

***BMP4* loss-of-function mutations in developmental eye disorders including SHORT syndrome**

Linda M. Reis · Rebecca C. Tyler · Kala F. Schilter · Omar Abdul-Rahman · Jeffrey W. Innis · Beth A. Kozel · Adele S. Schneider · Tanya M. Bardakjian · Edward J. Lose · Donna M. Martin · Ulrich Broeckel · Elena V. Semina

Received: 21 December 2010 / Accepted: 9 February 2011 / Published online: 22 February 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract *BMP4* loss-of-function mutations and deletions have been shown to be associated with ocular, digital, and brain anomalies, but due to the paucity of these reports, the full phenotypic spectrum of human *BMP4* mutations is not clear. We screened 133 patients with a variety of ocular disorders for *BMP4* coding region mutations or genomic deletions. *BMP4* deletions were detected in two patients: a patient affected with SHORT syndrome and a patient with anterior segment anomalies along with craniofacial dysmorphism and cognitive impairment. In addition to this, three intragenic *BMP4* mutations were identified. A patient with anophthalmia, microphthalmia with sclerocornea, right-sided diaphragmatic hernia, and hydrocephalus was found to have a c.592C>T (p.R198X) nonsense mutation in *BMP4*. A frameshift mutation, c.171dupC (p.E58RfsX17), was identified in two half-siblings with anophthalmia/microphthalmia, discordant developmental delay/postaxial

polydactyly, and poor growth as well as their unaffected mother; one affected sibling carried an additional *BMP4* mutation in the second allele, c.362A>G (p.H121R). This is the first report indicating a role for *BMP4* in SHORT syndrome, Axenfeld–Rieger malformation, growth delay, macrocephaly, and diaphragmatic hernia. These results significantly expand the number of reported loss-of-function mutations, further support the critical role of *BMP4* in ocular development, and provide additional evidence of variable expression/non-penetrance of *BMP4* mutations.

Introduction

Bone morphogenetic protein 4 (*BMP4*) is a member of a large cytokine family related to the transforming growth factor-beta proteins. *Bmp4* is an important regulator of

L. M. Reis · R. C. Tyler · K. F. Schilter · U. Broeckel · E. V. Semina
Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

L. M. Reis · R. C. Tyler · K. F. Schilter · U. Broeckel · E. V. Semina
Children's Research Institute, Children's Hospital of Wisconsin, Milwaukee, WI, USA

K. F. Schilter · E. V. Semina
Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI, USA

J. W. Innis · D. M. Martin
Department of Pediatrics and Human Genetics, The University of Michigan Medical Center, Ann Arbor, MI, USA

O. Abdul-Rahman
Department of Pediatrics, University of Mississippi Medical Center, Jackson, MS, USA

B. A. Kozel
Division of Genetics and Genomic Medicine, Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, USA

A. S. Schneider · T. M. Bardakjian
Division of Genetics, Department of Pediatrics, Albert Einstein Medical Center, Philadelphia, PA, USA

E. J. Lose
Department of Clinical Genetics, University of Alabama, Birmingham, AL, USA

E. V. Semina (✉)
C3520, Translational and Biomedical Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509, USA
e-mail: esemina@mcw.edu

normal development causing numerous embryonic defects when mutated or misexpressed in vertebrates (Chang et al. 2001; Furuta and Hogan 1998; Hogan 1996; Jiao et al. 2003; Solnica-Krezel 1999).

In humans, disruption of *BMP4* by deletion or mutation was shown to be associated with ocular, digital, and brain anomalies. *BMP4* is located at chromosome 14q22–q23, near the *OTX2* gene, which is a well-established cause of anophthalmia/microphthalmia (Ragge et al. 2005; Schilter et al. 2010). Deletion of *BMP4* was first demonstrated in combination with *OTX2* deletion in three patients with anophthalmia, pituitary defects, developmental delay, and structural brain anomalies with syndactyly, brachydactyly, and genitourinary anomalies in one (Bakrania et al. 2008; Nolen et al. 2006). A patient with de novo deletion of *BMP4* but not *OTX2* was subsequently reported with congenital glaucoma and sclerocornea, postaxial polysyndactyly, brain anomalies, and developmental delay (Hayashi et al. 2008). Two families with anophthalmia/microphthalmia and intragenic mutations in *BMP4* have been identified (Bakrania et al. 2008). The first mutation, c.226delAG (p.S76CfsX29), was identified in a proband with unilateral anophthalmia and coloboma, retinal dystrophy, and small anterior segment of the contralateral eye, along with postaxial polydactyly, structural brain anomalies, and learning difficulties. The c.226delAG mutation was seen in three additional family members affected with high myopia and/or polydactyly. The second, c.278A>G (p.E93G), was identified in a proband with bilateral microphthalmia, broad hands with low-placed thumbs, brain anomalies, and developmental delay. The p.E93G mutation was inherited from the father, whose only anomaly was mild inferior pigmentation of both retinas. A third variant in *BMP4*, c.751C>T (p.H251Y), was detected in a proband with mild microphthalmia (corneal diameter 10.7 mm; axial length 20.2 mm) and anterior segment anomalies as well as his unaffected brother (Zhang et al. 2009). The role of *BMP4* in anterior segment development/glaucoma is further supported by animal studies demonstrating anterior segment dysgenesis and elevated intraocular pressure in *Bmp4*^{+/-} mice and defects in *BMP4* signaling in experimental glaucoma models (Chang et al. 2001; Wordinger et al. 2007).

In addition to ocular phenotypes, *BMP4* was shown to be associated with cleft lip and palate (Suzuki et al. 2009), renal malformations (Weber et al. 2008), and colorectal cancer (Lubbe et al. 2011). Heterozygous missense mutations in *BMP4* were identified in seven probands with subepithelial, microform, and overt cleft lip/palate, and a nonsense mutation, p.R198X, was identified in another proband with overt cleft lip and palate; no information was provided regarding presence or absence of additional anomalies in these patients. All of the missense mutations

were inherited from either mildly affected (three cases) or unaffected parents (four cases); inheritance of the nonsense mutation was not examined (Suzuki et al. 2009). Heterozygous missense mutations in *BMP4* were also identified in four probands with renal agenesis, dysplasia, or hypoplasia, and a homozygous missense mutation was seen in one proband with cystic dysplasia of the kidneys; again, no details were provided regarding presence or absence of additional anomalies. Three of the mutations were inherited from unaffected parents (including both parents of the homozygous case): one was de novo, and the parents were not tested in the remaining two cases (Weber et al. 2008). Finally, *BMP4* coding region variants were identified in six probands with colorectal cancer in a case/control mutation screen (Lubbe et al. 2011). Based on in silico analysis and control data, the authors classified two missense and one nonsense (p.R286X) variants as pathogenic mutations; no inheritance pattern analysis was performed, and no additional systemic anomalies were documented for any of these cases (Lubbe et al. 2011).

