Mutation of the bone morphogenetic protein GDF3 causes ocular and skeletal anomalies

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Ocular mal-development results in heterogeneous and frequently visually disabling phenotypes that include coloboma and microphthalmia. Due to the contribution of bone morphogenetic proteins to such processes, the function of the paralogue Growth Differentiation Factor 3 was investigated. Multiple mis-sense variants were identified in patients with ocular and/or skeletal (Klippel–Feil) anomalies including one individual with heterozygous alterations in GDF3 and GDF6. These variants were characterized, individually and in combination, through integrated biochemical and zebrafish model organism analyses, demonstrating appreciable effects with western blot analyses, luciferase based reporter assays and antisense morpholino inhibition. Notably, inhibition of the zebrafish co-orthologue of GDF3 accurately recapitulates patient phenotypes. By demonstrating the pleiotropic effects of GDF3 mutation, these results extend the contribution of perturbed BMP signaling to human disease and potentially implicate multi-allelic inheritance of BMP variants in developmental disorders.

INTRODUCTION

Amongst key steps in ocular development, fusion of the edges of the optic cup’s embryonic fissure is essential to formation of the future spherical eye. Perturbation of this process results in a spectrum of malformations that include colobomata (ocular fissure closure defects), microphthalmia (reduced ocular size) and in extreme cases anophthalmia (absent eyes); disorders that account for substantial proportions of paediatric blindness (1). These conditions, hereafter abbreviated microphthalmia, anophthalmia and colobomata (MAC), frequently exhibit non-Mendelian patterns of inheritance. These disorders have broad relevance to human genetics since they are commonly associated with systemic anomalies (2) and share genetic aetiologies with conditions ranging from vertebral fusions (3–5) to cleft palate (6). This enables extreme ocular phenotypes to be used as a starting point for elucidating the molecular basis of systemic disease. In this manner, we and other laboratories have implicated Growth Differentiation Factor 6 (GDF6), in ocular and skeletal anomalies (2,3,7) with GDF6 mutations identified in ~2% of patients with MAC phenotypes (3).

GDF6 is a member of the Transforming growth factor β (TGF-β) family of secreted signaling ligands, whose diverse functions include regulation of embryonic development and organogenesis. The more than thirty TGF-β ligands can be classified according to their signaling mechanisms with the TGF-β/activin/nodal subfamily activating Smads 2 and 3, although the Bone Morphogenetic Proteins (BMPs) and Growth Differentiation Factors (GDFs) activate Smads 1, 5

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and 8. The signal transduction pathway is notable for convergence of a large number of ligands onto a restricted number of type I and type II serine/threonine receptors, and is attributable to disproportionate evolutionary expansion of ligand numbers. This permits partitioning of function between paralogues (sub-functionalization), which potentially may buffer the effects of individual mutations. Anticipated clinical manifestations of such buffering would include incomplete penetrance, a feature of BMP-attributable MAC phenotypes (3,8,9), and notably comparable effects have also been observed with animal models of reduced Gdf6 function (3,10). In this context, determining the contribution of other TGF-β ligands to MAC disorders would be expected to provide novel insight into their broader functions.

