was filled with a transparent liquid substance. Histological sections through the eyes showed that almost the entire vitreous chamber was filled by the extensively folded retina, which appeared to be closely associated with the posterior of the lens. The mole-rat retina was folded to the extent never seen in the mouse, as if it was too big for the size of the eyeball (Fig. 3-2E). Because this condition of the retina was common in the adult mole-rat, but was never seen in the mouse, it was considered a true difference between these species rather than a histological artefact. The general organisation of the neural retina was similar to that of the mouse, which is composed of nine layers, namely the photoreceptor layer, the external limiting membrane, the outer nuclear layer, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the optic nerve fibre layer and internal limiting membrane (Fig. 3-3F). All except two of the retinal layers were well defined. The nerve fibre layer was absent in all adult mole-rats examined and the ganglion cell layer appeared to be progressively reduced as the animals age. This reduction in the number of ganglion cells was apparent from examination of histological sections, and these observations have to be confirmed by ganglion cell counts. Besides the intensely staining ganglion cell bodies, the paler-staining large "frothy" cells, identified as microglial cells, were seen in increased numbers in the ganglion layer of the mole-rat retina (Fig. 3-3F, arrowheads). The optic nerve contains cells arranged in an irregular pattern, which is different to the regular, more parallel arrangement seen in the mouse (Fig. 3-3C, D). A prominent distension of the optic nerve head (the connection between the optic nerve and the retina) is seen in almost all adult mole rat specimens (data not
Table 3-1: Comparative measurements of the body length and eye size of the mouse and the naked mole-rat.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Body length, cm (tail not included)</th>
<th>Eye diameter (wet specimen), mm</th>
<th>Eye diameter (histological sections), mm</th>
<th>Average volume of the eye, mm³</th>
<th>Ratio of eye diameter to body length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse neonate (n=3)</td>
<td>2.8</td>
<td>2.2</td>
<td>2.0</td>
<td>4.2</td>
<td>0.071</td>
</tr>
<tr>
<td>Mole-rat neonate (n=3)</td>
<td>3.2</td>
<td>1.0</td>
<td>0.9</td>
<td>0.39</td>
<td>0.028</td>
</tr>
<tr>
<td>Mouse adult (n=4)</td>
<td>10.5</td>
<td>3.2</td>
<td>2.7</td>
<td>10.3</td>
<td>0.026</td>
</tr>
<tr>
<td>Mole-rat adult (n=9)</td>
<td>10.5</td>
<td>1.9</td>
<td>1.2</td>
<td>0.90</td>
<td>0.011</td>
</tr>
</tbody>
</table>

*These values are calculated using the eye diameter measurements from histological sections.

Table 3-2: Comparative measurements of the eye structures.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Eye diameter (mm)</th>
<th>Lens diameter (mm)</th>
<th>Lens flattening ratio</th>
<th>Corneal thickness (µm)</th>
<th>Iris length (mm)</th>
<th>Ciliary body length (mm)</th>
<th>Lens/eye proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mole-rat neonate (n=3)</td>
<td>0.91</td>
<td>0.45</td>
<td>1.4</td>
<td>36</td>
<td>None</td>
<td>0.43</td>
<td>49%</td>
</tr>
<tr>
<td>Mouse neonate (n=3)</td>
<td>2.0</td>
<td>1.1</td>
<td>1.21</td>
<td>86</td>
<td>0.12</td>
<td>0.13</td>
<td>55%</td>
</tr>
<tr>
<td>14-day old mole-rat (n=1)</td>
<td>1.0</td>
<td>0.50</td>
<td>1.24</td>
<td>44</td>
<td>0.23</td>
<td>0.19</td>
<td>50%</td>
</tr>
<tr>
<td>21-day old mole-rat (n=2)</td>
<td>1.1</td>
<td>0.48</td>
<td>1.4</td>
<td>24</td>
<td>0.24</td>
<td>0.29</td>
<td>44%</td>
</tr>
<tr>
<td>32-day old mole-rat (n=1)</td>
<td>1.1</td>
<td>0.50</td>
<td>1.6</td>
<td>28</td>
<td>0.25</td>
<td>0.27</td>
<td>45%</td>
</tr>
<tr>
<td>Adult mouse (n=4)</td>
<td>2.7</td>
<td>1.84</td>
<td>1.07</td>
<td>111</td>
<td>0.62</td>
<td>0.21</td>
<td>68%</td>
</tr>
<tr>
<td>Adult mole-rat (n=9)</td>
<td>1.2</td>
<td>0.84</td>
<td>1.2</td>
<td>52</td>
<td>0.40</td>
<td>0.29</td>
<td>70%</td>
</tr>
</tbody>
</table>
Figure 3-1: The eye size and external appearance of the naked mole-rat. (A) The head of a neonate naked mole-rat. Eyes still fused. (B) Adult naked mole-rats (Photograph courtesy of J O’Riain). Eyes open because alarmed. Note the extremely small eye size of these animals.

Figure 3-2: Comparative histology of the mouse (A and C) and the naked mole-rat (B, D, E) eyes. (A) Neonate mouse has a well-developed lens (l), anterior chamber (ac), distinct ciliary body (cb) and iris (i), and an undifferentiated retina (r). (B) Fourteen day old mole-rat showing what appears to be the developing iris (arrowheads). (C) Adult mouse eye with round lens, typical elongated iris, ciliary body with evident ciliary processes and differentiated layers in the retina. (D) Four-year old naked mole-rat eye, showing flat ciliary body without ciliary processes, very large round lens with vacuole-like structures at the posterior margin (arrowheads), iris undergoing thinning and depigmentation and pigment-filled trabecular meshwork (arrows). (E) Twelve-year old naked mole-rat with extremely large irregularly shaped lens, ciliary body abnormally associated with sclera (arrow), and degenerate iris. Scale bars = 200µm (A, D, E); 100µm (B); 500µm (C).
shown). Thus, while the retinal structure in the naked mole-rat appears to be essentially normal, certain histological features of the optic nerve are reminiscent of reactive gliosis, a process glial scar formation that occurs in response to the neural tissue damage (Omlin, 1997).

3.4 The naked mole-rat ciliary body and iris

One of the most prominent features of the mole-rat eye is the large size and abnormal shape of the ciliary body. In the adult mouse eye, the ciliary body is folded into processes, and covered by two layers of columnar epithelium, the outer layer of which is pigmented and the inner unpigmented (Fig. 3-3K). The border between the ciliary body and the iris is clearly distinguishable. In the mole-rat eye, however, typical ciliary processes were not observed, and there was no clear border between the ciliary body and the iris (Fig. 3-3L, M; also see Appendix B). Overall, the ciliary body appeared flattened and elongated. In some specimens, extensive peripheral anterior synchiasis (abnormal attachment of the ciliary body to trabecular meshwork and to the peripheral posterior corneal surface) were observed (Fig. 3-2D; 3-3L, M, arrows). Both the ciliary body and iris of all specimens were highly pigmented. The outer (pigmented) layer of the ciliary body shows slight folding, while the inner layer remains unfolded.

The differences in the iris structure between the mole-rat and the mouse became apparent during the examination of the freshly dissected eyes. Contrary to what is observed in the mouse, the shape of the pupillary border is irregular and not clearly defined. Moreover, brown pigment granules are often observed in the cornea and within the anterior chamber, suggesting that adult mole-rats exhibit iris degeneration and dispersion of pigment. These observations were confirmed by histological and ultrastructural analysis. In the mouse, the iris is composed of three layers, the anterior pigmented border, the stroma and the inner pigmented epithelium. The iris muscle is clearly visible in the anteriormost tip of the iris (Fig. 3-3I, arrowhead). In some mole-rats, the iris had the characteristic elongate shape and well-defined iris muscle, while in other specimens the decrease in the thickness of the iris and degeneration of the pigmented epithelium were observed (Fig. 3-3J, black arrowheads). The pigment, presumably released from these degenerating epithelial cells, was found in the anterior chamber angle, and sometimes appeared to fill the trabecular meshwork (Fig. 3-2D, arrows). The degree of iris thinning varied greatly among the mole-rats examined, and did not appear to be directly related to their age.
Figure 3-3: Comparative histology of the mouse and mole-rat cornea (A, B), optic nerve (C, D), retina (E, F) and iris and ciliary body (G-M). (A) Adult mouse cornea with clearly defined epithelium (e), stroma (s) and endothelium (ce). (B) Adult (12-year old) mole-rat cornea with obvious epithelium and stroma, but no visible endothelium (arrowhead). (C) Optic nerve of an adult mouse has a regular arrangement of nuclei. (D) Optic nerve of an eight-year old mole-rat exhibits an irregular arrangement of nuclei. (E) Adult mouse retina with characteristic retinal architecture. Note nerve fibre layer (arrowheads). (F) Adult (12-year old) mole-rat retina, with no nerve fibre layer, and with ectopic cell bodies in the inner plexiform layer (white arrow) and possible microglial cells in the retinal ganglion cell layer (arrowheads). (G) Neonate mouse ciliary body and iris. (H) Juvenile (14-day old) mole-rat ciliary body and iris. (I) Adult mouse iris with characteristic elongated shape and visible iris muscle (arrowhead). (J) Four-year old mole-rat iris exhibiting thinning and depigmentation (arrowheads). Red arrows indicate pigment-filled particles in the anterior chamber. (K) Adult mouse ciliary body. (L) Twelve-year old and (M) three-and-a-half year old mole-rat ciliary body. Arrows indicate line of adhesion of iris to cornea. Scale bars =50μm (C, D); 20μm in all the rest.
The trabecular meshwork of some adults is very extensive, spanning the whole length of the elongated ciliary body. The trabecular beams of a four-year old specimen are pigmented and run almost parallel to each other, and large and well-developed aqueous channels are visible (Fig. 3-2D). In 8-12-year old specimens, the size of the trabecular meshwork decreases dramatically, the trabecular beams are compacted and highly pigmented, and the spaces between them seem to be severely reduced (Fig. 3-2E). In some specimens, pigment-filled round structures were seen floating in the anterior chamber, or trapped in the trabecular meshwork (Fig. 3-2D; Fig. 3-3J, red arrows). A TEM micrograph of a conglomerate of these pigmented particles in a single cell, located within an inter-trabecular space, is shown in Figure 3-5C. The finger-like projections on the surface of the plasma membrane of this cell, the shape of its nucleus and the presence of a large number of lysosomes in the cytoplasm suggest that it is a macrophage.

3.5 The lens of the naked mole-rat

The most striking difference between the mouse and the mole-rat eye is the structure of the lens. The mouse lens is positioned in the anterior hemisphere of the eyeball, and is held in place by the zonula fibres, which are also attached to the ciliary body. It is typically round in shape with a characteristic pattern of nuclear distribution; a single layer of nucleated epithelial cells covers its anterior surface, and a group of nuclei belonging to the differentiating fibre cells are found at the equator (the "bow region") (Fig. 3-2C). The interior and posterior edges of the mouse lens are free of nuclei. In the adult mole-rat, the lens appears to occupy most of the interior of the eyeball, and our observations during dissections and the absence of zonula fibres from all histological sections suggests that the lens floats freely inside the eyeball, rather than being attached to the ciliary body. In the majority of the specimens studied the lenses were round, but often exhibited various irregularities in shape at the posterior margin or at the equator (Fig. 3-2D, E, arrowheads). A prominent lens capsule, up to 23 μm thick in older specimens, was present. The nuclei are found in a thin layer surrounding the entire lens. It can be deduced that it corresponds to the epithelial layer of the mouse lens, but it appears to cover a much more significant proportion of the lens surface. The "bow region" of fibre differentiation is often absent or very poorly developed in adult mole-rats, and nuclei can be altogether absent from the region of the lens epithelium closest to cornea (Fig. 3-2D). When TEM was used to examine epithelial cell morphology, it was observed that the cells appear to be very flattened and resemble squamous epithelium, rather than cuboidal epithelium that is found in adult mouse lens (Fig. 3-5E). The nuclei of these cells had very flat, pancake-like appearance. Since an obvious "bow region" was not seen in the adult mole-rat lens, BrdU incorporation assays were performed to establish whether new fibres could still be formed. These results (Fig. 3-6C, D) indicate that
some of the cells located within the posterior third of the epithelium, were dividing, and thus that the mole-rat epithelium was still mitotically active even in 12-year old mole-rats. TEM of the lens equator in a four-year old mole-rat confirmed the presence of maturing lens fibres, which still contained some of the organelles and exhibited highly variable thickness and length (Fig. 3-5B). Therefore, despite its abnormal architecture, the naked mole-rat lens appears to maintain its growth and fibre differentiation activity throughout the life of the animal.

3.6 Postnatal development of the naked mole-rat eye

In order to begin to define the sequence of the major developmental events in the postnatal mole-rat eye, the structure and histology of the naked mole-rats of different ages, ranging from neonate to 34-day old, were examined. Due to the peculiar social structure of the naked mole-rats, breeding females are essential for the survival of the colony and consequently could not be sacrificed to obtain embryos. Therefore, with the exception of one batch of embryos at a very late developmental stage, the neonate was the earliest developmental stage available for these investigations.

3.6.1 Morphogenesis of the anterior chamber and the corneal endothelium

In newborn mice, the anterior chamber is fully formed, and the squamous endothelium is clearly visible on the inner surface of the cornea (Fig. 3-2A). In the newborn mole-rat eye, as previously reported, there was no space separating the cornea from the lens. In fact, in all three neonate specimens examined, the cornea appeared to be attached to the anterior surface of the lens and the ciliary body (Nikitina et al., 2004; see Appendix A). In order to establish when the anterior chamber is first formed, histological sections from juvenile mole-rats of various ages were examined. The presence of a space between the cornea and the lens was noted in a 14-day and three 32-34-day old mole-rats, while no such space was observed in a 21-day old mole-rat (Fig. 3-2C). Therefore, it was deduced that the time of anterior chamber formation is variable, and that the process is usually completed by the time mole-rats are 32 days of age. It has been proposed that the formation of the anterior chamber in the mouse is concurrent with the corneal endothelium formation (Kidson et al., 1999; Reneker et al., 2000). In an attempt to determine whether these processes also occur concurrently in the mole-rat, semi-thin (0.5 μm thick) resin sections of a late embryonic, five-day old, 12-day old, 14-day old and two 34-day old mole-rats eyes were examined (Fig. 3-4). Unfortunately, no anterior chamber could be observed in any of these sections, because the lens was always found to have changed its position considerably with respect to other ocular structures, sometimes being turned so that its anterior epithelium was facing the retina. Thus the space between the lens and the cornea in these specimens appears to
be a resin-embedding artifact (except in the embryo, where the lens is not displaced, and still adheres to the cornea).

Since such lens displacement was never seen when mouse eyes were processed in an identical way, it appears that the mole-rat lens is not firmly attached to the cornea even in those specimens which apparently lack anterior chamber. The typical flattened endothelial cells filled with transcellular vacuoles were observed in both the central and the peripheral regions of the cornea in the 34-day old and the 14-day old specimen (Figure 3-4C, E). In the 12-day old mole-rat, the flattened endothelial cell layer was observed, but transcellular vacuoles were not found. In the five-day old specimen, corneal endothelial cells with transcellular vacuoles were seen in the peripheral regions of the cornea, but not in the centre (Fig. 3-4D). No endothelial layer was seen in the embryonic specimen, and the space between the lens and the cornea contained stromal cells, which appeared to adhere to the lens (Fig. 3-4B). TEM was used to confirm these observations. True corneal endothelial cells were seen in the peripheral regions of the TEM sections of a five-day old specimen, and in the cornea of a 32-day old specimen (data not shown). These findings suggest that, contrary to what is observed in the mouse, the formation of the anterior chamber in the naked mole-rat occurs after the formation of the corneal endothelium.

3.6.2 Development of the ciliary body and iris

In the mouse, the ciliary body and iris morphogenesis starts prenatally, at about E17 (Theiler, 1989), and continues until P10, when both of these structures are morphologically mature (Monaghan et al., 1991; Smith et al., 2001). The ciliary body and iris primordia are distinguishable by E18, and there is a clear boundary between these structures in the neonate mouse (Fig. 3-3H). Contrary to what is observed in the mouse, there are no clearly distinguishable ciliary body and iris primordia in the eye of the neonate naked mole-rat, though a highly pigmented structure covered with a non-pigmented epithelial layer is clearly visible (Nikitina et al., 2004; Appendix B), and probably represents the common primordium of the iris and ciliary body. This ciliary body-iris complex of the mole-rat is greatly enlarged, making up about 30% of the eye circumference; while in the mouse the combined lengths of the ciliary body and the iris make up only 8% of the circumference (Table 3-2). By the time the naked mole-rat is 14 days old, the ciliary body appears to have become distinct from the iris (Fig. 3-2B). Interestingly, the combined length of the iris and the ciliary body at that age is approximately equal to the length of the neonate ciliary-body-like complex (Table 3-2), suggesting that in the mole-rat, part of this initial structure differentiates to form the iris.
Figure 3-4: Semi-thin (1μm) sections through the corneas of the mouse (A) and the naked mole-rat (B-E) showing the development of corneal endothelium. (A) P7 mouse. (B) Naked mole-rat embryo, showing absence of identifiable corneal endothelial layer. (C) P14 naked mole-rat. (D) P5 naked mole-rat, peripheral region of the cornea. (E) P34 naked mole-rat. e-corneal epithelium, s-stroma of the cornea, ce-corneal endothelium, ps- mesenchymal cells that will form corneal stroma. Black arrowheads indicate transcellular vacuoles in the corneal endothelium.
Figure 3-5. Transmission electron micrographs of various structures of the naked mole-rat eye. (A) Corneal endothelium of a four-year old mole-rat, showing characteristic transcellular vacuoles (v). Insert: semi-thin section through the central region of four-year old mole-rat cornea. dm- Descemet's membrane; s- corneal stroma. (B) Equatorial lens fibres (lf) in a four-year-old mole-rat. lc- lens capsule. (C) Pigment-filled cell in the angle of the eye of a four-year-old mole-rat. (D) Anterior lens epithelium of a five-day old mole-rat with rounded nuclei and cubic-shaped cells. (E) Posterior lens epithelium of a four-year-old mole-rat, with flattened cells. Scale bars: 2 μm
Ciliary fold morphogenesis in the mouse begins around E18, and the elongated adult-like ciliary processes are evident by P4 (Theiler, 1989; Smith et al., 2001). In the neonate mole-rat, there is no evidence of the ciliary fold formation: only the outer (pigmented) layer shows some folding, while the inner (non-pigmented) layer remains flat, and accommodates the folds of the pigmented layer by the decrease in the height of the cells lying directly above the folds (Nikitina et al., 2004; Appendix B). True folding of the ciliary epithelium, similar to that of the neonate mouse, is observed in the 14-day old mole-rat (Fig. 3-2B). BrdU-labelling experiments show large numbers of proliferating cells in the inner (non-pigmented), but not in the outer (pigmented) epithelial layer of the ciliary body in the 14-day old mole-rat (Fig. 3-6A, B, arrowheads), suggesting that increased levels of cellular proliferation in the inner epithelial layer can be responsible for the ciliary folding. However, as the animal grows, the ciliary processes appear to become “flattened out”.

3.6.3 Development of the trabecular meshwork

In the mouse, formation of the trabecular meshwork is a postnatal event. Trabecular meshwork morphogenesis starts around P6, and this structure reaches its full structural and functional maturity by P21-P42 (Smith et al., 2001). The mole-rat trabecular meshwork appears to form at about the same time as the anterior chamber, when the ciliary body separates from the cornea. Well-defined trabecular beams and aqueous channels were visible in the eye angle of a 32-day old mole-rat, but in younger specimens, the state of differentiation of this structure cannot be easily observed due to the adherence of the iris and ciliary body to the cornea, and consequent absence of the iridocorneal angle. The trabecular meshwork in all juvenile mole-rats examined remained unblocked, and no signs of pigment dispersion are visible, suggesting that this phenotype develops slowly as the animal ages.

3.6.4 Development of the lens

Maughan-Brown reported that the lens of the naked mole rat, which has a regular oval shape at birth, develops an irregular, collapsing posterior margin by P21 (Nikitina et al., 2004; see Appendix B). The goal of this work was to corroborate these observations on a number of additional juvenile mole rat specimens, and to ascertain when the degeneration is initiated. Examination of the lens morphology of several additional mole rat specimens revealed that the posterior of the lens was smooth in 1-14 day old specimens, but developed sharp, irregular folds by third week after birth (see Fig. 3-12, 3-14 and 3-15).
Maughan-Brown also reported an abnormal accumulation of the nuclei in the centre of the juvenile and neonate mole rat lenses (Nikitina et al., 2004; Appendix B). Observations on additional mole rat specimens confirm these findings (Figures 3-12A, B; 3-14 and 3-15). In the mouse, a layer of nucleated epithelial cells covers the anterior surface of the lens, and there are accumulations of nuclei at the “bow region”, located at the equator of the lens. In the neonate mole-rat, the epithelial cell layer appears to extend over a significantly larger area of the lens. The region of fibre differentiation (the “bow region”), identified by the elongated shape and the clustered arrangement of their nuclei, is shifted towards the posterior pole of the lens (Nikitina et al., 2004; Appendix B). Interestingly, in juvenile mole-rats, the centre of the lens gradually becomes nuclei-free, suggesting that the fibre maturation process is delayed in this animal compared to the mouse. The lens epithelium in both the neonate mouse and the mole-rat is a simple cuboidal epithelium. However, in the mole-rat, the morphology of the lens epithelial cells changes from regular cuboidal, with round nuclei in a five-day old specimen (Fig. 3-5D), to squamous epithelium in the adult (Fig. 3-5E).

3.7 The adult eye morphology of the Cape dune mole-rat, Bathyergus suillus

The major disadvantage of working with the naked mole rats, as became clear when this work was in progress, is the inaccessibility of the embryonic developmental stages for histological investigations. While anterior chamber and trabecular meshwork develop postnatally in the naked mole rats, and can therefore be studied in this model, lens formation and the specification of the ciliary body zone occur before birth. In order to study these processes, a different but related model had to be sought. Serendipitously, we were able to obtain a number of specimens of a very common mole rat species, the Cape dune mole rat, which were captured and culled in the Cape Town airport as part of the pest control programme. This species belongs to the same rodent family (Bathyergidae) as the naked mole-rat, and similarly has extremely small eyes relative to the body size (Fig. 3-7). Large Cape dune mole-rat males can reach up to 60cm in length and weigh up to 1.8 kg. The absolute eye size of these animals is therefore larger than that of the naked mole-rat, but at 2.7-3.0 mm in diameter, it is still minute in relation to their body size.

Nothing has been reported on the Cape dune mole rat eye structure. It was not clear whether these animals had abnormal eye features similar to those seen in the naked mole rats. Therefore, it was necessary to perform a detailed histological investigation of this animal’s eye. After dissection, the eyes of the Cape dune mole-rat appear flattened in the cornea-retinal direction, making them somewhat disk-shaped (Fig. 3-7B). Unlike the naked mole-rat eyes, these eyes
Figure 3-6. Cellular proliferation in various tissues of the naked mole-rat eye. (A) Fourteen-day old mole-rat showing BrdU-positive cells (green) in the lens (arrows), ciliary body (arrowheads) and cornea. The yellow signal is due to autofluorescence of red blood cells. (B) Phase-contrast image of the section shown in (A). (C) Phase-contrast image of a BrdU-labelled section through the eye of a 12-year old mole-rat. (D) Fluorescent image of a region boxed in (C), showing a BrdU-positive nucleus (green) in the lens epithelium (arrow). The nuclei are stained blue with DAPI stain.