We present results from screening of *BMP4* in patients with various ocular conditions. Five new deletions/mutations in *BMP4* were found in four families. Our screening identified two deletions of *BMP4* but not *OTX2*, including one in a patient with SHORT syndrome, one nonsense mutation, and one frameshift mutation which was detected in two half-siblings with discordant phenotypes with an additional missense mutation in one.

Materials and methods

This human study was approved by the Institutional Review Boards of Children's Hospital of Wisconsin, the University of Michigan, and Albert Einstein Healthcare Network with written informed consent obtained from every subject. Genomic DNA was extracted using standard procedures from blood or buccal samples. Complete sequence of the *BMP4* coding region (reference sequence NM_001202.3) was obtained for 133 patients including 60 with anophthalmia/microphthalmia (34 syndromic), 38 anterior segment disorders (29 syndromic) including 3 with SHORT syndrome, 16 cataracts, 4 coloboma, 5 high myopia, and 10 other disorders using the following primers: set 1 forward, ctgatcttctgacctgct, and reverse, ttcttgaggtaagcagctc, PCR product equal 656 bp and set 2 forward, attgcccaacctgagctat, and reverse, ccagctataaggaagcagctc, PCR product equal 1,069 bp. Patients with a genetic etiology identified in previously reported and unreported screens were excluded. Sequences were reviewed manually and using Mutation Surveyor (Soft-Genetics, State College, PA, USA). All mutations were confirmed by independent sequencing reactions using new

PCR products. Screening for deletions of *BMP4* was performed using Affymetrix Genome-Wide Human SNP Array 6.0 or TaqMan assays in 89 of the above patients, including 40 patients with anophthalmia/microphthalmia and 26 with anterior segment disorders. In addition, all deletions were confirmed using TaqMan assays [*BMP4* probes Hs02617539_cn (5') and Hs00282899_cn (3') that recognize sequences located within the *BMP4* first and last exons, respectively, and *OTX2* probe Hs01323383_cn designed against *OTX2* exon 3 (first coding exon) sequence]. In order to determine the orientation of the *BMP4* mutations identified in Patient 5, PCR product obtained with set 1 primers and the patient's DNA was cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) followed by DNA sequencing of nine independent clones corresponding to single *BMP4* alleles.

A total of 179 Caucasian, 89 African American, 91 Asian, and 93 Hispanic controls described previously (Reis et al. 2010) were screened for mutations in *BMP4*.

Results

A deletion of *BMP4* was detected in two unrelated patients (Patients 1 and 2), a nonsense mutation was detected in a third patient (Patient 3), and a frameshift mutation was detected in a pair of affected half-siblings (Patients 4 and 5) with an additional missense mutation in one (Patient 5). The previously reported c.455T>C (p.V152A) polymorphism was also seen in cases and controls.

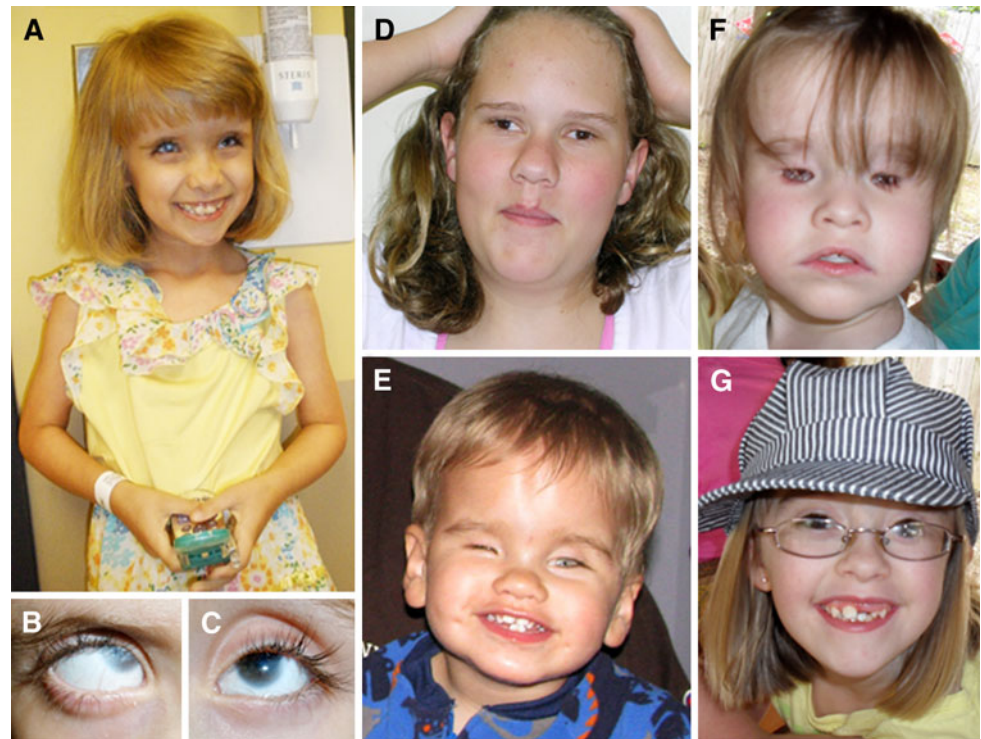
Patient 1 (Fig. 1a–c; Table 1) is a 6-year-old Caucasian female with a diagnosis of SHORT syndrome. Her ocular features include Rieger anomaly, congenital glaucoma, microcornea, and nystagmus. She has short stature, poor weight gain, and macrocephaly with measurements of 106.4 cm (3rd centile), 14.9 kg (<3rd centile), and 55 cm (>95th centile), respectively. The patient also has hyperextensible joints, delayed eruption of teeth (first teeth appeared at nearly 2 years of age), decreased subcutaneous fat in upper trunk and head, and dysmorphic facial features including prominent forehead, sunken eyes, small chin, and hypoplastic nares. Hearing is normal; hands and feet appear small with normal structure. Umbilicus is described as 'a bit pouchy' with mildly increased skin. She has a history of mildly delayed motor milestones felt to be related to her joint hypermobility; she currently receives special education services related to attention deficit hyperactivity disorder and vision problems. Brain magnetic resonance imaging (MRI) showed normal structures. Peripheral blood chromosomes showed 46,XX (580 bands); previous screening of *PITX2* showed normal sequence and copy number (data not shown). The patient was found to have a 2,263-kb deletion (1,508 probes in haploid state; minimum