Of the extensive TGF-β family, GDF3 is one of the most incompletely characterized ligands. Originally identified by homology to Xenopus Vgl (11), GDF3 is differentiated by an atypical cysteine knot configuration, where the fourth cysteine is missing. Two groups have studied GDF3 function during embryogenesis, reaching conflicting results regarding its specificity for activating signaling pathways. Levine and Brivanlou (12) demonstrated that mammalian GDF3, when overexpressed in Xenopus embryos, functions as a novel BMP antagonist, blocking Smad1/5/8 phosphorylation. In contrast, Chen et al. (13) used both cell culture and Xenopus systems to show that GDF3 requires the CRIPTO co-receptor and activates the Nodal pathway. Andersson et al. (14) have argued that GDF3 is poorly processed and its signaling activity is only detectable when combined with other Tgfβ ligands, such as Nodal. Certainly, the partial penetrance of phenotypes in Gdf3 null mutants (13) accords with the presence of other ligands with comparable function(s) (14). At present, although the role of GDF3 (located on 12p13.1) in disease is largely incompletely characterized ligands. Originally identified by homology to Xenopus Vgl (11), GDF3 is differentiated by an atypical cysteine knot configuration, where the fourth cysteine is missing. Two groups have studied GDF3 function during embryogenesis, reaching conflicting results regarding its specificity for activating signaling pathways. Levine and Brivanlou (12) demonstrated that mammalian GDF3, when overexpressed in Xenopus embryos, functions as a novel BMP antagonist, blocking Smad1/5/8 phosphorylation. In contrast, Chen et al. (13) used both cell culture and Xenopus systems to show that GDF3 requires the CRIPTO co-receptor and activates the Nodal pathway. Andersson et al. (14) have argued that GDF3 is poorly processed and its signaling activity is only detectable when combined with other Tgfβ ligands, such as Nodal. Certainly, the partial penetrance of phenotypes in Gdf3 null mutants (13) accords with the presence of other ligands with comparable function(s) (14). At present, although the role of GDF3 (located on 12p13.1) in disease is largely unknown, the association of large chromosome 12p duplications and deletions with ocular defects, provides some circumstantial evidence that altered dosage of GDF3 might result in ocular phenotypes.

In this report, we have investigated GDF3’s contribution to ocular and skeletal development, identifying a series of missense mutations. The effects of these variants, studied with western blot analysis, gene reporter assays and molecular models of the mature TGF-β domain, provide insight into how the mutations may affect GDF3 protein function. In order to extend these analyses to model organisms, and characterize the consequences of a double heterozygous BMP mutation identified in one patient, morpholino inhibition of the zebrafish orthologues was undertaken. The combined results of these experiments demonstrate an important and evolutionarily conserved role for GDF3 in oculo-skeletal development and provide insights into novel multi-allelic inheritance of BMP variants in human developmental disease.

## RESULTS

### Identification of patient GDF3 sequence variants

Thirty three DNA samples from patients with MAC phenotypes were initially screened for mutations by sequencing GDF3’s two exons and splice sites (Table 1), identifying a c.796C→T nucleotide change that is predicted to result in a R266C alteration. Alteration of cysteine residue number has profound effects in other molecular pathways (15–17), and the phenotypes of the proband (Table 2) accorded with those associated with BMP mutation (3,7,18,19). Assessment of the proband’s relatives revealed co-segregation of R266C with ocular and or skeletal phenotypes in three generations (Fig. 1A), and due to the pedigree’s size, the probability that this inheritance pattern occurred by chance is low (P = 0.015). The R266C variant was associated with a spectrum of ocular and or skeletal phenotypes, including: lumbar and thoracic scoliosis (I-1); Kippel–Feil type cervical vertebral fusions (II-2 and II-3); unilateral iris and retino-choriald coloboma, rudimentary 12th ribs and mild scoliosis in the proband (III-2).

Sequencing was subsequently expanded, bringing the total number of DNA samples screened to 472 North American (n = 51), European (n = 327) or Asian (n = 94) probands with either MAC (n = 449), skeletal (n = 9) or oculo-skeletal (n = 14) phenotypes. This identified four heterozygous nucleotide changes (c.584G→A, c.796C→T, c.820C→T and c.914T→C), each absent from dbSNP and 480 control DNA samples, that alter amino acid residues in the pre-pro (p.R195Q) and mature domains (p.R266C, p.R274W and p.L305P), respectively. The R266C and L305P variants were identified in three and two unrelated probands, respectively (Table 2), who do not share common haplotypes for the region encompassing GDF3 (data not shown). The distribution of these variants in MAC patients (7/472), and their absence from controls (0/480), is statistically significant (P = 0.007, t-test). Derivation of the majority of the DNA samples from collections established one to two decades ago, severely limited opportunities to re-contact families for additional samples to study the inheritance of these variants. Where this was possible, in contrast to the local pedigree (#1) that exhibits multi-generational inheritance, pedigree #2 illustrates incomplete penetrance (Fig. 1B), recapitulating features previously observed with GDF6 (3) and BMP4 (9). Accordingly all individuals with GDF3 sequence variations were screened for GDF6 mutation (3), identifying one proband (Fig. 1B #3.1) with heterozygous variation of both genes (GDF3-R266C and GDF6-A199T). Notably, this individual exhibits a more severe phenotype than the affected parent (#3.2) carrying GDF3-R266C alone (data not shown) (#3.1: mixed horizontal and rotary nystagmus, bilateral iris coloboma, severe colobomatous microphthalmia, bilateral foveal hypoplasia, abnormally small optic discs with reduced optic nerve diameters (on MRI), 20/200 acuity and abnormal electroretinograms (ERG) with decreased ‘a’ and ‘b’ wave amplitude; #3.2: mild bilateral iris colobomata, mild microphthalmia, normal optic discs, ERGs and 20/40 acuity in each eye).

