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Clinical and Molecular Findings in Osteoporosis-Pseudoglioma Syndrome

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Mutations in the low-density lipoprotein receptor–related protein 5 gene (*LRP5*) cause autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG). We sequenced the coding exons of *LRP5* in 37 probands suspected of having OPPG on the basis of the co-occurrence of severe congenital or childhood-onset visual impairment with bone fragility or osteoporosis recognized by young adulthood. We found two putative mutant alleles in 26 probands, only one mutant allele in 4 probands, and no mutant alleles in 7 probands. Looking for digenic inheritance, we sequenced the genes encoding the functionally related receptor *LRP6,* an LRP5 coreceptor *FZD4,* and an LRP5 ligand, *NDP,* in the four probands with one mutant allele, and, looking for locus heterogeneity, we sequenced *FZD4* and *NDP* in the seven probands with no mutations, but we found no additional mutations. When we compared clinical features between probands with and without *LRP5* mutations, we found no difference in the severity of skeletal disease, prevalence of cognitive impairment, or family history of consanguinity. However, four of the seven probands without detectable mutations had eye pathology that differed from pathology previously described for OPPG. Since many *LRP5* mutations are missense changes, to differentiate between a disease-causing mutation and a benign variant, we measured the ability of wild-type and mutant LRP5 to transduce Wnt and Norrin signal ex vivo. Each of the seven OPPG mutations tested, had reduced signal transduction compared with wild-type mutations. These results indicate that early bilateral vitreoretinal eye pathology coupled with skeletal fragility is a strong predictor of *LRP5* mutation and that mutations in *LRP5* cause OPPG by impairing Wnt and Norrin signal transduction.

Introduction

Osteoporosis-pseudoglioma syndrome (OPPG [MIM 259770]) is an autosomal recessive disorder generally characterized by congenital or infancy-onset visual loss and skeletal fragility recognized during childhood. Mutations in the low-density lipoprotein receptor–related protein 5 (encoded by *LRP5*) cause OPPG (Gong et al. 2001). OPPG is a rare disorder. With the assumption of an estimated population incidence of 1 per 2,000,000 and a carrier frequency of 1 per 700, ~380,000 U.S. citizens are predicted to be carriers of deleterious *LRP5* mutations. Heterozygous carriers of OPPG-causing mutations have reduced bone-mineral density (BMD) compared with age- and sex-matched controls (Gong et al. 2001; Lev et al. 2003), and *LRP5* mutations have been found among individuals with "idiopathic" osteoporosis and/or skeletal fragility (Hartikka et al. 2005). Population-based studies suggest that common variants of *LRP5* contribute to the normal population variation in BMD (Ferrari et al. 2004; Mizuguchi et al. 2004; Bollerslev et al. 2005). Two other phenotypes have been attributed to a mutation in *LRP5*. Heterozygous missense mutations in the receptor's first six-bladed propeller domain can cause autosomal dominant disorders of high bone mass (HBM), in which BMD is several SDs above the mean (Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003). These mutations may cause a gain of function in the receptor by altering a binding site for the receptor's endogenous inhibitors (Boyden et al. 2002; Ai et al. 2005). Heterozygous and homozygous mutations have also been described in some patients with the eye disease familial exudative vitreoretinopathy (FEVR) (Jiao et al. 2004; Toomes et al. 2004; Qin et al. 2005), a locus heterogeneous disorder that can also be caused by mutations in a secreted ligand, Norrin (*NDP*) (Chen et al. 1993), and an LRP5 coreceptor, Frizzled 4 (*FZD4*) (Robitaille et al. 2002).

LRP5 is a member of the low-density lipoprotein receptor superfamily of cell-surface receptors. Similar to the prototype family member LDLR, LRP5 has been implicated in plasma lipid homeostasis in mice (Fujino et al. 2003; Magoori et al. 2003). However, the relevance

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Table 1

Characteristics of Study Families

Mutation Type and Family[a]	Consanguinity[b]	DNA Change[c]	Protein Change	Sex of Proband	Phenotype (Age First Recognized)		Mental Retardation[d]	Atypical Findings of Affected siblings or parents[e]
					Eye	Skeletal		
Homozygous mutations:								
OP236*	Y	29G→A	W10X	F	Pseudoglioma (1 mo)	Craniotabes (birth)	No	
OP342*,[f]	Y	3804delA	T1268fsX1438	M	Hyperplastic vitreous (3 mo)	Osteoporosis and fractures (10–11 years)	No	
OP642*	N	3804delA	T1268fsX1438	M	Vitreoretinal dysplasia (birth)	Fractures (3 years)	Moderate	Mother has a mild degree of degenerative change in her retina
OP345*,[g]	N	1467delG	L489fsX529	M	Microphthalmia (4 mo)	Fractures (1 year)	No	
OP346*,[h]	Y	1708C→T	R570W	F	Rentrolental fibroplasia (5 years)	Osteoporosis (8 years)	No	Brother was blind at birth and had vertebral compression fractures noted in adulthood
OP352*	Y	2151_2152insT	D718fsX718	M	Pseudoglioma (3 mo)	Craniotabes (9 mo)	Moderate	
OP417*,[i]	Y	2557C→T	Q853X	M	Pseudoglioma (1 mo)	Fractures (4 years)	No	
OP445*	N	1481G→A	R494Q	F	Posterior synechiae (birth)	Fractures (7 years)	No	
OP457*	Y	1282C→T	R428X	M	Pseudoglioma (birth)	Fractures (<10 years)	Mild	
OP502[j]	Y	1058G→A	R353Q	M	Blind (6 years)	Osteoporosis (20 years)	No	Brother was blind at age 8 mo and had osteoporosis noted in adulthood
OP616	N	920C→T	S307F	M	Blind (birth)	Fractures (5 years)	Severe	Sister had retrolental fibroplasia at 1 year, fractures, and severe retardation
OP637	Y	1000_1004dupAGGAC	T335fsX385	M	PHPV (birth)	Fractures (1 year)	No	
Compound heterozygous mutations:								
OP238	N	1210 G→A 2718_2721delTATG	G404R C906fsX958	M	Exudative retinopathy (2 years)	Fractures (3 years)	No	
OP344*,[k]	N	765G→A 1067C→T	W255X S356L	F	Retrolental mass (1 mo)	Fractures (2 years)	No	
OP347*,[l]	N	789C→A 2202G→A	C263X W734X	M	Turbidity (2 mo)	Fractures (4 years)	No	
OP348*	N	1453G→T 4600C→T	E485X R1534X	F	Vitreoretinal disease (6 wk)	Fractures (2 years)	No	
OP349[m]	Y	1300G→A 1559G→T	D434N G520V	F	PHPV (birth)	Fractures (9 years)	No	
OP450[m]	N	1042C→T in *cis* with 3337C→T 2047G→A	R348W in *cis* with R1113C D683N	F	Retinal detachments (3 mo)	Compressed vertebrae (11 years)	No	
OP482*	N	2197T→C 2247delG	Y733H G749fsX797	F	Vitreous hemorrhages (birth)	Craniotabes (birth)	No	Mother with Y733H has subtle evidence of FEVR
OP524	N	731C→T 3763+2T→C	T244M splice mutation	M	Retrolental mass (birth)	Compressed vertebrae (4 mo)	No	
OP535	N	1750C→T 4512_4517delGGCCAC, insTGTACAACAT	Q584X P1504fsX1550	M	Retrolental mass (5 wk)	Fractures (2 years)	Moderate	

