WNT1 Mutations in Early-Onset Osteoporosis and Osteogenesis Imperfecta

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SUMMARY

This report identifies human skeletal diseases associated with mutations in *WNT1*. In 10 family members with dominantly inherited, early-onset osteoporosis, we identified a heterozygous missense mutation in *WNT1*, c.652T→G (p.Cys218Gly). In a separate family with 2 siblings affected by recessive osteogenesis imperfecta, we identified a homozygous nonsense mutation, c.884C→A, p.Ser295*. In vitro, aberrant forms of the WNT1 protein showed impaired capacity to induce canonical WNT signaling, their target genes, and mineralization. In mice, *Wnt1* was clearly expressed in bone marrow, especially in B-cell lineage and hematopoietic progenitors; lineage tracing identified the expression of the gene in a subset of osteocytes, suggesting the presence of altered cross-talk in WNT signaling between the hematopoietic and osteoblastic lineage cells in these diseases.

O STEOPOROSIS IS A COMMON SKELETAL DISORDER CHARACTERIZED BY low bone mineral density (BMD), impaired bone quality, and fragility fractures.[1] Although multiple genetic loci, including those for WNT ligands, have been defined on the basis of genomewide association studies in patients with osteoporosis, the known loci are generally associated with odds ratios for fracture that are below 1.1.[2] Recently, novel metabolic pathways in bone cells have been discovered in patients with osteogenesis imperfecta, a mendelian disease characterized by brittle bones.[3] The role of the WNT pathway in bone formation and maintenance has been extensively studied since the identification of mutations in key signaling WNT mediators (low-density lipoprotein receptor–related protein 5 [LRP5] and sclerostin) in diseases with high or low bone-mass phenotypes.[4,5] Despite numerous studies in cell and mouse models, however, the key WNT ligand that signals through LRP5/6 in the formation of human bone has not been identified.[6]

CASE REPORTS

FAMILY 1

We evaluated 16 members of a Finnish family with severe early-onset and dominantly inherited osteoporosis (Fig. 1A). Clinical and radiologic evaluation confirmed a diagnosis of osteoporosis with low BMD and low-impact vertebral and peripheral fractures in 10 family members (Table 1 and Fig. 1B). Affected persons had no extraskeletal abnormalities. Serum and urine markers of calcium homeostasis and bone turnover were normal (see Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Histomorphometric analysis of biopsy specimens of transiliac bone in 2 adults showed severe osteoporosis, with low rates of bone turnover and bone formation; a 14-year-old boy in the family had normal bone mass but a low rate of bone formation and remodeling for his age (Fig. S1 and Table S2 in the Supplementary Appendix).

FAMILY 2

We also evaluated a Lao Hmong family with two severely affected sisters who had what was presumed to be a recessive form of osteogenesis imperfecta (Fig. 1C). In the older of the two affected children, the first fracture was documented at 1 month of age. Radiographs in both children showed severe osteopenia, with multiple fractures and sequelae over time, including vertebral compression fractures, kyphoscoliosis, severe short stature, and deformities of the long bones (Fig. 1D). The older sister, now 26 years of age, is wheelchair-bound because of her bone disease but is able to perform most activities of daily living and is intellectually normal. Her head circumference is at the 25th percentile, as is her weight, but her height is more than 2 SD below the mean (she is less than 100 cm [3 ft] tall, and she has severe long-bone deformities) (Fig. S2 in the Supplementary Appendix).

In the younger of the two affected siblings, prenatal ultrasonography performed during the third trimester revealed multiple femoral and rib fractures. She has severe intellectual disability, with absence of speech, and has been quadriplegic since toddlerhood. Magnetic resonance imaging performed at 20 months of age revealed severe hypoplasia of the left cerebellar hemisphere with a short midbrain (Fig. 1E). This sister is now 23 years old, has no functional use of her hands, does not have language, and has met none of the milestones associated with the development of motor skills. She has ptosis and exotropia of the left eye. The circumference of her head is just below the 3rd percentile (51.5 cm) and her weight is at the 25th percentile. Her height is more than 2 SD below the mean (<100 cm), and she has severe long-bone deformities (Fig. S2 in the Supplementary Appendix).