Figure 3-7: The external appearance and eye size of the Cape dune mole-rat. (A) Adult Cape dune mole-rat. Note the extremely small size of the eyes, which are kept closed most of the time. (B) External appearance of the dissected out eyes of the mole-rat. Clear cornea, highly pigmented iris and the layer of fat covering the posterior hemisphere of the eye are distinguishable. Each division in the scale is equal to 0.1 mm, thus the eye is about 3 mm in diameter.
appear to maintain their shape due to internal turgidity, and only collapsed when pierced. The Cape dune mole-rat eyes have very large fluid-filled anterior chamber, clear cornea, highly pigmented iris. Like in the naked mole-rat eye, the posterior of the eyeball is covered with a thick layer of adipose tissue, and pigment spots are found all over the surface of the sclera (Fig. 3-7B).

The internal organisation of the Cape dune mole-rat eye resembles that of the naked mole-rat eye, with some important differences. The adult cornea, like that of the mouse, is composed of three clearly distinguishable layers, the epithelium, the stroma, and the endothelium (Fig. 3-8B). Large anterior chamber is evident in histological sections, in fact the anterior chamber of this eye is actually larger than that of the mouse eye.

The retina of the Cape dune mole-rat resembles greatly that of the naked mole-rat, though it appears thinner due to the difference in the eye size between the two species. All of the retinal layers are visible, except the optic nerve fibre layer, which is absent. The number of ganglion cells appears to be dramatically reduced even in comparison to the 12-year-old mole-rat (compare Fig. 3-3F to 3-8J), so much so that the cells no longer form a continuous layer. Interestingly, the inner nuclear layer in the Cape dune mole-rat retina is reduced in thickness to about one third of the outer nuclear layer, while in the naked mole-rat eye these two layers are of similar thickness. These important differences in retinal architecture indicate that the visual function in the Cape dune mole-rat is significantly compromised.

The lens of *Bathyergus suillus* is smaller relative to the eye size, and is positioned in the anterior half of the eye. The unobstructed vitreous chamber is clearly seen in this eye. The lens is somewhat elliptical in shape, and, similarly to what is seen in the naked mole-rat eye, exhibits irregularities in shape at the equator. The lens epithelium extends about two thirds of the way around the lens, and the equatorial region is irregular and poorly defined (Figure 3-8D). However, the lens epithelium, while more extensive than that of the mouse, does not cover the entire surface of the lens, as it does in the naked mole-rat (Figure 3-8E).

The ciliary body of this species is extremely large and highly pigmented, but, unlike that of the naked mole-rat, it is folded into large clearly defined processes. The well-developed stroma of *Bathyergus suillus* ciliary body is covered with pigmented and non-pigmented epithelial layers (Fig. 3-8C), and contains many capillaries. There is a clear border between the iris and the ciliary body, and the *pars plana*, the region between the retina and the ciliary body, is also easily
Figure 3-8: Histology of the adult Cape dune mole-rat eye. (A) Cross-section through the centre of the 2-year-old mole-rat eye, showing very large anterior chamber (ac), abnormally shaped lens (l) and large and highly pigmented iris (i) and ciliary body (cb). (B) Mole-rat cornea showing well-defined epithelium (e), stroma (s) and endothelium (ce). (C) Ciliary body of the Cape dune mole-rat, showing clear pigmented outer (oe) and non-pigmented inner epithelial layers (ie), and well-developed ciliary folds. Note large number of circular pigmented granules that appear to fill up the trabecular meshwork. (D) Equatorial region of the lens (enlargement of the area boxed in (A)). Lens capsule (lc) is clearly visible. (E) Posterior region of the lens, enlarged from the bottom box in (A). Arrowheads indicate epithelial nuclei, lc – lens capsule. (F) Cape dune mole-rat retina. All layers of the neural retina are present. RPE – retinal pigmented epithelium, pr – photoreceptors, onl – outer nuclear layer, opl – outer plexiform layer, inl – inner nuclear layer, ipl – inner plexiform layer, gcl – ganglion cell layer, nfl – nerve fibre layer. (G) The iris of the mole-rat. Note the well-developed muscle at the tip of the iris (m). Scale bar=500μm.
distinguishable (Figure 3-8A). Despite its abnormally large size, the ciliary body of the Cape dune mole-rat bears more resemblance to the corresponding structure of the mouse. The iris of this mole-rat species has the characteristic elongated shape, and the iris muscle, the stroma and the epithelial layers can be clearly identified (Fig. 3-8G). Despite the relatively young age of this specimen, degeneration of pigmented epithelium and pigment dispersion is also seen in this eye. Like in the naked mole-rat, a large number of pigment-filled macrophages can be seen filling up the large trabecular meshwork (Fig 3-8C).

It was suggested that the microphthalmic phenotype evolved only once in the common ancestor of the family Bathyergidae. Comparison of the adult ocular phenotype of these mole-rats allows one to distinguish the ancestral features, such as the greatly enlarged ciliary body size, the hyper-pigmented iris and ciliary body, and the abnormal shape and cellular organization of the lens, which are shared by these species, from the later appearing features, which have evolved independently in the ancestors of Bathyergus and Heterocephalus. It was therefore concluded that it is feasible to investigate the development of the above ancestral features using Bathyergus embryos.

3.8 Embryonic eye development in the Cape dune mole-rat

Unlike the naked mole-rat, the Cape dune mole-rat is a solitary animal, and is very common in the Cape Town area. The breeding season of these animals is from early June till September, with younger females becoming pregnant later in the season. While the exact duration of the pregnancy in these animals is not known, observations of wild-caught pregnant females suggest that it is at least 60 days (J. Jarvis, personal communication). Average litter size is 1-3 pups.

The embryos of a range of different ages were obtained from wild-caught pregnant Bathyergus suillus females. In order to be able to compare accurately the development of this animal to the mouse, a reliable staging system for this species had to be established. To this end, the crown-to-rump length of every embryo was recorded, and the Theiler staging system for mouse was used to identify stage-specific features. Embryos up to 30mm in length (equivalent to about E16.5 of the mouse) could be reliably staged using such diagnostic features as the presence and length of the limbs, presence/absence of the fingers and toes, whether their eyelids were fused or not. My observations suggest that early development in these animals proceeds at the rate comparable to that of the mouse, so that most of the morphogenesis is completed early during gestation. During the rest of the longer gestation period, the embryos greatly increase in size and develop a full
coat of hair. The largest embryos that were obtained were about 83 mm in length, virtually identical to the size at birth (Bennett and Faulkes, 2000).

In the mouse, eye development starts with the lateral outgrowth of the optic vesicles from the diencephalon, which reach the overlying surface ectoderm, flatten and then invaginate, forming the optic vesicle by E10.5 (Pei and Rhodin, 1970). By E11.0, the lens vesicle is formed by invagination and “pinching-off” from the overlying ectoderm. These initial stages of eye morphogenesis appear to occur similarly in the Cape dune mole-rat eye (Fig. 3-9A, B). The only differences between the mole-rat and mouse eye morphogenesis at this stage was the premature appearance of pigmentation in the prospective RPE, and somewhat flattened and misshaped lens primordium, though this may be a processing artefact.

As soon as the lens vesicle is formed, the cells at its posterior hemisphere (closer to the optic cup) begin to elongate to form the lens fibres, and by E13.0 the lumen of the lens vesicle is completely filled in. The lens continues to increase in size by formation of new fibres from the epithelial cells located at the equatorial region of the lens. The inner fibres start to undergo terminal differentiation, which results in denucleation and loss of other organelles. Because the fibre elongation is synchronised, the nuclei of the fibre cells become arranged in very neat and regular pattern. One of the earliest developmental abnormalities noticeable in the mole-rat eye is the decreased size of the lens primordium, and irregular arrangement of the nuclei throughout the centre of the lens (Fig. 3-9D, E). At later stages, as the central lens fibres began to differentiate, the bow region of the lens becomes distinguishable. As in the naked mole-rats, the bow region is clearly located closer to the posterior pole of the lens (Fig. 3-10A, C, E). The lens nucleus, composed of the primary lens fibres, is more flattened than round, like in the mouse, and the secondary lens fibres are irregular and misshaped. Therefore, abnormalities in the shape of the lens, reminiscent of those seen in the naked mole-rat juveniles, appear early during embryonic development.

Surprisingly, it was found that only the largest of the Cape dune mole-rat embryonic specimens (83 mm) had an anterior chamber. In all other embryonic stages, the lens epithelium appeared to be attached to the cornea, strongly resembling the phenotype of the juvenile naked mole-rats (Fig. 3-9 and 3-10). This was completely unexpected, because the adult Cape dune mole-rats possess a very large and well-developed anterior chamber. The corneal endothelium could not be identified in the wax sections. The cornea of all embryos appeared to be thinner relative to the eye size than that of the mouse of a corresponding stage. This difference could be traced to the
Table 3-3: Embryonic eye development of *Bathyergus* as related to the mouse development.

<table>
<thead>
<tr>
<th>Event in eye development</th>
<th><em>Bathyergus suillus</em>: embryo crown-rump length</th>
<th>Mouse developmental stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outgrowth of the optic vesicle</td>
<td>9 mm</td>
<td>E9.5</td>
</tr>
<tr>
<td>Formation of the optic cup</td>
<td>9-10 mm</td>
<td>E10.0-10.5</td>
</tr>
<tr>
<td>Closure of the lens vesicle</td>
<td>10 mm</td>
<td>E11.0</td>
</tr>
<tr>
<td>RPE pigmentation visible</td>
<td>10 mm</td>
<td>E11.5</td>
</tr>
<tr>
<td>Lumen of the lens vesicle filled by fibres</td>
<td>15-16 mm</td>
<td>E13.0</td>
</tr>
<tr>
<td>Ingression of the periocular mesenchyme</td>
<td>Between 9 and 15 mm</td>
<td>E12.5-E13.0</td>
</tr>
<tr>
<td>Formation of the anterior chamber</td>
<td>Between 65 mm and 83 mm</td>
<td>E14.0</td>
</tr>
<tr>
<td>Retina begins to differentiate</td>
<td>15 mm</td>
<td>E15.5</td>
</tr>
<tr>
<td>Ciliary body-retina border distinguishable</td>
<td>15 mm</td>
<td>E15.5-E16.0</td>
</tr>
<tr>
<td>Formation of the ciliary folds</td>
<td>29 mm</td>
<td>P0</td>
</tr>
<tr>
<td>Retinal differentiation completed</td>
<td>After birth</td>
<td>P16</td>
</tr>
</tbody>
</table>

earliest stage of corneal development, the ingression of the periocular mesenchyme cells; their number in the prospective cornea of the mole-rat appears to be reduced by half compared to the mouse (3-9D, E).

One of the most prominent features of the mole-rat eye is the incredibly large and highly pigmented ciliary body. In the mouse, ciliary body morphogenesis commences at around E15.5 with the slight thinning of the non-pigmented tip of optic cup and thickening of the corresponding region of the pigmented epithelium. By E16.5 the iris primordium can be distinguished from the prospective ciliary body, and slight folding of the ciliary processes is evident. The morphogenesis of the ciliary body and the outgrowth of the iris continues postnatally as described in section 3.6.2. In the Cape dune mole-rat, a much larger area of the optic cup becomes specified as the prospective ciliary body(CB)/iris region. This is evident very early during development, as soon as the prospective CB/iris becomes distinguishable from the rest of the optic cup in 15mm embryo (Fig. 3-9F). This large CB primordium undergoes rapid growth and fold morphogenesis. The outer epithelium thickens and becomes highly pigmented.
Figure 3-9: Embryonic eye development in the Cape dune mole-rat. Mouse eyes (C, E and G) of similar stages are used for comparisons. (A) Nine-mm mole-rat embryo optic vesicle, beginning to invaginate to form an optic cup. (B) The eye of a 10-mm mole-rat embryo. The lens vesicle has separated from the prospective cornea, and the elongating fibres are beginning to fill up the lumen of the vesicle. (C) E11.5 mouse embryo. (D) Sixteen-millimeter mole-rat embryo. Note prominent RPE pigmentation and irregular arrangement of nuclei in the lens. (E) E12.5 mouse embryo. (F) Fifteen-millimeter mole-rat embryo, with no anterior chamber, very large ciliary body/iris primordium and small lens. (G) E14.5 mouse embryo. Scale bars=100μm. Abbreviations: OC-optic cup; LV-lens vesicle; pom-periocular mesenchyme; L-lens; R-retina; RPE-retinal pigmented epithelium; CB-ciliary body.
Figure 3-10: Embryonic eye development of the Cape dune mole-rat (continued). (A) Mole-rat embryo 29 mm in length. (B) E16.5 mouse embryo. (C) Fifty-six mm long mole-rat embryo. (D) Neonate mouse. (E) Sixty-five mm long mole-rat embryo. (F) P5 mouse pup. In A, C and E note the absence of the anterior chamber, the extremely large size and high pigmentation of the ciliary body and iris, and the irregular shape of the lens. Scale bars = 100μm (A, C); 200μm (B, E, F); 500μm (D). Abbreviations: L- lens; CB- ciliary body; i-iris; ac-anterior chamber.
The fold morphogenesis appears to be driven by the growth of the pigmented epithelium, which, at some stages during development appears to be multilayered (Fig 3-10A, C), but undergoes thinning and becomes single-cell thick by the time the fold morphogenesis is completed. The non-pigmented CB epithelium stays single-layered, and maintains its thickness while growing in length to accommodate the enlargement and folding of the underlying pigmented epithelium. Interestingly, the iris primordium is not identifiable in the eye of the Cape dune mole-rat until the ciliary fold morphogenesis is well under way (Fig. 3-10A). Unlike the mouse or the naked mole-rat, the ciliary body and iris morphogenesis is completed in this animal by the time of birth.

In the mouse, the first signs of the retinal morphogenesis are apparent by E 16.5, when the ganglion cell layer becomes distinguishable form the uniform non-pigmented neuroblastic layer (Theiler, 1989; Pei and Rhodin, 1970). The retinal morphogenesis is completed postnatally, by P14. The pigmentation of the RPE layer is first noticeable in the mouse by E11.5, and reaches its full level by E16.5. In the Cape dune mole-rat, the retinal differentiation occurs much slower than the development of the anterior chamber structures. By the time the iris muscle starts to develop, the retina is still double-layered, resembling that of the E16.5 mouse (compare Fig. 3-10E to Fig. 3-10B). The reverse is true for the retinal pigmented epithelium. Highly pigmented RPE layer, exceeding in thickness that of the mouse, is clearly visible in the eye section of the 15-mm embryo (Fig. 3-9D).

3.9 Molecular basis of abnormalities in the naked mole-rat lens

As described above, the lens of the naked mole rats demonstrates extensive structural abnormalities at its posterior margin, which seem to develop around the third week of postnatal life (section 3.6.4). In animals with reduced eyes, the lens often undergoes inappropriate apoptosis (Jeffery and Martasian, 1998; Sanyal et al., 1990). In order to find out whether apoptosis was responsible for the abnormal lens morphology in the naked mole rats, Maughan-Brown performed TUNEL assays; however, she found no evidence of cells undergoing programmed cell death in the lens in any of the neonate or P21 eye sections examined (Nikitina et al., 2004, see Appendix D). Therefore, cell death by apoptosis did not appear to be responsible for the lens degeneration.

In the mouse eye, the central lens fibre cells loose their nuclei during embryonic development; no nuclei are present in the centre of this structure after birth (3-10D). The only nucleated fiber cells are those located at the equator of the lens. The presence of the nucleated cells in the central compartment of the naked mole rat lens in neonates and even older
juvenile specimens was therefore quite unexpected. It is possible that the fibre cells in the naked mole rat fail to exit the cell cycle and remain proliferative. In order to ascertain if this is indeed the case, BrdU-labelling studies were carried out on several naked mole-rat specimens. Eyes from three juvenile mole-rats were examined (n=2 (32-day old mole-rats); n=1 (14-day old mole-rat)). In all of the mole-rats examined, there was evidence of dividing cells in the region of the epithelium located just posteriorly to the lens equator. None of the nuclei in the centre or posterior border of the lens were BrdU-positive. These results suggest that the overall pattern of proliferation is similar in the mole-rat and in the mouse, but the position of the proliferative compartment (equatorial region) in the mole-rat is shifted towards the posterior end of the lens (Fig. 3-6A, B). It was concluded that the presence of nuclei in the centre of the mole-rat lens can most likely be attributed to a delay in the lens fibre denucleation process.

Lens fibre differentiation involves a series of closely co-ordinated steps, including cell cycle exit, degradation of the nuclei and other organelles and simultaneous synthesis and accumulation of fibre-specific structural proteins, including gamma-crystallins. In the naked mole rat, at least one aspect of lens fibre differentiation – denucleation – appears to be compromised. This fact, taken together with the collapse of the lens posterior margin that is often seen in these animals, strongly suggests that other aspects of the lens differentiation process could be abnormal as well. Therefore, investigation of γ-crystallin expression in the naked mole rat eye was undertaken.

In the mouse, the synthesis of γ-crystallins commences at about day 14 of embryonic development, and continues until the mouse reaches reproductive age (about P40), after which it gradually declines (Goring et al., 1992). The proteins persist in the lens fibres throughout the life of the animal, and their presence in the lens at appropriate concentrations is essential for maintaining its transparency (Piatigorsky, 2003). SDS-PAGE and western blotting were performed to analyse the two-day old and the six-year old naked mole-rat lens for the presence of γ-crystallins. The adult (six month old) and two-day old mouse lens extracts were used as a comparison. A protein band of the expected molecular weight of 21-22kDa, corresponding to all six γ-crystallin proteins (Siezen et al., 1988), was observed in the immunoblot of the adult and 2-day old mouse lens extracts (Figure 3-11, lanes 7 and 8). A band of the same size was also seen in the immunoblot of the proteins from the 2-day old naked mole rat lens (Figure 3-11, lane 4), but not in that of the adult (6-year-old) mole-rat, (Figure 3-11, lanes 1 and 2), despite repeated analysis. Because all six γ-crystallins migrate to the same position on the gel, and are all recognised by the polyclonal antibody that was used in this experiment, these results do not allow us to determine if all six gamma-crystallin proteins are transcribed in the juvenile mole rat lenses. All that can be concluded is that at least some of the γ-crystallins are present in the
soluble protein fraction from the lenses of a two-day old mole-rat, but not in the adult mole rat lenses. The diffuse appearance of the six-year-old mole-rat protein bands on the gel (Figure 3-11, gel A, lane 2) is an indication of increased protein degradation, and thus lends additional support to this conclusion.

The results of the western blot analysis demonstrate the presence of γ-crystallins in the 2-day old naked mole rats, but do not exclude the possibility that only some of the naked mole rat lens cells synthesise this protein. The distribution pattern of γ-crystallins within the lenses of naked mole-rats of different ages was therefore analysed by immunocytochemistry (ICC), and the results are shown in Figure 3-12. Sections of adult and neonate mouse eyes were used for comparisons. In the mouse, γ-crystallins are expressed throughout the lens, with the exception of the epithelium and the equatorial region (which includes fibres that have just began differentiating; Fig. 3-12F, G). The pattern of γ-crystallin expression in the naked mole-rat was found to be different to that of the mouse. In the neonate mole-rat, most of the lens fibres stain positive for γ-crystallins, leaving the epithelium and a thin circle of two to three fibre layers extending around the exterior of the lens (presumably, corresponding to the new differentiating fibres) signal-free (Fig. 3-12B, arrow). In the 21-day old mole-rat, this γ-crystallin-free zone was extended to seven to ten layers (Fig. 3-12C, arrow). These γ-crystallin free fibres appear to be mainly those formed after birth, suggesting that these fibres do not differentiate properly. Only the centre of the lens of a three-year old mole-rat stained positive for γ-crystallin (Fig. 3-12D), while no staining was observed in the lens of the 12-year old adult (Fig. 3-12E). It can be concluded that the synthesis of γ-crystallins in the mole-rat lens is terminated around the time of birth, and that the γ-crystallin produced earlier undergoes degradation as the animal ages. This process could be responsible for the irregular shape and fibre arrangement observed in the mole-rat lenses.

Abnormalities in lens shape are also seen in the eyes of adult Cape dune mole-rats. However, the lenses of this mole-rat species are never quite as irregular in shape as those of the juvenile naked mole rats. Because these two mole rat species belong to the same family, it is possible that the expression of gamma-crystallins is also abnormal in the Cape dune mole rats. To find out if γ-crystallins are present in the lens of this species, western blot analysis of protein extracts from two-year old and 8-month old Cape dune mole-rat lenses were performed. Adult mouse lens proteins were used as positive controls, while Cape dune mole-rat retinal protein extract was used as a negative control. The results showed that γ-crystallins were expressed normally in the adult Cape dune mole-rat lenses (Fig 3-13, lanes 2, 3, 7, 8). A single γ-crystallin band was clearly seen in the water-soluble fraction of the mole-rat lens extract, while multiple bands
Figure 3-11: SDS-PAGE and western blot analysis of γ-crystallin expression in the naked mole-rat. (A) SDS-PAGE of proteins from mouse and mole-rat eyes. Lane 1: proteins from a six-year old mole-rat retina; soluble (lane 2) and insoluble (lane 3) proteins from a six-year old mole-rat lens; soluble (lane 4) and insoluble (lane 5) proteins from a two-day old mole-rat lens; lane 6: molecular weight markers: ovalbumin and carbonic anhydrase; lane 7: soluble proteins from the two-day old mouse lens; lane 8: soluble proteins from the adult mouse lens. (B) Western blot of the above gel, showing γ-crystallin expression.
Figure 3-12: Expression of γ-crystallins in the naked mole-rat (A-E) and in the mouse (F and G). (A,B) Neonate mole-rat lens; (A) negative control (no primary antibody added), (B) expression of γ-crystallins (brown). (C) Juvenile (21-day old) mole-rat. (D) Three-year old mole-rat. Note absence of γ-crystallin expression. (E) Twelve-year old mole-rat. (F) Neonate mouse and (G) adult mouse showing a typical expression of γ-crystallins. Arrow in (B) and (C) indicate the γ-crystallin-free lens fibres.
Figure 3-13: SDS-PAGE and western blot analysis of γ-crystallin expression in the Cape dune mole-rat. (A) Ponceau S-stained blotted membrane. Lane 1: water-soluble proteins from adult mouse lens; soluble lens proteins from 2-year-old (lane 2) and 8-months old (lane 3) Cape dune mole-rat; lanes 4 and 5: molecular weight markers: ovalbumin (45kDa), carbonic anhydrase (29kDa) and egg lysozyme (14.3kDa); lane 6: water insoluble proteins from adult mouse lens, water-insoluble lens proteins from 2-year-old (lane 7) and 8-months old (lane 8) Cape dune mole-rat. (B) Western blot of the above gel, showing γ-crystallin expression.
Figure 3-14: Expression of γ-crystallins in the Cape dune mole-rat. See page 68 for the legend.