OP579	N	4202G→A 4586+2T→C	G1401D splice mutation	F	Blind (birth)	Fractures (10 mo)	No	
OP608	N	2409_2503+79del 174 3232C→T	G804_G835delfsX49 R1078X	M	Blind (birth)	Fractures (2 years)	Moderate	
OP610	N	2247delG 2737_2738insT	G749fsX797 C913fsX985	F	Pseudoglioma (birth)	Fractures (6 years)	No	
OP612	N	209_210TC→AA 1378G→A	F70X E460K	M	Blind (3 mo)	Compressed vertebrae (11 years)	No	
OP654	N	607G→A 1199C→A	D203N A400E	M	Pseudoglioma (birth)	Fracture (1 year)	No	
Single heterozygous mutations:								
OP240	N	1169C→A	T390K	M	Pseudoglioma (5 wk)	Vertebral compression (2 years)	No	
OP343[k]	N	4105_4106delAT	M1369fsX1370	F	Posterior vascular anomaly (birth)	Fractures (11 years)	No	
OP350	N	3295G→T	D1099Y	F	Blind (3 years)	Fractures (<9 years)	Mild	
OP547	N	1828G→C	G610R	M	PHPV	Fractures (4 years)	Mild	
No identified mutations:								
OP354	Y	Heterozygous for *LRP5* polymorphisms		F	Pseudoglioma (2 mo)	Fracture and pseudarthrosis (birth)	Mild	Brother had congenital heart defect, fractures, pseudarthroses, mild retardation, and retinopathy with temporal vascular changes detected at age 2 years
OP474	N	Homozygous for LRP5 polymorphisms		M	Congenital retinal folds (birth)	Fractures with osteoporosis (14 years)	No	
OP549	N			M	Bilateral glaucoma (birth), unilateral visual loss (34 years)	Fractures (7 years)	No	
OP555	N			M	Phthisis bulbi (birth)	Osteoporosis (2 years)	Mild	
OP618	N			M	Peters anomaly (birth)	Fractures (4 years)	No	
OP624	N			M	Retinal coloboma (birth)	Fractures (<15 years)	No	
OP641	N			M	Retinal detachment (5 years)	Fracture and osteoporosis (31 years)	Mild	

[a] Families marked with an asterisk (*) had their mutations published by Gong et al. (2001).

[b] Consanguinity is considered present when reported by the proband's parents.

[c] DNA mutations were identified by sequencing amplified genomic DNA fragments but are described relative to the *Lrp5* cDNA sequence, with A as the first nucleotide of the ATG methionine translation initiation codon. Potential consequences at the protein level, if there was no nonsense-mediated mRNA decay, are described relative to the translated protein, with the methionine representing the first amino acid residue. Missense mutations in the underlined protein were tested for Wnt and Norrin signal transduction in 293T cells.

[d] Mental retardation is considered to be independent of the proband's visual loss.

[e] Not all affected siblings are listed.

[f] Clinical features of the participating probands were initially described by Beighton et al. (1985).

[g] Clinical features of the participating probands were initially described by Frontali et al. (1985).

[h] Clinical features of the participating probands were initially described by Somer et al. (1988).

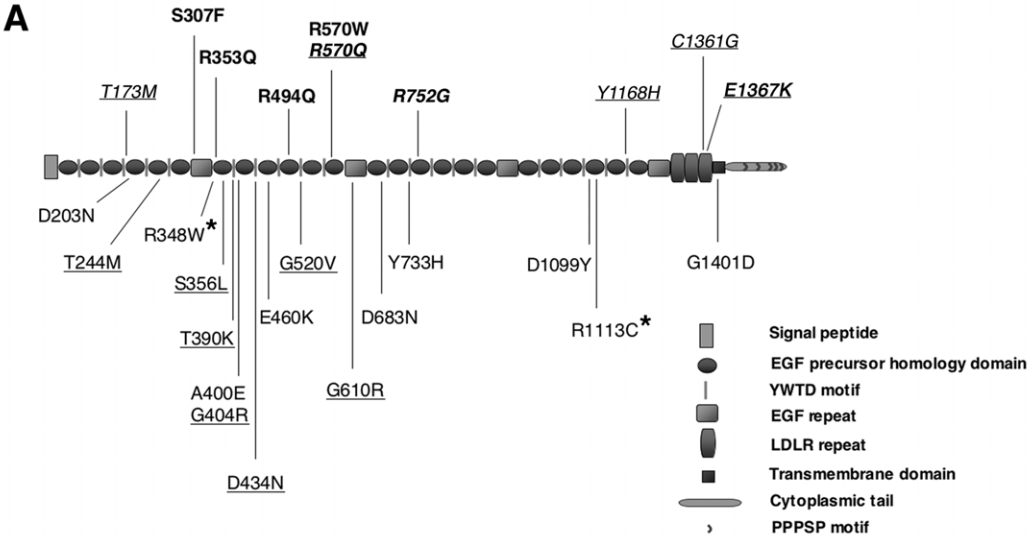
[i] Clinical features of the participating probands were initially described by Superti-Furga et al. (1986).

[j] Clinical features of the participating probands were initially described by Lev et al. (2003).

[k] Clinical features of the participating probands were initially described by De Paepe et al. (1993).

[l] Clinical features of the participating probands were initially described by Swoboda and Grill (1988).

[m] Clinical features of the participating probands were initially described by Zacharin and Cundy (2000).