Both sisters had severe dental caries as young children that necessitated the extraction of all deciduous teeth. Permanent dentition is relatively normal in both women, with few caries and no signs of dentinogenesis imperfecta. Their hearing is also normal. Their sclerae are white. Both sisters have asynchronous eye blinking. Both also have mild restrictive airway disease. Their hands are markedly hyperextensible, with marked laxity at the interphalangeal joints. Fibroblast collagen studies were normal in both sisters (data not shown).
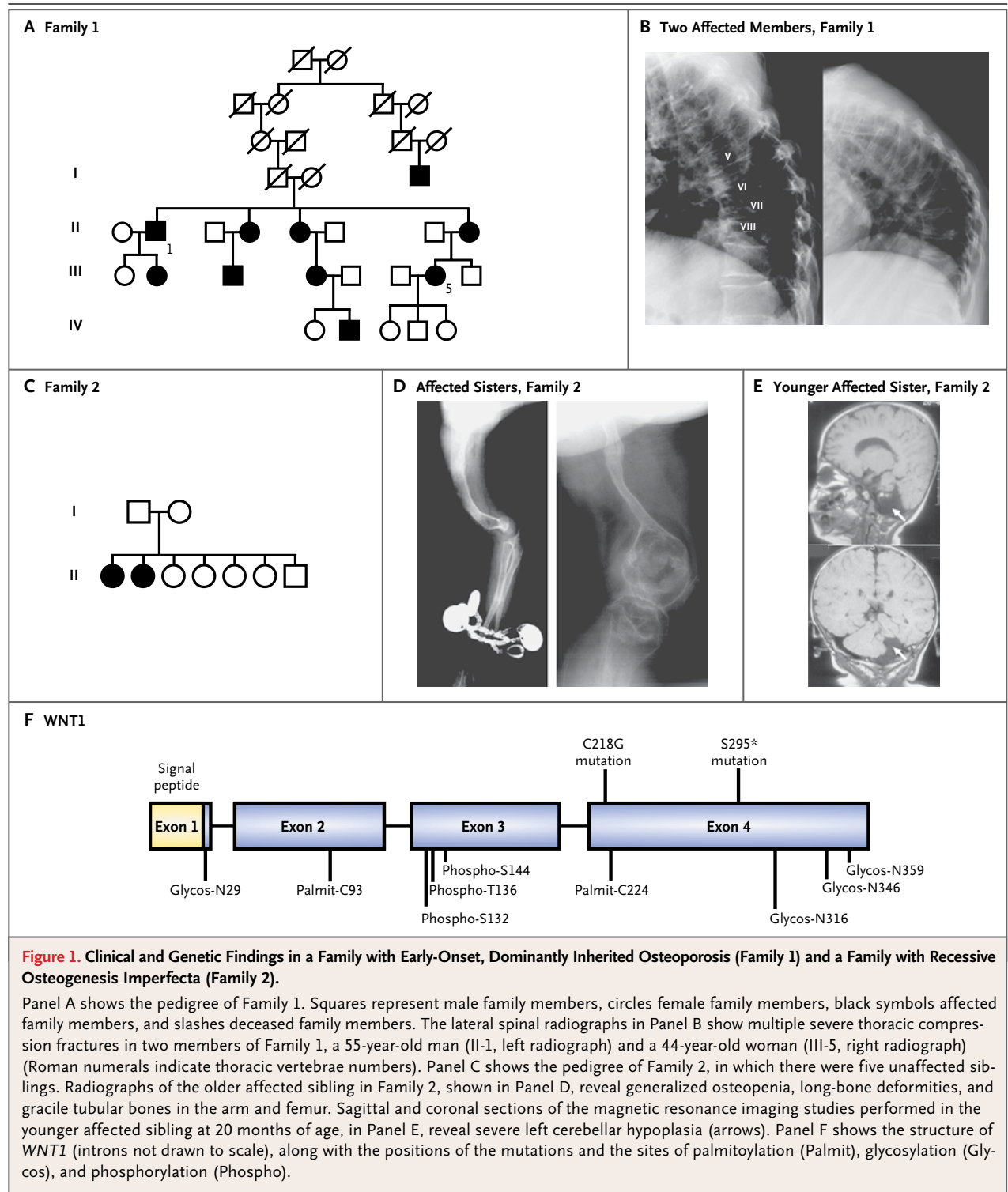
The other siblings in this family had no features of osteogenesis imperfecta or any neurologic disease. The mother, who is 44 years of age, was found to have normal BMD on dual-energy x-ray absorptiometry (DXA) and had normal spinal radiographs. The father, who is 43 years of age, had normal femoral BMD but had a z score of –1.8 for BMD of the lumbar spine (vertebral bodies L1 through L4). His height is normal, at 160 cm (5 ft 5 in.). The father's spinal radiographs showed a mild compression deformity involving the superior end plate of the L5 vertebral body.

