Published in final edited form as:

Ophthalmic Genet. 2008 March ; 29(1): 1–9. doi:10.1080/13816810701651241.

Developmental Basis of Nanophthalmos: *MFRP* is Required for both Prenatal Ocular Growth and Postnatal Emmetropization

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Abstract

Background—Nanophthalmos is a genetic disorder characterized by very small, hyperopic eyes that are without gross structural defects. Recessive nanophthalmos is caused by severe mutations in the *MFRP* gene, which encodes a Frizzled-related transmembrane protein that is selectively expressed in the retinal pigment epithelium (RPE) and ciliary body.

Results—For two *MFRP* $-/-$ adults, we have obtained records of refraction that begin in early childhood. At the age of 6 months, one patient's eyes already had a refractive error of +12.25 D, and over the next 20 years this slowly increased to +17.50 D. Adults homozygous for null mutations in *MFRP* have eyes with axial lengths shorter than those of normal newborns. Furthermore, the unusually high curvature of their corneas is consistent with eyes that had been smaller than normal during late fetal development. *MFRP* protein was first detected at 14 weeks of gestation, when it was restricted to the posterior pole RPE. By 20 weeks gestation, *MFRP* expression had spread laterally, and was found throughout the RPE. *MFRP* protein was detected in both posterior and lateral RPE of the adult eye.

Conclusions—Embryonic function of the *MFRP* gene appears necessary for the eye to reach its full size at birth. Its onset of expression in the RPE during mid-gestation suggests that *MFRP* does not participate in early formation of the optic cup, and is consistent with a role in later growth and development of the eye. Patients without *MFRP* gene function exhibit no correction of refractive error during childhood, which suggests that this gene is essential for emmetropization, a complex process by which vision regulates axial growth of the eye.

Keywords

hyperopia; microphthalmia; nanophthalmos development

INTRODUCTION

Microphthalmia is one of the most prevalent congenital disorders of the eye (1,2), with an incidence greater than 15 per 100,000 live births (3). It is defined by a small eye that is usually affected by one or more gross structural defects. Among these are coloboma, Axenfeld-Rieger's anomaly, cystic outgrowths of retina or surface epithelia, and optic nerve hypoplasia

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(1,2). These malformations all arise from a diverse set of genetic defects that produce anomalies of early embryonic induction and pattern formation of the optic cup (1,4). The few known genes involved in the etiology of human microphthalmia are transcription factors that play important roles in the early formation of the eye, which take place during the first two months of development (5). Nanophthalmos is a special subtype of microphthalmia in which the eye is small yet “well formed” and lacks overt structural defects (6,7,8). In children, both eyes typically exhibit extreme axial hyperopia and are highly functional. Adults with the condition often develop secondary complications such as angle closure glaucoma and retinal detachment (7,9). Although this disorder has a number of synonyms, including posterior microphthalmia, most patients share a set of distinctive features. They have an axial length of 21 mm or less, a high corneal curvature, with surrounding choroidal and scleral layers that are much thicker than normal (6–9).

We have recently identified recessive mutations in the *MFRP* gene at 11q23.3 as the cause of classic non-syndromic Mendelian recessive nanophthalmos (10). This gene encodes a glycosylated transmembrane protein with an extracellular Frizzled-related cysteine-rich domain (11); it is specifically expressed in the RPE and ciliary body (12). Patients completely lacking MFRP protein have no obvious pathology outside the eye, suggesting that its function is restricted to ocular development and physiology. The specific cellular processes in which MFRP participates remain unknown.

An important question that has not been addressed by earlier work is when *MFRP* is required for human ocular growth. Although data on temporal expression of *Mfrp* have been reported for the mouse (12), genetic data indicates that mouse *Mfrp* is not involved in ocular growth (13,14,15). In this report we evaluate data of adult biometry and the postnatal progression of refractive error in *MFRP* nanophthalmos. The results suggest a role for this gene during human embryonic and postnatal development. Our histochemical results indicate that MFRP protein is not yet expressed during formation of the optic cup, but is expressed in the RPE during later embryonic and postnatal growth of the eye.