B

Mutations:	T244M	S356L	T390K
Human LRP5	PFALTLSGD	LRRISLDTP	YVYWTDDEV
Mouse LRP5	PFALTLSGD	LRRISLDTP	YVYWTDDEV
Rat LRP5	PFALTLSGD	LRRISLDTP	YVYWTDDEV
Xenopus LRP5	PFALTLTGK	LRRISLDMP	YIYWTDDEV
Human LRP6	PFALTLFED	LRRISLDTP	YIYWTDDEV
Mouse LRP6	PFALTLFED	LRRISLDTP	YIYWTDDEV
Drosophila arrow	PFSLTFYDD	ISKISLDSP	HIYWSDVET

Mutations:	G404R	D434N	G520V	G610R
Human LRP5	AYLDGSGAQ	LYWTDTGTD	KLYWGDAKT	ADRNGGCSH
Mouse LRP5	AYLDGSGAQ	LYWTDTGTD	KLYWGDAKT	ADGNGGCSH
Rat LRP5	AYLDGSGAQ	LYWTDTGTD	KLYWGDAKT	ADGNGGCSH
Xenopus LRP5	AYLDGSGVE	LYWTDTGTD	KLYWGDAKT	ADNNGGCSH
Human LRP6	SFIDGSGSQ	LYWTDTGTD	KIYWGDAKT	AEENGGCSH
Mouse LRP6	SFIDGSGSQ	LYWTDTGTD	TIYWGDAKT	AEDNGGCSH
Drosophila arrow	AHADGTGVT	LYWTDTVTD	AIYWCDGKT	AVRNGGCSH

Mutations:	D203N	S307F	R348W	R353Q
Human LRP5	GLTIDLEEQ	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Mouse LRP5	GLTIDLEEQ	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Rat LRP5	GLTIDLEEQ	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Xenopus LRP5	GLTIDLEEQ	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Human LRP6	GLTLDYEEQ	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Mouse LRP6	GLTLDYQER	NGGCSHLCL	LLLARRTDL	RTDLRRISL
Drosophila arrow	GLAVDLKNE	NGNCSHLCL	MFIVQRTQI	RTQISKISL

Mutations:	A400E	E460K	R494Q	R570W
Human LRP5	AIRRAYLDG	EDLDEPRAI	DGQERRVLV	RSIERVHKV
Mouse LRP5	AIRRAYLDG	EDLDEPRAI	DGRDRHVLV	RSIERVHKV
Rat LRP5	AIRRAYLDG	EDLDEPRAI	DGRDRHVLV	RSIERVHKV
Xenopus LRP5	AIRRAYLDG	ENLDEPRAI	DGSERLVLV	RSIERVHKV
Human LRP6	AIRRSFIDG	EDLEEPRAI	DGSDRVVLV	RSIERVHKR
Mouse LRP6	AIRRSFIDG	EDLEEPRAI	DGSDRVVLV	RSIERVHKR
Drosophila arrow	TIKRAHADG	EHLEEPRAI	DGSERVVLV	RSIDRAHKI

Mutations:	D683N	Y733H	D1099Y	R1113C	G1401D
Human LRP5	ALDFDVSNN	GKNLYWADT	RAALDGTER	TGLIRPVAL	LFVMGGVYF
Mouse LRP5	ALDFDVSNN	GKNLYWADT	RASLDGTER	TGLIRPVAL	LFVMGGVYF
Rat LRP5	ALDFDVSNN	GKNLYWADT	RASLDGTER	TGLIRPVAL	LFVMGGVYF
Xenopus LRP5	ALDFDVSDN	GKNLYWSDT	RAAFDGTER	TGLIRPVAL	VFVMGGLYF
Human LRP6	ALDFDVTDN	GKNLYWADT	RAALDGTER	SGLSKPIAL	IFVSGTVYF
Mouse LRP6	ALDFDVTDN	GKNLYWADT	RAALDGTER	SGLSKPIAL	IFVSGTIYF
Drosophila arrow	ALDVSVAER	ANNIYWSDA	RARVDGNER	K-LEGVTAL	ITIFSIVYL

Figure 1 Disease-associated missense mutations in LRP5. *A*, Schematic depiction of LRP5 protein and the sites of missense amino acid substitutions that have been associated with OPPG. Homozygous mutations in patients with OPPG are noted in bold above the protein. Heterozygous mutations in patients with OPPG are shown below the protein. Two missense mutations that occur in *cis* on a single allele are marked with an asterisk (*). Missense mutations that are associated with autosomal dominant FEVR are shown in italics, and mutations associated with autosomal recessive FEVR are in bold italics. All underlined mutations were tested ex vivo. *B*, Single-letter amino acid ClustalW alignments of residues surrounding the 20 OPPG-associated missense mutations for *LRP5*, *LRP6*, and *arrow*. The amino acid residues altered by the missense mutations are shaded in the species in which the residue is evolutionarily conserved. The seven putative OPPG-causing mutations that were tested ex vivo are shown in the upper half of the panel.

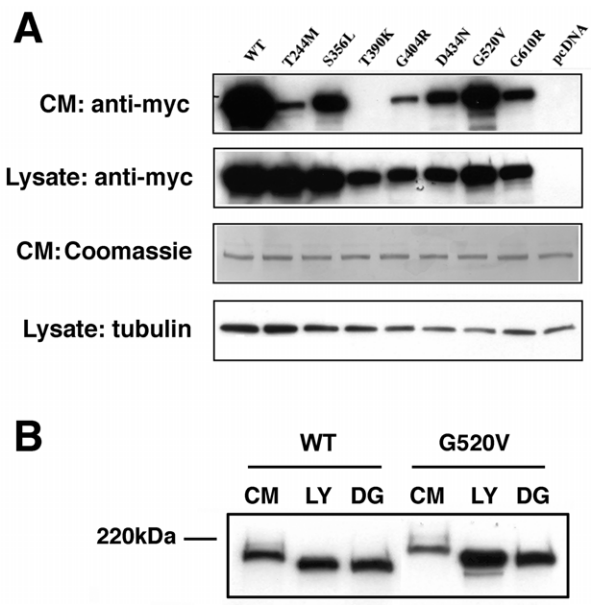


Figure 2 Trafficking and posttranslational modification of OPPG-LRP5 mutants in 293T cells. *A,* WT and OPPG-causing LRP5 constructs that express a myc-tagged, truncated polypeptide lacking the transmembrane and cytoplasmic domains (LRP5N-myc) were transiently transfected into 293T cells. Western-blot analyses were performed to detect the recombinant protein in the conditioned medium ("CM") and cell lysate ("Lysate") with use of an anti-myc antibody. Note the relative efficiencies of different mutants to transit the cell and be secreted into the conditioned medium compared with the WT protein. T390K is present in the cell lysate but is not secreted into the conditioned medium, whereas G520V is secreted into the conditioned medium at rates comparable to WT. Equal loading of cell lysate and conditioned medium in each lane is demonstrated by immunodetection of cell lysate with an anti-tubulin antibody, D-10-HRP (Santa Cruz Biotechnology), and Coomassie staining of conditioned medium, respectively. *B,* Western-blot analysis of conditioned medium ("CM") and cell lysate ("LY") from 293T cells expressing WT or G520V LRP5N-myc protein. Note that, when the conditioned medium was digested with N-glycosidase ("DG"), the molecular weight of the secreted protein was decreased. Other OPPG-associated mutant proteins were tested in the same assay and gave similar results (data not shown).