METHODS

CLINICAL AND GENETIC ANALYSES

The families provided written informed consent to participate in studies approved by the ethics committee of the Helsinki University Central Hospital (Family 1) and by the institutional review board of the Baylor College of Medicine (Family 2). The fourth, penultimate, and last authors take responsibility for the integrity of the data and the analyses.

The members of Family 1 completed questionnaires and underwent physical examinations and DXA studies. Biopsies of transiliac bone were performed after tetracycline double labeling in three family members. Genomewide scanning was conducted with the use of 384 microsatellite markers, followed by fine mapping for chromo-

Figure 1. Clinical and Genetic Findings in a Family with Early-Onset, Dominantly Inherited Osteoporosis (Family 1) and a Family with Recessive Osteogenesis Imperfecta (Family 2).

Panel A shows the pedigree of Family 1. Squares represent male family members, circles female family members, black symbols affected family members, and slashes deceased family members. The lateral spinal radiographs in Panel B show multiple severe thoracic compression fractures in two members of Family 1, a 55-year-old man (II-1, left radiograph) and a 44-year-old woman (III-5, right radiograph) (Roman numerals indicate thoracic vertebrae numbers). Panel C shows the pedigree of Family 2, in which there were five unaffected siblings. Radiographs of the older affected sibling in Family 2, shown in Panel D, reveal generalized osteopenia, long-bone deformities, and gracile tubular bones in the arm and femur. Sagittal and coronal sections of the magnetic resonance imaging studies performed in the younger affected sibling at 20 months of age, in Panel E, reveal severe left cerebellar hypoplasia (arrows). Panel F shows the structure of *WNT1* (introns not drawn to scale), along with the positions of the mutations and the sites of palmitoylation (Palmit), glycosylation (Glycos), and phosphorylation (Phospho).

some 12 with the use of 29 additional markers, and finally, a targeted next-generation–sequencing strategy (described in the Supplementary Appendix) to sequence the exons and flanking intron bases in the linkage region.

For Family 2, apart from performing the standard clinical care for patients with osteogenesis imperfecta and, in the case of the second affected sister, neurologic disease, we obtained DXA scans and spinal radiographs for the par-

Table 1. Clinical and Bone Densitometry Findings in the 10 Affected and 6 Unaffected Members of the Family with Early-Onset, Dominantly Inherited Osteoporosis (Family 1).*

Family Member†	Sex	Age	BMD z Score			Multiple Vertebral Fractures	Low-Impact Peripheral Fractures	Height Loss
			Lumbar Spine	Hip	Whole Body			
		yr					*no.*	*cm*
Affected members								
I-1	Male	68	0.5	0.1	NA	Yes	1	NA
II-1	Male	55	−2.2	−1.3	−3.5	Yes	3	7
II-2	Female	63	−1.5	−1.6	NA	Yes	6	13
II-3	Female	68	−0.6	−0.6	−1.3	No	2	2
II-4	Female	65	−0.8	−1.6	NA	Yes	5	17
III-2	Female	26	−1.5	−2.2	−2.2	No	1	0
III-3‡	Male	44	−2.8	−1.3	−2.3	No	2	0
III-4‡	Female	36	−2.5	−2.2	−1.6	No	4	0
III-5	Female	44	−2.0	−0.8	−1.1	Yes	0	4
IV-2‡	Male	12	−1.7	−1.8	−1.8	Yes	0	NA
Unaffected members								
III-1	Female	28	−0.4	−0.2	−0.6	No	0	0
III-6	Male	34	−0.8	−1.0	NA	No	0	0
IV-1	Female	14	−0.4	−0.4	−0.5	No	0	NA
IV-3	Female	22	0.7	0.6	0.5	No	0	0
IV-4	Male	15	−0.2	−0.5	−0.5	No	0	NA
IV-5	Female	10	0.1	0.2	−0.4	No	0	NA