interval chr14: 51,402,258–53,665,008; maximum interval chr14: 51,400,039–53,667,259; based on UCSC 2006 hg18 assembly) of 14q22.1–14q22.2 which deletes one copy of *BMP4* as well as *GNG2*, *NID2*, *C14orf166*, *PTGDR*, *PTGER2*, *TXNDC16*, *GPR137C*, *ERO1L*, *PSMC6*, *STYX*, *GCPNAT1*, *PLEKHC1*, and *DDHD1* (Fig. 2a). *BMP4* is the most distal gene included in the deletion, but the deletion extends past the end of the coding region of *BMP4*. Quantitative PCR data obtained with TaqMan probes confirmed deletion of one copy of *BMP4* (Fig. 2b) and presence of both copies of *OTX2* (data not shown). The mother (noted to have high myopia but otherwise normal ocular and other systemic features) was tested by TaqMan assay and showed no evidence of *BMP4* deletion; the unaffected father is not available for testing.

Patient 2 (Fig. 1d; Table 1) is a 12-year-old Caucasian female with right corectopia, bilateral microphthalmia, bilateral persistence of the pupillary membrane, high myopia, strabismus, and nystagmus. The patient also has a history of hypotonia and mild–moderate cognitive impairment. The patient has dysmorphic facial features including maxillary hypoplasia with midface flattening, thin upper lip, broad nasal bridge and tip, telecanthus, and had a preauricular ear tag on the right. She has normal growth, head circumference, umbilicus, hands, and feet. Head imaging studies are not available. Peripheral blood chromosome analysis showed 46, XX (550 bands), and subtelomeric FISH was normal. The patient is adopted, so information about family history is limited, and no family members are available for testing. The patient was found to have a 158-kb deletion (122 probes in haploid state; minimum interval chr14:53,361,728–53,520,165; maximum interval chr14:53,352,059–53,520,859; based on UCSC 2006 hg18 assembly) involving 14q22.2 which deletes one copy of *BMP4* (Fig. 2a). No other genes are present in the deleted region. Quantitative PCR data obtained with TaqMan probes confirmed deletion of one copy of *BMP4* (Fig. 2b) and presence of both copies of *OTX2* (data not shown).

Patient 3 (Fig. 1e; Table 1) is a 19-month-old Caucasian male with right anophthalmia/severe microphthalmia, left mild microphthalmia with sclerocornea, facial asymmetry, and right-sided diaphragmatic hernia. His height and weight were normal at 11 weeks of age (60.4 cm/25th–50th centile and 6.8 kg/75th–90th centile, respectively), and by 19 months he was large for his age (90 cm/>97th centile and 13.3 kg/75th–90th centile). He has mild–moderate laryngomalacia with indentation from the innominate artery and bilateral inguinal hernias. He is developmentally normal with some minimal correction for his vision deficits. He has macrocephaly (43.5 cm at 11 weeks/ 90th–97th centile; 51 cm at 19 months/ >97th centile), a large anterior fontanelle, and hydrocephalus treated with a

Fig. 1 Patient 1 with SHORT syndrome demonstrating short stature, macrocephaly, decreased subcutaneous fat in upper trunk and head, prominent forehead, sunken eyes, small chin, and hypoplastic nares (a) along with Rieger anomaly, congenital glaucoma, and microcornea (b, c). Patient 2 with bilateral microphthalmia, maxillary hypoplasia with midface flattening, thin upper lip, broad nasal bridge and tip, and telecanthus (d). Patient 3 with right anophthalmia/severe microphthalmia, left mild microphthalmia with sclerocornea, facial asymmetry macrocephaly (e). Patient 4 with bilateral clinical anophthalmia (f) and Patient 5 with left anophthalmia (prosthesis in place) (g)



subdural-peritoneal shunt. Brain MRI at 4 months of age confirmed the ocular findings and showed macrocrania with very prominent subarachnoid spaces, superimposed overlying subdural collections as well as diffuse cerebral atrophy with ventricular prominence. Clinical chromosomal microarray (using Affymetrix Whole Genome-Human SNP Array 6.0) was normal. He was found to have a heterozygous *c.592C>T* (p.R198X) mutation, previously reported in cleft lip/palate (Suzuki et al. 2009) and predicted to result in premature termination of the BMP4 protein (Fig. 3a). Neither parent carries the mutation.

Patient 4 (Fig. 1f; Table 1) is a 3.5-year-old Caucasian female with bilateral clinical anophthalmia, small ears, and a small left renal cyst. Her birthweight was 3.35 kg (25th–50th centile), birth length was 49.5 cm (50th centile), and birth head circumference was 34 cm (50th centile). At 36 months, her weight was 11 kg (<3rd centile), and height was 93 cm (25th–50th centile). She has normal development, craniofacial features, hands, and feet. Head CT in the neonatal period showed significantly small globes, minimal ocular tissue, and absent optic nerves but otherwise normal brain structures. She was found to have a heterozygous *c.171dupC* (p.E58RfsX17) mutation (Fig. 3a).