MATERIALS AND METHODS

Human Subjects

Informed consent was obtained and patient research conducted according to procedures established by IRB committees at The Johns Hopkins University. These were based on international principles of the Declaration of Helsinki and on current United States HIPAA regulations regarding patient confidentiality. Homozygosity for the *MFRP*^{1143insC} null mutant allele was identified previously in these patients by PCR amplification of MFRP exon 10 human genomic DNA from buccal epithelium, followed by automated ABI dye-terminator sequence analysis (10). These homozygotes were designated patients #5,6,7 and #8, and the affected null/h heterozygote designated #14 in a previous publication (10). The two normal heterozygotes were designated #1 and #13.

Ocular measurements

Standard clinical eye exams were used to obtain visual acuity, cycloplegic refraction by retinoscopy, manual keratometry to determine corneal curvature. Immersion A-scan ultrasonography was employed to reveal length of the visual axis and ocular compartments. Refractive values were obtained from archived glasses by manual lensometry. Statistical analysis of significance employed Student's t-test, and the derivation of one or two-tailed P-values, as appropriate.

Human Tissue Samples

Normal human adult eye bank eyes were obtained from the Maryland Eye Bank. Embryonic eyes were obtained from Advanced Bioscience Resources, Inc (Alameda, CA), as described previously (16). Utilization of this human tissue was in accordance with the Declaration of Helsinki with approval of the JHMI-IRB at Johns Hopkins University School of Medicine. To localize human MFRP protein, we used a commercial goat polyclonal antibody to human MFRP (# AF1915 R&D Systems Inc., Minneapolis, MN). Briefly, 8- μ m frozen sections of 2% paraformaldehyde-fixed adult or embryonic human retina were blocked and incubated with a 1/3,000 dilution of 200 μ g/ml goat anti-MFRP. This was followed with biotinylated rabbit anti-goat, streptavidin alkaline phosphatase, and then was detected by a 30 minute BCIP-NBT reaction. Treatment with peracetic acid was then used to fully uncover the BCIP reaction product by bleaching RPE melanin (17).

RESULTS

We have obtained biometric data from a group of four related nanophthalmos patients, each of whom is homozygous for the recessive *MFRP*^{1143insC} exon 10 frameshift allele. This allele truncates the protein within a Cubilin domain, which results in complete loss of a conserved C-terminal Frizzled cysteine-rich domain (10). Such null-homozygotes are extremely hyperopic, and differ by over 10 standard deviation units from the general population mean (18). The hyperopia is associated with an unusually short axial length, which averages 16.1 mm, in contrast to a population mean of 23.6 mm (Fig. 2B). These patients also have a very high corneal surface curvature, which confers on average an 8.5 diopter greater than normal refractive power to the anterior segment. This corrects roughly a third of the hyperopic error caused by the very short axial length. Yet another feature of these patients is a lens that is about 50% thicker than normal for individuals of this age. The data for these patients indicates that either the lens is unusually large, or that it has a normal volume but is extended further along the visual axis due to a more spherical shape. Both conditions may apply. This lens is also centered at a more forward position than normal, and produces an unusually shallow anterior chamber, in the range of 1.6mm. These young adult patients already have very narrow irido-corneal angles, and one of them developed angle closure glaucoma in her mid twenties.

Although these biometric differences of *MFRP*-null homozygotes are all highly significant, the heterozygotes differ in some features, but not others. In the case of refraction, heterozygotes show no tendency towards hyperopia. It remains to be determined whether the significant ($P = 0.012$) shift towards myopia is a population-specific artifact or truly due to genotype (Fig. 2A). In axial length, the two heterozygotes measured show no significant ($P = 0.15$) deviation from the general population (Fig. 2B). However, other features appear to exhibit semidominance with regard to *MFRP* function. In the case of corneal curvature and anterior chamber depth, the *MFRP*-null heterozygotes show significant differences from the general Caucasian population described by Lyhne (18), with P values of 0.013 and 0.0003 respectively.