Figure 3-15: Distribution of the γ-crystallin transcripts in the lens of the mouse, the naked mole-rat and the Cape dune mole-rat. See page 68 for the legend.
appeared to be present in the insoluble fraction of the lens extract. The size of the larger bands (approximately 44 and 65 kDa) indicated that they must be the products of covalent cross-linkage of individual γ-cryrtallin molecules (Fig. 3-13, lanes 7 and 8). Such polymers have been characterized in aging mouse and human lenses and are often associated with cataract formation. The smaller band (14 kDa) probably represents degraded crystallins. None of these bands were seen in the mouse lens protein extract (Fig. 3-13, lane 6).

Bent and misshapen lens fibres are occasionally seen in the lenses of older Cape dune mole rat embryos (see Figure 3-10E). Also, the persistence of the nuclei is seen in the central and posterior fibres of the late embryonic stages of Cape dune mole rats, suggesting possible delay in differentiation of these fibres. ICC was performed to determine the pattern of gamma-cryrtallin expression in the lenses of embryos of several different stages. The results of this analysis suggest that these proteins begin to be expressed in lens fibres as soon as they have finished elongating (Fig. 3-14A), at a developmental stage corresponding to E13.5-14.0 in the mouse, when γ-cryrtallin synthesis normally commences. The expression continues throughout embryonic development, and is restricted to the fibre compartment of the lens (Fig. 3-14B, C). Therefore, it appears that a delay in γ-cryrtallin expression is not responsible for the lens malformation in the Cape dune mole-rats.

Figure 3-14: Expression of γ-cryrtallins in the Cape dune mole-rat. (A) 15 mm long embryo. Gamma-cryrtallin expression has just begun and is restricted to the central fibres. (B) 56 mm long mole-rat embryo. (C) 65-mm long mole-rat embryo. (D) Adult (2-year old) mole-rat, showing uniform distribution of γ-cryrtallins throughout the fibre mass, but not the lens epithelium.

Figure 3-15: Distribution of the γ-cryrtallin transcripts in the lens of the mouse (A-C), the naked mole-rat (F-I) and the Cape dune mole-rat (D, E), as determined by in situ hybridization with dig-labelled riboprobe. (A) γ-cryrtallin transcripts are detected in the central lens fibres of E13.5 mouse embryo (dark blue), but not in the newly formed fibres on the periphery of the lens or in the epithelium. (B) No signal was detected when the section was hybridized to the sense probe. (C) In P7 mouse pup, γ-cryrtallin RNA in the central fibres was degraded, but significant amount of the transcript was still present in peripheral lens fibres. (D) γ-cryrtallin transcripts were detected in the mature lens fibres, but not in the forming fibres of the bow region, or the epithelium of 35-mm Bathyergus suillus embryo. (E) The lens of the 35-mm Bathyergus suillus embryo hybridized to the sense probe. (F) Gamma-cryrtallin transcripts are detected in a few sub-surface lens fibres of a 2-day-old Heterocephalus glaber (G) The lens of 2-day-old Heterocephalus glaber hybridized to the sense probe. (H) Very small number of lens fibres maintain γ-cryrtallin expression in the lens of 21-day-old Heterocephalus glaber (I) The lens of 21-day-old Heterocephalus glaber hybridized to the sense probe.
This led to the next question of whether the cessation of the γ-crystallin expression in the naked mole-rats occurs as the result of the termination of transcription, or a block in protein synthesis. To answer this question, RNA in situ hybridization was carried out using a mouse-derived gamma-crystallin RNA probe. To confirm that the probe was effectively binding to the mole-rat γ-crystallins, Bathyergus samples were also included in this experiment, because these proteins are known to be transcribed in the Cape dune mole-rat lenses. The specificity of the signal was confirmed by using an antisense probe. In the mouse, gamma-crystallin transcripts were clearly seen in the central fibres of an E13.5 lens (Fig. 3-15A). RNA transcripts disappear from the central fibres (the embryonic nucleus) of the lens; this occurs by P7 in the mouse (Fig. 3-15C). As expected, γ-crystallin mRNA was present throughout the fibre compartment of a 35mm Bathyergus embryo. Unexpectedly, the γ-crystallin mRNA synthesis continued to be initiated in a thin ring of the newly differentiating peripheral lens fibres in the naked mole rat lenses, even at P21, when these fibres were no longer translating the message, as shown by ICC (Figure 3-12). It appears that in the naked mole-rat, transcription of the gamma-crystallins is initiated normally, but the message does not persist long enough for it to be translated.

In the mouse, gamma-crystallin mRNA appear to be exceptionally stable; full-length transcripts could be detected in the core of the lens up to P40 (Goring et al., 1991). Therefore, the instability of the γ-crystallin mRNAs of the naked mole rat was surprising. It is possible that the instability of the mRNA is due to a lack of RNase inhibitors that have been found in the mouse lenses and are thought to be responsible for preventing the RNA degradation. If the general levels of RNA turnover are elevated in the naked mole rats, then transcription of other abundant lens proteins should also be affected. Therefore, expression pattern of αA and αB crystallins was investigated in the naked mole rat eyes by ICC (Figure 3-16). The results of this experiment clearly demonstrated the presence of these proteins throughout the lenses of the naked mole rats (Figure 3-16G, H, I). In fact, the alpha-crystallin expression patterns in the mouse eyes and the eyes of the two mole rat species were identical. Thus, it appears that γ-crystallin transcripts are specifically targeted for degradation in the naked mole rat eye.

3.10 The increased ciliary body size in the Cape dune mole-rat is associated with the downregulation of Ptmb4

The most striking feature of the Cape dune mole-rat eye is the extremely large ciliary body. This feature becomes evident in the embryonic specimens as soon as the prospective ciliary zone can be distinguished from the retina (Figure 3-9F). This increased ciliary body size in the adult mole-
Figure 3-16: Expression of α-crystallins (brown) in the mouse (A-C), Cape dune mole rat (D-F) and naked mole-rat (G-J) lenses. (A) E13.5 mouse, showing α-crystallin expression throughout the lens epithelium and fibres, and in the cornea. P7 mouse lens: (B) α-crystallin expression (C) negative control (no primary antibody added). (D) 83mm Cape dune mole rat. (E) 38mm Cape dune mole rat: α-crystallin expression (F) 38mm Cape dune mole rat: negative control (G) 2-day old naked mole rat (H) 14-day old naked mole rat: α-crystallin expression. (I) 21-day old naked mole rat. (J) 14-day old naked mole rat: negative control.

Rats could be caused either by a larger part of the optic cup being specified as CB, or by the relatively higher proliferation rate in that area resulting in increased growth of CB. In order to distinguish between these two possibilities, the length of the ciliary zone/prospective ciliary body in a number of adult and embryonic Cape dune mole-rat and mouse specimens was measured, and expressed as a percentage of the total length of the optic cup (i.e., the sum of the lengths of the retina and the ciliary body). The iris was not included in the measurements, because it grows dramatically during later embryogenesis (Pei and Rhodin, 1970), and this would complicate the
interpretation of the results. For consistency, all measurements were done on central sections through the eye, where the optic nerve was visible. The results of these measurements (Figure 3-17A) suggest that indeed a three times larger region of the optic cup in the mole-rat begins to differentiate as the ciliary zone, compared to the developmental age-matched mouse embryo (Figure 3-17B). This increased to 25% in late embryos and 27% in the adults, which indicates that the proliferation rate in this region is also higher then that of the prospective retina. In the mouse, a comparable dramatic growth of the ciliary zone relative to the retina is not observed, and the size of the ciliary zone relative to the retina remains virtually the same throughout the embryonic development. It could therefore be concluded that both the increased proliferation of the ciliary body zone and the initial specification of a larger area of the optic cup as the prospective CB are responsible for the formation of the enlarged CB in the Cape dune mole rats.

3.10.1 Cloning and analysis of the Bathyergus homologues of Ptmb4 and Tgfb1i4

In has been known for over 40 years that the lens in a vertebrate eye is the source of the instructive signal that induces the subterminal tip of the optic cup to adopt the ciliary body fate (Coulombre and Coulombre, 1964; Stroeva 1967; Genis-Galvez, 1966; Beebe, 1986). Therefore, one could hypothesize that the large CB size in the Cape dune mole-rat is caused by the increase in the area of the optic cup that receives or is responsive to these lens-derived signals. The exact molecular identity of this CB-inducing signal remains unknown. However, Thut et al. (2001) demonstrated that two ciliary body specific genes, Ptmb4 and Tgfb1i4, are upregulated in the tip of the optic cup in response to a diffusible lens-derived signal. These genes can therefore be used as the earliest markers for the prospective ciliary body zone. The above hypothesis could therefore be tested by comparing the area of Ptmb4 and Tgfb1i4 expression in the mole-rat optic cup to that in the optic cup of the mouse. If the above hypothesis is correct, the area expressing these markers in the mole-rat eye should be increased compared to that in the mouse.

Because molecular studies have never been performed on the Cape dune mole-rat, and no genomic resources exist for this animal, the degree of sequence conservation between the mouse and Cape dune mole-rat genes was not known. Therefore, in order to obtain suitable probes, degenerate PCR primers were designed. This was done by aligning the Ptmb4 and Tgfb1i4 sequences from chicken and a number of different mammalian species available in the NCBI Nucleotide database (mouse, rat, human, cow, Chinchilla), and then selecting the regions within the sequence with the least amount of interspecies sequence variation. Total RNA extracted from the adult eye or embryonic heads was used as the template for RT-PCR reaction. The products
of these PCR reactions were expected to be similar in size to those obtained when using total RNA isolated from E19.5 mouse eye as a template. A single prominent band of 170nt in length was obtained with \( \text{Ptmb4} \) primers, and a single band of approximately 1.1 kb was obtained with \( \text{Tg/bl4} \) degenerate primers (Figure 3-18). These sizes of the PCR products derived from the mouse and the mole-rat material were identical, suggesting that the expected products were amplified. The bands were excised from the gel, purified, cloned into pGemTEasy and sequenced. The sequencing results were analyzed using NCBI BLAST nucleotide-nucleotide alignment software (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analysis confirmed that the cloned RT-PCR products had high sequence similarity, but were not identical to the mouse...
and human Ptmb4 and Tgfl114. This excluded the possibility that a human or mouse-derived contaminating DNA was amplified, and confirmed that the RT-PCR products were most likely mole rat homologues of Ptmb4 and Tgfl114. In the case of Tgfl114, the cloned fragment contained complete coding sequence and most of the 3' untranslated sequence (UTR). The nucleotide sequences of Bathyergus homologues of Ptmb4 and Tgfl114 were deposited in the GenBank database and assigned the accession numbers DQ400347 and DQ400348.

The nucleotide sequence of Bathyergus Ptmb4 clone (thereafter referred to as BsPtmb4) was found to be 98% identical to the homologous gene of Chinchilla lanigera, 95% to the human, mouse and rat Ptmb4 and 87% to the chicken Ptmb4, showing a very high level of conservation (Figure 3-19). This clone was then translated using BioEdit software and the resulting protein sequence analysed using protein-protein BLAST. The partial BsPtmb4 sequence was 38 amino acids long, missing six N-terminal amino acids, and showed 100% identity to the mouse, human and chick protein sequences.

Two variants of Tgfl114 mRNA, about 1.8 kb and 5.5kb in length, have been identified in mouse and human tissues (Jay et al., 1996). The smaller, 1.8kb transcript is by far the most abundant in both adult and embryonic human tissues, while the 5.5kb transcript is found as a minor fraction in adult muscle and testis and fetal brain. The primers used to clone the BsTgfl114 were designed to amplify only the more universally transcribed 1.8kb variant, because this form was found to be expressed in the optic cup tip (Thut et al., 2001). The sequencing confirmed that Tgfl114 variant 2 has been isolated. BLAST alignment of the BsTgfl114 nucleotide sequence of the coding region revealed that it was 94% identical to the human, 92% to the mouse and 78% to the chicken Tgfl114. To further assess the degree of conservation of this gene between different vertebrate species, the cDNA was translated and the resulting protein sequence analysed. It showed 97% amino acid identity and 98% similarity to the human, mouse and rat proteins, and 84% identity and 90% similarity to the chicken protein. Interestingly, this protein was more divergent from the mouse and the human proteins than either of these proteins was from each other (human and mouse Tgfl114 proteins are 98.5% identical; Hamil et al., 1994; Jay et al., 1996). Further analysis of the protein sequence identified the leucine zipper and TSC-box motifs that are highly conserved in all members of TSC-22 family of transcription factors (Kester et al., 1999; Uchida et al., 2003; Shibanuma et al., 1992; Fiorenza et al., 2001) (Figure 3-22).
Figure 3-18: RT-PCR amplification of the Bathyergus-specific homologues of Ptmb4 (gel A) and Tgfb1i4 (gel B) using degenerate primers. (A) Lane 1: λVIII molecular weight marker; lane2: Ptmb4 band obtained by RT-PCR on mouse E19.5 whole eye RNA; lane3: control for DNA contamination (no reverse transcriptase included in the RT mixture); lane 4: Ptmb4 band obtained by RT-PCR amplification of adult Bathyergus whole eye RNA, lane 5: control for DNA contamination – Bathyergus sample, lane 6: no RNA control. Note the presence of a few smaller bands in lane 4 that are identical to those in lane 5, indicating that these bands result from amplification of contaminating DNA. (B) Lane 1: POX molecular weight marker; lanes 2-4: Tgfb1i4 amplified from total RNA isolated from 25mm mole-rat embryonic eyes (lane 2), 16mm mole-rat embryo head (lane 3) or E19.5 mouse whole eye (lane 4); lane 5: no RNA PCR control.
Figure 3-19: Alignment of nucleotide sequences of the PtmB4 protein-coding region of Bathyergus, rat, mouse and Chinchilla. Nucleotides in Bathyergus sequence that differ from those in the mouse sequence, but are identical to Chinchilla are highlighted green. Nucleotide substitution that is different from Chinchilla, but identical to the mouse is shaded in blue. Unique Bathyergus nucleotide substitution (A for G at position 121) is shaded in turquoise.

Figure 3-20: Nucleotide and deduced partial amino acid sequence of the clone BsPtmB4. The asterisks indicate two consecutive stop codons.
Figure 3-21: Alignment of nucleotide sequence of the protein-coding region and the 3' untranslated region of Tgfb14 (transcription variant 2) from Batrachurus sturlus, mouse, human and rat. The start (ATG) and the termination (TAG) codons are in bold. Stars underneath the aligned sequences indicate nucleotides that are conserved among these four species. Positions of the Tgfβ-responsive elements are highlighted in dark grey, while Shaw-Kamens sequences are shaded in light grey (Uchida et al., 2003). Two putative polyadenylation signals (AUUAAA) are underlined and in bold.
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Figure 3-22: Nucleotide and deduced amino acid sequence of clone BsTgfb1i4. Leucine zipper motif is shaded in darker grey, and the central leucine zipper residues (one valine and four leucines) are boxed (Shibanuma et al., 1992; Kester et al., 1999). TSC-box is indicated by light grey shading (Fiorenza et al., 2001). The asterisk indicates the stop codon.

The 3' UTR of BsTgfb1i4 was significantly less conserved in sequence than the coding region of the gene (see multiple alignments in Figure 3-21). It was previously demonstrated that TGFβ1 can stabilize Tgfb1i4 mRNA through a trans-acting factor that is able to bind to conserved elements within the 3'UTR of Tgfb1i4 (Uchida et al., 2003). A putative Tgf β1-responsive sequence was identified at nucleotide positions 535-541, which would suggest that BsTgfb1i4, like its human homologue, can be regulated by Tgf β1 at the transcriptional level (Figure 3-22). An AU-rich Shaw-Kamen’s sequence, which is found in many transiently expressed genes and appears to target this mRNA for turnover (Shaw and Kamens, 1986), was found at positions 1060-1064.

3.10.2 Ptmb4 and Tgfb1i4 are not expressed in Bathyergus prospective ciliary body

The Bathyergus-specific probe templates described above were used to prepare dig-labelled RNA probes, which were then hybridized to the mole-rat embryonic eye sections. The mouse-derived probes of the same genes were used on mouse eye sections to optimize the conditions for the procedure and to investigate in detail the expression of these genes during the mouse eye development.
In the mouse, *Ptmb4* expression is first seen at E12.5 at the tip of the optic cup. At later developmental stages, strong signal appears in the tip of the optic cup, the prospective ciliary zone, while weaker signal is identifiable in the inner retina and in the cells at the equator of the lens (Thut *et al.*, 2001; Figure 3-23). This pattern of expression persists until at least P2. In the Cape dune mole-rat, *Ptmb4* expression was not seen at earlier developmental stages (Figure 3-23A). Low levels of expression were apparent in the inner layer of the retina in a 16-mm embryo, which is developmentally equivalent to mouse E12.5/13.0 embryo. In a later-stage, 25-mm embryo, the retinal and lens equatorial *Ptmb4* expression was very similar to that of a corresponding mouse embryonic stage, but no *Ptmb4* signal in the ciliary body region was found.

*Tgfbr1i4* expression in the eye commences at about E13.5 in the mouse (Thut *et al.*, 2001), in the non-pigmented epithelium of sub-terminal optic cup (excluding the prospective iris epithelium). By E16.5, *Tgfbr1i4* transcripts are found in the lens epithelium, non-pigmented ciliary body epithelium, and the inner layer of the retina (Figure 3-24E, F, G). The optic cup expression pattern of this gene in mouse embryos strongly resembles that of *Ptmb4*. In the mole-rat, *Tgfbr1i4* expression was observed in the lens epithelium and in the retina, but elevated levels of *Tgfbr1i4* expression were not observed in the non-pigmented CB endothelium (Figure 3-24). In 22-mm embryo, this gene appeared to be expressed uniformly throughout retina (Figure 3-24A), while in 38-mm embryo inner retina appeared to have higher levels of expression than outer, more differentiated retina (Figure 3-24D). Ciliary body-specific expression of this gene was not seen in any of the specimens (Figure 3-24H).

### 3.10.3 Expression of other ciliary body markers

Because neither *Tgfbr1i4* nor *Ptmb4* exhibit ciliary body-specific expression in the mole rat, it is not possible to use these genes to investigate CB formation. Therefore, alternative CB markers were sought. *Mx1* and *Otx1* are known to be expressed in CB-specific fashion in the mouse eye. *Lmx1b* is a marker for periocular mesenchyme, part of which contributes to the ciliary stroma. Expression pattern of these genes in the mole rat could provide useful information about the dynamics of early ciliary zoning, and the role of the periocular mesenchyme in the mole rat ciliary body and cornea morphogenesis. Sequence analysis of the mole rat homologues of *Ptmb4* and *Tgfbr1i4* suggested that there are high levels of sequence similarity between the mole rat and the mouse mRNAs, at least in the protein-coding regions. Therefore, an attempt was made to use
Figure 3-23: Thymosin B4 (Ptbmb4) expression in the mouse (D, F, G) and the Cape dune mole-rat eye (A-C, E, H, I), detected by in situ hybridization. (A) No Ptbmb4 transcripts are detected in the eye of the ten-mm mole-rat embryo. (B) The ten-mm Cape dune mole-rat eye hybridized to the sense probe. (C) 16-mm mole-rat embryo. Ptbmb4 is beginning to be expressed in the differentiating inner retina, but no expression can be detected in the prospective ciliary body. (D) E16.5 mouse embryo showing Ptbmb4 expression in the non-pigmented epithelium of the ciliary body (arrowheads), and lower levels of expression in the inner layer of the retina (arrows) and the equatorial lens fibres. (E) In a 25-mm mole-rat embryonic eye Ptbmb4 expression is observed in the inner retina (arrows) and the equatorial lens fibres, but not in the ciliary body. (F) Higher magnification of the mouse E16.5 ciliary body in (D). (G) Higher magnification of the mouse retina in (D). (H) 25-mm mole-rat ciliary body and iris, showing the absence of Ptbmb4 expression. (I) 25-mm mole-rat retina. Cb(i) – inner layer of the ciliary body; r(i) – inner layer of the retina; i-iris.
Figure 3-24: Comparison of the distribution of Tgfb1i4 transcripts in the mouse (C, E-C') and the Cape dune mole-rat eye (A, B, D, H, I). (A) Expression of Tgfb1i4 in the eye of a 22-mm Cape dune mole-rat. (B) Eye section from the 22-mm Cape dune mole-rat eye hybridized with the antisense probe, demonstrating the specificity of the signal. (C) Tgfb1i4 expression in E13.5 mouse eye is observed in the inner ciliary body epithelium, corneal stroma and endothelium, periocular mesenchyme and inner retina. (D) Expression of Tgfb1i4 in the eye of a 38-mm Cape dune mole-rat is confined to the lens epithelium and inner layer of retina (arrows). (E) In E15.5 mouse eye Tgfb1i4 is strongly expressed in the inner ciliary body epithelium (arrowheads) and lens epithelium; lower levels of expression are observed in the inner retina (arrows). Higher magnification micrographs of the E15.5 mouse ciliary body and retina are shown in (F) and (G). Higher magnification of the 38-mm Cape dune mole-rat ciliary body (H) and retina (I).
mouse-specific *Msx1, Otx1* and *Lmx1b* probes (complementary to the coding regions of the corresponding genes) to determine the expression pattern of these genes in the mole rat eye.

Preliminary experiments were performed to determine the optimal conditions for hybridization using these probes. *Msx1* and *Otx1* produced a specific and characteristic expression pattern, when hybridized to mouse embryonic eye sections (Figure 3-25A, B) at 58°C, while higher temperatures (70°C) were required to obtain a background-free expression pattern when using *Lmx1b* probe (Figure 3-25C). However, when *in situ* hybridizations were performed using these probes on the mole rat eye sections, no signal was obtained (data not shown). Lowering the hybridization temperature to 55°C or changing the stringency of the wash solution resulted in the appearance of extensive background (data not shown). It was therefore concluded that these probes were not suitable for use in the Cape dune mole rat.

### 3.11 Function of *Ptmb4* in the anterior optic cup morphogenesis

The absence of *Ptmb4* expression from the tip of the optic cup in Cape dune mole-rats was rather unexpected, since this gene is expressed in the prospective ciliary body-specific manner in all vertebrates examined to date including chick (Thut *et al.*, 2001; Kubo *et al.*, 2003), mouse (Thut *et al.*, 2001) and zebrafish (Roth *et al.*, 1999). The role of *Ptmb4* in ciliary body morphogenesis was not understood. However, investigations in non-ocular cell types have identified several functions for this molecule. Intracellular *Ptmb4* facilitates cellular motility and cell shape changes by maintaining an intracellular pool of monomeric actin (Sanders *et al.*, 1992; Yu *et al.*, 1993). *Ptmb4* can also be secreted and taken up by cells in culture (Bock-Marquette *et al.*, 2004). Secreted *Ptmb4* was shown to play important role in angiogenesis by interacting with extracellular actin (Philp *et al.*, 2003), and can promote cell survival and migration (Bock-Marquette *et al.*, 2004). The onset of *Ptmb4* expression in the prospective ciliary body (E12.5 in the mouse) occurs just before or concurrently with several important morphological changes in this area of the optic cup. The ciliary body becomes distinct from the retina after E15.5, because the rate of cell division slows down in this area of the optic cup. Next, the capillaries are formed, and the overlying layers of the optic cup begin to undergo cell shape changes in order to accommodate the forming vessels. *Ptmb4* function could be important for any of these processes. From the expression pattern in the eye of animals such as mouse and chick, it could be hypothesised that: 1) intracellular *Ptmb4* (in the prospective CB epithelium) could be important.