Her 9-year-old maternal half-sister, Patient 5 (Fig. 1g; Table 1), is affected with unilateral anophthalmia (left), blepharophimosis and telecanthus and bilateral postaxial polydactyly of both hands. Her birthweight was 2.72 kg (25th centile), her length was 48 cm (25th centile), and her

head circumference was 34 cm (50th centile). At 9 years, she demonstrates poor growth with height and weight <3rd centile (121 cm and 20 kg) and relative macrocephaly (75th centile) with frontal bossing. Her development is appropriate for age. Head CT showed atrophic left globe and small left orbit. Patient 5 was found to carry the same heterozygous *c.171dupC* (p.E58RfsX17) mutation along with a second variant, *c.362A>G* (p.H121R), in *BMP4* (Fig. 3a, b). This variant was found to alter a conserved amino acid (Fig. 3b) and is predicted to be tolerated by SIFT (Ng and Henikoff 2003; <http://sift.jcvi.org/>) while probably damaging by PolyPhen-2 (Adzhubei et al. 2010; <http://genetics.bwh.harvard.edu/pph2/>) analysis. Both the p.E58RfsX17 and p.H121R mutations affect exon 2 of *BMP4* and were determined to be positioned on different alleles by PCR cloning and DNA sequencing of isolated alleles. The unaffected mother of Patients 4 and 5 was found to carry the p.E58RfsX17 mutation with no evidence of mosaicism (data not shown); the mutation apparently occurred de novo since the unaffected maternal grandparents of Patients 4 and 5 were found to carry wild-type *BMP4* alleles. The unaffected father of Patient 5 was not available for testing. Neither parent was available for formal ophthalmological examination, so mild ocular anomalies could not be ruled out.

Screening of 179 Caucasian, 89 African American, 91 Asian, and 93 Hispanic control individuals did not identify any of the three coding region mutations discussed above.

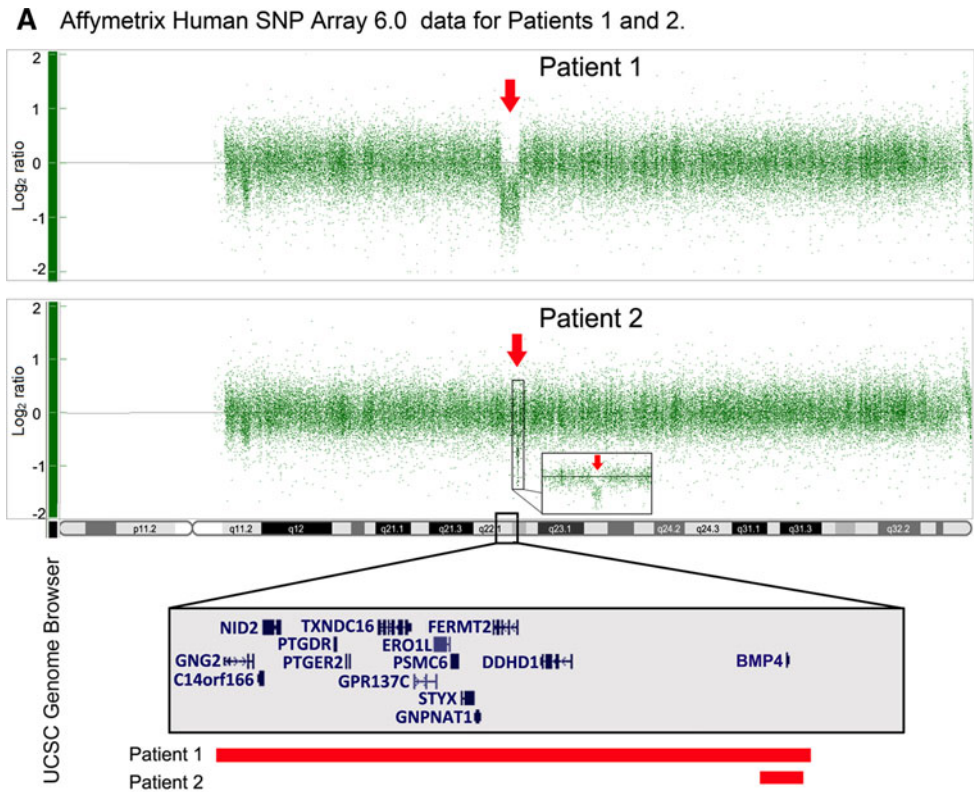
Table 1 Summary of *BMP4* loss-of-function mutations and patient features

<i>BMP4</i> mutation ^a	Eye	Digit	Brain/neuro	Craniofacial	Growth	Other	Family history	
Deletions^b								
Patient 1, this study	2,263-kb deletion (+ 13 genes)	Rieger anomaly, congenital glaucoma, microcornea, nystagmus	Hands/feet appear small	Mild gross motor delay with hypotonia; normal brain structures	Macrocephaly, prominent forehead, sunken eyes, small chin, hypoplastic nares	Height and weight <3rd centile	SHORt syndrome; hyperextensible joints, teething delay, lipodystrophy, umbilical anomaly	Parents unaffected; mother does not carry deletion; father not tested
Patient 2, this study	158-kb deletion (<i>BMP4</i> only)	Microphthalmia, persistence of pupillary membrane, high myopia, strabismus, nystagmus, corectopia (right)	WNL	Mild–moderate cognitive impairment, hypotonia; no imaging studies	Maxillary hypoplasia, midface flattening, thin upper lip, broad nasal bridge and tip, telecanthus, preauricular ear tag	WNL	None	Not available
Hayashi et al. (2008)	2,700-kb deletion (+ 17 genes)	Congenital glaucoma, sclerocornea	Bilateral postaxial polydactyly (feet)	Global delay; Decreased brain white matter, lateral ventricular dilatation	Micrognathia	Weight <3rd centile	None	De novo deletion
Intragenic mutations								
Patient 3, this study	c.592C>T (p.R198X)	Anophthalmia; microphthalmia with sclerocornea	WNL	Development normal; hydrocephalus, diffuse cerebral atrophy	Facial asymmetry, macrocephaly, large anterior fontanelle	Height >97th centile	Right sided diaphragmatic hernia, laryngomalacia, inguinal hernia	De novo mutation
Patient 4, this study	c.171dupC (p.E58RX17)	Anophthalmia	WNL	Development normal; Normal brain structures	Small ears	Height <3rd centile	Small renal cyst (left)	Affected half-sister (Patient 5) and unaffected mother carry the mutation
Patient 5, this study	c.171dupC (p.E58RX17); c.362A>G (p.H121R) in trans	Anophthalmia (left), blepharophimosis	Bilateral postaxial polydactyly (hands)	Development normal; normal brain structures	Telecanthus, relative macrocephaly (75 th centile), frontal bossing	Height and weight <3rd centile	None	Above; Father not available
Bakramia et al. (2008)	c.226delAG (p.S76CfsX29)	Anophthalmia; Microanterior segment, iris and chorioretinal coloboma, retinal dystrophy	Bilateral postaxial polydactyly (feet)	Learning difficulties; hypoplastic corpus callosum, enlarged trigones, sulcal widening	WNL	Not reported	None	Mutation seen in mother, grandmother, and great aunt with polydactyly and/or high myopia
Suzuki et al. (2009)	c.592C>T (p.R198X)	Not reported	Not reported	Not reported	Cleft lip and palate	Not reported	Not reported	Parents not tested
Lubbe et al. (2011)	c.856C>T (p.R286X)	None	None	None	None	Not reported	Colorectal cancer diagnosed at 42 years	Parents not tested; no first degree relative with colorectal cancer