of this function to human plasma lipid homeostasis has not yet been evaluated. The most important role of LRP5 in humans is as a cell-surface signaling receptor. LRP5 serves as a coreceptor with members of the frizzled family of seven-pass membrane receptors in transducing signal by two extracellular ligand classes, Norrin and Wnts. Evidence to support LRP5 function as a Norrin coreceptor derives from ex vivo cell signaling assays (Xu et al. 2004) and the aforementioned observation that mutations in *NDP, FZD4,* or *LRP5* can each cause FEVR. Evidence to support LRP5 function as a coreceptor in canonical Wnt signaling also derives from ex vivo signaling assays (Tamai et al. 2000; Gong et al. 2001) and from observations of phenotypic overlap between knockout and transgenic mouse models involving *Lrp5* (Kato et al. 2002; Babij et al. 2003; Holmen et al. 2004), its closely related family member *Lrp6* (Holmen et al. 2004), and members of the Wnt ligand superfamily (Bennett et al. 2005). Current data suggest that the skeletal effects of *LRP5* mutations result from altered Wnt signaling and that the altered visual effects result from altered Norrin signaling. However, this hypothesis has not been definitively proven.

Most clinical descriptions of patients with OPPG were published prior to the discovery of *LRP5* (summarized by Gong et al. [1996]). With the identification of *LRP5* as the responsible gene, it is now possible to define phenotypic features that are common to patients with mutation-confirmed OPPG, to describe the disease course in these individuals, to correlate their phenotype with their genotype, and to address whether OPPG exhibits locus heterogeneity. Since there is the prediction of a large number of OPPG carriers who are at increased risk of osteoporosis (Gong et al. 2001), it is also important to determine the functional consequences of specific missense variations in *LRP5*—in particular, whether they interfere with Wnt and/or Norrin signaling and whether their mechanism of mutational effect is to cause a simple loss of function or an interfering function within the signaling complex. We report the clinical features and the results of mutation detection by direct sequencing of PCR amplimers from genomic DNA in a cohort of 37 probands/families in whom OPPG was clinically suspected. We also describe the functional consequences of several missense mutations found in patients with OPPG on LRP5 receptor trafficking and on Wnt and Norrin signal transduction, and we compare them with wild-type (WT) protein and missense mutants that have been associated with FEVR.

Material and Methods

Recruitment of Study Participants

Individuals clinically suspected of having OPPG were invited to participate in the present study. Informed consent was obtained from all study participants. The institutional review board at University Hospitals of Cleveland approved the study. All participants, or their physicians, were asked to complete a detailed questionnaire about clinical features and disease course (the clinical data form used in the study is accessible from the Warman lab Web site).

Mutation Detection in LRP5, LRP6, FZD4, *and* NDP *by Use of Genomic DNA*

Participant DNA was extracted and quantified using standard techniques. Genomic DNA (50 ng) was ampli-

fied by PCR in 25-μl reactions by use of intronic primers that flank each exon. Primer sequences and locations relative to the exon splice sites for the 23 *LRP5* coding exons are available on request. Standard PCR conditions (94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 50 s, and 72°C for 50 s, followed by a single 72°C 10-min extension) were used, with the following exceptions: reactions for exons 1, 4, and 5 contained 10% dimethyl sulfoxide, and the reaction for exon 21 contained 10% enhancer solution (Invitrogen). Primer annealing temperatures were 70°C for exons 1 and 23 and were 60°C for exons 4, 5, and 21. Amplification primer pairs for exons 1 and 3–9 were designed to avoid amplifying the *LRP5* pseudogene on chromosome 22.

Amplicons were sequenced with BigDye 1.1 or 3.1 chemistry (Applied Biosystems) on an ABI 3100 or 3730 sequencer. Sequencing primers were usually the same primers used in PCR. Internal primers were used in some sequencing reactions. The ABI Sequence Analysis Software (v5.1) was used, and sequence electropherograms were also visually inspected for quality and for evidence of heterozygous changes and were electronically aligned with genomic sequences from human clones AC024124 and AC024123, to look for other types of mutations. Mutations identified in each proband were confirmed by sequencing amplimers from the proband's parents or a sibling. For two mutations for which a relative was unavailable, the probands' mutations were confirmed by sequencing independent amplimers, to exclude the possibility that the mutations were created during PCR. We did not look for mutations in ethnically/geographically matched controls, to differentiate a disease-causing mutation from a low-frequency polymorphism.

In four probands for whom only one putative disease-causing allele in *LRP5* was identified, the 23 coding exons of *LRP6,* the two coding exons of *FZD4,* and the two coding exons of *NDP* were also PCR amplified and sequenced (primers and conditions are available on request). *FZD4* and *NDP* were also sequenced for the seven probands for whom no *LRP5* mutations were found.

Creation of Expression Constructs for LRP5

Construction of full-length human LRP5 (WT-LRP5) and a truncated form of human LRP5 that lacks the transmembrane and cytoplasmic domains but has a myc and His_6 epitope at the C-terminus (LRP5N-myc) have been described elsewhere (Ai et al. 2005). Putative disease-causing missense mutations were introduced into WT-LRP5 by site-directed mutagenesis (Quickchange [Stratagene]). Smaller restriction fragments were subcloned from the expression construct, were used as the template for mutagenesis, were sequence verified, and were then shuttled into the original expression vectors. For example, the T244M mutation was introduced into a 1.3-kb *Eco*RI/*Sal*I restriction fragment containing the first epidermal growth factor–like (EGF-like) domain, and the S356L, T390K, G404R, D434N, G520V, and G610R mutations were individually introduced into a 700-bp *Sal*I/*Xho*I restriction fragment containing coding sequence for part of the second EGF-like domain. FEVR-associated mutations T173M, R570Q, Y1168H, C1361G, and E1367K were made in a similar manner. Other expression vectors used in this study were mouse Wnt1-v5 (Ai et al. 2005), Wnt 10b (Bennett et al. 2005), Norrin and Frizzled 4 (Xu et al. 2004), MESD-C2 and RAP (Hsieh et al. 2003), Topflash (Korinek et al. 1997), and pRL-TK (Upstate Biotechnology).