The adult eyes in these patients have a volume roughly 25% of the mean for normal adults. They are so small that their mean axial length is shorter than that of normal newborns. When the distribution of adult *MFRP*-homozygotes is compared with similarly plotted distributions for normal newborns 5 days or younger (Fig. 3, refs 19,20), a 2-tailed Student's t -test gives a P value of 1.1×10^{-4} . This indicates that adult *MFRP* $-/-$ eyes have axial lengths that are significantly shorter than those of normal newborns. If we assume that the eye does not grow during childhood, these nanophthalmic eyes are already unusually small at birth. This may not seem surprising, given the congenital nature of other types of microphthalmia. Nanophthalmos is typically apparent when extreme hyperopia is measured at a child's first eye exam. Refractive errors in the range of +8 to +12 diopters have surprisingly little effect because of an infant's considerable capacity for accommodation. The eyes also appear superficially normal at birth,

since they lack external malformations. It should be noted that these eyes do have some distinctive internal retinal malformations. Earlier retinoscopic observations of 6 of the *MFRP*^{1143insC} eyes showed an abnormal optical reflex in the macula, one indication of a malformed or absent foveal pit, and showed evidence of para-macular retinal folds (10). These malformations are often observed in nanophthalmos (21,22), and are thought to occur during morphogenesis of the fovea, which occurs late during fetal development (4).

In the case of this family, we were able to follow postnatal development of the nanophthalmic eye because the mother kept all old glasses of a daughter and son (affected individuals #5 and #7, ref. 10). Beginning at age 3 for the eldest affected child, and beginning at 6 months for the youngest, new glasses were fitted at regular intervals by an optometrist. Lensometer measurements from these sets of glasses have provided a record of the early progression of refractive error, and merge well with later clinical retinoscopic measurements (Fig. 4). These results show that both children were already extreme hyperopes (+11.00 D, +13.25 D) at an early age, and that their refractive error slowly drifted upwards by roughly 5 diopters over the next 20 years. This change could be due to a gradual decrease in lens power, which is observed during childhood due to growth and shape changes of the lens (23,24). Therefore it is possible that there was little or no postnatal axial extension of these eyes. Such axial growth is the mechanism by which emmetropization corrects refractive error during the first 6 years of life, as shown in the green curve. In these *MFRP*-nanophthalmos patients there is no evidence of such a corrective mechanism, only a continued slow progression towards more severe hyperopia. In summary, it appears that these eyes have major growth defects in both the prenatal and postnatal period, with the most marked effect being the absence of postnatal emmetropization.

We further investigated the prenatal role of *MFRP* by determining when it is expressed in the embryonic RPE. Sensitive detection of MFRP protein was achieved by alkaline phosphatase-coupled immunocytochemistry, followed by a bleach of overlying melanin pigment (17). We first examined RPE of a 67 year-old eye donor in which the retina had detached before sectioning. In this specimen, strong staining for MFRP was apparent only in the RPE (Fig. 5). Signal was concentrated towards the apical side of the RPE, with virtually none in Bruch's membrane. No signal was apparent in the detached neural retina. In a second, better preserved adult specimen, most of the retina was intact at the time of fixation. This specimen also showed a similar localization to the apical RPE, but the signal was weaker and more variable, possibly because antibody staining was partially blocked by the dense interphotoreceptor matrix (Fig. 6G). In the same specimen, much stronger RPE signal was obtained at sites of retinal detachment (Fig. 6E). Interestingly, in this case we found significant signal in the matrix surrounding the distal portion of the outer segments (Fig. 6D). It should be noted that the MFRP antibody is directed entirely to the C-terminal Frizzled-type CRD domain. Therefore, this matrix-associated signal could represent partially proteolyzed MFRP protein that is no longer anchored to the RPE plasma membrane.