* Several other probes recognizing developmentally important anterior chamber genes were also tested, including *Tgfβ1, Bmp4, Mitf* and *Otx2*, but these did not show specific expression patterns even on mouse sections, and were not used in further work.
for the cell shape changes that accompany the folding of CB into the processes or 2) secreted Ptmb4 could promote the formation of the capillaries in the ciliary stroma. In order to establish the function of Ptmb4 in normal CB morphogenesis, it was necessary to 1) investigate the ocular distribution of the Ptmb4 protein to find out whether it is secreted; 2) develop a suitable experimental approach to investigate the function of Ptmb4.

3.11.1 Endogenous Ptmb4 expression in the chick and mouse embryo

In order to establish whether Ptmb4 is secreted in the eye, immunocytochemical study of Ptmb4 distribution within the eye was performed using frozen sections of P0 mouse eye. It was found that Ptmb4 protein was not confined to the tissues that transcribe *Ptmb4* mRNA (compare Figure 3-23F, G to Figure 3-26C, D). Ptmb4 was found in both the inner and the outer layers of the prospective ciliary body as well as in the prospective iris (Figure 3-26D); while the corresponding mRNA is seen only in the inner CB epithelium (Figure 3-23F). Ptmb4 was also found in the inner layer of the retina (Figure 3-26C) and in the corneal epithelium and endothelium, where the mRNA is also normally present (Figure 3-23G). In addition, high levels of Ptmb4 protein were observed in the hair follicles (Figure 3-26E). Dathe and Brand-Saberi (2004) report elevated levels of Ptmb4 mRNA in the embryonic chick feather buds, which are homologous to the mammalian hair follicles, while Philp et al. (2004) demonstrated that a subset of hair follicular keratinocytes in the mouse skin express Ptmb4. The presence of Ptmb4 in the hair follicles serves as an additional confirmation that the signal was specific. Since the outer ciliary body and the iris do not transcribe *Ptmb4* RNA, the presence of the protein in these tissues can best be explained by it being secreted from the inner CB layer. These results suggest that secretion of Ptmb4 might take place during development of the ciliary body.

Because both intracellular and secreted Ptmb4 appear to be present during eye morphogenesis, several experimental approaches were considered to investigate the role of Ptmb4 in this process. The most meaningful approach would be a loss-of-function experiment where Ptmb4 is knocked out. However, such systems (e.g., transgenic mice) are not easily available, and do not provide conditional knockout. Therefore, two gain-of-function approaches were attempted: overexpression of Ptmb4 within the cells of the optic cup, and introduction of cells secreting Ptmb4 into the optic cup.

In order to ensure the success of the overexpression experiment, the exogenous Ptmb4 should be introduced into cells that are normally capable of expressing high levels of Ptmb4 before endogenous expression is initiated. However, the expression pattern of Ptmb4 in the chick embryo was not well established. Kubo et al. (2003) demonstrated that Ptmb4 is expressed
Figure 3-25: Expression of selected anterior segment genes in the mouse eye. (A) Msx1 expression in the eye of E16.5 mouse is confined to the prospective ciliary body region. (B) Otxl expression in E16.5 mouse eye. (C) Expression of Lmx1b in the cornea and periocular mesenchyme of E15.5 mouse.

Figure 3-26: Distribution of Ptmb4 protein in the eye of P0 mouse, assayed by immunocytochemistry. (A) Whole eye, showing signal (brown staining) in the corneal epithelium and endothelium, lens, inner layer of the retina, ciliary body and iris. (B) Negative control (no primary antibody added). (C) Ptmb4 distribution in the retina. (D) Ciliary body and iris, showing Ptmb4 localised predominantly to the inner layer of CB, but also in the iris and the outer CB epithelium. (E) Keratinocytes of the hair follicles are also Ptmb4-positive. r(i) – inner layer of the retina; r(o) – outer layer of the retina; cb(i) – inner layer of the ciliary body; cb(o) – outer layer of the ciliary body; i – iris.
strongly and specifically in the optic cup tip of 4-day old chick embryos using section in situ hybridization, while Thut et al. (2001) showed the same using whole mount in situ hybridisation. However, there are no reports on the time when Ptmb4 expression commences in the chick eye, and nothing is known about the dynamics of this gene’s expression during development. In order to establish when and where Ptmb4 expression in the chick eye is initiated, whole mount in situ hybridization experiments on chick embryos of HH16 (56hrs of incubation) to HH27 (5 days of incubation) were performed.

Ptmb4 expression was first observed around the eye and in what in the whole-mount view appears to be the surface of the lens of HH16 (56hrs) embryos (Figure 3-26 A, B). This corresponds to E11 of mouse embryogenesis (see Appendix E). However, when these specimens were cryosectioned, it became obvious that the signal was confined to the neural-crest derived periocular mesenchyme cells, which would form the future corneal stroma and endothelium (Figure 3-27C, arrow). Other neural crest derived mesenchymal populations throughout the embryo’s head and trunk also showed strong Ptmb4 expression (Figure 3-27E). This neural crest-specific expression has not been reported in the mouse or any other animals. Ptmb4 expression in the prospective ciliary body was first seen at HH21 (E3.5) embryos, and became progressively stronger until HH25 (E4.5) (Figure 3-28B-E). It was not possible to determine from the surface view of the eyes whether Ptmb4 expression was confined to the prospective CB or extended all the way to the tip of the iris. In order to visualize the internal expression pattern, the eyes were frozen in OCT and cryosectioned. Unfortunately, due to the large size of the eye at these advanced embryonic stages, the signal was too faint to be seen in the sections.

Non-ocular expression of Ptmb4 was seen in the somites and the mesenchyme of the trunk at HH16 (Figure 3-27A). At HH20-22, high levels of Ptmb4 expression were observed in the somites and trunk mesenchyme, as well as in the developing blood vessels of the brain and allantois (Figure 3-28B, C).

Since it was determined that the ciliary body specific expression of Ptmb4 in the chick embryos commences at HH21, it was decided that overexpression experiments should be conducted using younger embryos (before HH 20), which do not yet transcribe large amounts of endogenous Ptmb4.
Figure 3-27: Wholemount in situ hybridization showing Ptmb4 expression in the HH16 chick embryo. (A) Strong Ptmb4 expression is seen in the mesenchyme and somites of a HH16 chick embryo. Line indicated the plane of sectioning for (C). (B) Higher magnification of the area boxed in (A); Ptmb4 signal is seen in the periocular mesenchyme and on the surface of the lens. (C) Cryosection through the embryonic head. Ptmb4 signal is confined to the ingressing neural-crest derived mesenchymal cells (arrow). (D) Embryo hybridized to a sense probe, showing absence of background. (E) Section through the trunk of the same embryo showing neural crest expression of Ptmb4 (arrows).
Figure 3-28: *Ptmb4* expression during chicken embryonic development. (A) HH20 embryo; the signal is seen in the trunk mesenchyme and the midbrain. Note the absence of the signal in the eye. (B) Embryo at HH21 showing *Ptmb4* expression in the midbrain, trunk and anterior eye. (C) Embryo at HH22. Note the reduction in the signal in the trunk at the level of the limbs. (D) Higher magnification of the eye in (B). (E) Higher magnification of the eye in (C). Note prominent *Ptmb4* expression in the prospective ciliary body (arrows).
Figure 3-29: Distribution of Ptmb4 protein in the eye of E4.5 (A-D) and E8.5 (E-H) chick embryos, assayed by immunocytochemistry. (A) Lens of E4.5 chick embryo, showing signal (brown staining) in the lens epithelium (arrows). Corneal epithelium also demonstrates strong positive signal for Ptmb4 (arrowhead). (B) Retina of E4.5 chick embryo, with the inner cell layer positive for Ptmb4. (C) Ciliary body of E4.5 chick. (D) Negative control (no primary antibody added). (E) Ptmb4 distribution in the lens and the cornea of E8.5 chick embryo. Strong signal is detected in the lens epithelium (arrows) and in the corneal epithelium (arrowheads). (F) Ciliary body and iris, showing Ptmb4 localized predominantly to the inner layer of CB and the iris (arrows). (G) Ptmb4 localization in the E8.5 chick pars plana. (H) Ptmb4 expression in the retina of E8.5 chick embryo.
In order to investigate whether Ptmb4 secretion occurred in the chick eye, ICC was performed on cryosections of E4.5 and E8.5 chick eye. The results were consistent with the observations on the mouse eye sections. Ptmb4 protein was found in the corneal epithelium, and the epithelium and peripheral fibres of the lens both in the E4.5 and in the E8.5 eye (Figure 3-29A, E). At E4.5 Ptmb4 was also found throughout the retina, the RPE and the prospective ciliary body and iris, although the signal appeared stronger at the tip of the optic cup (Figure 3-29B, C). At E8.5 strong Ptmb4 expression was detected in the iris and both layers of the ciliary body, both in the pars plana and at the level of the ciliary folds, but not in the retina (Compare Figure 3-29F, G to 3-29H). Because Ptmb4 mRNA has been detected only in the prospective ciliary body region, but not throughout the retina (Figure 3-28D, E; Kubo et al., 2003), these results possibly suggest that Ptmb4 is secreted during chick embryonic eye development.

3.11.2 Cloning of the chick-specific Ptmb4 expression construct

The full coding sequence of the chick Ptmb4 was PCR-amplified using a clone from the chick neural crest cDNA library (V. Lee, unpublished) as a template, and primers incorporating XhoI and EcoRI restriction sites to facilitate directional cloning. The resulting 120bp DNA fragment (Figure 3-30A) was then ligated into XhoI and EcoRI sites of the chicken-specific expression vector pCIG. Individual colonies were screened for the presence of the insert by colony PCR using pCIG sequencing primers that flank the multiple cloning site of pCIG. When empty pCIG was used as the template, 150bp PCR products were generated, while amplification from the insert-containing colonies yielded products of 270bp in length (Figure 3-30B, arrowheads). The final construct (Figure 3-30) contained the chick Ptmb4 full coding sequence under the control of beta-actin promoter and CMV enhancer. The vector also contained the coding sequence for green fluorescent protein downstream from the inserted Ptmb4 and separated from it by an internal ribosomal entry site (IRES). The GFP and Ptmb4 would be transcribed as a single polycistronic mRNA, and both of the open reading frames would be translated. The IRES served to prevent the mRNA from dissociating from the ribosome. The absence of amplification errors was verified by sequencing the insert in both directions. This expression construct was named pCIG+Ptmb4.

3.11.3 In ovo Ptmb4 overexpression experiments

One approach to overexpressing Ptmb4 in the eye is to introduce the overexpression construct into the chick embryonic optic cup by in ovo electroporation. To determine if this approach was technically possible, a series of preliminary experiments were performed. Empty pCIG was injected into the chick eye at HH20 (E3.5), followed by 5 electric pulses at 25V.
However, at that stage of development, vitreous chamber has not yet appeared, and the posterior of the lens is in close contact with the retina. Injecting high-concentration and therefore very viscous DNA solution resulted in the rupture of the eye, and death of all the embryos within 24 hrs after the procedure.

Next, the same approach was attempted using slightly older embryos, with larger eyes with well-developed vitreous chamber (E4.5, HH25). The posterior retina, which does not express high levels of Ptmb4, was targeted in these experiments. However, it was found that only a very small proportion of the embryos (1 out of 10) showed GFP expression; also, only a few of the retinal cells took up the plasmid, resulting in a localised, low-level expression (data not shown). Because electroporation of the eye is extremely time and labour-consuming, and the results were not encouraging, it was concluded that an alternative method for overexpressing Ptmb4 had to be sought.
3.11.4 Expression of Ptmb4 in Cos7 cells

One approach to this problem is to introduce Ptmb4-expressing and secreting cells into the vitreous chamber of the chick eye and study the resulting phenotype. Monkey kidney Cos7 cell line was chosen for use in these experiments, because these cells are very fast-growing, can be cultured in standard DMEM medium and can be transfected extremely efficiently. The Ptmb4 overexpression construct was cloned using the expression vector specifically designed for use in chick cells, and it was therefore important to ascertain if it is efficiently expressed in Cos7 cells.

A series of transfections were performed using various concentrations of a mammalian GFP-expressing vector pEGFP-N1 (positive control), pCIG and pCIG+Ptmb4. Green fluorescence resulting from the transcription of GFP was observed in a significant proportion (30%) of cells transfected with pCIG+Ptmb4 construct (Table 3-4). Because Ptmb4 and GFP are translated from a single mRNA transcript, and the protein-coding sequence for GFP is located closer to the 3' end of the transcript than the Ptmb4 coding sequence (Figure 3-30C), the fact that GFP could be observed was an indication that Ptmb4 must have been translated as well.

After performing a range of transfections using varying concentrations of the plasmids and FuGENE to plasmid ratios (as recommended by the manufacturers), optimal conditions for Cos7 transfection were established. It was determined that using 10μg of DNA and 15μl of FuGENE transfection reagent per 35mm plate resulted in the highest efficiency of transfection (50%), when pCIG and pCIG-Ptmb4 were used. Lower DNA concentrations resulted in lower numbers of transfected cells (27-30%, Figure 3-31C). It is interesting to note that the vector pEGFP-N1, which contains GFP under control of a mammalian promoter, could be used.

Table 3-4: Optimization of the Cos7 transfection conditions.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>5μg DNA</th>
<th>10μg DNA</th>
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<tbody>
<tr>
<td>pEGFP-N1</td>
<td>50%</td>
<td>38%</td>
</tr>
<tr>
<td>pCIG</td>
<td>30%</td>
<td>43%</td>
</tr>
<tr>
<td>pCIG+Ptmb4</td>
<td>27%</td>
<td>49%</td>
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</table>
Figure 3-31: Optimization of Cos7 cell transfection. (A) and (B) Cos7 cells transfected with 5μg/plate and 10μg/plate of pEGFP. (C) and (D) Cos7 cells transfected with 5μg/plate and 10μg/plate of pCIG-cPmbH4. (E) Cos7 cells transfected with 10μg of pCIG. Note that EGFP expressed from pCIG contains nuclear localisation signals, and therefore is confined to the nuclei of the transfected cells.
to transfect Cos7 cells efficiently at much lower concentrations (3-5 µg per plate), and using higher DNA concentrations was in fact, resulting in the loss of transfection efficiency. This difference could be attributed to the fact that GFP is not expressed as efficiently from chicken promoter in the monkey-derived Cos7 cells, as it is when a mammalian promoter is used.

To determine whether the Cos7 cells transfected with pCIG+Ptmb4 were able to express Ptmb4, the transfected cells were immunostained with anti-Ptmb4 antibody. The results demonstrated that low levels of Ptmb4 were present in some non-transfected cells (this was most likely the endogenous Ptmb4, Figure 3-32B, E), but that the cells transfected with pCIG+Ptmb4 construct were expressed much higher levels of this protein (Figure 3-32E, blue arrows). The transfected cells were identified by the nuclear-localised GFP expression (Figure 3-32A, D). When the Cos7 cells were transfected with the control plasmid (pCIG), the amounts of Ptmb4 expressed by transfected and non-transfected cells (as judged by the intensity of the fluorescent signal in the red channel, Figure 3-32B, E) were identical. However, in cells transfected with Ptmb4, the levels of red fluorescence were dramatically increased. Overlaying of the signals from the red and the green channel demonstrated that this was not due to the bleed-through of the GFP signal from the green channel, as the elevated levels of Ptmb4 were present throughout the cytoplasm of the Ptmb4-transfected cells (Figure 3-32F). Therefore it was concluded that Cos7 cells were indeed expressing and processing Ptmb4 from the chick-specific expression construct.

In order to establish whether that Ptmb4 was being secreted by Cos7 cells, proteins were isolated from the culture medium and analysed by western blot. Because the immunostaining results discussed above demonstrated the presence of intracellular Ptmb4, total proteins extracted from the cells were used as the positive control. However, no signal was detected in either the intracellular or the secreted protein lanes, suggesting that the anti-Ptmb4 antibody used was not suitable for western blots.

Despite the fact that the western blot results were inconclusive, it was possible to determine that Ptmb4 was synthesized by the transfected Cos7 cells. Since Bock-Marquette et al. (2004) used a very closely related cell line Cos1 for their experiments and have shown that these cells were successfully secreting Ptmb4, it was deemed worthwhile to proceed with the injection experiments using transfected Cos7 cells.

3.11.5 Does secreted Ptmb4 play a role in the morphogenesis of the optic cup?

In order to find out whether secreted Ptmb4 has any function during the morphogenesis of the chick eye, two experimental series were attempted. In the first, Cos7 cells transfected with
Figure 3-32: Immunofluorescence images showing the levels of Ptmb4 protein in Cos7 cells transfected with pCIG (A-C) and pCIG+Ptmb4 (D-F). (A) and (D): nuclei of the transfected cells can be identified by the expression of GFP. (B) and (E): Ptmb4 expression in the cells. Blue arrows indicate the cells transfected with Ptmb4 expression construct, showing high levels of Ptmb4 expression. (C) and (F): merged images.

pCIG+Ptmb4 were injected next to the RPE of 3-day-old chick embryos. Cells transfected with the empty pCIG were injected in the same manner to control for non-specific effects that the cells could have on the eye morphogenesis. The injected eggs were re-sealed and incubated for further 24 to 48 hrs to allow the embryos to develop to the stage when ciliary body/iris becomes distinguishable from the rest of the RPE. The eyes were then dissected out, sectioned and examined for abnormalities. Out of 50 embryos injected with Ptmb4-expressing cells (aggregate
of two separate experiments), 43 survived for at least 24 hrs, and 12 of those contained significant amounts of injected cells in close proximity to either the retina or the RPE. However, no alterations in the morphogenesis of either the RPE or the retina as the result of the injection of Ptnb4-expressing cells were observed (Figure 3-33).

It is possible that no effect was observed, because the Ptnb4-expressing cells were introduced after the responsive tissues were specified. Therefore, in the next experiment, the cells were injected at an earlier stage, as soon as the optic vesicle is formed. In chick, the optic vesicle becomes completely separated from the midbrain by 48hrs of incubation (HH stage 12). The transfected cells were delivered to the OV by injecting the brain at HH11 or by injecting cells directly into the eye by HH12. In the former case, it was found that the cells stayed in the brain rather than the optic vesicles, probably because of the pressure generated during the folding of the optic cup (Figure 3-34A). When the optic vesicles were injected directly, the cells were mostly trapped between the RPE and the retina when the optic cup morphogenesis was completed (Figure 3-34B, C, D). Fortunately, it appeared that these ectopic cells did not prevent the optic cups from folding normally (Figure 3-34B, D). However, due to the small size of the optic cup at the time of injection, only a few cells were eventually trapped inside the eye and these did not appear to increase in number, even after 3 days of incubation. It is possible that close juxtaposition of the retina and the RPE prevented these cells from proliferating.

The effect of Ptnb4 on eye morphogenesis was assessed on cryosectioned eyes 72 hrs after the injection. In total, 30 embryos were injected in this experiment, of which 20 were allowed to develop for 72 hrs after the operation (the rest were fixed at earlier stages to monitor the success of the procedure). There appeared to be no morphological differences between the eyes injected with Ptnb4-expressing cells and those containing the empty plasmid (compare Figure 3-34F, G to Figure 3-34E). Figure 3-34E shows the space between the retina and the RPE that could have developed because the Cos7 cells prevented the retina from adhering properly to the RPE. This was not observed in any of the other injected eyes. In addition, a single eye in which the injected cells seemed to have leaked outside the optic cup and were positioned next to the RPE did not show any developmental defects.

Experimental evidence described above suggests that introducing ectopic extracellular Ptnb4 into the eye or optic cup does not affect the morphogenesis of the retina and the ciliary body. However, due to experimental constraints it was not possible to establish whether this phenotype could also be the result of the inability of Cos7 cells to secrete Ptnb4.
Figure 3-33: Effect of secreted Ptmb4 on RPE development in the chick embryo. (A) External appearance of a chick eye 24hrs after cell injection. (B) Higher magnification of the boxed area in (A), showing the location of the injected cells (green). (C) Cryosection through an eye injected with GFP-expressing Cos7 cells (negative control) after 48 hrs of incubation. Arrows indicate the part of the RPE in close contact with the injected cells. RPE morphology is normal. (D) Cryosection through an eye injected with Ptmb4-expressing Cos7 cells, 48hrs after injection. The RPE is not affected by the injected cells (arrows). (E) Cryosection through the eye in which the Ptmb4-expressing cells (arrows) were accidentally injected into the lumen of the eye.
Figure 3-34: Effect of secreted Ptmb4 on the development of the optic cup in the chick embryo. (A) Whole view of the chick embryo head injected with Ptmb4-expressing cells at HH11, 24 hrs after injection. (B) Cryosection through the eye 24 hrs after injection, showing that injected cells trapped between the developing retina and RPE (arrowheads) do not prevent the proper invagination of the optic cup. (C) Eye of an embryo injected at HH12, 48 hrs post-injection, showing trapped Cos7 cells. (D) Cryosection through the eye in (C). (E) Cryosection through an eye injected with the control Cos7 cells (white arrowhead), 72 hrs after injection. (F) Cryosection through an eye injected with Cos7 cells expressing Ptmb4 (white arrowhead), 72 hrs after injection. (G) Cryosection through an eye showing Cos7 cells expressing Ptmb4 (black arrows), that were accidentally localized next to the developing RPE, 72 hrs after injection.
4. Discussion

Vertebrate eye development is a complex, intricately coordinated process, which is still not completely understood. Modifications to this process are the "raw material" of evolution, resulting in diversification of ocular phenotypes, for example, in the formation of a microphthalmic eye in darkness-adapted animals. Very little is known about the developmental and molecular mechanisms responsible for the formation of the microphthalmic eye, but it is clear that eye reduction does not have the same underlying molecular basis in all subterranean/cave animals. In fact, it can be estimated from the phylogenetic relationships between different mammalian groups that the reduced eye phenotype must have evolved independently at least six times in the class Mammalia (Nevo, 1979). In order to gain a comprehensive understanding of natural eye reduction, new mammalian models must be sought. Therefore the first aim of this project was to conduct a detailed histological and ultrastructural investigation of the eye structure and development in the naked mole rat *Heterocephalus glaber*, and the Cape dune mole rat, *Bathyergus suillus*.

It was established that the eyes of these mole rats, while being very small relative to the body size, still retained the general internal organization typical of a vertebrate eye. The most prominent features for the two mole rat species were the irregular shape and abnormal persistence of the nucleated cells in the lens, the extremely large and highly pigmented ciliary body, and the delayed formation of the anterior chamber. The preservation of ocular organization in the mole rats is quite unlike the phenotype seen in other established blind vertebrate models (e.g. *Astyanax, Spalax*), where the lens degenerates and the anterior chamber structures fail to develop. These features make the mole rats uniquely suitable for investigations into the evolutionary and developmental basis of the abnormal patterning of the anterior eye. Because the development of the ciliary body is dependent on the signalling from the lens, and both of these structures exhibit pronounced abnormalities in the mole rats, it seems likely that the initial defect in the lens formation underlies the subsequent developmental abnormalities in the structures of the anterior chamber. Thus, African mole rats are also potentially very useful alternative models for addressing the questions pertaining to the role of the lens in the ciliary body morphogenesis.