Ex Vivo Reporter Assays for Wnt and Norrin Signal Transduction

HEK293T cells (American Type Culture Collection), cultured in Dulbecco's modified essential medium containing 10% fetal bovine serum, were plated at 2.5×10^5 cells per well in 24-well plates 24 h prior to transfection. Transfection was done in serum-free media with Lipofectamine Plus (Invitrogen), in accordance with the manufacturer's protocol. For Wnt-signaling assays, DNA transfections included the following expression constructs: Topflash (100 ng), pRL-TK (5 ng), MESD-C2 (20 ng), WT-LRP5 or LRP5 constructs containing missense mutations (30 ng), and Wnt1-v5 or Wnt10b (100 ng). For Norrin-signaling assays, DNA transfections included the following expression constructs: Topflash (100 ng), pRL-TK (5 ng), MESD-C2 (20 ng), WT-LRP5 or LRP5 constructs containing missense mutations (30 ng), Fzd4 (50 ng), and Norrin (50 ng). To assure that equal amounts of DNA were transfected in each experiment, pcDNA3.1-LacZ (Invitrogen) was added to make 255 ng the total amount of DNA per transfection. Cells were lysed 30 h after transfection. Firefly luciferase activity from the Topflash reporter was measured using a dual Luciferase assay kit (Promega) in a luminometer (Molecular Devices). Renilla luciferase activity from pRL-TK was measured, as an internal control for transfection efficiency. Each assay was performed in triplicate. Data from single experiments are reported in the "Results" section, but each experiment was performed three times with consistently reproducible results.

Assay to Assess Trafficking of WT and OPPG LRP5 Constructs That Lack the Transmembrane and Cytoplasmic Domains

WT-LRP5N-myc or LRP5N-myc constructs that contained OPPG missense mutations were used. These constructs lack the transmembrane and cytoplasmic domain and should be secreted into the conditioned medium of expressing cells if properly trafficked. HEK293T cells were cultured as described above. Cells were plated at

5×10^5 cells per well in six-well culture plates 24 h prior to transfection. Each well was transfected with LRP5N-myc (200 ng), MESD-C2 (200 ng), and RAP (200 ng), with use of Lipofectamine Plus, and was maintained in 1 ml of serum-free medium. LRP5N-myc protein was recovered from the medium after 48 h and from the cell lysate, by scraping the cell layer after the addition of 1 ml RIPA buffer (50 mM Tris; pH 8; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% sodium dodecyl sulfate). Twenty microliters of conditioned medium or cell lysate were mixed with 5 μl of 5 × SDS-PAGE–loading buffer, were subjected to reducing SDS-PAGE, and were detected by the monoclonal anti-myc antibody 9E10 (Santa Cruz Biotech). To demonstrate that the secreted forms of LRP5 are posttranslationally modified, we mixed 17.5 μl of LRP5N-myc that contained conditioned medium with 1 μl Tris (1 M; pH 8.0), 1 μl β-mercaptoethanol, and 0.5 μl SDS (10%) and heated at 99°C for 5 min. We then added 25 μl water, 5 μl NP-40 (6%), and 5 μl N-glycosidase F (1 unit/μl) (Roche) and incubated overnight at room temperature. A control reaction was performed by adding 5 μl water instead of 5 μl N-glycosidase. The control and N-glycosidase digested samples were mixed with 25 μl 4 × SDS-PAGE loading buffer, and 25 μl were subjected to reducing SDS-PAGE and were detected using the anti-myc antibody.

Results

LRP5 *Mutations in Patients Suspected to Have OPPG*

Probands from 37 families were suspected of having OPPG (table 1). PCR amplification and direct sequencing of amplimers for all *LRP5* coding exons led to the identification of 11 nonsense, 11 frameshift, 2 splice-site, and 20 missense mutations, all of which we assumed were disease causing (table 1 and fig. 1*A*). Several other missense mutations were identified that have an appreciable frequency in unaffected controls and were not considered to be disease causing (data not shown). Of the 37 probands, 12 were homozygous for the disease-causing mutations, and 14 of the 37 were compound heterozygous for two disease-causing mutations. A single heterozygous mutation was detected in 4 of the 37 probands; these mutations were unlikely to cause OPPG via a dominant mechanism, since they were also found in an asymptomatic carrier parent or sibling. Of the 37 probands, 7 had no identified mutant *LRP5* alleles.

LRP5 has a closely related paralog, LRP6, which also serves as a Wnt receptor. Mice completely lacking *Lrp6* are nonviable (Pinson et al. 2000); mice doubly heterozygous for mutations in *Lrp5* and *Lrp6* have low bone mass (Holmen et al. 2004). Therefore, we sequenced *LRP6* in the four probands with OPPG for whom we could detect only a single mutant *LRP5* allele, to look for digenic inheritance; we found no *LRP6* mutations. We did not sequence *LRP6* in the seven probands with no detected *LRP5* mutation, given the embryonic lethality in the *Lrp6* homozygous mutant mice and the lack of reported eye or severe skeletal phenotypes in the *Lrp6* heterozygous mice. Frizzled 4 and LRP5 act as coreceptors for Norrin, and mutations in either *LRP5* or *FZD4* have been found to cause FEVR (Toomes et al. 2004; Xu et al. 2004; Qin et al. 2005). Therefore, we also sequenced *FZD4* and *NDP* in the four probands with single *LRP5* mutations and in the seven probands who had no identified *LRP5* mutations. No *FZD4* or *NDP* mutations were found.

Clinical Characteristics in Probands With and Without LRP5 *Mutations*

Congenital, childhood-onset, or childhood-recognized ocular disease was reported for all 30 probands with identified *LRP5* mutations. The severity of ocular disease in patients for whom original ophthalmologic records were available ranged from phthisis bulbi to less-severe vitreoretinal findings, such as persistent hyperplasia of the primary vitreous (PHPV), congenital retinal folds, and exudative retinopathy. The majority of probands with mutations were congenitally blind in both eyes; some were congenitally blind in one eye and visually impaired in the other or were moderately visually impaired in both eyes. Most adult probands with *LRP5* mutations—and their affected adult siblings—were blind by age 15 years, and all were blind by age 25 years. Four of the seven probands without *LRP5* mutations had ocular defects that had not been previously described in OPPG, including isolated cataract, retinal coloboma, Peters anomaly, and unilateral eye involvement. The male:female ratio is 3:2 in probands with *LRP5* mutations and 6:1 in probands without identified mutations. It remains possible that mutation in an X-linked gene, such as *NDP,* may be responsible for some instances of disease in these probands. However, we did not find an *NDP* coding-sequence mutation in any of the patients, and osteoporosis and skeletal fragility have not been described in males with Norrie disease.