### Table 1. Sequencing primers

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<th>Reverse (5’→3’)</th>
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(continued...)

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The residues affected by the GDF3 amino acid alterations are differentially conserved between species (Fig. 1C). L305P affects a residue which is invariant in all vertebrates, adding a proline, whose cyclic structure can disrupt secondary structural elements (20). Similarly, R266C affects a residue conserved in all mammals and increases the number of cysteines in the TGF-β domain. Notably, the number of cysteine residues is highly conserved and forms the basis by which individual BMPs/GDFs are classified (21). R274W changes a hydrophilic residue that is constant through evolution to a hydrophobic residue, whereas R195Q, which affects the least conserved residue, results in a charge alteration. Therefore, the observed sequence changes would all be predicted to have an appreciable effect.

**In silico modeling of sequence variants**

In order to provide further insight into the significance of these sequence alterations, a homology model of GDF3 was created using the crystal structure of BMP6’s TGF-β domain, the closest defined paralogue, and in silico mutagenesis was performed. GDF3’s intra-molecular disulfide bonds occur between C264–C361, C293–C329 and C297–C363 (Fig. 2), and alteration of residue 266 to a cysteine is predicted to place the new side-chain in close proximity to that of C329 (Fig. 2), the green dashed line indicates potential hydrogen bonds. Comparison between the estimated mean atomic force potentials of the wild-type and R266C GDF3 models predicts that R266C perturbs non-local interactions (positive ANOLEA energy difference values) in four different areas of the protein (Fig. 2) (residues 263–270, 288–296, 328–335 and 358–361), signifying the importance of R266 in other non-covalent interatomic interactions that characterize the protein’s tertiary structure. In contrast, models for R274W and L305P predict only localized structural disturbances that may not have as profound effects as those predicted for R266C (Fig. 2).

**Functional effects of sequence variants**

In view of these modeling predictions, western blot analysis was performed to assess the functional effects of the GDF3 (R195Q, R266C, R274W and L305P) and GDF6 (A199T) variants on protein expression and secretion. In contrast to the levels of full-length (≈40 kDa) and mature (≈22 kDa) wild-type GDF3, the amount of full-length protein of the L305P and R195Q variants was mildly reduced in the cytosol with more appreciable reductions apparent in the amount of mature R266C and R195Q ligands. In the media, striking reductions were evident in the level of mature R266C and L305P ligand (Fig. 3A), with the milder changes in the amount of full length and mature R274W and R195Q ligands confirmed by analysis of multiple western blots (Image J software, NIH, Bethesda, USA) (data not shown). In addition, western analysis of the GDF6–A199T alteration revealed reduced amounts of protein in both whole cell lysate and media (Fig. 3B).

In view of the identification of a double GDF3-GDF6 heterozygote (#3.1, Fig. 1B), the effect of co-transfecting V5-tagged wild-type GDF3 and GDF6, and their mutant variants (GDF3–R266C and GDF6–A199T), was assessed by western blot analysis. Consistent with the earlier results (Fig. 3A and B), transfection of either GDF3–R266C or GDF6–A199T reduces the level of GDF3 or GDF6 ligand in the cytosol and media (Fig. 3C). Compared with wild-type, co-transfection of both variants leads to an increased amount of mature GDF6 in the cell lysate and a reduced amount of mature GDF6 ligand in the media, with no comparable changes in GDF3 ligand (Fig. 3C).