* Family members were considered to be affected if their bone mineral density (BMD) z score was below –2.0 (in the case of premenopausal women and all members younger than 50 years of age) or their T score was below –2.5 (in the case of postmenopausal women and all members 50 years of age or older). They were also considered to be affected if they had multiple vertebral compression fractures and a diagnosis of secondary osteoporosis could be ruled out. Height loss is provided only for family members who completed growth; data on height loss were not available for children and teenagers because they had not completed growth. A vertebral fracture was defined as a reduction in vertebral height of more than 20%.[7] NA denotes not available.

† Roman numerals indicate generation, and Arabic numbers the order of offspring within a generation. The number of individuals in the pedigree is shown in Figure S3 in the Supplementary Appendix.

‡ In this family member, osteoporosis was confirmed on bone histomorphometry, in addition to dual-energy x-ray absorptiometry.

ents. Given the family history of two affected siblings and the negative results on fibroblast collagen studies, whole-exome sequencing was performed, as previously described,[8] in an attempt to identify a new recessive gene for osteogenesis imperfecta. Variants were identified and analyzed with an in-house pipeline described in the Supplementary Appendix. The final variant-filtering scheme, which focused on rare recessive variants, is detailed in Table S3 in the Supplementary Appendix.

IN VITRO AND MOUSE EXPERIMENTS

A complementary DNA (cDNA) encoding WNT1 was cloned into mammalian expression plasmids, and the mutations were introduced with the use of standard techniques. The plasmids were transfected in HEK293T, MC3T3, and C57MG cells, and

the cells were tested for β-catenin activation, WNT1 protein expression, target-gene transcription, and differentiation. To profile *Wnt1* expression in vivo, we used quantitative real-time polymerase-chain-reaction (PCR) assays of tissue from wild-type mice and performed lineage tracing with the reporter mouse strain Rosa^mT/mG intercrossed with Wnt1-Cre transgenic mice, as previously described.[9,10] Details of these experiments are described in the Supplementary Appendix.

RESULTS

IDENTIFICATION OF *WNT1* MUTATIONS

In Family 1, a genomewide microsatellite linkage analysis with the use of DNA from 10 affected and 6 healthy family members revealed one putative linkage area of 25.5 Mb on chromosome

12 (P=0.01) (Fig. S3 and S4 in the Supplementary Appendix). Fine mapping and targeted next-generation sequencing allowed the identification of a single novel variant in *WNT1* (p.Cys218Gly) segregating with the phenotype. The mutation (c.652T→G) affects the first cysteine of the so-called WNT motif (C-[KR]-C-H-G-[LIVMT]-S-G-x-C), which is conserved across species and in 19 known human WNT family members.[11] The mutation substitutes the polar, sulfur-containing cysteine with the small, nonpolar amino acid glycine (Fig. S5A in the Supplementary Appendix).