Skeletal disease was apparent by adolescence in 29 of 30 probands with *LRP5* mutations and in nearly all affected siblings. One proband (family OP346) has an affected sibling in whom skeletal disease was recognized in his 20s (table 1). Another proband and his sibling (family OP502) were both found to be osteoporotic in their 20s (table 1). Among all patients with *LRP5* mutations, the skeletal disease was characterized by fractures and/or radiologically determined severe osteoporosis. Importantly, skeletal disease was recognized during the first 2 years of life in fewer than half of the probands with identified mutations. In several asymptomatic infants and

toddlers, osteoporosis or vertebral compressions were found incidentally when radiographs were obtained for other reasons (e.g., suspected pneumonia or constipation). We observed no consistent difference in clinical and radiographic skeletal features between probands for whom *LRP5* mutations were and were not found.

Cognitive impairment, independent of visual impairment, was reported for 8 of 30 probands with identified mutations. There were no significant differences in the rates of cognitive impairment between probands with homozygous (4 of 12 probands), compound heterozygous (2 of 14 probands), or single identified (2 of 4 probands) *LRP5* mutations. Three of seven probands without identified mutations were also reported to be cognitively impaired.

Functional Analysis of LRP5 Receptors with Missense Mutations

We hypothesized that most nonsense and frameshift mutations would result in nonsense-mediated mRNA decay or would produce truncated proteins that would be improperly trafficked within the cell. The consequences of missense mutations, which accounted for 20 of the 44 putative disease-causing mutations, were less clear. Alignment of the amino acid residues affected by the missense mutations against *LRP5* sequence from mouse, rat, and *Xenopus laevis, LRP6* sequence from human and mouse, and *arrow* sequence from *Drosophila melanogaster* demonstrated complete conservation of these residues within *LRP5* orthologs and near-complete conservation within *LRP6* across vertebrates and *arrow* in fruit fly (fig. 1*B*).

Since conservation can suggest but neither prove causality of a missense change nor predict the precise mechanism by which a missense change will exert an effect, we studied several of the mutant proteins in transiently transfected 293T cells, to determine their ability to traffic through the cell and to transduce Wnt and Norrin signal. We expressed a myc-epitope–tagged version of LRP5 that lacks the transmembrane and cytoplasmic domains and should be secreted into the conditioned medium when normally trafficked. The version of this construct that contained the WT sequence was able to traffic through the cell, be posttranslationally modified by N-linked glycosylation, and be secreted (fig. 2). We then studied seven different mutant proteins whose missense mutations affect the first or second six-bladed propeller domains within the protein. One mutant, T390K, was expressed within the cell but was unable to traffic normally. Several other mutants—T244M, G404R, D434N, and G610R—appeared to traffic less well than did the WT protein. Two mutants, S356L and G520V, appeared to traffic comparably to the WT protein (fig. 2*A*). All secreted OPPG mutant proteins appeared to be posttranslationally modified, similar to WT LRP5 (fig. 2*B* and data not shown).

To assess the effect of these mutations on signal transduction, we coexpressed full-length, untagged WT LRP5 (or full-length untagged mutant LRP5) with Wnt ligand (or Norrin ligand) and measured β-catenin–mediated signaling, using the Topflash reporter assay (white bars in fig. 3*A* and 3*B*). WT-LRP5 was able to transduce Wnt signal, as indicated by the large fold increase in Topflash reporter activity. However, the mutant LRP5 receptors T244M, S356L, T390K, and G520V were unable to transduce Wnt1 or Wnt10b signal. The mutants G404R and D434N had <50% the activity, and the mutant G610R had 60% the activity of WT-LRP5. The phenotype of the probands with these latter three mutations was not clinically milder than that of probands with other *LRP5* mutations; each was blind and had severe skeletal disease.

Carriers of OPPG mutations often have significantly reduced BMD. Furthermore, some *LRP5* mutations that cause FEVR appear to act in a dominant manner. Therefore, we coexpressed WT and mutant LRP5, to determine whether the heterozygote phenotype of *LRP5* mutations was likely to be due to functional haploinsufficiency or a dominant negative effect. When coexpressed with WT LRP5, none of the mutant proteins interfered with WT Wnt signal transduction (*gray bars* in fig. 3*A* and 3*B*). This would argue against a dominant negative effect for these mutations on Wnt signaling.

We also assessed the effects of the *LRP5* mutations on Norrin signaling. Norrin also activates the β-catenin–mediated signaling pathway, for which the Topflash reporter assay is a useful readout (Xu et al. 2004). Each of the LRP5 mutants, including those that appear to traffic normally through the cell, had a significantly reduced ability to transduce Norrin signal (fig. 3*C*). This result is consistent with the hypothesis that visual loss in patients with OPPG results from defective Norrin signaling, although this result does not preclude defective Wnt signaling from also affecting eye development.

Three *LRP5* missense mutations have been identified in patients with autosomal recessive FEVR who were not reported to have skeletal involvement. We tested two of these mutants, along with three missense mutants that have been associated with dominantly inherited FEVR, for their ability to transduce Wnt and Norrin signal (fig. 4). One dominant mutant, Y1168H, was unable to transduce Wnt or Norrin signal. One recessive mutant, R570Q, had significantly reduced Wnt and Norrin signal transduction, and one dominant mutant, C1361G, had mildly reduced Wnt and Norrin signal transduction. However, the remaining dominant and recessive mutants behaved like WT LRP5 in these assays.

Discussion

We sequenced *LRP5* in 37 probands who had been referred with a suspected diagnosis of OPPG. We found