At 7 weeks gestation, a stage when the eye has a well developed optic cup and heavily pigmented RPE layer (4,16), we observed no MFRP signal, with the possible exception of occasional small foci in the RPE-retina interface (arrow, Fig. 6A). We found no indication that the absence of MFRP signal is a sample preparation artifact, since adjacent sections from the same specimen stained readily with antibodies to CD31 and Hb-epsilon (see ref 16). At 20 weeks, however, all RPE throughout the eye revealed strong expression of MFRP (Fig. 6C). As in the adult eyes, MFRP appeared distributed on or near the apical face of the RPE, with no detectable signal in Bruch's membrane. At 20 weeks, photoreceptor inner segments have normally begun to form and protrude from the neural epithelium, while some outer segments are beginning to appear (4,16,25). In regions where retinal detachment had taken place, the apical face of the 20 week neural retina revealed distinct MFRP-positive signal. It is unclear

whether this MFRP signal associated with the apical retinal surface is integral to the plasma membrane or part of the adjacent extracellular matrix (26). At an intermediate time point, 14 weeks of gestation, only the posterior pole sector of the RPE shows strong MFRP expression (Fig. 7) while the lateral and anterior RPE show little or no signal. This posterior-first gradient of expression parallels that of the neural retina, and has been observed for several other landmarks of RPE development (27). The pattern of expression strongly suggests that the onset of MFRP expression is coupled to a particular stage of retinal histogenesis. Hematoxylin-eosin histology of an adjacent section reveals that the lateral retina (inset, Fig. 7A) has a well developed inner plexiform and ganglion cell layer prior to the appearance of major MFRP expression. These results indicate that expression of MFRP begins relatively late during formation of the eye, during the second trimester of gestation.

DISCUSSION

Severe mutations in the coding region of *MFRP* account for roughly 20 % of nanophthalmos patients, and it is clear that other genes underlying this disorder remain to be identified (28). Still, it is of interest that several distinctive features of this defined subset of patients are typical of this genetically heterogeneous disorder (7,8,9). This unity of phenotype suggests that nanophthalmos mutations may affect several different components of a single ocular growth mechanism. An important feature of the disorder is that the delineation of nanophthalmos from high hyperopia is one of degree, and that common hyperopia may represent a less severe form of the same condition. In this sense, nanophthalmos represents a biometric extreme of the spectrum of axial myopia and hyperopia, and its origin is fundamentally different from the structural malformations that underly most types of microphthalmia.

While prenatal and postnatal ocular growth are both affected in individuals without *MFRP* function, it is still not clear how the growth defect is partitioned between these two phases of development. The simplest hypothesis is that a deficiency of prenatal ocular growth causes enough lateral crowding and thickening of the normal-sized retina to interfere with formation of the foveal pit, and to produce para-foveal retinal folds (21,22). The magnitude of this prenatal growth deficiency is unknown, but we can make estimates of eye size based on curvature of the cornea, which is locked in place by maturational crosslinking of the collagenous stroma and basement membranes during late embryogenesis (29). Prior to this stromal maturation, the cornea is extensible, and its curvature reflects the radius of the whole ocular globe (4). Subsequently, corneal curvature undergoes little change from birth to adulthood (23). For our patients, the ratio of adult *MFRP* $-/-$ and normal corneal radii is 0.837. If this embryonic scaling factor is multiplied by the normal newborn axial length, one obtains an estimated axial length of 14.05 mm for *MFRP* $-/-$ newborns. Given this estimate, only 2.05 mm of axial growth is required to reach the mean adult axial length of 16.1 mm for *MFRP* $-/-$ patients. This is much less than the 6.5 mm of axial growth required for a normal eye to reach the adult length of 23.55 mm. The estimated ~2 mm of postnatal axial extension could be due to continued growth of the anterior segment and lens, and it is possible that these patients exhibit no axial growth of the vitreal chamber.