A luciferase assay employing a SOX9 responsive reporter was next used to quantify the effects of the GDF3 and GDF6 variants. Due to its central role in chondrogenesis, SOX9 is extremely sensitive to, and can be used to accurately quantify, the status of BMP/GDF signaling (22,23). The luciferase assays, performed in triplicate on three separate occasions, yielded consistent results. In accordance with the western blot data, transfection of either GDF3–R266C or GDF6–A199T, results in significantly reduced luciferase

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**Table 2. Summary of GDF3 mutations**

<table>
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<th>Mutation</th>
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<th>Inheritance</th>
<th>Designation</th>
<th>Region</th>
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<td>–</td>
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<tr>
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<td>Autosomal dominant</td>
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*Original proband.
*Patient also carries GDF6 mutation A199T.

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Figure 1. GDF3 variants in ocular and skeletal patients. (A) Three generation pedigree illustrating segregation of R266C with ocular and/or skeletal diseases. Note the contrast between the skeletal phenotypes in the first two generations [I-1 cervical and thoracic scoliosis, II-2 Klippel–Feil type cervical fusion, II-3 Klippel–Feil type cervical fusion C3–C4 (data not shown)] and the ocular and skeletal phenotypes of the proband III-2 [ocular: unilateral iris (g) and retinocchoroidal colobomata (not shown); skeletal: rudimentary 12th ribs (h, arrow) and mild scoliosis (~5°)]. Restriction enzyme TspRI digestion confirms the segregation of R266C with the skeletal and ocular phenotypes (inset); for clarity, only a subset of the chromatograms is displayed. Individual II-4 has yet to be ascertained. (B) Structure of the remaining pedigrees with chromatograms provided for every available DNA sample (inset, restriction enzyme BsaXI digests). #2 illustrates the presence of an L305P mutation in an unaffected parent, demonstrating incomplete penetrance for this mutation. Note the proband (#3.1) carries mutations in GDF3 (R266C) and GDF6 (A199T) whereas the unaffected parent (#3.2) only carries GDF3-R266C. (C) Mutational analysis of GDF3 together with ClustalW alignment of selected amino acids encompassing the regions affected in patients with ocular/skeletal phenotypes.
expression compared with wild-type GDF3 ($P < 0.01$, t-test) or GDF6 ($P < 0.001$, t-test) (Fig. 3D). Co-transfection of both GDF3–R266C and GDF6–A199T results in significantly decreased luciferase activation compared with co-transfection of one wild-type and either variant ($P < 0.005$, t-test) or both wild types ($P < 0.001$, t-test, Fig. 3D), indicating that the mutations have additive effects.