In Family 2, we first excluded mutations in the known recessive genes for osteogenesis imperfecta with Sanger sequencing, and we then performed whole-exome sequencing. Analysis of the rare or novel genetic variations revealed two potential candidates. The first was a variant in *COL1A2* (NM_000089.3:c.3200G→A, p.Arg1067His). However, this variant was not considered to be causal, since the father, in whom no evidence of osteogenesis imperfecta was detected, had the mutation in his blood and fibroblasts and both affected children were also heterozygous for this mutation. In addition, this *COL1A2* variant would not be expected to cause the severe osteogenesis imperfecta phenotype in the two sisters in this family (one of whom also had severe neurologic abnormalities) because the amino acid change is within the X residue of the G-X-Y collagen triplet, and the results of collagen studies in fibroblasts were normal. The other variant found was a homozygous nonsense mutation in *WNT1* (NM_005430.3:c.884C→A, p.Ser295*) (Fig. S5B and S5C in the Supplementary Appendix). Both affected children were homozygous for the change, and both parents were heterozygous. The mutation resides in the last exon of *WNT1* and thus escapes nonsense-mediated decay, which allows for the expression of a WNT1 protein in which the last 76 amino acids are truncated (Fig. S5D and S6 in the Supplementary Appendix).

CANONICAL WNT SIGNALING AND BONE MINERALIZATION

To evaluate the functional consequence of the *WNT1* mutations, we used C57MG cells for the expression of mutant and wild-type WNT1; the cells do not express endogenous WNT1.[12] All proteins showed a similar cellular distribution, indicating that the mutant proteins were stable and the mutations did not alter the intracellular targeting of WNT1 (Fig. S7 in the Supplementary Appendix). We next assessed the induction of canonical WNT signaling. Activation of this pathway results in the accumulation of nonphosphorylated active β-catenin in the nucleus, where it induces target gene expression in cooperation with TCF/LEF transcription factors.[6] In contrast with wild-type WNT1, WNT1[C218G] and WNT1[S295*] did not induce marked accumulation of nonphosphorylated or total β-catenin in either cytosolic or nuclear fractions (Fig. 2A and 2B). We also found that in a superTOPFLASH-luciferase reporter assay,[13] both mutant proteins had significantly reduced capacity to activate canonical WNT signaling as compared with wild-type WNT1. On cotransfection, WNT1[C218G] did not interfere with the induction of superTOPFLASH reporter by wild-type WNT1, which suggests that WNT1[C218G] does not function in a dominant negative manner; however, the WNT1[S295*] protein seemed to have mild, context-dependent dominant negative activity (Fig. S8 in the Supplementary Appendix). To assess the effect of the mutations on endogenous targets of WNT signaling, we expressed the mutant proteins in MC3T3 osteoblastic cells. The expression of the downstream β-catenin targets (*Axin2* and *Lef1*) were significantly induced by wild-type WNT1 but not by the two mutants (Fig. S9 in the Supplementary Appendix). In addition, stable expression of the abnormal proteins stimulated less mineralization than did wild-type WNT1 (Fig. 2C). These assays reveal the markedly diminished capacity of the abnormal proteins to induce canonical WNT signaling and associated osteoblast differentiation.