^a Nucleotide numbering is relative to reference sequence NM_001202.3 where +1 is the A of the ATG initiation codon

^b Patients with deletion of *OTX2* in addition to *BMP4* were excluded as *OTX2* is a well-established cause of ocular and pituitary defects; therefore, the contribution of *BMP4* to the phenotype could not be determined

Fig. 2 Affymetrix Genome-Wide Human SNP Array 6.0 microarray analysis (a) and TaqMan assay data (b) for Patients 1 and 2. Heterozygous *BMP4* deletions (arrows in a and b) were detected. The UCSC Genome Browser (<http://genome.ucsc.edu>) view of the deleted region indicating positions of genes is shown with rectangular bars indicating the extent of the deletion in each patient



Discussion

This is the first report of *BMP4* deletion in a patient with the complete constellation of features comprising SHORT syndrome (Patient 1). SHORT syndrome was first described in 1975 and is characterized by Short stature, hyperextensibility of joints or inguinal Hernia, ocular depression, Rieger anomaly, and delay in dental eruption (Teeth) (Gorlin et al. 1975; Sensenbrenner et al. 1975). Autosomal dominant inheritance has been suggested (Koenig et al. 2003), but the genetic basis of SHORT syndrome is currently poorly understood. One case report identified a familial translocation, t(1;4)(q31.2;q25), presumably disrupting the *PITX2* locus, in a child with SHORT syndrome and his mother with Axenfeld–Rieger syndrome and polycystic ovary syndrome (Karadeniz et al. 2004), but

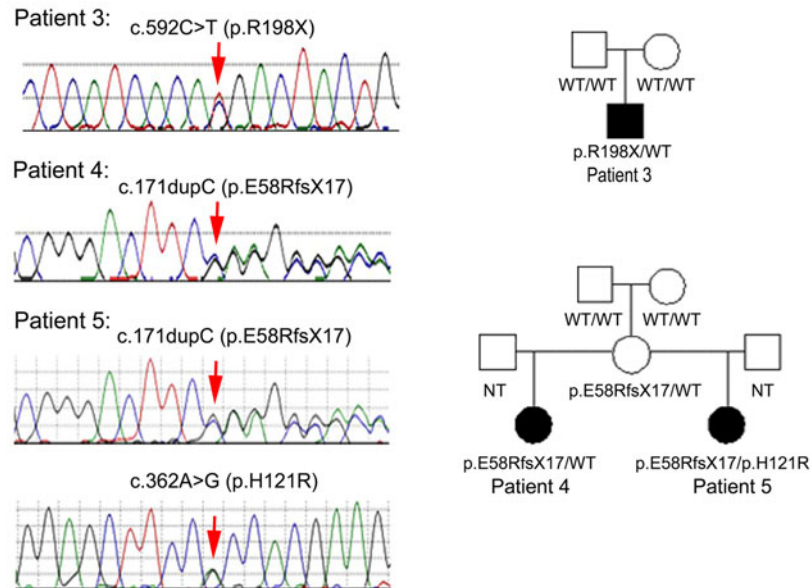
no other studies have replicated involvement of *PITX2* in SHORT syndrome, which was also excluded in Patient 1 in this study.

Since the patient with SHORT syndrome (Patient 1) is affected with a deletion of the *BMP4* region, we compared the “SHORT” deletion with other patient deletions of this region. Both *BMP4* deletions reported in this study are smaller than the previously reported deletion of *BMP4* without *OTX2* involvement (Hayashi et al. 2008). Patient 2 (reported in this manuscript) with deletion of *BMP4* only and the previously reported patient with a 2.7-Mb deletion involving *BMP4* (Hayashi et al. 2008) did not demonstrate a SHORT syndrome phenotype. There are seven genes which are deleted in Patient 1 with SHORT syndrome, but not in Patient 2 or the patient presented by Hayashi et al. (2008): *GNG2*, *NID2*, *C14orf166*, *PTGDR*, *PTGER2*,

Fig. 3 a Intragenic *BMP4* mutations and affected pedigrees; mutation sites indicated with *arrows*.

b Multiple sequence alignment of *BMP4* amino acid sequences demonstrating conservation of the histidine affected in the identified H121R mutation. *Shaded areas* indicate conserved amino acids. GenBank accession numbers are as follows: *Homo sapiens* NP_570911.2, *Mus musculus* AAH34053, *Danio rerio* AAH78423, *Gallus gallus* NP_990568

A Intragenic *BMP4* mutations and affected pedigrees



B Multiple sequence alignment showing conservation of H121R region

<i>H. sapiens</i>	60	TLLQMFGLRRRPQPSKSAVDPDYMRDLYRLQSG-EEEEEQIHSTGLEYPERPASRANTVRSFHHEEHLENI	132
<i>M. musculus</i>	60	TLLQMFGLRRRPQPSKSAVDPDYMRDLYRLQSGEEEEEQIHSTGLEYPERPASRANTVRSFHHEEHLENI	133
<i>D. rerio</i>	53	TLLHMFGLQRRRPSSHSAVVPQYLLDLYRLQSG-ELEEAGAHSVDFYPERSTSRANTVIRGFHHEEHLELQSD	125
<i>G. gallus</i>	61	TLLQMFGLRRRPQPSKSAVPSYMLDLYRLQSG-EEESLQELISLQ-YPERSTSRANTVRSFHHEEHLESVPGP	132
	H121R	-----R-----	

TXNDC16, and *GPR137C*. Although none of these genes represents an obvious candidate for the observed phenotype based on current data (<http://www.ncbi.nlm.nih.gov/omim>), SHORT syndrome may be a contiguous gene deletion syndrome which requires deletion of one (or more) of these genes in addition to *BMP4*.