These estimates fit well with the observed upward drift in refractive error during childhood, which can be explained entirely by changes in the anterior segment. For normal individuals, the change in anterior segment refraction from the ages of 9 months to 15 years is dominated by a 14 diopter decrease in refractive power caused by decreased curvature of the growing lens. A major portion of this decrease is due to lens flattening, thought to be the result of increased zonule tension caused by growth of the globe (23,24). In nanophthalmos, stretch-flattening seems less likely to take place in the absence of significant expansion of the globe. In this case, we would expect normal lens growth to contribute a more modest -7 diopters

decrease in anterior segment refraction. This is roughly the amount observed, and is compatible with a virtual absence of postnatal axial growth of the eye.

For *MFRP* $-/-$ individuals, we observe no emmetropic correction of the infant's initial refractive error, and the likely underlying explanation is a near-absence of axial growth. During normal emmetropization, visual input to the retina is somehow evaluated to extract information concerning focus, and signals that speed up or slow down growth of the eye are produced by the retina (30,31). It has been suggested that these signals are received by the RPE, which then relays this information to the choroid and sclera (32). How this signaling is achieved and how *MFRP* is involved in this process remains completely unknown. Since the loss of *MFRP* function appears to affect both embryonic and postnatal ocular growth, it seems reasonable that this same *MFRP*-dependent growth mechanism operates during both phases of development. It receives developmentally-regulated input in the embryo, and visually-regulated input during infancy and childhood. It is important to note that secondary embryonic growth of the eye does not stop in the absence of *MFRP* function; there is just less of it. One possible explanation is that *MFRP* is not an essential component of secondary growth, but rather part of an overlying regulatory mechanism. In the case of postnatal growth, however, the mutation of *MFRP* has a much more severe effect on axial growth, possibly because other regulatory mechanisms are no longer active.

Our results on the histochemistry of human eyes strongly support the notion that *MFRP* is required for normal growth of the embryo, but that it is not expressed until the second trimester of development. This late onset of *MFRP* expression is consistent with the lack of colobomas or other defects in early pattern formation of the globe. These eyes, however, do show defects of the fovea, a structure that forms during the third trimester of development (4). Whether these foveal defects are related to lateral crowding of the retina or to functions of *MFRP* in the retina-RPE interface remains to be determined.

Acknowledgments

This work was supported by NIH grants EY013610 to O.S., EY016151 to G.L., and a Lew Wasserman Award to O.S. from Research to Prevent Blindness. Core facilities were supported by NIH EY01765 and by an unrestricted grant from Research to Prevent Blindness to the Wilmer Eye Institute. Takuya Hasegawa was a Bausch and Lomb Japan Research Fellow.

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Figure 1. Structural features of hyperopic and myopic eyes

Diagrams of **A.** nanophthalmic, **B.** normal and **C.** myopic eyes are shown in horizontal section. The nanophthalmic eye is a composite of biometric features of MFRP $-/-$ homozygotes (10) and histological data reviewed earlier (6,7,8). Relative biometric and histological features of eyes from emmetropes and high axial myopes (6) were the basis of B. and C.

Figure 2. Biometry of *MFRP*-null homozygotes and heterozygotes

Biometric measurements of Amish-Mennonites ages 18–64 with the 1143insC frameshift, a null mutation of *MFRP*, compared with the Caucasian population described by Lyhne (18). Solid arrows: null homozygotes (–/–). Open-headed arrows: null heterozygotes(–/+). The –/h heterozygote (only in panel A) is a compound heterozygote for the 1143insC allele and HM-1, a presumed hypomorphic allele of *MFRP*. **A.** Spherical equivalent refractive error, determined with cycloplegia, and averaged of both eyes. **B.** Axial length (corneal surface to retina), by ultrasound. **C.** Corneal radius of curvature, R, in mm. The conversion to K-value (diopters of corneal refractive power) is: $K = 337.5/R$. **D.** Anterior chamber depth, from endothelium to lens surface (ultrasound). **E.** Axial lens thickness (ultrasound).