WNT1 EXPRESSION PROFILING

To gain insight into how WNT1 modulates bone mass, we analyzed its expression pattern. We did not detect notable expression of *Wnt1* messenger RNA (mRNA) in mouse calvarial osteoblasts or osteoclasts, or of *WNT1* mRNA in human mesenchymal stromal cells (data not shown). In a panel of 19 murine tissue samples, we consistently detected *Wnt1* mRNA expression in the brain, femur, and spleen with the use of real-time PCR; the brain had the highest relative expression of *Wnt1* mRNA (Fig. S10 in the Supplementary Appendix). We also found clear *Wnt1* expression in the hematopoietic bone marrow (Fig. 2D). We used fluorescence-activated cell sorting to isolate major hematopoietic lineages (B-cell, monocyte and macrophage, and erythrocyte) present in bone marrow and real-time PCR assays to ana-
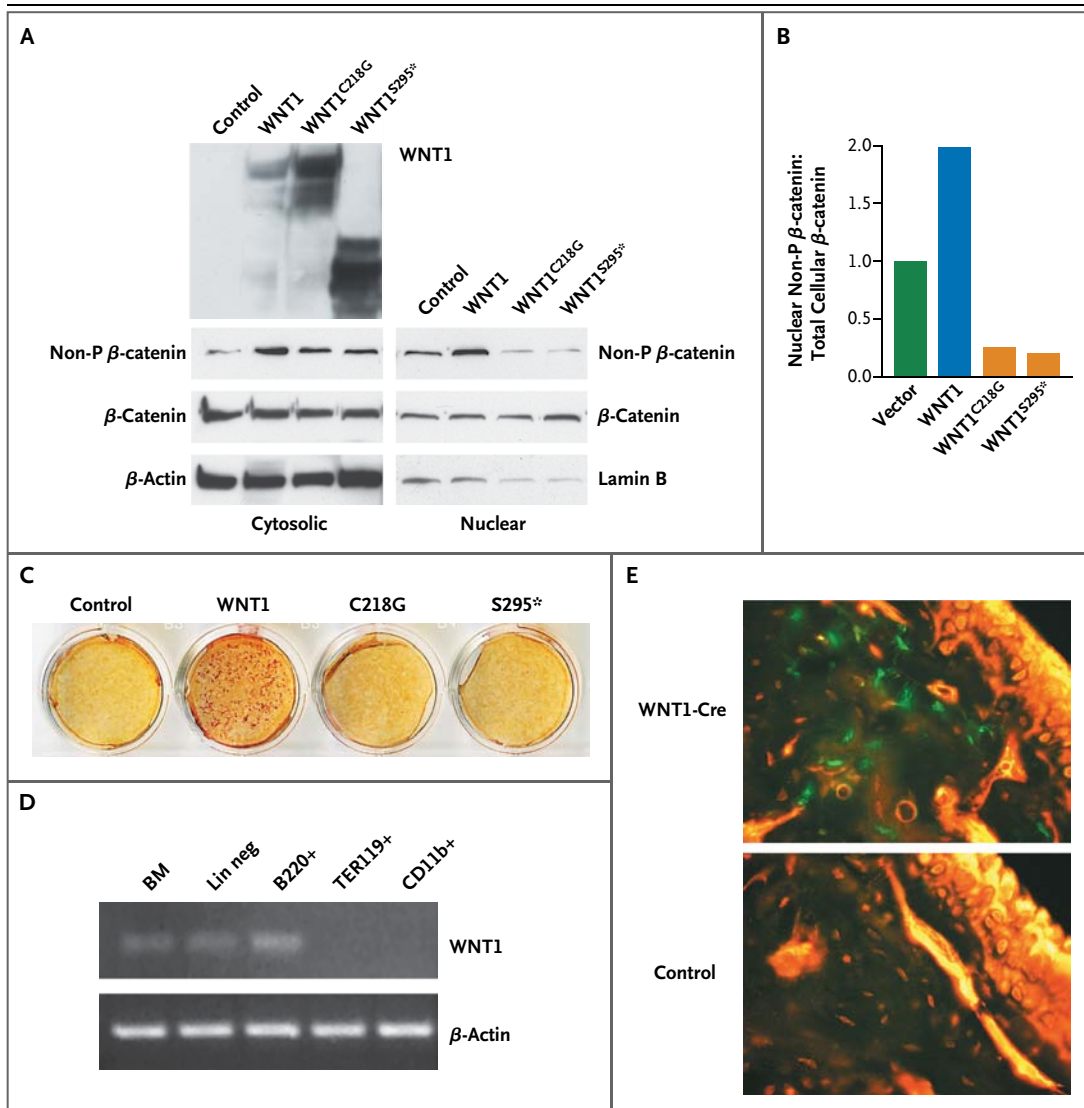
Figure 2. Functional Consequences of *WNT1* Mutations.