Analysis of other phenotypic information presented in this manuscript as well as previously reported data further supports the role of *BMP4* in SHORT syndrome. The association of *BMP4* with anterior segment dysgenesis (Patients 2 and 3; Chang et al. 2001; Hayashi et al. 2008; Zhang et al. 2009), poor growth with height and/or weight measurements below the 3rd percentile (Patients 4 and 5; Hayashi et al. 2008), macrocephaly (Patients 3 and 5) and craniofacial/dental development in human patients (Patient 2; Suzuki et al. 2009) and animal models (Fujimori et al. 2010; Vainio et al. 1993; Zhang et al. 2000) is consistent with features of SHORT syndrome. The fact that other patients with *BMP4* deletions/mutations do not demonstrate the full SHORT syndrome phenotype may be explained by variable phenotypic expressivity of *BMP4* mutations and modification of their effect(s) by other genetic factors located elsewhere in genome. The observed incomplete penetrance/variable expressivity of *BMP4* mutations and their wide phenotypic spectrum are in agreement with this possibility (Bakrania et al. 2008; Lubbe et al. 2011; Suzuki et al. 2009; Weber et al. 2008;

Zhang et al. 2009). Screening of additional patients with SHORT syndrome is needed to determine the role/frequency of *BMP4* mutations in this phenotype; in our study, one out of three patients diagnosed with this rare condition demonstrated *BMP4* deletion.

The *BMP4*-positive phenotypes reported in this manuscript also show overlap with the Axenfeld–Rieger spectrum (Alward 2000; Rieger 1934, 1935; Shields et al. 1985) caused by mutations in the *PITX2* and *FOXC1* genes (Semina et al. 1996; Tümer and Bach-Holm 2009). This spectrum is characterized by ocular findings that include posterior embryotoxon, hypoplastic iris, irido-corneal adhesions and glaucoma and additional systemic defects such as craniofacial dysmorphism, dental hypoplasia and redundant periumbilical skin. Patient 1 (SHORT syndrome) has the characteristic Rieger anomaly in combination with atypical dental and umbilical anomalies, Patient 2 shows anterior segment dysgenesis and characteristic craniofacial dysmorphism including maxillary hypoplasia, and Patient 3 demonstrates anterior segment dysgenesis. Taken together with the potential role of both *PITX2* (Karadeniz et al. 2004) and *BMP4* (this paper) in SHORT syndrome, these data strongly suggest involvement of both factors in the same developmental processes. This is supported by previous studies in animal models which have shown that *Bmp4* and *Pitx2* act in a common pathway in craniofacial/dental and left–right asymmetry

development (Liu et al. 2003; Lu et al. 1999; St Amand et al. 2000; Tsiairis and McMahon 2009). Specifically, *Pitx2* was shown to be a repressor of *Bmp4* expression (Liu et al. 2003; Lu et al. 1999), but *Bmp4* was also able to repress *Pitx2* expression (St Amand et al. 2000).

The c.171dupC (p.E58RfsX17) mutation identified in Patients 4 and 5 represents the most 5' nonsense mutation reported to date and is expected to result in a complete loss of function. This allele is likely to result in an absence of protein product due to nonsense-mediated (NMD) decay of the mutant mRNA since the stop codon associated with this mutation is located more than 55 nt (152 nt) from the end of the second to last exon (Holbrook et al. 2004; Khajavi et al. 2006). If present, the mutated protein is predicted to be 14% of its normal length and lack the transforming growth factor beta (TGF β)-like domain and 91% of the TGF β propeptide.

Our results further support the variable expressivity/incomplete penetrance of *BMP4* mutations, as has been shown in previous publications (Bakrania et al. 2008; Suzuki et al. 2009; Weber et al. 2008; Zhang et al. 2009). While one previous family demonstrated a highly variable phenotype in association with a frameshift mutation (Bakrania et al. 2008), this is the first report of a loss-of-function mutation in an apparently unaffected parent (mother of Patients 4 and 5). In addition, only one patient in our group demonstrated digit anomalies and only one had a brain anomaly, both commonly reported in previously described cases with deletion/nonsense mutations. At the same time, new syndromes/features not described in earlier reports, such as SHORT syndrome, poor growth, macrocephaly, hydrocephalus, and diaphragmatic hernia, were identified. In addition, the nonsense mutation p.R198X, previously seen in a patient with cleft lip and palate and no details about additional features (Suzuki et al. 2009), was also seen in Patient 3 without cleft lip/palate in our study. None of our patients or the previously reported ocular cases demonstrated cleft lip/palate or family history of colorectal cancer, which have been previously reported in association with *BMP4* alterations, primarily missense mutations (Lubbe et al. 2011; Suzuki et al. 2009).

The significance of the p.H121R variant seen in Patient 5 in combination with the p.E58RfsX17 is unclear. The histidine at position 121 is conserved (Fig. 3b), and the variant was neither seen in 452 controls reported here nor in 1,053 previously reported controls (Suzuki et al. 2009; Lubbe et al. 2011). Interestingly, Patient 5, with two mutations in *BMP4*, displays a milder ocular phenotype (unilateral anophthalmia) than her half-sister with the p.E58RfsX17 mutation only (bilateral anophthalmia), but has more severe digit involvement (polydactyly vs. normal hands/feet in Patient 4). Overall, the ocular and digit

phenotypes observed in these half-siblings are consistent with the previously reported *BMP4* spectrum, and therefore, it is difficult to determine the contribution of the p.H121R allele; it is possible that this variant represents a rare polymorphism. The unaffected father of this patient was not available for testing to determine whether the allele was inherited from an unaffected parent or occurred de novo.