4.1 Cellular organization in the mole rat lens is abnormal

The histological studies presented here demonstrate that the nuclear distribution in the naked mole rat lens differs significantly form that of the mouse lens. In the neonate and juvenile mole
rats, the nuclei are found in a thin layer covering about two thirds of the anterior surface of the lens (presumably, corresponding to the nuclei of the lens epithelium in the mouse), as well as within the posterior hemisphere of the lens, which in the mouse is occupied only by non-nucleated lens fibre cells. In order to ascertain whether this peculiar nuclear distribution is the result of the failure of the prospective lens fibre cells to exit cell cycle, the levels of cellular proliferation were assayed using BrdU incorporation. No BrdU-positive cells were found within the fibre cell compartment. This suggests that the abnormal presence of the nucleated cells within the centre of the lens is due to the delayed nuclei degradation in the maturing fibres, rather than to continuous cell proliferation and failure to exit the cell cycle. The only proliferating cells that were found in the mole rat lenses were localized at the equator. The lens proliferative region, therefore, appears to be localized posteriorly in the mole rat, compared to the mouse.

In the mouse, lens polarity is established early in development, around E12, when the posterior cells of the lens vesicle are instructed to elongate and form lens fibres, while the anterior cells remain epithelial. Classic transplantation experiments have demonstrated that molecular signals originating from the retina and present in the vitreous humour of the eye are responsible for the establishment of lens polarity (Coulombre and Coulombre, 1964; Coulombre and Coulombre, 1957). More recent tissue culture-based studies identified that a high concentration of FGFs is able to cause lens fibre differentiation, and a lower concentration can initiate proliferation of lens epithelial cells (McAvoy and Chamberlain, 1989; Lovicu and McAvoy, 2005). Since the role of FGFs in lens morphogenesis has been documented in a range of mammalian species (human, rat, mouse, cow; Chamberlain and McAvoy, 1997), it seems likely that a similar mechanism operates in the mole rats. It is possible that the altered localisation of the proliferative region towards a more posterior location in the naked mole rats is caused by the decreased FGF concentration in the vitreous humour. Further investigations are necessary in order to ascertain what role FGFs and their receptors play in the establishment of the lens polarity in this animal.

As can be clearly seen on histological and even on ICC sections, the lens fibre arrangement in the naked mole rat lenses, especially at the posterior margin in 15-30 day old juveniles is abnormal. The newly differentiated lens fibres, which do not express γ-crystallins, do not form concentric circles around the embryonic nucleus, as in the mouse, but are predominantly located in the posterior hemisphere of the lens (Fig. 3-12B,C, D). It is possible that such an irregular arrangement of lens fibres could result from the altered localisation of the "equatorial region". A consequence of this displacement is that the elongating secondary lens fibres are forced to grow into the posterior lens hemisphere, rather than to elongate equally in both the anterior and the
into the posterior lens hemisphere, rather than to elongate equally in both the anterior and the posterior directions. The embryonic lens nucleus is thus progressively pushed towards the lens epithelium, rather than remaining in the centre of the lens, with the result that the lens fibres become organized into the irregular, bent arrangement, observed in the naked mole rat (Fig. 4-1).

The adult Cape dune mole rat lens also demonstrates the extension of the epithelial cell layer over a larger proportion of the lens surface than in the mouse. However, abnormal persistence of the nuclei in the centre of the lens was not detected. When the embryonic development of the lens was investigated, it became clear that the posterior shift in the position of the equatorial

![Figure 4-1: Model for the development of lens abnormalities in the mole rats. Blue – lens epithelium; yellow – proliferative region of the epithelium; purple – primary lens fibres; green – secondary lens fibres. Top row: specification of the “bow region” and development of the secondary fibres in the mouse. Middle and bottom row: a shift in the position of the “bow region” towards the posterior pole of the lens in the naked and the Cape dune mole rats.](image-url)
region develops very early, when the primary lens fibres start to elongate. This elongation pattern could be caused by a decreased concentration or availability of the differentiation inducing factor(s) (possibly FGF or IGF) within the embryonic eye cup. This observation suggests that the model of lens fibre differentiation in the naked mole rat is also appropriate for the Cape dune mole rat (Figure 4-1). However, the shape of the individual lens fibres in the adult *Bathyergus* lens is much more regular than in the lens of the naked mole rat. This could be attributed to the presence of γ-crystallins in the Cape dune mole rat lens; high concentration of these proteins in the core of the mouse lens is responsible for the hardness of this structure.

### 4.2 Gamma-crystallin expression is regulated differently in the two mole rat species

The unusual persistence of nuclei in the central fibres of the naked mole rat lens led to the question of whether other aspects of lens fibre differentiation are abnormal. ICC and western blots were therefore used to investigate the expression of the lens specific proteins γ-crystallins, which are synthesized only in lens fibre cells, and thus can be used as a marker of lens differentiation. In the western blot experiments, γ-crystallins were detected in the lenses of two-day old mole rat pups, but not in the lens of the adult (six year old) mole rat. This suggests that either the synthesis of this protein is turned off in the adult lens, or the protein undergoes degradation. This result was confirmed by the ICC, which showed that at birth, γ-crystallin proteins were found throughout the entire fibre compartment of the lens, with the exception of a few lateral fibres, which appear to still be undergoing the differentiation process. The same pattern of the γ-crystallin staining is observed in the mouse eyes. However, as the mole rats matured, the area of the γ-crystallin-free fibres was expanded. Moreover, only a very weak signal was detected in the centre of the three-year old mole rat lens, and no γ-crystallin positive fibres were observed in a 12-year old mole rat. It therefore appears that the synthesis of γ-crystallin proteins is downregulated soon after birth, and also that the protein that was already synthesized, becomes degraded as the animals age.

Although the posterior shift in the position of the lens equatorial region was also seen in the Cape dune mole rat embryonic and adult lenses, the shape of the fibres and their organization appeared much more regular. In order to find out whether these lenses also lacked γ-crystallin expression, western blots and ICC were performed. The results clearly demonstrated that γ-crystallin proteins were present in the lens of this animal. Since six individual γ-crystallin proteins are synthesized in the mouse and rat lenses (Breitman *et al.*, 1984; Lok *et al.*, 1984), and all of these are recognized by the polyclonal antibody that was used in this work (Linlin Ding,
personal communication), it is possible to conclude that at least one functional γ-cystallin gene is present in the Cape dune mole rat.

These results lead to the question of whether the shutdown of γ-cystallin expression observed in the naked mole rat lens is a transcriptional or a translational event. The distribution of γ-cystallin mRNAs was assessed by in situ hybridization using a dig-labelled RNA probe transcribed from a full-length mouse crystallin γD cDNA clone. It has been previously reported that such full-length probes can bind to all six mouse γ-cystallins (Goring et al., 1992; Murer-Orlando et al., 1987). Because sequence divergence between different γ-cystallin orthologs is greater than between the homologous genes in different mammalian species, it was assumed that mouse-derived probe would bind successfully at least to the more similar γD/E/F mRNAs. Indeed, the probe was able to recognize γ-cystallins from both mole rat species. Surprisingly, transcription of γ-cystallins in the peripheral sub-surface lens fibres was initiated even in the lens of a 21-day old mole rat, even though no protein could be detected in these fibres by ICC. The γ-cystallin mRNAs were seen only in a thin ring of fibres, which appear to correspond to the ones in which γ-cystallin synthesis is normally initiated; the transcripts disappeared from the more cortical fibres. This pattern of expression suggests that transcription of these genes is initiated, but the protein synthesis does not occur, most likely because the transcripts are rapidly degraded before they can bind to the ribosome and be transcribed. In an indirect attempt to find out whether the general levels of mRNA turnover are elevated in the naked mole rat lens, the expression of another class of abundant lens proteins (α-cystallins) was investigated by ICC. It was found that αA, B-cystallin proteins were produced throughout the lens, and that their synthesis was not affected even in the fibres that have stopped making γ-cystallins. These observations strongly suggest that general levels of mRNA turnover are not affected, and that γ-cystallin transcripts are specifically eliminated from the naked mole rat lens.

A recent paper by Faulkner-Jones et al. (2003) reported that in the chick lens, the persistence of the delta-cystallin transcripts within the central lens fibres does not result from an increased stability of these transcripts, but from their exceptional abundance. It was demonstrated that the rate of delta-cystallin mRNA turnover was the same as that of all other transcripts, but the initial concentration of these mRNAs was 10-23 times higher than that of the next most abundant transcript, ribosomal RNA. It is not clear at present whether a similar mechanism is responsible for the persistence of γ-cystallins in the mammalian lenses. Therefore, it is also possible that the transcription levels of γ-cystallins are reduced in the naked mole rat lens, thus resulting in a much smaller initial amount of the transcript, which can be degraded faster. Sequence analysis of
the γ-crystallin mRNAs and investigation of the levels of transcription of these genes should allow one to distinguish between these two possibilities.

The expression pattern of the γ-crystallins in the naked mole rat is unusual even when compared to that of other blind subterranean mammals. For example, the common mole, *Talpa europaea*, despite having a small poorly differentiated lens, consisting of what appears to be nucleated primary fibres, shows a typical lens expression of α, β and γ-crystallins (Quax-Jeukens *et al.*, 1985). The undifferentiated lens of the blind mole rat, *Spalax ehrenbergi*, does not express either β or γ-crystallins, but does exhibit α-crystallin expression (Quax-Jeukens *et al.*, 1985; Hough *et al.*, 2002). In this animal, lens development appears to be arrested earlier than in the mole, so that no fibre differentiation (and thus no synthesis of fibre-specific proteins) takes place. In other subterranean mammals, the expression of γ-crystallin is either never initiated because of an early defect in fibre differentiation, or continues throughout its lifetime. The initiation of crystallin synthesis followed by turning it off at the transcriptional level, as seen in the naked mole rat, is unique among mammals studied so far. It is rather difficult to speculate why there is a “switching-off” of γ-crystallin expression in the naked mole rat, unless we assume that formation of a lens that allows at least some of the light through, gives an evolutionary advantage to the naked mole rat. Gamma-crystallins differ from all other crystallin types by having a relatively high phase-separation temperature, and thus a tendency to precipitate when the body temperature is slightly lowered, as observed in “cold cataract” formation in neonate rats and mice (Siezen *et al.*, 1988). The naked mole rats, unlike any other mammals, are poikilothermic, i.e. have a variable body temperature (Bucken and Yahav, 1992). It is possible therefore, that switching off the expression of γ-crystallins was an evolutionary solution to producing a lens that would not become completely opaque when the body temperature of the mole rat fluctuates. The finding that γ-crystallin expression is present in the lenses of *Bathyergus suillus*, a closely related species with much better temperature-regulatory ability, lends strong support to this hypothesis. Observation of cold-induced cataracts in the lenses of both types of mole rats could help to further clarify this matter.

4.3 The ciliary body and iris of the mole rats are disproportionately large

Both the iris and the ciliary body of the naked and the Cape dune mole rats are highly pigmented, and the ciliary body is large relative to the size of the eye. Extreme pigmentation and enlargement of these anterior chamber structures has been reported in other mammals adapted to a fossorial lifestyle. This phenomenon is most noted in *Spalax ehrenbergii*, where the pupil is completely obliterated by a mass of pigmented tissue (Sanyal *et al.*, 1990). The presence of
enlarged ciliary body could be of adaptive value to these animals, as it probably protects the retina from sudden exposure to bright light when the animal emerges from its burrow during the day (Sanyal et al., 1990). Alternatively, the increase in the relative ciliary body size might not, in itself, confer any evolutionary advantage, but result from the selective pressure to decrease the size of the retina. As has been pointed out previously, neuronal tissue uses larger amounts of energy than most other tissues, and therefore maintenance of excess neurons is an evolutionary luxury that is strongly selected against (Nevo, 1998). Another possibility is that the increased size of this structure is a secondary downstream consequence of an alteration in the regulatory network involved in eye formation. A number of transcription factors (e.g., Msx1, Otx1) expressed in the prospective ciliary zone and known to be essential for its morphogenesis are also crucial for the development of the brain and parts of the cranial skeleton (Acampora et al., 1996; Martinez-Morales et al., 2001). It is therefore conceivable that alterations in the expression domains of these genes were selected for because the resulting changes in the brain or mandibular structures were advantageous to these animals (for example, as an adaptation to digging), and that the extended ciliary body is a neutral "side effect". A similar pleiotropic effect appears to be responsible for the eye regression in the blind cave fish, where the expansion of the expression domain of Shh, a developmentally important molecule involved in the regulation of the tooth, tastebuds and craniofacial structure development, also results in the reduction of the eye primordia (Jeffery, 2005).

In the mouse, the iris and ciliary body epithelia are formed from the tip of the optic cup, while the iris stroma and the ciliary muscle are derived largely from the cephalic neural crest (Beebe, 1986). A part of the anterior optic cup is specified as the ciliary epithelium at around E12 by as yet unidentified signals from the lens (Stroeva, 1967; Genis-Galvez, 1966; Beebe, 1986; Thut et al., 2001). The morphologically distinct ciliary body is first formed in the mouse around E14.5, and the formation of the ciliary processes and its functional maturation are completed postnatally (Theiler, 1989; Napier and Kidson, 2005). In the neonate mole rat, the size of the ciliary body and iris, relative to the eye circumference, is three times greater than in the newborn mouse (Table 3-2). In the Cape dune mole rat embryos, the ciliary body takes up about 17% of the optic cup compared to 6% in mouse embryos of comparable developmental stage; the relative size of the ciliary body increases throughout embryogenesis. This suggests that, in the two mole rat species, a greater proportion of the anterior optic cup is instructed to adopt the ciliary body fate during embryogenesis. There is abundant experimental evidence that the lens epithelium is the source of the signal that instructs part of the optic cup to form the ciliary body. The region of close apposition of the lens and the anterior optic cup is also significantly enlarged. Therefore, it
can be hypothesized that the enlarged ciliary zone observed in the mole rats could be caused by a larger portion of the optic cup receiving the signal from the lens.

The ciliary body of all adult and most juvenile naked mole rat specimens is flat and elongated in shape, and the ciliary processes are absent. This lack of the ciliary processes is another feature that naked mole rats share with other subterranean mammals (e.g., the common mole (Quilliam, 1966)), but not with any other vertebrate models exhibiting normal visual acuity, i.e. chick, mouse, frog *Rana* (Beebe, 1986). It is currently not clear whether this lack of the ciliary processes affects the functioning of the ciliary body in a way that is somehow advantageous to these animals, or whether it is simply a developmental consequence of the altered signalling within the eye, leading to the increased ciliary body size. The Cape dune mole rats, however, appear to develop prominent ciliary processes despite having an enlarged ciliary body, suggesting that the lack of the ciliary processes in the naked mole rats is not caused by the same molecular mechanisms that are responsible for the increased ciliary body size, and that this phenotype evolved relatively recently.

Ciliary fold morphogenesis in chick appears to be dependent on the intraocular pressure (Bard and Ross, 1982). Observations during dissections of the naked mole rat eyes suggest that the intraocular pressure is reduced, which could be another factor contributing to the absence of the ciliary processes. Incidentally, intraocular pressure is also not visibly reduced in the Cape dune mole rat eyes. It would be very interesting to compare the expression of genes implicated in the ciliary body morphogenesis between the two mole rat species in order to understand the molecular basis of these phenotypic differences.

### 4.4 Expanded ciliary zone of the Cape dune mole rat: the role of the lens

The next question that was addressed in this study concerned the role of the lens-derived signalling in the determination of the ciliary body zone in the Cape dune mole rat. Even at the earliest stages of the CB morphogenesis, as this structure becomes distinct from the retina and the iris, the proportion of the optic cup that is taken up by the prospective ciliary body in the mole rat embryo is about three times greater than in the mouse embryo of a corresponding age. This increased CB zone could be established in one of the following ways: 1) a greater proportion of the optic cup could be specified as the prospective ciliary body; 2) the increased size of the CB could be due to the differential growth after the CB primordium is specified, but before it is morphologically distinct. In the first case, it could be hypothesized that the CB size is determined by the larger area of the optic cup receiving the lens-derived inductive signal, or a
larger portion of the optic cup being able to respond to it. If the latter possibility is true, then the area of the optic cup that is specified as the CB in the mole rat is the same as in the mouse, but the rate of cell division in this area is increased. To distinguish between these two possibilities, in situ hybridization with CB specific markers Ptnb4 and Tgfb114 was performed on the mole rat eye. These molecules were chosen because they are among the earliest and the most specific CB markers known, and also because it was previously demonstrated that they were upregulated in the subterminal optic cup by the signals from the lens (Thut et al, 2001). Therefore using these two markers of the ciliary zone makes it possible to address the role of the lens in the CB morphogenesis in the Cape dune mole rats.

It was expected that if the increased CB size in the mole rats results from the larger proportion of the optic cup receiving the lens-derived signal, then a larger area of the optic cup would exhibit Ptnb4 and Tgfb114 expression at all embryonic stages. However, if the large size of the CB is due to differential growth, a similar zone of expression of these markers in the early embryos of the mouse and the mole rat would be seen, while at later stages the area of expression should increase.

Because little was known about the degree of sequence conservation between the mouse and the Cape dune mole rat genes, the appropriate mole rat-derived probe templates for riboprobe synthesis were obtained by RT-PCR. Surprisingly, in situ hybridization results revealed that neither of these genes was expressed in a CB-specific manner in the Cape dune mole rat. In fact, Ptnb4 was absent from the CB zone altogether, while Tgfb114 was expressed at much lower levels compared to the mouse. Even though the expression pattern was investigated in a wide range of embryonic ages, no CB-specific expression was seen in any of the specimens. Therefore, it is not possible at present to resolve the question of the role of the lens in the enlarged CB morphogenesis in the Cape dune mole rat. Both of the above possibilities may be true. In order address this question further, it is essential to search for additional CB-specific markers that are downstream from the lens-derived signals. A more direct experimental approach to investigate whether the mole rat lens can induce the formation of a larger CB is to transplant the mole rat lens into the chick optic cup and study the resulting phenotype. However, this latter approach is rather difficult to accomplish due to the very narrow timeframe in which the mole rat embryos of the age appropriate for such experiments are available (not more then 2 weeks per year).
Because neither \textit{Ptmb4} nor \textit{Tgfb1i4} were found to be expressed in the prospective ciliary body in the Cape dune mole rat, other CB-specific markers were used in an attempt to determine the size of the early CB zone relative to the rest of the optic cup. \textit{Otx1} and \textit{Msx1} are known to be expressed specifically in the prospective ciliary body (Monaghan \textit{et al.}, 1991; Zhao \textit{et al.}, 2002; Simeone \textit{et al.}, 1993; Martinez-Morales \textit{et al.}, 2001). Both of these are developmentally important transcription factors, and \textit{Otx1} was demonstrated to be essential for the ciliary body morphogenesis. \textit{Otx1}\textnormal{-}/- mice have a hypotrophic CB and do not develop ciliary processes (Acampora \textit{et al.}, 1996). While the relationship between the expression of these genes in the ciliary body and the signalling from the lens has not been established, these are still useful as markers for the ciliary body zone prior to its morphological differentiation. Investigation of the expression pattern of these genes can help understand the dynamics of the CB growth. Because it was established that Cape dune mole rat gene coding sequences appear to be very highly conserved with those of the mouse genes, mouse-derived \textit{Msx1} and \textit{Otx1} probes were used. These were first tested on the mouse tissue and found to produce a clear and specific expression pattern. Unfortunately, these probes did not work on the mole rat tissue despite repeated attempts. Both \textit{Msx1} and \textit{Otx1} contain homeobox sequences that may hybridize to other homeobox genes. It is therefore likely that even small mismatches between the mouse-derived probe and the mole rat target RNA can result in non-specific binding and high background. It appears that it is essential that suitable mole rat specific probes are used for \textit{in situ} hybridization.

4.5 Enlarged CB zone in the Cape dune mole rat correlates with the absence of \textit{Tgfb1i4} expression

The absence of the ciliary body-specific expression of \textit{Ptmb4} and \textit{Tgfb1i4} in the Cape dune mole rat led to the next question: could the absence of these molecules be responsible for the extended size of the Cape dune mole rat ciliary body? Several experiments were performed to explore the role of \textit{Ptmb4} in the morphogenesis of the chick ciliary body, and these are discussed below. The function of \textit{Tgfb1i4} in CB morphogenesis has not as yet been established. However, some of the developmental aspects of \textit{Tgfb1i4} function have been investigated in other systems; it is therefore possible to speculate about the role of this molecule in the eye morphogenesis.

\textit{Tgfb1i4} is a potent negative regulator of cell proliferation (Hino \textit{et al.}, 2000; Nakashiro \textit{et al.}, 1998; Gupta \textit{et al.}, 2003). Tight coordination of cell proliferation and differentiation are essential for proper embryonic development, and alterations in the \textit{Tgfb1i4} expression are known to have profound phenotypic consequences for the embryo. Morpholino-mediated knockdown of \textit{Tgfb1i4} expression appears to affect the gastrulation movements in \textit{Xenopus} by promoting inappropriate
proliferation of the ectoderm. Overexpression of this molecule resulted in cessation of proliferation and inappropriate apoptosis (Hashiguchi et al., 2004). It is possible that the role of Tgfb1i4 in the prospective ciliary zone is to downregulate cellular proliferation in the epithelial layers. The observation that decreased levels of Tgfb1i4 expression in the prospective CB in the Cape dune mole rat correlate with increased growth of this tissue appear to support this notion. It would be interesting to establish whether the ciliary body size and the levels of cell proliferation are decreased in the optic cups expressing high levels of Tgfb1i4, and if the opposite is seen when this molecule is knocked down.

4.6 Role of Ptmb4 in eye morphogenesis

Studies on cultured mammalian cells demonstrated that Ptmb4 plays an important role in cytoskeletal rearrangement by sequestering actin monomers and inhibiting actin polymerization (Huff et al., 2001; Bubb, 2003; Sanders et al., 1992). This ability to modify cytoskeleton is essential for various developmentally important cellular processes, such as cell migration and cell shape changes during morphogenesis (Grant et al., 1999; Malinda et al., 1997). Ptmb4 can also be secreted and taken up by cells, though the exact mechanism by which this occurs is still not understood (Bock-Marquette et al., 2004; Al-Nedawi et al., 2004). Secreted Ptmb4 has a number of functions distinct from those of the intracellular Ptmb4, for instance it can promote angiogenesis through interactions with extracellular actin (Phelp et al., 2003), and stimulate migration and survival of embryonic cardiac cells (Bock-Marquette et al., 2004). The expression pattern of Ptmb4 in ocular tissues suggests that it could be important for several morphogenetic processes occurring in the eye. First, it is possible that high intracellular concentrations of Ptmb4 are necessary for the rearrangement of the cytoskeleton during the growth and folding of the ciliary processes. Second, Ptmb4 might play a role in the formation of the capillaries in the ciliary stroma. Comparison between the intraocular distribution of Ptmb4 protein and mRNA in the mouse and chick eye suggests that Ptmb4 is secreted. Therefore, it is possible that Ptmb4 performs both of these roles during CB morphogenesis.

In order to investigate the role of Ptmb4 during CB development, several approaches could be used. Downregulation of Ptmb4 expression would provide the most meaningful result. The most obvious model system for this approach – to create a Ptmb4 knockout mouse - would not work in this case, because Ptmb4 is expressed in a range of embryonic tissues and knocking it out would likely result in embryonic death. Chick embryos are much more available and amenable to experimental manipulation. However, gene knockout techniques that could be used to downregulate Ptmb4 are not yet established in this model system. This technical problem was
addressed in Chapter 5-7 of this thesis, where the construction of a suitable chick-specific siRNA expression vector is described. While this work was in progress, overexpression of Ptmb4 in the eye was attempted.