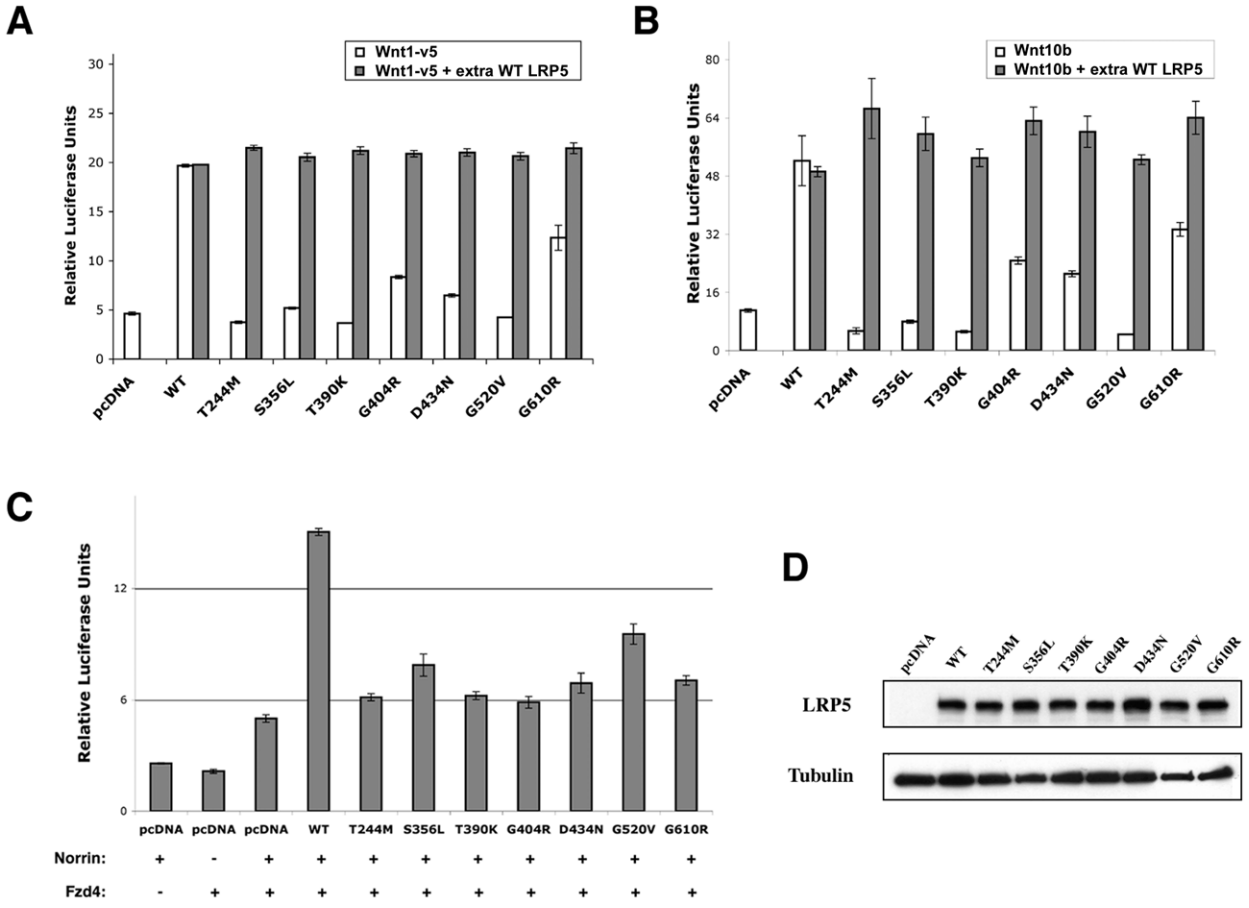


Figure 3 OPPG-causing missense mutations impair Wnt and Norrin signaling. Fold-induction of luciferase activity in 293T cells expressing WT or OPPG-causing missense mutants are expressed in combination with Wnt1-v5 (*A*), Wnt10b (*B*), and Norrin and Fzd4 (*C*). Thirty hours after transfection, firefly luciferase activity was measured and normalized to Renilla luciferase activity. Note that four mutants have no signal transduction and that three (G404R, D434N, and G610R) have markedly reduced signal transduction (*white bars*); WT-LRP5 signal transduction was not inhibited when coexpressed with an OPPG mutant (*gray bars*), which indicates that the OPPG mutants do not exert a dominant negative effect (*A* and *B*). *C,* OPPG mutants have reduced ability to transduce Norrin signal. *D,* Western blot of cell lysates from transfected 293T cells used in the Wnt1-v5 signal transduction assay shown in panel A, demonstrating comparable expression of WT and OPPG-causing LRP5 receptors. Lysates were separated by SDS-PAGE and were immunodetected with an anti-LRP5/LRP6 antibody, 3801–100 (Biovision). An anti-tubulin antibody was used to demonstrate that comparable amounts of cell lysates were loaded.

44 likely disease-causing mutations of 64 anticipated mutant alleles (we anticipated fewer mutant alleles because several families were consanguineous), for a mutation-detection rate of ~70%. For those four probands in whom only one heterozygous change was identified, we assume that an undetected second mutation exists in the other allele. Mutations affecting *LRP5* that would have been missed in our mutation-detection strategy include exon deletions, intron mutations that affect splicing, coding mutations that could not be PCR amplified because of primer annealing-site polymorphisms, and mutations that affect regulatory regions. Another possible explanation for the presence of only a single mutant allele is digenic inheritance, which has been observed in other human diseases (Kajiwara et al. 1994; Katsanis et al. 2001; Gabriel et al. 2002), including FEVR (Qin et al. 2005). We sequenced three additional genes, *LRP6, FZD4,* and *NDP,* that have skeletal or ocular findings when disrupted in mice (Richter et al. 1998; Holmen et al. 2004; Xu et al. 2004), but we found no additional mutations. However, it remains possible that mutant alleles of genes encoding other Wnt signaling components, such as Wnt ligands and other Frizzled receptors, could cause OPPG in combination with a heterozygous mutation in *LRP5*.

In addition to finding likely loss-of-function alleles, such as those with nonsense and frameshift mutations, we identified 20 missense mutations in *LRP5*. We assume that these are disease-causing because they alter amino acid residues that are highly conserved across species. However, this does not prove causality, since mutations affecting highly conserved amino acid resi-

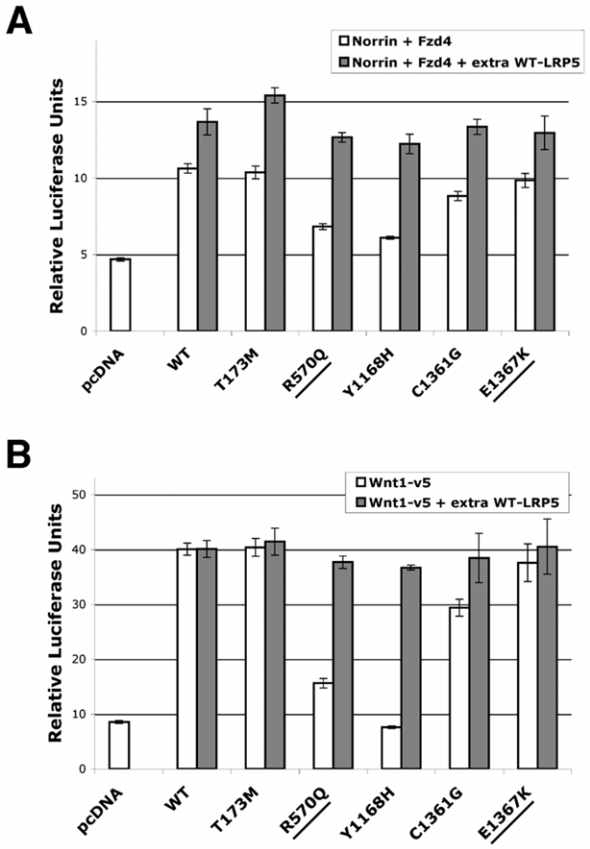


Figure 4 FEVR-causing missense mutations variably affect Wnt and Norrin signaling. Fold-induction of luciferase activity in 293T cells expressing WT or FEVR-causing missense mutants in combination with Norrin and Fzd4 (*A*) or Wnt1-v5 (*B*). Mutants associated with autosomal recessive FEVR are underlined. Note that the Y1168H mutant could not transduce Norrin or Wnt signal, and mutants T173M and E1367K could transduce signal from both ligands (*white bars*). Coexpression of WT and FEVR-causing mutant receptors did not interfere with the WT receptor's ability to transduce Norrin or Wnt-v5 signal (*gray bars*), which indicates that these mutations do not have dominant negative effects.