A wide range of phenotypes have been reported in association with various mutations in *BMP4*, ranging from anomalies evident at birth to adult onset colorectal cancer. Since *Bmp4* has been shown to have various roles in embryonic and adult tissues (Chang et al. 2001; Furuta and Hogan 1998; Hogan 1996; Jiao et al. 2003; Solnica-Krezel 1999; Wordinger et al. 2007), its involvement in multiple phenotypes is highly possible. Our data provide additional support for loss-of-function *BMP4* mutations to be associated with syndromic ocular phenotypes. Each of the probands reported here and all but one of the previously reported cases with ocular phenotypes have mutations which result in lack of functional protein product (deletion or nonsense/frameshift mutations); in all but one of these cases in which family members were tested, the mutation was de novo or seen in affected family members only and loss-of-function *BMP4* mutations have never been reported in control populations. In contrast, the majority of mutations associated with cleft lip and palate, renal anomalies, or colorectal cancer were missense mutations (Weber et al. 2008; Suzuki et al. 2009; Lubbe et al. 2011). While most of the reported missense mutations were not seen in control individuals and several modify conserved amino acids, in more than half of the cases in which family members were tested, the missense mutation was seen in an unaffected relative (8 of 13). This suggests the possibility that some of these missense variants represent rare polymorphisms rather than pathogenic mutations. At the same time, the number of rare *BMP4* variants seen in patients with variable phenotypes may suggest a contributory/sensitizing role for *BMP4* missense mutations leading to different phenotypes depending on other genetic variants/mutations present in the affected individuals. Additional mutational screens and identification of factors that may be involved in modification of the phenotypic expression of *BMP4* mutations are needed to provide insight into the complexity of human phenotypes associated with *BMP4* genotypes.

The results of this study confirm the role of *BMP4* in developmental ocular anomalies, particularly anophthalmia/microphthalmia and anterior segment defects, and suggest that limb and brain anomalies may not be informative for determining which patients will benefit from molecular screening. Further screening for *BMP4* mutations/deletions in patients with SHORT syndrome and a variety of ocular disorders will be important to further

define the range of anomalies associated with mutations in/deletions of this gene.

Ethical standards All experiments performed comply with the current laws of the United States of America.

Acknowledgments The authors would like to thank the patients and their families for their participation in research studies. We are also grateful to Robert Owens for helping with photography and to Rachel Lorier, Stephen Hall, Katie Felhofer and Andrea Lenarduzzie for assistance with Affymetrix array CNV analysis. This project was supported by award EY015518 from the National Eye Institute, and 1R21DC010912 from the National Institute of Hearing and Communication Disorders, National Institutes of Health (EVS) and grant 1UL1RR031973 from the Clinical and Translational Science Award (CTSA) program of the National Center for Research Resources, National Institutes of Health as well as two grants from the Mellon Mid-Atlantic Charitable Trusts, the Albert B. Millett Memorial Fund and the Rae S. Uber Trust, and a grant from the Gustavus and Louis Pfeiffer Research Foundation.

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7:248–249. doi:10.1038/nmeth0410-248
- Alward WL (2000) Axenfeld–Rieger syndrome in the age of molecular genetics. *Am J Ophthalmol* 130:107–115
- Bakrania P, Efthymiou M, Klein JC, Salt A, Bunyan DJ, Wyatt A, Ponting CP, Martin A, Williams S, Lindley V, Gilmore J, Restori M, Robson AG, Neveu MM, Holder GE, Collin JR, Robinson DO, Farndon P, Johansen-Berg H, Gerrelli D, Ragge NK (2008) Mutations in BMP4 cause eye, brain, and digit developmental anomalies: overlap between the BMP4 and hedgehog signaling pathways. *Am J Hum Genet* 82:304–319. doi:10.1016/j.ajhg.2007.09.023
- Chang B, Smith RS, Peters M, Savinova OV, Hawes NL, Zabaleta A, Nusinowitz S, Martin JE, Davisson ML, Cepko CL, Hogan BL, John SW (2001) Haploinsufficient Bmp4 ocular phenotypes include anterior segment dysgenesis with elevated intraocular pressure. *BMC Genet* 2:18
- Fujimori S, Novak H, Weissenbock M, Jussila M, Goncalves A, Zeller R, Galloway J, Thesleff I, Hartmann C (2010) Wnt/beta-catenin signaling in the dental mesenchyme regulates incisor development by regulating Bmp4. *Dev Biol* 348:97–106. doi:10.1016/j.ydbio.2010.09.009
- Furuta Y, Hogan BL (1998) BMP4 is essential for lens induction in the mouse embryo. *Genes Dev* 12:3764–3775
- Gorlin RJ, Cervenka J, Moller K, Horrobin M, Witkop CJ Jr (1975) Malformation syndromes. A selected miscellany. *Birth Defects Orig Artic Ser* 11:39–50
- Hayashi S, Okamoto N, Makita Y, Hata A, Imoto I, Inazawa J (2008) Heterozygous deletion at 14q22.1–q22.3 including the BMP4 gene in a patient with psychomotor retardation, congenital corneal opacity and feet polysyndactyly. *Am J Med Genet A* 146A:2905–2910. doi:10.1002/ajmg.a.32519
- Hogan BL (1996) Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 6:432–438
- Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE (2004) Nonsense-mediated decay approaches the clinic. *Nat Genet* 36:801–808. doi:10.1038/ng1403
- Jiao K, Kulessa H, Tompkins K, Zhou Y, Batts L, Baldwin HS, Hogan BL (2003) An essential role of Bmp4 in the atrioventricular septation of the mouse heart. *Genes Dev* 17:2362–2367. doi:10.1101/gad.1124803
- Karadeniz NN, Kocak-Midillioglu I, Erdogan D, Bokesoy I (2004) Is SHORT syndrome another phenotypic variation of PITX2? *Am J Med Genet A* 130A:406–409. doi:10.1002/ajmg.a.30206
- Khajavi M, Inoue K, Lupski JR (2006) Nonsense-mediated mRNA decay modulates clinical outcome of genetic disease. *Eur J Hum Genet* 14:1074–1081. doi:10.1038/sj.ejhg.5201649
- Koenig R, Brendel L, Fuchs S (2003) SHORT syndrome. *Clin Dysmorphol* 12:45–49. doi:10.1097/01.mcd.0000046501.50562.59
- Liu W, Selever J, Lu MF, Martin JF (2003) Genetic dissection of Pitx2 in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. *Development* 130:6375–6385. doi:10.1242/dev.00849
- Lu MF, Pressman C, Dyer R, Johnson RL, Martin JF (1999) Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. *Nature* 401:276–278. doi:10.1038/45797
- Lubbe SJ, Pittman AM, Matijssen C, Twiss P, Olver B, Lloyd A, Qureshi M, Brown N, Nye E, Stamp G, Blagg J, Houlston RS (2011) Evaluation of germline BMP4 mutation as a cause of colorectal cancer. *Hum Mutat* 32:E1928–E1938. doi:10.1002/humu.21376;10.1002/humu.21376
- Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res* 31:3812–3814
- Nolen LD, Amor D, Haywood A, St Heaps L, Willcock C, Mihelec M, Tam P, Billson F, Grigg J, Peters G, Jamieson RV (2006) Deletion at 14q22-23 indicates a contiguous gene syndrome comprising anophthalmia, pituitary hypoplasia, and ear anomalies. *Am J Med Genet A* 140:1711–1718. doi:10.1002/ajmg.a.31335
- Ragge NK, Brown AG, Poloschek CM, Lorenz B, Henderson RA, Clarke MP, Russell-Eggitt I, Fielder A, Gerrelli D, Martinez-Barbera JP, Ruddle P, Hurst J, Collin JR, Salt A, Cooper ST, Thompson PJ, Sisodiya SM, Williamson KA, Fitzpatrick DR, van Heyningen V, Hanson IM (2005) Heterozygous mutations of OTX2 cause severe ocular malformations. *Am J Hum Genet* 76:1008–1022. doi:10.1086/430721
- Reis LM, Tyler RC, Schneider A, Bardakjian T, Stoler JM, Melancon SB, Semina EV (2010) FOXE3 plays a significant role in autosomal recessive microphthalmia. *Am J Med Genet A* 152A(3):582–590. doi:10.1002/ajmg.a.33257
- Rieger H (1934) Verlagerung und Schlitzform der Pupille mit Hypoplasie des Irisvorderblattes. *Z Augenheilkd* 84:98–103
- Rieger H (1935) Beitrage zur Kenntnis seltener Missbildungen der Iris: ueber Hypoplasie des Irisvorderblattes mit Verlagerung und Entrundung der Pupille. *Albrecht von Graefes Arch Klin Exp Ophthalm* 133:602–635
- Schilter KF, Schneider A, Bardakjian T, Soucy JF, Tyler RC, Reis LM, Semina EV (2010) OTX2 microphthalmia syndrome: four novel mutations and delineation of a phenotype. *Clin Genet* 79(2):158–168. doi:10.1111/j.1399-0004.2010.01450.x
- Semina EV, Reiter R, Leysens NJ, Alward WL, Small KW, Datson NA, Siegel-Bartelt J, Bierke-Nelson D, Bitoun P, Zabel BU, Carey JC, Murray JC (1996) Cloning and characterization of a

- novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat Genet* 14:392–399. doi:[10.1038/ng1296-392](https://doi.org/10.1038/ng1296-392)
- Sensenbrenner JA, Hussels IE, Levin LS (1975) A low birthweight syndrome, ? Rieger syndrome. *Birth Defects Orig Artic Ser* 11:423–426
- Shields MB, Buckley E, Klintworth GK, Thresher R (1985) Axenfeld–Rieger syndrome. A spectrum of developmental disorders. *Surv Ophthalmol* 29:387–409
- Solnica-Krezel L (1999) Pattern formation in zebrafish—fruitful liaisons between embryology and genetics. *Curr Top Dev Biol* 41:1–35
- St Amand TR, Zhang Y, Semina EV, Zhao X, Hu Y, Nguyen L, Murray JC, Chen Y (2000) Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. *Dev Biol* 217:323–332. doi:[10.1006/dbio.1999.9547](https://doi.org/10.1006/dbio.1999.9547)
- Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A, Natsume N, Shimoizato K, Ohbayashi N, Suzuki Y, Niimi T, Minami K, Yamamoto M, Altannamar TJ, Erkhembaatar T, Furukawa H, Daack-Hirsch S, L'heureux J, Brandon CA, Weinberg SM, Neiswanger K, Deleyiannis FW, de Salamanca JE, Vieira AR, Lidral AC, Martin JF, Murray JC (2009) Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *Am J Hum Genet* 84:406–411. doi:[10.1016/j.ajhg.2009.02.002](https://doi.org/10.1016/j.ajhg.2009.02.002)
- Tsiairis CD, McMahon AP (2009) An Hh-dependent pathway in lateral plate mesoderm enables the generation of left/right asymmetry. *Curr Biol* 19:1912–1917. doi:[10.1016/j.cub.2009.09.057](https://doi.org/10.1016/j.cub.2009.09.057)
- Tümer Z, Bach-Holm D (2009) Axenfeld–Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *Eur J Hum Genet* 17:1527–1539. doi:[10.1038/ejhg.2009.93](https://doi.org/10.1038/ejhg.2009.93)
- Vainio S, Karavanova I, Jowett A, Thesleff I (1993) Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* 75:45–58
- Weber S, Taylor JC, Winyard P, Baker KF, Sullivan-Brown J, Schild R, Knuppel T, Zurowska AM, Caldas-Alfonso A, Litwin M, Emre S, Ghiggeri GM, Bakkaloglu A, Mehls O, Antignac C, Network E, Schaefer F, Burdine RD (2008) SIX2 and BMP4 mutations associate with anomalous kidney development. *J Am Soc Nephrol* 19:891–903. doi:[10.1681/ASN.2006111282](https://doi.org/10.1681/ASN.2006111282)
- Wordinger RJ, Fleenor DL, Hellberg PE, Pang IH, Tovar TO, Zode GS, Fuller JA, Clark AF (2007) Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Invest Ophthalmol Vis Sci* 48:1191–1200. doi:[10.1167/iovs.06-0296](https://doi.org/10.1167/iovs.06-0296)
- Zhang Y, Zhang Z, Zhao X, Yu X, Hu Y, Geronimo B, Fromm SH, Chen YP (2000) A new function of BMP4: dual role for BMP4 in regulation of Sonic hedgehog expression in the mouse tooth germ. *Development* 127:1431–1443
- Zhang X, Li S, Xiao X, Jia X, Wang P, Shen H, Guo X, Zhang Q (2009) Mutational screening of 10 genes in Chinese patients with microphthalmia and/or coloboma. *Mol Vis* 15:2911–2918