**Effect of inhibition of the zebrafish orthologue dvr1**

A zebrafish model was next used to study the expression and function of decapentaplegic and Vg-related 1 protein (dvr1), a zebrafish ortholog of mammalian Gdf1/3. *In situ* hybridization demonstrated modest dvr1 expression in the head and somitic regions at 14–18 h post-fertilization (hpf), with higher levels detected in tail bud and presumptive heart tissue (Fig. 4A). Subsequently, two distinct morpholino oligonucleotides [dvr1$^{MO1}$, dvr1$^{MO2}$] targeting the translation start site of dvr1 were used to determine the effect of inhibiting dvr1 function, with embryos co-injected with a morpholino to $p53$, to minimize non-specific apoptotic cell death (24). As is evident (Fig. 4B), embryos injected with either dvr1$^{MO1}$ or dvr1$^{MO2}$ exhibit comparable ocular and skeletal anomalies that recapitulate key elements of the patient phenotypes (Fig. 1; Table 2). Ocular features apparent at 48 hpf include colobomata and reduced ocular size (Fig. 4B), with significantly higher rates of malformations observed in morphants receiving the active morpholinos compared with the mismatch morpholino (dvr1$^{MO-MM}$) that contains five base pair substitutions, or the $p53^{MO}$ control [dvr1$^{MO1}$ 143/220 ocular defects; dvr1$^{MO2}$ 61/137; dvr1$^{MO-MM}$ 0/206; $p53$ 5/145 (t-test, $P < 0.0001$) (Fig. 4C)]. Similarly, injection of either dvr1$^{MO1}$ or dvr1$^{MO2}$ resulted in prevalent skeletal defects ranging from mild to severe tail curvature and reduced tail length (Fig. 4B) [dvr1$^{MO1}$ 169/220; dvr1$^{MO2}$ 43/137; dvr1$^{MO-MM}$ 6/206; $p53^{MO}$ 3/145 (t-test, $P < 0.0001$)]. Histological sections performed to investigate ocular development at a cellular level revealed consistent reductions in ocular, lenticular and retinal size, that accord with the microphthalmia seen at earlier stages in live embryos (Fig. 4B). In addition, there were prominent defects in both lens and retina including retention of nuclei in the lens (data not shown), and shortened or missing photoreceptor outer segments (Fig. 5A).
Figure 3. Biochemical analyses of wild-type and mutant GDF3 proteins. (A) Western blot analyses illustrating the effect of sequence variants on full length (~40 kDa) and mature (~22 kDa) GDF3 protein. Note in the cell lysate, mildly reduced levels of full length protein for all four variants and more significant reductions in mature R266C and R195Q, whereas in the medium, striking decreases in the level of mature R266C and L305P plus milder changes in full length and/or mature R274W and R195Q ligands are evident. (B) Reduced expression of GDF6-A199T compared with wild-type in both cell lysate and medium. (C) Western blot analysis illustrating the effect of co-transfecting the combinations shown of either wild-type or mutant GDF3 and GDF6. Transfection of either GDF3-R266C or GDF6-A199T reduces the levels of mature GDF6 or GDF3, respectively [also shown in (A) and (B)]. Transfection of both variants leads to an increased level of GDF6 in the cell lysate and a reduced level in the media compared with the double wild-type. Alpha-tubulin and secreted alkaline phosphatase (SEAP) represented controls for cytosolic and secreted proteins, respectively. (D) SOX9 luciferase reporter assay illustrating the effects of different combination of wild-type and mutant GDF3 or GDF6 with reporter gene activity expressed in relative light units (pcDNA 3.1 represents empty vector). Transfection of either GDF3-R266C or GDF6-A199T significantly reduces luciferase reporter activity compared with wild-type, whereas co-transfection of both mutations significantly reduces luciferase reporter activity compared with the combination of one mutation and its fellow wild-type. Relative light unit (RLU) = Mean ± SE. For each comparison probabilities were calculated using a student t-test.
Figure 4. Zebrafish phenotypes induced by \textit{dvr1} morpholino inhibition. (A) Lateral and dorsal views of \textit{dvr1} \textit{in situ} hybridizations in 16 and 18 h post fertilization (hpf) embryos illustrating generalized low-level expression, with increased expression in tail bud and presumptive heart tissue [The apparent increase in the region of the developing eye is secondary to the overlap of multiple tissues (eye plus forebrain)]. (B) Representative images of phenotypes induced by injection of \textit{dvr1}^{MO1} and \textit{dvr1}^{MO2} in zebrafish embryos (i, ii, iv, v) compared with control (p53^{MO}) embryos at 48 and 72 hpf. Morphants exhibit both ocular and skeletal defects including reduced ocular size (i, ii, iv, v), loss of ventral eye tissue (i, red bar), defects in ventral retinal fissure closure (ii, iv, v, red arrow) as well as skeletal defects (i, ii, iv, v, blue arrow). (C) Graphs illustrating the prevalence of phenotypes in morphants at the same timepoints: note the minimal level of ocular or skeletal phenotypes in zebrafish embryos injected with either \textit{dvr1}^{MO-MM} or p53^{MO}, whereas injection of \textit{dvr1}^{MO1} or \textit{dvr1}^{MO2} resulted in appreciably perturbed ocular and skeletal development.
Effect of co-inhibition of dvr1 and gdf6a in zebrafish