Panel A shows a representative Western blot image from one of the three independent experiments. The transient transfection of wild-type WNT1 to HEK293T cells led to the accumulation of active nonphosphorylated (non-P) β-catenin and total β-catenin in both cytosolic and nuclear protein fractions, which was not observed with the vector control, WNT1[C218G], or WNT1[S295*]. Because of glycosylation heterogeneity, WNT1 migrates as a doublet band. β-Actin and lamin B served as cytosolic and nuclear-protein loading controls, respectively. Panel B shows the ratio of nuclear non-P β-catenin to total cellular β-catenin from one of the representative experiments. Scanning densitometry of band intensities confirmed that WNT1[C218G] and WNT1[S295*] did not lead to the accumulation of active non-P β-catenin that was observed with wild-type WNT1. The experiment was performed three times with similar results. Panel C shows the results of a mineralization assay with MC3T3 cells that express WNT1, WNT1[C218G], and WNT1[S295*]. The MC3T3 cells expressing the mutant proteins showed reduced mineralization. The overexpression of the WNT1 proteins was confirmed with the use of a real-time polymerase-chain-reaction assay (PCR) and Western blot analysis (data not shown). Panel D shows the results of an analysis of *Wnt1* messenger RNA (mRNA) in mouse tissues by means of real-time PCR. *Wnt1* mRNA was detected in unsorted bone marrow (BM) and in lineage-negative (Lin neg) hematopoietic progenitor cells. The greatest *Wnt1* expression was seen in the B220-positive cells of the B-cell lineage. Panel E shows frozen sections of distal tibial subchondral bone from a Wnt1-Cre transgenic Rosa[mT/mG] mouse (top) and a control reporter mouse (bottom). The section from the Wnt1-Cre transgenic mouse has osteocytes that are positive for Cre deletion, as indicated by protein expression (fluorescent green). The osteocytes in the section from the control reporter mouse are negative for Cre deletion.

lyze *Wnt1* expression (Fig. 2D, and Fig. S11 in the Supplementary Appendix). *Wnt1* was expressed in B220-positive cells of the B-cell lineage and to a lesser extent in the lineage-negative cells that represent hematopoietic progenitor cells, but not in the myeloid and erythroid lineages. We performed additional lineage-tracing experiments using Wnt1-Cre transgenic mice intercrossed with Rosa[mT/mG] reporter mice, which express cell-type specific green fluorescent protein on Cre-mediated activation.[9,10] Fluorescence analysis showed strong expression in a subset of osteocytic cells in the subchondral bone and weaker expression in cortical bone (Fig. 2E, and Fig. S12 in the Supplementary Appendix).

DISCUSSION

Several lines of evidence indicate that canonical WNT signaling is essential for normal skeletal development and homeostasis.[6,14] WNT signaling induces osteoblast differentiation and bone formation in early osteoblast progenitors and regulates osteoblast-dependent osteoclastogenesis in mature osteoblasts and osteocytes.[15-18] Moreover, mice lacking the WNT receptor Fzd9 have a cell-autonomous defect in bone formation.[19]

The patients described here have a form of autosomal dominant osteoporosis caused by a missense mutation in *WNT1* and a severe form of osteogenesis imperfecta (which could be viewed as prenatal-onset osteoporosis) caused by a homozygous truncation mutation in *WNT1*. Both mutations interfere with WNT1 signaling and impair bone formation, and the truncating mutation appears to have mild, context-dependent dominant negative activity in vitro. The severe intellectual disability with cerebellar malformation in one of the affected sisters in Family 2 and the severe clinical osteogenesis imperfecta in both sisters suggest that homozygous mutations in *WNT1* may variably affect other signaling pathways that are critical for central nervous system development, as was found in the *Wnt1* knockout mouse phenotype.[20]

The communication of osteoblasts and stromal cells with hematopoietic cells in the hematopoietic stem-cell niche of the bone marrow is essential for normal hematopoiesis, and WNT signaling plays a role in this cross-talk.[21] Conversely, it has been suggested that hematopoietic cells, especially B cells, regulate bone formation.[22] When considered in the light of these observations, our data suggest that *WNT1* has a complex expression pattern that is probably both temporally and spatially dynamic. The net effect of the identified mutations appears to be complex, interfering with WNT signaling between different cellular compartments, including the hematopoietic and osteocytic lineages and possibly different WNT coreceptor transactions, in a context-dependent fashion. These data support a role of hematopoietic cells in regulating bone formation and implicate WNT1 as a key signaling molecule that mediates these effects. Our findings suggest that WNT1 is an important ligand in the regulation of bone mass in humans and thus may serve as a biomarker of skeletal health and a therapeutic target in osteogenesis imperfecta and osteoporosis.

APPENDIX

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