Figure 3. Adult *MFRP* null-homozygote have eyes shorter than those of normal newborns
Distributions of individual ocular axial lengths, normalized to percent of total and assigned to 0.5 mm wide bins. Open circles: 7 eyes of adults homozygous for null mutation *MFRP*^{1143insC}. Caucasians, aged 20 to 32. (10). Solid squares: 160 eyes of normal Caucasian newborns 1 to 5 days (from Larsen et al. ref. 18). A 2-tailed Student's t-test gives a P-value of 1.1×10^{-5} , which allows us to confidently exclude the null hypothesis that these populations are identical. Solid circles: 228 normal Caucasian adult eyes (from Lyhne et al. ref. 17).

Figure 4. Developmental changes in refractive error in the absence of MFRP

Spherical equivalent refractive error was obtained for two siblings featured in Figs 2 and 3. Each is homozygous for the 1143insC null allele of *MFRP*. **Red curve:** patient #5 (ref. 10), better eye; **Blue curve:** Patient #7 (ref. 10), average of both eyes. **Points without circles:** lensometry values from the child's glasses, collected by the mother. The earliest set was prescribed at 6 months. **Circled points:** retinoscopic cycloplegic refraction from our study or from medical records. **Green curve:** average refractive error in the general population (ref. 30).

Figure 5. MFRP protein in the adult RPE

A. Neural retina, detached from RPE. **Ros-arrow:** Rod outer segments. **B.** Retinal pigment epithelium, detached from neural retina. Blue BCIP-alkaline phosphatase reaction product. **Br-arrow:** Bruch's membrane. **C.** Control, without primary antibody. **RPE:** Faint golden color in RPE of control remains after bleach. **Bar:** 20 μm. A–C are at the same magnification.

Figure 6. MFRP protein in the embryonic eye at 7 and 20 weeks

Immunocytochemical localization of MFRP in retinal sections as in Fig. 5. **A**, 7-week neural retina (Ret), with RPE attached. No signal visible in RPE, which retains a faint golden color after bleaching of melanin. Underlying mesenchyme (Mes) shows no signal. **B, C**: 20-week neural retina and RPE+choroid (Chor), respectively. **s**: space between neural retina and RPE, resulting from post-mortem detachment. **nos**: Nascent rudiments of photoreceptor outer segments, showing signal. **D, E**: Adult retina, neural retina and RPE, respectively, in region of detachment. Signal appears associated with extracellular matrix surrounding the distal portion of photoreceptor outer segments (OS). **F**: Control section of detached adult RPE+choroid, without primary antibody. **G**: Region in which neural retina and RPE remained

attached, same retinal section shown in panels D,E. **Bar:** 20 μ m. A–G are at the same magnification.

Figure 7. Expression of MFRP in the human eye at 14 weeks

Prior to sectioning, the dissected optic cup fragment extended from one margin of the optic cup, and contained the posterior pole. Sections included retina, RPE, developing choriocapillaris, and sclera. **A. Hematoxylin and eosin staining.** A gradient of retinal development is visible from the margin to the posterior pole, with the inner plexiform layer visible in lateral and posterior retina. The RPE is heavily pigmented, and detached at both edges of the specimen. *Gcl*: ganglion cell layer. *Nbl*: neuroblastic layer. *RPE*: retinal pigment epithelium. **B. MFRP protein.** Signal is intense in the posterior pole sector of RPE, intermittently visible in the lateral RPE, and absent in anterior and marginal RPE (insets 1, 2, 3). All observed MFRP expression is located towards the apical side of the RPE.