In light of the GDF3 and GDF6 sequence alterations in #3.1 (Fig. 1B), the effect of co-inhibiting gdf6a and dvr1 was investigated to determine if this generated more severe phenotypes than dvr1 inhibition alone. To ensure uniform amounts of morpholino (8 ng) were injected, a control morpholino with no ocular or skeletal effects (unpublished data) was co-injected to balance the varying combinations of the other morpholinos used (dvr1MO1 2 ng; gdf6aMO1 4 ng; p53MO 2 ng; controlMO 0–4 ng). Injection with dvr1MO1 (31/103 ocular defects, 25/103 skeletal defects) or gdf6aMO1 (61/147 ocular, 16/147 skeletal) generated significantly lower prevalences of phenotypes than use of both morpholinos (103/141 ocular, 75/141 skeletal) (P < 0.0001, t-test) (Fig. 5B). In view of the more severe and more prevalent phenotypes generated by dvr1 and gdf6a co-inhibition, the in situ expression patterns of dvr1 in gdf6a morphants, and the converse, were evaluated to elucidate whether they lie in the same pathway. Their essentially unaltered expression (data not shown) implies that dvr1 and gdf6a do not directly regulate each other in zebrafish development. Accordingly, we next analyzed selected genes with known, spatially restricted, retinal expression patterns to determine the effect of dvr1 inhibition on dorso-ventral and naso-temporal patterning. As is evident, in addition to its normal nasal retinal expression, the forkhead transcription factor foxg1 is ectopically expressed in the developing lens of dvr1 morphants (Fig. 6A and B). In contrast, the expression of axon guidance molecules epha3 and efna5a remains unchanged as do the dorso-ventral retinal markers examined.
(vax2, aldh1a2 and tbx5) (Fig. 6C–L). In view of the induced lenticular expression of foxg1, two lens markers (cx23 and lim2.3) were also examined, but their unaltered expression suggests that dvr1 inhibition does not induce widespread changes in lenticular expression.

**DISCUSSION**

To date, the molecular basis of microphthalmia/anophthalmia and coloboma phenotypes have been defined in some 10% of patients, and combined with incompletely penetrant cases this low proportion complicates efforts to determine the recurrence risk in families. Since these disorders impair vision, and are present in an appreciable proportion (3–11%) of blind children, identifying new causes of MAC phenotypes affords clinical insight into important causes of pediatric vision loss. Colobomatous disorders have equal research significance, providing molecular entry points into key developmental pathways. As this study demonstrates, these prominent markers of aberrant ocular development can be used to inform understanding of a range of human disease.

This study has defined novel developmental roles for GDF3, a relatively unstudied BMP previously implicated in regulation of stem cell differentiation (12). With experiments encompassing human genetics, molecular modeling, biochemistry and zebrafish analyses, strong evidence is provided that GDF3 is a key developmental regulator and that mutations result in a range of ocular and skeletal disorders. The predominantly ocular phenotypes observed, likely reflects the patient panel’s composition (Table 2). The variants identified in 1.5% (7/472) of MAC patients, and their absence from a substantial cohort of controls (n = 480), makes it statistically improbable that these amino acid alterations are associated by chance with disease (P = 0.007, t-test). Each variant generates an appreciable amino acid alteration, with those in the mature TGF-β domain affecting residues demonstrating considerable evolutionary conservation. Amongst these, R266C is notable for increasing the number of cysteine residues by which the BMPs are classified, positioning this ligand variant in a new BMP sub-clade.

In contrast to other TGF-β domain family members which contain at least three cysteine pairs (sequences summarized as C1x[LIVM]x2Px[F,Y]x4xC2xGxC3x4xC5xC6xC7x4xC8xC9x), and form disulfide bonds between C1–C7, C2–C6 and C3–C7; GDF3, GDF9 and BMP15 are the only BMPs lacking an unpaired cysteine in the mature domain. This fourth cysteine (C4) is involved in an inter-molecular bridge to stabilize the active form of homodimeric or heterodimeric signaling molecules. Its absence from wild-type GDF3 suggests GDF3 may not form covalently-bonded active dimers. In this context, it is intriguing to propose that R266C, by introducing an extra cysteine in the TGF-β domain, may alter inter-molecular interactions. Thus, instead of affecting protein dimerization, this model predicts that the R266C amino acid change may allow the formation of an aberrant disulfide bridge between C266 and C329, disrupting an important stabilizing interaction of the TGF-β fold. In view of this prediction it is noteworthy that R266C segregates in an autosomal dominant manner with pleiotropic phenotypes (pedigree #1),