It is easier to interpret the results of overexpression experiments if the gene is introduced into the cells before its endogenous expression is initiated, because then the observed phenotype would be due to the exogenous gene. Before overexpression of Ptmb4 in the chick eye could be attempted it was therefore essential to investigate the ocular expression pattern of this gene. The results of the whole-mount Ptmb4 \textit{in situ} hybridization reported in this thesis suggest that Ptmb4 expression in the ciliary body commences at HH21. Before that, Ptmb4 was detected in the periocular mesenchyme and prospective corneal stroma and endothelium. This expression domain has not been reported before, and suggests the possibility of using it as a novel molecular marker for these cells, which could increase the sparse number of markers for undifferentiated corneal precursor cells currently available. Ptmb4 can also be detected in the trunk and head neural crest cells, and in the neural-crest derived periocular mesenchyme, which further underscores the importance of this protein for cell motility. Local decrease in the somitic Ptmb4 expression at the level of the forming limb buds by about HH22 has also not been previously reported (Dathe and Brand-Saberi, 2004). This could be caused by the migration of some of the Ptmb4-expressing somatic cells into the limb buds to form the skeletal muscle.

To investigate the effect of secreted Ptmb4 on the morphogenesis of the outer layer of the optic cup, Ptmb4-expressing Cos7 cells were injected into the developing eye or optic cup of the chick eye prior to the onset of the endogenous expression of this gene. Both injecting near the RPE of the eye at E3 and delivering the cells directly into the developing optic vesicle at E2 (HH11-12) was attempted. The results of the above experiments demonstrated that intraocular injection of Ptmb4-expressing cells has no effect on the morphogenesis of either of the two layers of the optic cup. Even though it was clearly demonstrated that Cos7 cells transfected with Ptmb4 expression vector were synthesizing increased amounts of Ptmb4, it was not possible to establish whether these cells were secreting Ptmb4. Thus, the observed lack of phenotype could be caused by inability of the Cos7 cells to secrete Ptmb4. However, Bock-Marquett \textit{et al.} used a very similar cell line (Cos1), and observed high levels of Ptmb4 in the cell culture supernatant, suggesting that these monkey kidney cell lines are secretory.

It is also possible that the role of secreted and internalized Ptmb4 in the prospective ciliary body is mainly structural, unlike its function in the embryonic heart cells (Bock-Marquett \textit{et al.}, 2005). If the main function of Ptmb4 is the stabilization of actin monomers, then the presence of excess
Ptmb4 would result in a larger pool of monomeric actin available for cytoskeleton re-arrangement, but will not necessarily translate into observable alterations of the cell shape or cytoskeleton, because these shape changes are likely to be controlled by a different regulatory network. It is of interest that the only other experimental study on the role of Ptmb4 during embryogenesis reported no significant developmental abnormalities resulting from overexpression of this gene (Roth et al., 1999). In general, it is a known limitation of overexpression studies that, while they are often much easier to perform technically, no obvious phenotypic change may be observed when the gene that is overexpressed has a permissive, rather than instructive function. Down-regulation experiments are necessary to ascertain the role that Ptmb4 plays during the development of the ciliary body. It was also concluded that cell injection into the developing chick eye could be a very useful technique for studying the effects of diffusible factors on eye morphogenesis.

The functional significance of the absence of Ptmb4 from the prospective ciliary zone of the Cape dune mole rat is therefore still unclear. Ptmb4 might facilitate cell shape changes accompanying the folding of the epithelial cell layers of the ciliary body into processes or it might play a role in the formation of the capillaries. However, the observation that both the CB folding and the angiogenesis in the CB stroma occur in the Cape dune mole rat despite the absence of Ptmb4 expression contradicts this hypothesis. It is possible that another actin-sequestering molecule functionally substitutes for Ptmb4 in the mole rat eye. Alternatively, it is possible that, due to the large extent of the prospective CB that undergoes folding, individual cells do not need to undergo such dramatic shape changes as, for instance, when mouse CB folding occurs. Therefore, there could be no need for elevated Ptmb4 expression.

4.7 The cornea and anterior chamber of the mole rats

Studies to date on chick (Hay and Revel, 1969) and mouse eyes (Kidson et al., 1999) suggest that the formation of the anterior chamber is coupled to the morphogenesis of the corneal endothelium. Thus, the anterior chamber forms as the neural crest cells closest to the lens undergo mesenchymal-epithelial transformation and become the tightly packed corneal endothelial layer, which separates the extracellular matrix of the corneal stroma from the surfaces of the lens and iris (Kidson et al., 1999; Reneker et al., 2000). Interestingly however, in the naked mole rats the anterior chamber appears to form significantly later than the corneal endothelium. A similar phenotype is seen in the Cape dune mole rat. The corneal endothelium appears to be present in the Cape dune mole rat embryos 65 mm to 83 mm in length, while the anterior chamber is only seen in the oldest (83 mm long) embryonic specimens. Because neither
TEM nor ICC for corneal endothelial specific markers (such as ZO-1 or N-cadherin) were performed on the Cape dune mole rat, it was not possible to establish with certainty when the corneal endothelium is formed. However, it is clearly present in the adult Cape dune mole rats.

Current theories on the mechanism of anterior chamber formation are based on studies in chick and mouse, where the corneal endothelial formation is closely followed by the formation of the anterior chamber. Mutant mouse models that lack anterior chamber all appear to do so because the corneal endothelium is not present, and the anterior surface of the lens adheres to the sticky corneal stroma (Kidson et al., 1999; Reneker et al., 2000; Saika et al., 2001). To this day, no other mammalian species or mouse mutant is known to exhibit a phenotype similar to that observed in the mole rats. Further studies on these animals could significantly extend our knowledge of the mechanism of anterior chamber formation.

Why is the development of the anterior chamber in the mole rats delayed so long after the physical barrier between the surface of the lens and the corneal stroma (the corneal endothelium) is formed? It is possible that the anterior surface of the ciliary body, which in the mole rats is greatly increased in size, adheres to the cornea and prevents the space from forming between the cornea and the lens until the trabecular meshwork develops in the angle of the eye, and causes the angle to open up. This notion is supported by the observation that in the naked mole rat eye, the appearance of the trabecular meshwork coincides with the development of the anterior chamber.

4.8 The structure of the iris and pigment dispersion in the mole rat eye

As in the mouse, the ciliary body and iris of the naked mole rats are not mature at birth. In fact, there is no distinguishable border between the ciliary body and the iris in the neonate mole rat. The measurements (Table 3-2), however, indicate that the iris primordium is present in the neonate mole rats, even if it is not yet morphologically distinguishable from the CB. The length of the ciliary body, as measured on the juvenile and adult mole rats is 0.4 to 0.7 times that of the ciliary body-like structure of the neonates (Table 3-2). This data suggests that part of this structure thins out and differentiates to form the iris. As in the mouse, most of the growth of the iris in the mole rats occurs postnatally. In the Cape dune mole rat, the iris morphogenesis occurs in a very similar way to what is observed in the mouse, except that most of it occurs before birth, due to the differences in the gestation length between these species.
Thinning of the iris stroma and loss of the iris pigment in many old naked mole rat specimens, as well as in adult (2-year old) Cape dune mole rats was observed. This interesting phenotype could be a consequence of the elevated levels of melanogenesis in the iris and the pigmented epithelium of the ciliary body. The increased eye pigmentation in the mole rats suggests that the melanocytes of the anterior surface of the iris, which are not active in the adult mouse, are still producing pigment in the adult mole rats. These greatly increased levels of pigment synthesis could lead to the melanocytes becoming filled up with pigment, bursting and releasing their contents into the anterior chamber. The excessive pigmentation and pigment dispersion seen in the mole rat eyes resembles the phenotypic features of the mouse models of pigment-dispersion glaucoma (John et al., 1998; Anderson et al., 2002). Genetic dissection of the mechanisms regulating pigment synthesis in the naked mole rats could provide additional insights into the aetiology of human pigmentary glaucoma.

The mouse trabecular meshwork is derived from a group of mesenchymal cells situated in the angle between the base of the iris and the cornea. These cells undergo differentiation to form trabecular beams separated by channels, which serve to allow the exit of the aqueous fluid from the anterior chamber. The process of trabecular beam differentiation commences around P10 in the mouse, and the mature meshwork is established by P21 (Smith et al., 2001). In the mole rat, the formation of the trabecular meshwork cannot be assessed at early postnatal stages due to the absence of the anterior chamber and the consequent adherence of the iris and ciliary body to the inside of the cornea, obliterating the iridocorneal angle. It appears, however, that in the five-day old specimen, no identifiable trabecular beams and channels are yet evident, making the state of differentiation of that specimen comparable to P6-P10 mouse. At 30-34 days of age, the trabecular meshwork (TM) of the mole rat is extensive and well-differentiated and comparable to the mouse TM at P21-30. A very unusual feature of the naked mole rat eye, often observed in mature specimens, is a closure of the eye angle and degeneration of the trabecular meshwork. These changes could be related to the unusual longevity of the mole rats (Buffenstein and Jarvis, 2002), but the extensive tissue loss from the trabecular meshwork observed in these animals, is not a feature of the normal aging process even in humans (Oates and Belcher, 1994). A similar deterioration of the trabecular meshwork and blockage of it with excessive pigment is seen in two-year old Cape dune mole rats. It is possible, that increased macrophage activity associated with the pigment dispersion results in the deterioration of the trabecular meshwork.
4.9 The mole rat retina and optic nerve

In contrast to what was reported earlier by Maughan-Brown (2001), who studied only 3 adult naked mole rat specimens, the structure and retinal layer organisation in the mole rat eyes was found to be essentially normal, except that, in the adults, no nerve fibre layer and an apparent decrease in the density of the retinal ganglion layer was observed. A very similar retinal architecture is observed in the Cape dune mole rat eye. Retinal ganglion cells appear to be the most susceptible to apoptosis as the result of increased intraocular pressure, as seen in glaucomas (John et al., 1998). It is possible that, in the mole rats, the impaired circulation of the aqueous fluid due to the blockage and degeneration of trabecular meshwork, causes the loss of retinal ganglion cells. However, the reduction in the numbers of ganglion cells have been observed in other completely or partially subterranean mammals (Herbin et al., 1994), and is not accompanied by the "pigment dispersion" phenotype seen in the naked mole rats. Other factors, perhaps related to decreased exposure to light in subterranean habitats, could therefore play a role. In order to further investigate the age-related changes in retinal architecture, ganglion cell counts and extensive quantitative studies of apoptosis in the retina of mole rats of different ages will need to be performed. These experiments are currently under way in our department.

4.10 Can the mole rats see?

The results of this study allow one to speculate on the degree of visual function of the microphthalmic eyes of the mole rats. Behavioural observations on the members of the family Bathyergidae suggest that these animals rely almost solely on olfactory and tactile cues to navigate and when interacting with conspecifics. There is no evidence that they rely on visual information for any aspect of their daily lives (Narins et al., 1997). Histological examination of the adult naked mole rat and Cape dune mole rat eye lends support to these observations. The small eye size of these animals probably results in their having a very restricted visual field. In addition, the irregular shape of the lens, and the presence of cellular nuclei along the visual axis, might suggest that light scattering prevents clear images from forming on the retina. The absence of the zonula fibres and the reduced ciliary muscle of most naked mole rat specimens suggest that light focusing on the retina does not occur. However, the presence of rudimentary iris muscle indicates that the naked mole rats are able, at least to a degree, to regulate the amount of light that enters the eye. This is definitely true for the Cape dune mole rat, where a well-defined iris muscle is present. Thus it can be concluded that the visual abilities of the mole rats are limited to judging the intensity of the surrounding light (i.e., being able to distinguish between night and day, or between being inside the burrow or outside it), and, possibly, to seeing the
shadows cast by large moving objects, without being able to see their details. These conclusions, drawn on the basis of eye histology, are in agreement with those of several other groups, who investigated primarily the retinal and optic nerve structure and physiology of light response (Peichl et al., 2004; Hetling et al., 2005). The Cape dune mole rat eye appears to be more morphologically normal than that of the naked mole rat; this may result in the preservation of a greater degree of visual perception. Before definite conclusions can be reached, further investigations involving retrograde tracing of the optic nerve projections, and measurements of the retinal response to light are necessary.

4.11 Why are the eyes still retained?

The above findings led to the question of why, after at least 25 million years of subterranean evolution (Bennett and Faulkes, 2000), the naked mole rats still retain all of their ocular structures and apparently a degree of visual ability? The conservation of the eye architecture of this species is especially surprising when compared to the regressed ocular phenotypes seen in other dark-adapted vertebrates. For instance, the eyes of the blind cavefish Astyanax mexicanus do not develop a cornea, an iris, secondary lens fibres or differentiated retina (Jeffery, 2001; Yamamoto and Jeffery, 2000). The cornea, iris and ciliary body epithelia, differentiated retinal layers, as well as the vitreous and aqueous chambers are altogether absent from the eyes of the marsupial mole Notoryctes typhlops (Sweet, 1906), and the insectivorous moles Scalopus aquaticus and Eremotalpa granti (Slonaker, 1902; Gubbay, 1956). The comparatively well-preserved eye phenotype of the mole rats can not be explained by the length of time passed since their ancestors have become adapted to the fossorial habitat, since both Astyanax and Notoryctes, which have arguably the most "degenerate" eyes among any vertebrates, have had only 1-2 million years evolution in the light-free environment (Jeffery, 2001; Nevo, 1979).

The extremely reduced eyes seen in some darkness-adapted vertebrates are thought to have evolved in response to the evolutionary pressure to decrease the metabolic expenditure, associated with the formation and maintenance of the organ that is no longer used (Nevo, 1998). However, the naked mole rats, as well as a number of other fossorial mammals, retain much of the normal ocular architecture, and in particular, an apparently normal retina. This suggests that retaining the capacity for light-dark discrimination is important for the survival of these animals. The soil-removal activity of the naked mole rats results in their direct exposure to sunlight, as the animals kick soil out of an open mound. The open mound poses a further threat of exposure to above ground predators (Sherman et al., 1991). An ability to detect light and dark and sudden
transitions associated with the arrival of a predator at well-lit burrow entrance may confer a survival advantage and hence be maintained by natural selection.

What can the results of this investigation tell us about the evolutionary mechanism responsible for the eye degeneration? The size of the ciliary body is greatly increased at the expense of the retina, which is the tissue of the eye that consumes the most metabolic energy. The decrease in the size of the retina in these animals appears to have adaptive value, as it would lower the energy expenditure of the eye. This observation supports the energy conservation hypothesis of Nevo as one of the driving forces behind the regressive evolution of the mole rat eye (1998). The loss of gamma-crystallins from the lens of the naked mole rat, while they are still expressed in the Cape dune mole rat lens would appear to provide an illustration of the neutral mutation followed by selection. Indeed, the neutral mutation hypothesis predicts that the downstream genes that are expressed in localized fashion during embryonic development are likely to be among the first lost as the result of the accumulation of random mutations. Gamma-crystallins appear to be good examples of such genes. However, there are several gamma-crystallins, and their simultaneous loss in the naked mole rat seems to be more likely the result of an alteration to an upstream regulatory factor. Further investigations of the gamma-crystatin gene cluster and the regulation of their expression in these animals can help resolve this matter and might uncover the underlying pleiotropic effect. The loss of Ptmb4 expression from the prospective ciliary body, while its expression is maintained in other parts of the eye, does not support the random mutation hypothesis either. If random mutation was in operation, the expression of this gene would have been lost altogether, while the actual expression changes of this gene appear to be tightly regulated. While further work is definitely needed, the results discussed in this thesis appear to suggest that most of the evolutionary changes seen in the mole rat eyes are adaptive, and not the result of random mutation.

It is interesting to notice that the degree of eye reduction in various burrowing species correlates very well with the method they use for soil digging. Thus, the eyes of the animals that use their head to push the excavated soil (Spalax), or to force their body forward through the soil loosened by their forelimbs (Notoryctes, insectivorous moles), are much more reduced than those of the animals that use both their incisors and forelimbs to scrape off the substrate, and push the loosened soil backwards with their limbs (Bathyergidae, Rhizomyidae) (Vaughan, 1978; Webb et al., 1979; Nevo, 1979; Bennett and Faulkes, 2000). The “forelimb-diggers” appear to be more at risk of eye damage and infection than the “teeth-diggers”. It is possible that one of the major selective forces favouring the reduction in the eye structures in subterranean mammals is the need to protect this soft and sensitive organ from the abrasive effects of soil.
4.12 Conclusion and future perspectives

In this thesis the microstructure of the eye and its embryonic or postnatal development, as well as the expression of a number of developmentally important genes in two mole rat species are reported. This is one of the first works of this kind aimed at understanding the molecular and embryological basis of the regressive eye evolution in a mammalian system. While the results presented here revealed many hitherto unknown aspects of eye regression in the mole rats, many questions still remain. Some of the more exciting future perspectives, the yet unanswered questions, as well as the experimental approaches that could be used to tackle these questions are discussed below.

4.12.1 The lens and crystallins

Why are $\gamma$-crystallins degraded in the naked but still maintained in the Cape dune mole rat? One of the possible explanations is that some of these proteins in mammals are cryoproteins, i.e., they tend to precipitate and form light-scattering aggregates at high concentrations or low temperatures, thus rendering the lens opaque. The lack of these proteins in the mole rat lens would thus be necessary to ensure that the lens is light permeable even when the body temperature of these poikilothermic animals drops. To test this hypothesis, individual naked mole rat crystallins should be purified and their solubility at different concentration and temperatures (co-existence curves, Siezen et al., 1988) established, and compared to the mouse and the Cape dune mole rat proteins.

What substitutes for gamma-crystallins in the lens of the naked mole rat? Lens transparency depends on the regular arrangement of the crystallin molecules in the fibre cytoplasm, so that if one or more of them are missing or mutated, as happens in mouse mutants and certain human congenital defects, cataract is formed (Graw, 1997). The lens of the young adult mole rats is transparent, suggesting that the appropriate crystalline structure of the cytoplasm is maintained. It is possible that higher amounts of $\beta$-crystallins are made in this lens, or that a novel molecule has been recruited as a “taxon-specific” crystallin. To find the answer to this question, it would be interesting to investigate the crystallin content of the naked mole rat lenses.

What happens to the gamma-crystallin mRNA in the naked mole rat lenses? Can lower rates of gamma-crystallin transcription, rather than increased degradation, account for the absence of $\gamma$-crystallin mRNA? To resolve this, the rate of mRNA turnover in the naked mole rat should be determined and compared to that in the mouse and the Cape dune mole rat. Sequence analysis of the $\gamma$-crystallin mRNA, especially the 3' UTR, could help identify the cause of mRNA
instability. A number of transcription factors (such as Maf, Sox1, Prox1, Pax6) can bind directly to the promoters of γ-crystallin genes and regulate the rates of transcription. Investigation of the expression pattern and activity of the naked mole rat homologues of these proteins could be used to further understand the regulation of the gamma-crystallin levels in the naked mole rat eye.

Why is the lens epithelium in both species of the mole rat located more posteriorly? FGFs are important regulators of the lens polarity, and can promote, in different concentrations, cell proliferation, migration and elongation of the lens fibres. To find out whether alterations in FGF activity within the mole rat eye are responsible for the altered lens morphology, the level of FGF and the expression pattern of the FGF receptors in the mole rat lenses should be investigated.

4.12.2 The mole rat ciliary body

What is responsible for the extreme CB size in the mole rats? What is the function of the lens? My attempts to dissect the molecular basis of this phenotype in the Cape dune mole rat were hindered by the fact that even in the standard laboratory animal models (mice, rats, chickens) ciliary body development is so poorly understood. Thus, before our knowledge of the evolutionary alterations to the development of this ocular structure can be furthered, the regulatory network governing CB morphogenesis should be investigated in detail. For example, only two molecules (Ptmb4 and Tgfb1i4) regulated by the lens-derived factors are identified to date, and it has not been established whether they form part of the same or parallel regulatory pathways (i.e., is Tgfb1i4 turned on by the signals from the lens, and then activates the transcription of Ptmb4, or is the transcription of both of these molecules directly regulated by the lens-derived signal). Moreover, it is not clear what relationship the signalling from the lens bears to the expression of other developmentally important signalling molecules and transcription factors (Msx1, Otx1, Bmp4 and Bmp7), or what instructs the periocular mesenchymal cells to migrate between the ciliary epithelial layers and form the ciliary stroma. Once more is known about the developmental regulation of the ciliary body morphogenesis, the evolutionary variation of CB development can be studied further.
Part II: CONSTRUCTION OF THE CHICK-SPECIFIC siRNA VECTOR

5. Introduction

5.1 RNA interference: history of discovery and mechanism

RNA interference (RNAi) is a recently discovered cellular process, which occurs when double-stranded RNA specifically inhibits expression of a gene containing its complementary sequence. This phenomenon was first observed in transgenic purple petunias, engineered to contain multiple copies of chalcone synthase, an enzyme involved in the biosynthesis of the purple pigment. Instead of the deeper purple colour, these flowers turned out to be variegated or even white (Napoli et al., 1990). The mechanism of this process remained poorly understood, and it was thought to be a peculiarity of certain plants and fungi (Romano and Macino, 1992; Cogoni and Macino, 2000). It was not until the work by Fire and colleagues that the real understanding of the phenomenon and its significance emerged (Fire et al., 1998). When using antisense RNA to silence *unc-22* (myofilament) gene expression in *C. elegans*, Fire et al. discovered that inhibition of the target gene was seen also when the control, sense RNA strand, was injected. Since both RNA strands were transcribed *in vitro* using bacteriophage RNA polymerases, which can produce non-specific transcripts, they surmised that the apparent inhibitory activity observed when using the control preparations might have come from the contaminating antisense strand. This was tested by using chemically synthesized pure RNA. Surprisingly, Fire and colleagues found that double-stranded RNA was about 100-fold more efficient than the antisense strand alone (Fire et al., 1998). This unusual phenomenon attracted much scientific attention. Further experiments were conducted in order to unravel the molecular mechanism of this process. The first important clue came from the identification of short, about 25 nucleotides in length, RNA fragments in plants undergoing co-suppression. These nucleotides were complementary to both the sense and the antisense strands of the gene being silenced, suggesting that short RNA molecules serve as an intermediate in this process (Hamilton and Baulcombe, 1999). Similarly, in *Drosophila* cells treated with dsRNA, short RNA fragments were identified as the products of cleavage of the exogenous dsRNA, suggesting that a ribonuclease is involved in the silencing process, and that the small RNAs serve as guides for sequence-specific target RNA degradation (Yang et al., 2000; Hammond et al., 2000; Tuschl et al., 1999). Identification of the enzyme, Dicer, which is responsible for the generation of the short RNA fragments, soon followed (Bernstein et al., 2001). Dicer was found to belong to the RNase III ribonuclease family, which is highly conserved among all eukaryotes, emphasizing the universal nature of the phenomenon of RNA interference.
RNA-mediated gene silencing is a two-step process (Figure 5-1). In the initiator step, the dsRNA precursor is rapidly cleaved into short, about 21 nucleotides in length, RNA duplexes. This step is catalysed by Dicer in a highly processive, ATP-dependent manner (Lee et al., 2004; Nykanen et al., 2001). These short duplexes are then incorporated into a nucleoprotein complex called RNA-induced silencing complex (RISC), which contains a member of the Argonaute (Ago) protein family. Activation of the RISC complex involves unwinding of the RNA duplex and release of one of the RNA strands (Meister and Tuschl, 2004). Usually, only one (antisense) RNA strand is preferentially incorporated. The specificity of the RNA interference depends strongly on this step, which in turn, is dependent on the sequence of the RNA duplex. For example, the strand whose 5' end has a lower stability than 3' end is preferentially incorporated (Schwarz et al., 2003). Activated RISC then becomes associated with the homologous transcript by base pairing and cleaves the target RNA in the middle of the homologous region, about 12 nucleotides from the 3' terminus of the siRNA (Martínez and Tuschl, 2004; Martínez et al., 2002; Elbashir et al., 2001). Ago proteins of the RISC complex are proposed to catalyze this reaction (Meister and Tuschl, 2004).