dues can occur without any functional consequence. For example, residues in the highly conserved catalytic domain of *Thermus aquaticus* DNA polymerase I could be mutated without disrupting enzymatic activity (Patel and Loeb 2000). Additionally, two missense mutations, each affecting a conserved residue, are present on the same allele in siblings with OPPG (family OP450) (table 1). Therefore, to determine whether missense mutations in *LRP5* cause a loss of function, we utilized a cell-based reporter assay to measure the ability of mutant receptors to transduce Wnt signal. Although we have not tested all 20 missense mutants, the first 7 mutants we did test all had impaired Wnt signal transduction. This result supports the hypothesis that the skeletal phenotype in OPPG is due to reduction in Wnt signaling.

The hypothesis that impairment of Wnt signal transduction is specific to OPPG-causing missense mutations is supported by studies of LRP5 mutants that cause the opposite skeletal phenotype, autosomal dominant HBM. When seven HBM-causing LRP5 mutants were tested in this assay, none exhibited reduced Wnt signal transduction (Ai et al. 2005). Therefore, this assay should be useful for determining whether a missense mutation identified in a person with OPPG or idiopathic osteoporosis is disease causing.

The availability of an allelic series of mutations in *LRP5* that affect Wnt signal transduction enabled us to ask whether all OPPG mutations affect receptor function in the same way. By expressing mutant receptors in transiently transfected cells, we observed that some mutations impaired receptor trafficking, whereas others did not. These latter mutations may impair signal transduction at the level of ligand binding, coreceptor interaction, or recruitment of cytoplasmic factors. Importantly, we found no evidence of a dominant negative effect in any of the mutants tested thus far. Since low bone mass has been observed in obligate carriers of nonsense, frameshift, and missense mutations (authors' unpublished data), functional haploinsufficiency appears to be the common mechanism of mutational effect associated with isolated osteoporosis.

Within the developing eye, LRP5 may transduce Norrin signal rather than Wnt signal. Mutations in *NDP* cause blindness associated with PHPV (Sims 2004), which has also been observed in patients with OPPG (Steichen-Gersdorf et al. 1997). A second eye disease, FEVR, is locus heterogeneous, with mutations identified in *NDP, FZD4,* and *LRP5* (Toomes et al. 2004). In mice and in ex vivo studies these three proteins interact in a signal transduction pathway (Xu et al. 2004). This led us to determine whether OPPG-causing mutations affect Norrin-signal transduction and whether FEVR-associated mutations affect Wnt-signal transduction. All seven OPPG-causing missense mutants impaired Norrin signaling (fig. 3*C*). Surprisingly, there was great variability in the ability of the FEVR-causing mutant receptors to transduce Wnt and Norrin signal (fig. 4). For example, one mutant, Y1168H, which is associated with autosomal dominant FEVR, was unable to transduce Wnt and Norrin signal, whereas the mutant R570Q, which is associated with autosomal recessive FEVR, had residual Wnt- and Norrin-signal transduction. Another homozygous mutation affecting this residue (R570W) is present in siblings with OPPG (family OP346) (table 1). We suspect that, when evaluated, the patients with homozygous R570Q FEVR will have significantly reduced BMD. Several other FEVR mutations had no effect on Wnt or Norrin signaling. We do not know whether these results imply that these latter mutations are non–disease causing or that the cell-based

assay is insensitive. The T173M mutation may be an example of the former possibility, since it was found in an elderly patient with retinal folds who had no family history of FEVR (Toomes et al. 2004); additionally, the mutated residue is not evolutionarily conserved. In support of the latter possibility are several FEVR mutations that alter highly conserved residues and the observation that disease-causing mutations in Norrin have had variable effects on signal transduction in a similar assay (Xu et al. 2004).

Our results do not help elucidate the mechanism by which heterozygous mutations in *LRP5* cause autosomal dominant FEVR in some families but little eye disease among the carrier parents and siblings of patients with OPPG. Because obligate OPPG carriers have not complained of visual impairment, few eye exams have been performed (10 parents underwent formal ophthalmologic assessment in our study, and only 2 have subtle evidence of retinal disease [families OP642 and OP482] [table 1]). This contrasts with FEVR, which had been considered a highly penetrant disorder (Toomes et al. 2004). Penetrance could be higher in FEVR-affected families with *FZD4* mutations than in families with *LRP5* mutations or could seem to be higher because family members have undergone studies, such as fluorescein angiography, to detect subtle signs of disease. Similarly, we do not know which patients with FEVR may be at increased risk for osteoporosis as are OPPG carriers, although a recent study reported that five of seven patients with FEVR due to *LRP5* mutation had BMD >1 SD below the mean, whereas zero of five patients with FEVR due to *FZD4* mutation had comparably reduced BMD (Qin et al. 2005).

The results of this study have several practical implications for patients and families suspected of being affected by OPPG or of having deleterious *LRP5* mutations. First, infancy-onset visual loss that is not associated with vitreoretinal disease seems unlikely to be due to *LRP5,* since mutations were not found in the four probands who lacked vitreoretinal pathology. Second, radiographs and quantitative BMD measurements should be performed in infants and children who have eye features of OPPG, since skeletal features such as vertebral compressions may be present but may not be clinically apparent in young children; early diagnosis of skeletal disease is important so that affected individuals can take advantage of emerging therapies for improving bone strength (Zacharin and Cundy 2000). Third, cognitive problems that are independent of visual impairment occur in only a minority of individuals with OPPG and do not correlate with the type of *LRP5* mutation. Fourth, most patients with typical features of OPPG have detectable *LRP5* mutations; however, we cannot exclude locus heterogeneity or digenic inheritance as accounting for a minority of cases of OPPG. Last, the ability to functionally test a mutation's effect on Wnt- and possibly Norrin-signal transduction may help determine whether a variant identified in patient with OPPG, idiopathic osteoporosis, or FEVR is disease causing.

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Web Resources

The URLs for data presented herein are as follows:

Warman lab at Case Western Reserve University, http://genetics.case.edu/warmanlab/ (for the Department of Genetics Web site)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for OPPG)

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