5.2 RNA interference as a tool for investigating gene function

Almost as soon as the phenomenon of RNA interference was discovered, its incredible usefulness as a tool for specific silencing of gene expression was realized. Double-stranded RNA injection was successfully used to knock out target gene expression in organisms as diverse as C. elegans, Drosophila, Trypanosoma and zebrafish (Cogoni and Macino, 2000). In several worm phyla efficient RNAi can be induced by feeding the animals with E. coli cells expressing large quantities of dsRNA bearing homology to the target sequence (C. elegans - Timmons et al., 2001; planaria – Newmark et al., 2003). RNA interference works especially well in these animals, because the dsRNA can efficiently cross cellular boundaries and is inherited by the next generation through the germline (Fire et al., 1998). In Drosophila, attempts to induce RNAi by feeding the flies with similarly engineered yeast were unsuccessful, so currently injection of dsRNA remains the method of choice.

First attempts to establish RNA interference in mammalian cells failed, because transfection of exogenous dsRNA molecules results in non-specific suppression of gene expression. This occurs through either PKR-mediated shutdown of translation, or RNase L activation, which leads to non-specific RNA degradation. Both processes ultimately result in cell death (Minks et al., 1979; Hunter et al., 1975; Williams, 1997). However, it was soon discovered that introduction of short, 21 to 22 nucleotides in length, RNA duplexes (small interfering RNA or siRNA) evades this
Figure 5-1: Mechanism of the RNA-mediated gene silencing. Adapted from www.ambion.com

cytotoxic response and allows for strong and specific gene silencing to occur (Elbashir et al., 2001). While RNA is relatively easy to deliver to cells in high concentrations, which ensures effective silencing, using RNA for transfection has a host of disadvantages, with the inherent instability of the molecule resulting in the reduction in target gene expression being only transient. To overcome this problem, a number of plasmid and viral expression vectors, which encode siRNA-like transcripts were engineered (Brummelkamp et al., 2002; Sui et al., 2002; Paddison et al., 2002; Miyagishi and Taira, 2002; Hannon and Rossi, 2004). With these new
tools now commercially available, RNA interference is fast becoming a method of choice for study of gene function both in mammalian cell culture and \textit{in vivo}.

5.3 Mammalian plasmid systems for gene silencing

In order to effect a stable and efficient gene silencing, a number of plasmid vector systems for expression of short interfering RNA in mammalian cells have been designed. These vectors all contain several essential components: 1) a marker gene (GFP, RFP or beta-galactosidase), which would enable easy visualization of the cells that have been transfected with the plasmid; 2) a suitable promoter which would ensure a continuous transcription of the small interfering RNA within the transfected cells; 3) a multiple cloning site directly downstream from the promoter for cloning of the desired siRNA insert (see Figure 5-2). In addition, such vectors also contain all the normal components of eukaryotic expression vector systems, i.e. bacterial and eukaryotic origins of replication, an antibiotic resistance gene for selection in the bacterial cells, and an optional antibiotic resistance gene under the control of a eukaryotic promoter to enable selection of stable transfectants in mammalian cells. RNA polymerase III promoters (U6, H1) are most commonly used to drive siRNA expression from these vectors, as these promoters are naturally adapted for high-level expression of small non-coding RNAs. These promoter sequences are relatively simple, and the RNA transcribed from them is not polyadenylated. Transcription driven by type III promoters terminates when a stretch of 3-6 consecutive thymidines is encountered in the DNA template, which obviates the need for any terminator sequences to be incorporated at the 3' end of the siRNA template (Sui \textit{et al.}, 2002; Paddison \textit{et al.}, 2002; Paul \textit{et al.}, 2002). The absence of posttranscriptional modification and exogenous sequences in the final transcript is particularly important for siRNA applications, as the specificity of the response is highly sequence-dependent.

A number of RNA polymerase III promoters are known, but only a subset of them (those of class 3) are useful for siRNA vector construction. Three classes of RNA polymerase III promoters have been described. This classification is based on the composition of the promoter elements and their position relative to the transcription start site. For RNA polymerase promoters of classes 1 and 2, some of the promoter elements are located downstream of the start sites and are transcribed as the 5'end of the RNA. On the other hand, all of the active elements for class 3 promoters lie upstream of the transcription initiation sites, and so the activity of these promoters does not depend on intragenic sequences (Kunkel and Pederson, 1989; Gunnery \textit{et al.}, 1999; Lobo \textit{et al.}, 1990). Of these, U6 and H1 promoters are the simplest and the best studied. The H1 promoter directs the transcription of the RNA component of the RNase P enzyme, while a class
Figure 5-2: Plasmid maps of two commercially available vectors for small hairpin RNA expression in mammalian cells. (A) pSuper.gfp/neo (Brummelkamp et al., 2002), supplied by Oligoengine. (B) pSilencer 3.0-H1 (Sui et al., 2002), supplied by Ambion.
of small nuclear RNAs (U6 RNAs), which form a part of the spliceosome complex, are
transcribed from the U6 promoters. U6 genes number up to 821 in the human genome, though
most of these are pseudogenes (Hillier et al., 2004); five functional U6 genes have so far been
identified (Domitrovich and Kunkel, 2003). Only one H1 gene has been found in humans (Hillier
et al., 2004). Fifteen U6 genes were found in the chicken genome, most of which are predicted to
be functional (Hillier et al., 2004).

Several sequence elements essential for the promoter activity have been identified in polymerase
III promoters. U6 promoters are about 300 nucleotides in length, and contain a TATA box, a
proximal sequence element (PSE) at about -60 nt, an octamer (OCT) element in the distal,
enhancer-like region further upstream (about 220 bp 5' to the transcription start site), and an
SPH element adjacent to OCT (Domitrovich and Kunkel, 2003). All of these elements are
essential for the activity of the promoters, but are known to differ in sequence and relative
position between different active U6 promoters within the same genome (Carbon et al., 1987;
Domitrovich and Kunkel, 2003). PSE/TATA spacing is however highly conserved between the
expressed U6 genes, consistent with the central role for PSE in the control of the U6 promoter
activity.

The H1 promoter is shorter than the U6 promoters, with all the sequence elements essential for
the control of transcription efficiency being located within about 100bp upstream of the start site.
These are similar to the cis-regulatory elements of the U6 promoter, and include a TATA box, a
PSE and a DSE (distal sequence element) (Hannon et al., 1991; Baer et al., 1990).

5.3.1 Design of the small hairpin insert

Transcription of short double-stranded RNA duplexes from a plasmid can be achieved either by
placing individual DNA templates for the sense and the antisense strands under control of two
separate promoters (Miyagishi and Taira, 2002; Lee et al., 2004) or by transcribing a "hairpin"-
like structure, containing the sense and the antisense strands separated by a short non-pairing
"loop" sequence, from a single promoter (Brummelkamp et al., 2002; Sui et al., 2002; Paul et
al., 2002). When transcribed, this RNA will fold to form a "hairpin" structure that is resistant to
degradation by RNases (Figure 5-3). Both of these approaches have been attempted in
mammalian cells, and both were found to be successful; however, currently the preference is
given to the latter, more straightforward method.
The insert for siRNA vectors is a short, 80-100 nucleotides in length, double stranded DNA duplex. Each strand incorporates 19-29 nucleotides of the target gene (the gene to be silenced) sequence in the forwards and reverse orientation, separated by a 6-7 nucleotide “loop” sequence (Figure 5-3). When transcribed, the RNA folds into a “hairpin” structure, which is then cleaved at the loop region by Dicer forming the active siRNA.

5.4 RNA interference in the chick embryo

Because of its large size and easy accessibility to experimental manipulation, the chick embryo has been one of the most favoured models for experimental developmental biology for over a hundred years. However, the major disadvantage of the chick embryo is the limited range of tools for manipulation of gene expression. With the advent of electroporation as the method of in vivo gene delivery, gain-of-function studies in chick embryos have recently become possible (Muramatsu et al., 1997; Momose et al., 1999; Swartz et al., 2001). Down-regulation of gene expression has been more problematic and has depended on the availability of the dominant-negative forms of the gene product of interest. Loss of function can now be achieved using morpholino oligos, stable synthetic DNA analogues which have high affinity for RNA and therefore serve as efficient antisense reagents (Kos, 2001). Unfortunately, morpholinos are produced by a single company worldwide (Gene Tools, USA) and are therefore prohibitively
Part II: Construction of the siRNA vector

expensive. It is therefore highly desirable to develop new methods for efficient, specific and inexpensive gene knockout in chick embryos. As RNA interference already proved to be an invaluable tool for reverse genetic studies in a range of vertebrate and invertebrate organisms, attempts to adapt it for use in the chick are now under way.

In 2002, Pekarik et al. reported that efficient loss of gene function in ovo could be achieved by electroporating dsRNA into the neural tube of developing chick embryos (Pekarik et al., 2002). This was the first demonstration that RNAi could be effectively applied in an avian model. However, as the effect of RNA application was only transient, several labs began working on adapting the existing vectors for small hairpin RNA (shRNA) expression for use in the chick, because vector-mediated expression can confer more stable, long-term RNAi (Katahira and Nakamura, 2003; Chesnutt and Niswander, 2004; Sahin et al., 2005; Dai et al., 2005). Several different mammalian-specific vectors were introduced into the chick embryos by in ovo electroporation in an attempt to evaluate their ability to knock down expression of several endogenous and foreign genes. Two problems of using such vectors were immediately apparent. First, the marker gene (GFP) is under control of a mammalian promoter (see Figure 5-2), which results in inefficient transcription of this gene in chick cells. Thus to identify the transfected cells, either an additional plasmid containing a marker gene under the control of a chick-specific promoter has to be included into the electroporation mixture (Chesnutt and Niswander, 2004), or the promoter driving the GFP gene expression has to be replaced (Dai et al., 2005). Second, the degree of the target gene knockdown achieved by mammalian-specific vectors in the chick was much lower then when the same vectors were used in mammalian systems. For example, pSuper anti-Pax3 construct failed to reduce Pax3 RNA levels in the chick embryonic neural tube (Bronner-Fraser, personal communication). Katahira and Nakamura reported a very small, unquantifiable decrease in the amount of En2 RNA in chick midbrain and hindbrain, after electroporation of the neural tube with pSilencer U6-1.0 constructs containing different anti-En2 inserts (Katahira and Nakamura, 2003). Visible (over 60%) decreases in the amount of target RNA, as determined by in situ hybridization, were achieved when shRNA transcription was driven by mouse U6 or human U6 and H1 promoters (Chesnutt and Niswander, 2004; Dai et al., 2005). Unfortunately, however, when assessing the efficacy of the RNAi using both in situ hybridization and immunocytochemistry, it was found that the observed drop in the RNA levels was not accompanied by a corresponding reduction in the protein levels (Chesnutt and Niswander, 2004). Furthermore, none of the studies published to date report significant morphological alterations as would be expected to result from efficient target gene knockdown. Experimental evidence to date suggests that the use of commercially available mammalian
siRNA vectors in the chick tissues is unable to achieve a consistent high-level knockdown of a wide range of target genes. Therefore, further efforts are needed to develop a chick-specific system for efficient downregulation of target genes.

5.5 Aims and objectives

One of the most intriguing findings reported in the first section of this work was the absence of Ptmb4 expression in the ciliary zone of the Cape dune mole rats (see Section 3 of this thesis). Since the role of Ptmb4 in ocular development is not known, one of the best ways to investigate the function of this gene in eye development would be to downregulate its expression and study the resulting phenotype. As chick embryos are relatively cheap, available and have large eyes which are easily accessible to experimental manipulation, it would be an ideal model organism to study the role of Ptmb4 in ciliary body development. However, RNAi is not yet established in the chick model. Therefore, the general goal of the work described in this chapter was to engineer a chick-specific RNA interference vector, which could produce a stable knockdown of Ptmb4 expression in ovo, with a suitable fluorescent marker to enable identification of the transfected cells. Thus the specific aims were:

1) To replace the human PGK promoter directing the transcription of the green fluorescent protein (GFP) in the commercially available mammalian shRNA expression vector pSuper.gfp/neo with the chick beta-actin promoter and universal CMV enhancer in order to deliver more efficient expression of GFP in the chick cells.

2) To identify putative RNA polymerase III promoters (H1 and U6) in the chick genome, and replace the mouse H1 promoter of pSuper.gfp/neo with such chick-specific promoters to ensure high levels of small hairpin RNA transcription.

3) To test the new vector(s) in order to demonstrate that the system works in chick embryos.
6. Materials and Methods

6.1 Screening of the chicken genome assembly

The latest chick genome assembly (www.ensembl.org) was searched in order to identify candidate H1 and U6 chick promoter sequences. Conservation of genomes among different species is often confined to the coding sequences, with the promoters being the most divergent. The human H1 small RNA gene coding sequence (Hannon *et al.*, 1991) was used to screen the chick genome, the most promising match was identified, and 250 bp fragment of the putative promoter sequence upstream of the transcription initiation site was selected to be used in vector construction. In the mouse and human genome, there are ten U6 genes, which all contain a highly conserved 110 nucleotide domain. This conserved domain sequence was used to screen the chicken genome. The putative upstream promoter sequences were compared to those of the U6 genes in the mouse, the human and sea urchin, using the multiple sequence alignment tool CLASTALW (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl). Three sequences containing conserved essential promoter elements were used for subsequent vector construction.

6.2 PCR amplification

The chick-specific H1 and three U6 promoters were obtained by high-fidelity polymerase chain reaction (PCR) using genomic BAC clones, purchased from Children’s Hospital Oakland Research Institute (CHORI, Oakland, California), as templates. The BAC clones and primers used for each promoter are summarized in Table 6-1. In order to facilitate the directional cloning, appropriate restriction enzyme sites were incorporated into the primers (EcoRI into the 5’ primer and BglII into the 3’ primer).

The CMV enhancer, chick β-actin promoter and intron sequences were amplified from the chick expression vector pCIG (Megason and McMahon, 2002) using the following primers:

forward: CAGG+SalI-5’-NheI: 5’- TTT GCTAGC GTCGACATTGATTGTA -3’

reverse: CAGG-3’-AgeI: 5’-TTT ACCGTT TTGCCAAAATGATGAGA- 3’

The 150-bp sequence in pSuper.gfp/neomneo vector that separates the H1 and the PGK promoters (intervening sequence) was amplified using the following primer combination:

forward: Int-5’-NheI: 5’- TTT GCTAGC CCGGTGGATGGAATGT-3’
reverse: Int-3'-EcoRI: 5'-TTTGAATTCTACGGGTAGGGAGGC-3'

The optimal annealing temperatures for all of the above reactions were determined empirically using the theoretical melting temperature value of the primers as a guideline. This was carried out by setting up 5-6 PCR reactions with annealing temperatures varying over a range of 6°C above and below the theoretical melting temperature. The lowest annealing temperature at which the product of interest was amplified specifically (as judged by the absence of spurious bands) was used to set up the large-scale preparative PCR reactions.

Table 6-1: Chromosomal positions and primers used to amplify the chick RNA polymerase III promoters used in this work.

<table>
<thead>
<tr>
<th>Promoter name</th>
<th>Chromosomal location</th>
<th>Template (BAC clone)</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>Chr1: 91820715-91820849</td>
<td>Ch261-120K16</td>
<td>Forward - H1-5'-EcoRI: 5'-TTTGAATTCTCGCAACATTTACAGCT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse - H1-3'-BglII: 5'-TTTGAATCTAATTATTTGTATTCA-3'</td>
</tr>
<tr>
<td>U6.1</td>
<td>Chr28: 2784425-2784831</td>
<td>Ch261-96B15</td>
<td>Forward - U6.1-5'-EcoRI: 5'-TTTGAATTCGCAGCGCCGCGGGAAT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse - U6.1-3'-BglII: 5'-TTTGAATCTAAGCGGCTGTCCTC-3'</td>
</tr>
<tr>
<td>U6.2</td>
<td>Chr28: 2747326-2747732</td>
<td>Ch261-96B15</td>
<td>Forward - U6.2-5'-EcoRI: 5'-TTTGAATTCGCAGCGCCGCGGCTCCT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse - U6.2-3'-BglII: 5'-TTTGAATCTGACTAAGGCTCGGAC-3'</td>
</tr>
<tr>
<td>U6.3</td>
<td>Chr18: 3311444-3311750</td>
<td>Ch261-54N19</td>
<td>Forward - U6.3-5'-EcoRI: 5'-TTTGAATTCGTGCTTAAAGTCCCACC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse - U6.3-3'-BglII: 5'-TTTGAATCTGATATCTTGGACCCTCTA-3'</td>
</tr>
</tbody>
</table>

To ensure the highest possible accuracy of transcription, Expand High Fidelity PLUS PCR System (Roche, Germany) was used according to the manufacturer’s instructions. The PCR conditions for all of the above reactions were: denaturation - 94°C for 5 minutes; annealing and amplification – 24 cycles of 94°C for 30 seconds, Tₘₐₜ (appropriate annealing temperature as determined above) for 30 seconds, 72°C for 1-2 minutes (depending on the length of the
fragment to be amplified, usually 1 minute per kb template); final extension - 72°C for 10 minutes.

The fragments were separated on 1 or 2% agarose gel (at 100-120V), stained with EtBr and viewed under UV transilluminator.

6.3 DNA digestion

All PCR products were digested with the appropriate enzymes (discussed in the next section) to create appropriate sticky ends for cloning. The enzymes used in this work were obtained from Roche (Germany) or New England BioLabs (Ipswich, USA), and used according to the manufacturer's instructions. To minimize the possibility of star activity, the concentration of glycerol in the final mixture did not exceed 2.5%, and digestions were allowed to proceed for no longer than 2 hrs. For double digests, the restriction buffer compatible with the highest levels of activity of both enzymes was used. Enzymes were inactivated by incubation at 65°C for 15 minutes.

6.4 DNA purification

After PCR amplification of the intervening region from pSuper.GFP/neo (see Results for details on the cloning strategy), the product had to be purified to ensure that it is free from all traces of the template, because the PCR product will then be ligated and transformed into bacteria, and ampicillin-resistant transformants selected and screened. Presence of even the smallest traces of the template plasmid, which also encodes ampicillin resistance, may significantly affect the outcome of the bacterial transformation. The PCR products were separated on the 1.5 or 2% agarose gel, and the appropriate fragments excised and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, USA). The plasmids, if digested completely (as verified by gel electrophoresis), were column-purified using QIAquick PCR purification kit (Qiagen, Valencia, USA), otherwise the same procedure as for PCR products was followed.

6.5 Ligation

T4 DNA ligase (NEB, Ipswich, USA) was used for all ligations according to the manufacturer's instructions. Blunt-end ligations were performed overnight at 16°C. Fragments with compatible sticky ends were ligated for 1-2 hrs at room temperature.
6.6 Transformation of bacteria

Frozen chemically competent (MAX Efficiency DH5α Competent Cells, Invitrogen, Carlsbad, USA) or electocompetent (ElectroMAX DH10B Cells, Invitrogen, Carlsbad, USA) *Escherichia coli* cells were used for plasmid transformation or electroporation, as directed by the manufacturers. Electroporation was the preferred method for blunt-end cloning, as 10-100-fold increase in transformation efficiency can be achieved.

6.7 Screening for transformants

To differentiate between the colonies containing plasmids with the desired inserts, and those containing the empty plasmid, Colony Fast-Screen Kit (Epicentre Biotechnologies, Madison, USA) was used according to the manufacturer’s instructions. Briefly, a small amount of cells from individual colonies was lysed in EpiLyse solution at 70°C, thereby releasing the cellular DNA. The resulting solution was then loaded onto agarose gel along with suitable supercoiled molecular weight markers. Where the insert size was too small to allow the plasmids containing the insert to be distinguished from the empty plasmids by comparing the electrophoretic velocity of the supercoiled DNA (less than 500bp), plasmid mini-preparations from individual colonies were done using QIAprep Spin Miniprep Kit (Qiagen, Valencia, USA). Plasmid DNA was digested with the appropriate restriction enzymes and analysed by agarose gel electrophoresis.

6.8 Sequencing and sequence analysis

All sequencing was done by Davis Sequencing (Davis, CA, USA) and by Inqaba Biotech (Pretoria, South Africa). Sequences were assembled and aligned using BioEdit software or Clustalw (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl).

6.9 Anti-red fluorescent protein shRNA design and cloning

The sequence of the interfering RNA is crucial in determining its specificity. Ambion scientists (www.ambion.com) approximate that out of every four randomly designed siRNAs only one will typically give 75-95% reduction of target gene expression. In order to avoid results caused by sub-optimal efficiency of the siRNA, only experimentally validated shRNA sequences that were demonstrated to be very efficient against red fluorescent protein (RFP) were used (Ui-Tei et al., 2004). The appropriate restriction sites were incorporated into the 5' and 3' ends of all oligos (*BamHI* and *HindIII*), and the inverted repeats were separated by the 7-nucleotide loop sequence CAAGAGA as recommended by Oligoengine.
(http://www.oligoengine.com/pSUPER_New/pSUPER_Oligos.html). The sequences of the anti-RFP oligos were as follows:

Anti-RFP 277 sense:

5'-GATCCCCATGGGAGCGCGTGTGAACCTTCAGAGAAGTTCATACGCGCTC

CCATTTTTTA-3'

Anti-RFP 277 antisense:

5'-AGCTTAAAAACGGCCCCGTAATGCAGAGAGATCTTTGCTCTTTCTGGAATT

CGGGGCGGG-3'

The oligos were synthesized and supplied in column-purified form by IDT (Coralville, USA). Prior to cloning, the single-stranded oligos were allowed to hybridize under conditions which promoted formation of double-stranded duplexes rather than hairpins, i.e. they were resuspended at 40ng/μl final concentration in annealing buffer (10mM Tris, 50mM NaCl, pH 8.0) and cooled down in a step-wise fashion using the PCR machine. The annealed oligos were ligated into the BglII/HindIII-digested pSuper and pSuperChick plasmids. After ligation and transformation as described above, screening for positive colonies was done by digestion of purified plasmid DNA with BglII. Insert-containing colonies were resistant to BglII-digestion as the result of the formation of BamHII/BglII hybrid sites. The presence of the small hairpin insert was further confirmed by double-digestion with HindIII and EcoRI.

6.10 In ovo electroporation

Fertile chicken (Gallus gallus domesticus) eggs were obtained from commercial sources. The eggs were incubated horizontally at 38°C and 50% humidity until the embryos reached Hamburger-Hamilton stage 10 (HH10, 35-38 hrs incubation). To expose the embryos, 2-3ml of albumen was removed from the egg and elliptical windows were made in the eggshell directly above the embryo. A one in 25 dilution of India ink in chick Ringers (120mM NaCl, 1.5mM CaCl₂, 5.0mM KCl, 0.81mM Na₂HPO₄, 0.15mM KH₂PO₄, pH 7.4) was injected under the vitelline membrane to visualize the embryo. The plasmid was used in concentration 2-5μg/μl. To make it visible, it was mixed with 1 μl of non-toxic blue food colourant (CI 42090 and CI 14720). The plasmid-dye mixture was injected into the lumen of the neural tube using pulled glass needle. Two platinum wire electrodes, about 0.5mm in diameter, were then placed on top
of the vitelline membrane one on each side of the embryo, about 5 mm apart. Chick Ringers was used to wet the embryo and the electrodes. Five pulses of 25.0V (100ms in length with an interval of 900ms) were delivered to the embryo using a square-pulse electroporator (B.E.S. 2004, Caltech). The eggs were sealed with thick plastic tape, and re-incubated for a further 24hrs. The embryos were then dissected out, fixed in 4% PFA for 5-12 hrs, washed extensively in PBS, viewed under Zeiss Axiovert 200M inverted microscope and photographed using Axiocam HRm camera.
7. Results and Discussion.

7.1 Identification of *Gallus gallus* RNA polymerase III promoters

Most commercially available plasmids for RNAi use either human or mouse H1 and U6 promoters to direct the transcription of the small hairpin RNAs. One of the reasons why mammalian vectors do not perform consistently when used in chick cells is possibly because the mammalian H1 and U6 promoters are not efficient in chick cells. Therefore, replacement of the mammalian polymerase III promoters with their chick counterparts would be necessary to achieve the maximum efficiency of RNAi.

When this work was started, *Gallus gallus* genome project was nearing completion, and large portions of the assembled draft genome were available. However, nothing was yet reported on the number or genomic position of H1 or U6 promoters in the chick. Because the overall promoter sequences are generally poorly conserved between species, with the exception of short transcription factor binding motifs, the genome assembly was searched using the conserved elements of the corresponding small RNA sequence rather than the promoter sequence itself. One high-similarity hit for H1 was located on chromosome 1 (Table 6-1). As expected, a number of putative U6 hits were found throughout the genome when searching with the 110nt sequence conserved among all the human U6 genes (Domitrovich and Kunkel, 2003). Next, 200-300nt regions upstream of the putative target genes were selected and aligned with the corresponding promoters from human, mouse and sea urchin. Putative H1 and U6 promoters, which showed conservation of essential promoter elements PSE, DSE, the octameric sequence and the TATA box were chosen for further work. Figure 7-1 shows the sequences of the promoters chosen for cloning.

7.2 Construction of the vector

The RNAi plasmid pSuper.gfp/neo was chosen as the starting material for the construction of the chick-specific siRNA vector pSuper-Chick, because it was available in the laboratory. In order to make this vector suitable for use in the chick, both the mouse PGK promoter and the human H1 promoter of this vector had to be replaced with suitable chick counterparts.

An efficient chicken β-actin promoter coupled with the strong CMV enhancer and followed by an intron sequence (which improves the efficiency of mRNA processing in the chick cells) was obtained from the expression vector pCIG (Megason and McMahon, 2002). In order to
Figure 7-1: Alignment of the putative chick-specific H1 (A) and U6 (B) promoter sequences with the corresponding human promoter sequences. Conserved promoter elements are shown in blue, primer binding sequences— in purple. DSE-distal sequence element, PSE-proximal sequence element, OCT-octameric sequence. Note that the positions of SPH motif and OCT sequence are reversed in GgU6.2.
minimize the possibility that these two promoters (one of which also contains enhancer elements) would interfere with each other's activity, they had to be physically separated; therefore it was essential to retain the short nucleotide sequence that is positioned between the H1 and PGK promoters in pSuper.gfp/neo.

The initial plan for cloning included PCR amplification of the DNA fragment containing beta-actin promoter from pCIG as one of the initial steps. However, repeated attempts to obtain the chicken beta-actin promoter and CMV enhancer from pCIG by high-fidelity PCR amplification failed. Sequencing of part of this region revealed that the cause of the failure was due to the extremely high GC content of this sequence (higher than 80%). Therefore, an alternative cloning strategy (as outlined in Figures 7-2, 7-3 and 7-4) was adopted.

First, the PGK promoter and the 120bp sequence upstream (called the intervening sequence) were excised from pSuper.gfp/neo by double digestion with EcoRI and Nhel, and the vector purified (Figure 7-2, Step 1; Figure 7-2, Gel 1, lane 3). The intervening sequence was then PCR-amplified using an uncut pSuper.gfp/neo as the template, and primers incorporating the appropriate restriction sites (Figure 7-2, Step 2; also Gel 1, lane 2). Then, the intervening sequence was re-inserted into the vector in order to ensure that there would be an adequate distance separating the promoters (Figure 7-2, Step 3). The clones containing the IS insert were identified by EcoRI/Nhel digestion (Figure 7-2, Gel 2). Next, the human H1 promoter was removed using the closest unique restriction sites BglII and EcoRI, and the vector purified (Figure 7-3, Step 4; also gel 3, lane 6). The putative chick H1 and U6 promoters (identified as described in section 7-1) were obtained by PCR amplification from the appropriate BAC genomic clones (Table 6-1; Figure 7-3, Step 5). The reverse PCR primers used for this reaction were designed to incorporate a BglII site, while the forward primers contained an EcoRI site to facilitate directional cloning. Single bands of the expected size were obtained in each case (Figure 7-3, Gel 3, lanes 2-5). After ligation, the clones containing the desired inserts were identified by BglII/EcoRI digestion (see Figure 7-3, Gel 4). The four products of this cloning step were then digested with Nhel (Figure 7-4, Step 7), the sticky end overhangs filled in using T4 DNA polymerase and terminal phosphates removed by calf intestinal phosphatase treatment in order to prevent self-ligation of the plasmids. At the same time, pCIG was digested with a combination of Sall, XhoI and NotI (Figure 7-4, Step 8). Sall cuts pCIG twice, while XhoI site is unique in this plasmid sequence, the resulting fragments are 1720bp, 1990bp and 3kb in length. The first two fragments are very similar in size and thus would have been hard to
Figure 7-2: Cloning strategy employed to construct pSuper-Chick. Removal of the PGK promoter followed by replacement of the intervening sequence. Gel 1: IS - PCR-amplified intervening sequence; pSuper - EcoRI/NheI digested and purified pSuper.gfp/neo; MWM - extended 1kb DNA marker (Invitrogen). Gel 2: EcoRI/NheI digests of 4 individual clones from Step 3, blue arrow indicates 150bp band corresponding to the re-inserted intervening sequence; MWM - extended 1kb DNA marker (Invitrogen).
Figure 7-3: Cloning strategy employed to construct pSuper-Chick (continued). See page 134 for the legend.
Step 7: NheI

Gel 5

Step 8: Sall/Xhol NotI

Isolate 1720bp fragment

Fill in the ends: T4 DNA polymerase

Remove terminal phosphates: CIP treatment

Step 9: blunt-end ligation

Figure 7-4: Cloning strategy employed to construct pSuper-Chick (continued). See page 134 for the legend.
separate by gel electrophoresis. Therefore \textit{XhoI} was added to the restriction mix to cut the 1990bp fragment into two pieces, 1450 bp and 540bp in length (Figure 7-4, gel 5). Prior to ligation, the single-strand overhangs left by the digestion of the 1720bp insert were filled up with T4 DNA polymerase. In the final step, the chick beta-actin promoter-containing 1720bp fragment from pCIG was ligated into the filled-in \textit{NheI} site just upstream of the EGFP coding sequence (Figure 7-4, Step 9). To identify the insert-containing clones, individual plasmid mini-preps were digested with \textit{EcoRI} and \textit{AgeI} (the \textit{NheI} site was destroyed during the blunt-end cloning procedure). Clones containing the desired insert (see Figure 7-4, gels 6 and 7) were digested with \textit{XbaI} to determine the orientation of the insert (Figure 7-5). Each of the pSuper-Chick plasmids contained two \textit{XbaI} sites, one in the part of the vector derived from the original pSuper.gfp.neo plasmid, and the second – within the 1720bp fragment derived from pCIG (see Figure 7-5). Digestion of the plasmids containing the insert in the desired orientation resulted in two fragments of 1.9kb and 4.9kb (Figure 7-5A, 7-5C), while if the insert was in the opposite orientation, two fragments of 3.4kb were obtained (Figure 7-5B). After each cloning step, the sequence integrity and the orientation of the insert were confirmed by bi-directional sequencing.

### 7.3 Testing of the GFP promoter activity

In order to assess the efficiency of the GFP expression in the newly made vectors they were delivered to chick embryos by \textit{in ovo} electroporation of the neural tube. The parental plasmids, pCIG and pSuper.gfp.neo, were used as the positive and the negative controls, respectively. The concentrations of the plasmid solutions were the same. pSuper.gfp.neo produced very low-level, patchy GFP fluorescence (Figure 7-6A). In contrast, neural tubes electroporated with pCIG exhibited about 10-15 times more intense GFP signal (Figure 7-6B). Encouragingly, the EGFP fluorescence levels observed in the pSuper-Chick electroporated neural tubes were comparable to those obtained with pCIG (Figure 7-6 C-E). This result demonstrates that chick cells electroporated with pSuper-Chick vectors can be easily visualized.

**Figure 7-3:** Cloning strategy employed to construct pSuper-Chick (continued). Replacement of the human H1 promoter of pSuper.gfp.neo with chick specific H1 and U6 promoter sequences. Gel 3: PCR-amplified and purified chick H1, U6.1, U6.2 and U6.3 promoters; plasmid 1 - \textit{BglII/EcoRI} digested Plasmid 1; MWM - extended 1kb DNA marker (Invitrogen). Gel 4: \textit{BglII/EcoRI} digests of selected clones. Blue arrows indicate clones containing the desired inserts. MWM - extended 1kb DNA marker (Invitrogen).

**Figure 7-4:** Cloning strategy employed to construct pSuper-Chick (continued). Insertion of the chick-specific \textit{beta-actin} promoter and CMV enhancer. Gel 5 shows the sizes of DNA fragments obtained by \textit{SalI/XhoI/NotI} digests of pCIG. Gel 6 and 7: \textit{EcoRI/AgeI} digests of individual pSuper-Chick clones, blue arrows indicate the clones containing the desired 1720bp insert.
7-5: Selection of the clones containing the 1720bp insert in the desired orientation. (A) and (B) Restriction maps of pSuper-Chick, indicating the position of the XbaI sites in the vector containing the insert in the desired (A) and in the opposite (B) orientation. Note: the size of the insert is not drawn to scale with the rest of the vector. (C) XbaI restriction digests of individual pSuper-Chick clones. Blue arrows indicate the clones containing the 1720bp insert in the desired orientation. MWM - extended 1kb DNA marker (Invitrogen).
7.4 Testing of the shRNA activity

In order to assess the efficacy of the novel RNA interfering vectors, I first tested the ability of the new pSuper-Chick vectors to knock down expression of an exogenous, plasmid driven RFP (red fluorescent protein) gene. The strategy used in this work involved delivering RNAi vectors expressing anti-RFP siRNA to the chick cells together with the RFP-expressing target vector. Only the cells that receive the siRNA vector would exhibit green fluorescence, while those that contain only the target vector would be fluorescing red. The red fluorescence levels in the cells containing both plasmids should be reduced relative to the green signal, in proportion to the efficiency of the siRNA vector. This testing strategy was essentially the reverse of the one described by Sui et al., where the fluorescent marker incorporated into the siRNA vector was RFP, and GFP was the target gene (Sui et al., 2002).

To achieve this, the DNA template for the anti-RFP shRNA was prepared and inserted into all four pSuper-Chick vectors just downstream of their polymerase III promoters. Two complementary DNA oligonucleotides containing 19nt of the target gene (RFP) in the sense and the antisense orientation, separated by a loop sequence, and flanked by BamHI site at the 5’ end and the HindIII site at the 3’ end were designed. The 19-nt sequence of the oligos was identical to the sequence of the siRNA that was previously found to be efficient against RFP in mammalian cells (Ui-Tei et al., 2004). Prior to cloning, the two oligos were allowed to anneal to form a double-stranded DNA fragment, which was then digested with BamHI and HindIII, and ligated into the BglII and HindIII sites of the vectors. BamHI and BglII form compatible sticky ends, but the hybrid site formed upon ligation can no longer be cut with BglII. This permits an easy method of screening for insert-containing clones: those resistant to BglII digestion contain the insert. To confirm the presence of a single insert, the clones resistant to digestion with BglII were further tested by double-digestion with HindIII and EcoRI (Fig 7-7). This enzyme combination excises both the shRNA template and the promoter, giving a fragment of 300-360nt in length, which can be identified on the gel. Since the anti-RFP oligos were chemically synthesized, which is an error-prone method, and the efficiency of the RNAi can be affected even by a single mismatch between the siRNA and the target sequence, the integrity of the insert sequence in all the positive clones was verified by bidirectional sequencing.
Figure 7-6: Testing of the efficiency of the GFP expression in the pSuper-chick vector, as compared to the parental pCIG and pSuper.gfp/neo plasmids. A - pSuper.gfp/neo; B - pCIG; C - pSuper-Chick U6.1; D - pSuper-Chick U6.2; E - pSuper-Chick U6.3. The photographs are taken at the same exposure. Nt - neural tube; nc- neural crest cells; all embryos are oriented with their anterior end towards the top of the figure. Photographs in (B) and (D) depict the larger head portion of the neural tube (prospective brain), while the thinner trunk portion of the neural tube is shown (A), (C) and (E).

The target RFP-expressing plasmid (pCAGGS-RFP) is much smaller in size than pSuper-Chick vectors, which could affect the speed of plasmid replication and result in different copy number of the plasmids present in the same cells, and therefore different levels of expression of RFP and GFP. These two plasmids were therefore co-electroporated into chick neural tubes in varying relative concentrations to determine their optimal ratio for further work. The resulting levels of GFP and RFP expression were compared visually. These preliminary experiments suggested that 10-20 times greater concentration of pSuper-Chick (2μg/μl vs 0.1-0.2μg/μl pCAGGS-RFP) was required to achieve the same levels of intensity of GFP and RFP fluorescence.

To test the effect of pSuper-Chick+anti RFP on the RFP expression from pCAGGS in chick cells, 0.1 and 0.2μg/μl pCAGGS was then co-electroporated together with 2, 3 or 4μg/μl of all the pSuper-Chick-antiRFP plasmids. The same plasmids without anti-RFP inserts were used as the negative controls. The inhibition of RFP expression was visually assessed 24 hrs later. Only one of the plasmids (pSuper-Chick U6.2) inhibited RFP fluorescence to background level when used in concentration of 4μg/μl; the concentration of pCAGGS used in the experiment was 0.2μg/μl (Fig. 7-8 D-F). The control (pSuper-Chick U6.2 without the insert) was co-electroporated with pCAGGS in the same concentration and there was no decrease in RFP fluorescence (Figure 7-8 A-C).

These results demonstrate that at least one of the novel pSuper-Chick plasmids can be used to significantly reduce the levels of the target gene product in ovo. A number of factors could be responsible for the failure of the other three vectors to elicit any effect. First, the putative promoter sequences chosen for cloning may not be the true H1 or U6 promoters, but represent genomic regions located near inactive pseudogenes, the coding sequence of which may still maintain high degree of similarity to the active H1 or U6 genes (Domitro维奇 and Kunkel, 2003). Indeed, the putative chick H1 promoter identified by Dai et al., is different from the one reported here (Dai et al., 2005). Also, it has been previously observed that the efficiency of different RNA polymerase III promoters and cellular localization of their small RNA products can differ significantly in different cell types (Ilves et al., 1996). Both of these factors could contribute to the apparent lack of RFP inhibition observed when pSuper-Chick U6.1/U6.2+anti-RFP constructs were used. In addition, RFP is an exceptionally stable protein and is transcribed from a very efficient promoter, so that it could be synthesised before any shRNA has been transcribed, and the subsequent persistence of the protein in the cells may mask later effects of shRNA on the levels of RFP mRNA. To ascertain the exact reasons for the failure of pSuper-Chick H1/U6.1/U6.3 to knock down RFP, further testing is essential.
Figure 7-8: pSuper-Chick U6.2 successfully inhibits plasmid-driven RFP expression in chick embryo in ovo. The chick embryonic neural tube was co-electroporated with the empty U6.2 plasmid and pCAGGS (negative control –A, B, C) or with the U6.2-antiRFP shRNA and pCAGGS (D, E, F). The images obtained with the red channel are shown in A and D, while the GFP signal is shown in B and E. C and F: overlay of the images obtained in green and red channels.
Some of the experiments that should be performed next include testing of the pSuper-Chick-mediated shRNA activity against a range of endogenous target genes, both in ovo and in a range of different cell types. The inhibition of the target genes must be assessed at both the RNA (in situ hybridization, Northern blotting), and the protein levels (ICC, western blots). In this report, the first successful use of the chick RNA polymerase III promoters to achieve efficient knock-down of gene expression in ovo is described. The novel vector system, which uses a chick U6 promoter to drive shRNA expression, opens up new possibilities for gene function analysis in the chick model.

The original plan was to complete the testing of this vector and then to use the most efficient version to knock-down the expression of Ptmb4 in the eye of chick embryos in order to investigate the function of this molecule in the ciliary body development. However, due to time constrains, it was not possible to complete this work in time for it to be included in this thesis. However, this work will be followed up and completed in the near future.
Appendix A: Comparative histology of the mouse (A and F) and the naked mole-rat (B, C, D, E, G, H) eyes. (A) Neonate mouse has a well-developed lens (l), anterior chamber (ac), distinct ciliary body (cb) and iris (i), and an undifferentiated retina (r). (B) Neonate mole-rat has no anterior chamber, greatly enlarged and irregularly shaped ciliary body and retina with clearly defined layers. (C) Fourteen day old mole-rat showing what appears to be the developing iris (arrowheads). (D) Twenty one day old mole-rat has no anterior chamber and its lens is irregularly shaped at the posterior margin (arrowheads). (E) Thirty two day old mole-rat has anterior chamber (ac) and abnormally shaped lens (arrowheads). (F) Adult mouse eye with round lens, typical elongated iris, ciliary body with evident ciliary processes and differentiated layers in the retina. (G) Four-year old naked mole-rat eye, showing flat ciliary body without ciliary processes, very large round lens with vacuole-like structures at the posterior margin (arrowheads), iris undergoing thinning and depigmentation and pigment-filled trabecular meshwork (arrows). (H) Twelve-year old naked mole-rat with extremely large irregularly shaped lens, ciliary body abnormally associated with sclera (arrow), and degenerate iris. Scale bars: A, D, E, G, H 200μm; B, C 100μm; F 500μm
From Nikitina et al., 2004
Appendix B: Comparative histology of the mouse and mole-rat cornea (A-C), optic nerve (D, E), retina (F-I), iris and ciliary body (J-Q), and lens (R, S). (A) Adult mouse cornea with clearly defined epithelium (e), stroma (s) and endothelium (ce). (B) Adult (12-year old) mole-rat cornea with obvious epithelium and stroma, but no visible endothelium (arrowhead). (C) Neonate mole-rat cornea (c) is attached to the lens (l). (D) Optic nerve of an adult mouse has a regular arrangement of nuclei. (E) Optic nerve of an eight-year old mole-rat exhibits an irregular arrangement of nuclei. (F) Neonate mouse retina with undifferentiated photoreceptor layers. (G) Neonate mole-rat retina. All ten retinal layers are clearly identifiable at birth. (H) Adult mouse retina with characteristic retinal architecture. In (G) and (H), note nerve fibre layer (arrowheads). (I) Adult (12-year old) mole-rat retina, with no nerve fibre layer, and with ectopic cell bodies in the inner plexiform layer (white arrow) and possible microglial cells in the retinal ganglion cell layer (arrowheads). (J) Neonate mouse ciliary body and iris. (K) Neonate mole-rat ciliary body and iris. (L) Juvenile (14-day old) mole-rat ciliary body and iris. (M) Adult mouse iris with characteristic elongated shape and visible iris muscle (arrowhead). (N) Four-year old mole-rat iris exhibiting thinning and depigmentation (arrowheads). Red arrows indicate pigment-filled particles in the anterior chamber. (O) Adult mouse ciliary body. (P) Twelve-year old and (Q) three-and-a-half year old mole-rat ciliary body. Arrows indicate line of adhesion of iris to cornea. (R) Neonate mole-rat lens. “Bow regions” are boxed, and arrowheads point at the nuclei found at abnormal positions in the centre of the lens. (S) Neonate mouse lens. “Bow regions” are boxed. Scale bars: 20μm in all except D, K, M 50μm; R 100μm and S 200μm.

From Nikitina et al., 2004
Appendix C: Scanning electron micrographs of mouse and naked mole-rat corneal endothelium (A-C) and ciliary body (D-F). (A) Corneal endothelium of the adult mouse showing typical arrangement of hexagonal cells in a cobblestone pattern. (B) Juvenile (21-day old) mole-rat corneal endothelium. (C) Five-year old mole-rat corneal endothelium. (D) Mouse ciliary body showing characteristic ciliary processes (arrow). (E) Ciliary body in a five-year old mole-rat, showing an absence of ciliary processes. (F) Higher magnification of the region boxed in (E). Scale bars: A, D, E 10μm, B 2μm, C 4μm and F 1μm.

From Nikitina et al., 2004
Appendix D: TUNEL assay showing cell death in tissues of the naked mole-rat eye. (A) Neonate mole-rat eye. (B) Juvenile (21-day old) mole-rat eye showing a number of TUNEL-positive cells at the posterior margin of the lens (arrowheads).

From Nikitina et al., 2004
APPENDIX E

Comparison of the embryonic eye developmental stages between mouse and chick.

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<thead>
<tr>
<th>Event in eye development</th>
<th>Mouse developmental stage</th>
<th>Chick developmental stage (Hamburger-Hamilton)</th>
<th>Chick developmental stage (length of incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outgrowth of the optic vesicle</td>
<td>E9.5</td>
<td>HH11</td>
<td>40-45hrs</td>
</tr>
<tr>
<td>Formation of the optic cup</td>
<td>E10.0-10.5</td>
<td>HH14-15</td>
<td>50-55hrs</td>
</tr>
<tr>
<td>Closure of the lens vesicle</td>
<td>E11.0</td>
<td>HH16</td>
<td>51-56hrs</td>
</tr>
<tr>
<td>RPE pigmentation visible</td>
<td>E11.5</td>
<td>HH20</td>
<td>70-72hrs/E3</td>
</tr>
<tr>
<td>Formation of the anterior chamber</td>
<td>E14.0</td>
<td>HH30</td>
<td>E6.5</td>
</tr>
<tr>
<td>Ciliary body-retina border distinguishable</td>
<td>E15.5-E16.0</td>
<td>HH27</td>
<td>E5</td>
</tr>
<tr>
<td>Formation of the ciliary folds</td>
<td>P0</td>
<td>HH30-HH34</td>
<td>E6.5-E8</td>
</tr>
<tr>
<td>First signs of iris outgrowth</td>
<td>E16.5</td>
<td>HH35-HH36</td>
<td>E9-E10</td>
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</tbody>
</table>
References


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Ingolia TD, Craig EA. 1982. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc Natl Acad Sci USA 79:2360-2364.