![Figure 6. Analysis of the expression of selected lenticular and retinal patterning genes at 28 hpf in dvr1 morphants, illustrating mis-expression of foxg1 in the developing lens. Although foxg1 retinal expression is unaltered in dvr1MO injected embryos, in contrast to controls (p53MO injected embryos (A)), there is ectopic lenticular expression (B), delineated by the dashed red circle. In situ hybridization patterns of epha3 (C, D), and efn5a (E, F) as well as dorso-ventral retinal patterning genes vax2 (G, H), aldh1a2 (I, J), and tbx5 (K, L) remains unchanged, with no alteration in expression levels or domains observed. Similarly, expression of lens markers cx23 (M, N) and lim2.3 (O, P) were unaltered at the same 28 hpf timepoint.](image-url)
demonstrating that a single mutant allele is sufficient to cause disease. Western analysis performed to more directly characterize the variants’ effects demonstrates that each alteration reduces the amount of active ligand secreted. Comparable findings from a SOX9-based reporter assay, with transfection of GDF3–R266C resulting in significantly reduced luciferase expression compared with wild-type (Fig. 3D), provide additional support that these variants are functionally significant.

In order to derive further information regarding GDF3’s role in early development, morpholino antisense oligonucleotides were used to inhibit the function of the zebrafish co-orthologue, dvr1. To address the concern that these synthetic oligonucleotides may have off-target effects, overlapping translation blocking morpholinos were used that generated identical and spatially restricted phenotypes comparable to those seen in patients. Since a control morpholino (dvr1MO–MM) did not induce phenotypes and non-specific apoptosis was minimized by co-injection of p53MO, these data are consistent with dvr1 inhibition causing ocular and skeletal disorders. This is in turn supported by the prominent ocular histological changes that resemble those induced by gdf6a inhibition (4), with lenticular mis-expression of the ventral retinal patterning marker foxl1, highlighting the specificity of effects induced by dvr1 inhibition and being consistent with disrupted ocular patterning observed in patients. In this context, it is noteworthy that comparable mis-expression of a retinal patterning marker (vax2) was observed with gdf6a inhibition (25), compatible with loss of BMP signaling leading to aberrant lenticular development.

Since dvr1 is the co-orthologue of GDF1 and GDF3, a potential limitation of dvr1 morpholino inhibition is that the induced oculo-skeletal phenotypes might correspond with GDF1 mutation, rather than GDF3 as proposed. This is, however, addressed by the extensive characterization of GDF1 and Gdf1, demonstrating involvement in heart development (26) and left/right asymmetry (27), but not ocular/somatic or skeletal disorders. Indeed GDF1 mutation results in congenital defects ranging from tetralogy of Fallot to transposition of the great arteries (26), and combined with the lack of oculo-skeletal phenotypes in two Gdf1 murine mutants (27,28), confirms the validity of dvr1 inhibition as a model of human GDF3 mutation. In this context, recent description of a Gdf3 murine mutant characterized solely from a metabolic perspective (29) provides opportunities for examining this strain to determine whether oculo-skeletal phenotypes are also present.

A second limitation is the lack of DNA samples from some parents and siblings, restricting study of the segregation of mutations in four pedigrees. Although an unavoidable consequence of using long established DNA collections that predate current requirements for signed consent to re-contact families, this limits opportunities to comprehensively define GDF3’s role in disease. Notwithstanding this constraint, the available data illustrate incomplete penetrance, as seen with GDF6 and BMP4 (3.9), and fascinatingly have identified a double GDF3–GDF6 heterozygote (#3.1). Western blot analysis of the constituent GDF3–R266C and GDF6–A199T mutations reveals that their co-transfection leads to retention of mature GDF6 ligand in the cellular fraction and a corresponding reduction in mature GDF6 ligand in the media. These data demonstrate that sequence variation in one BMP may affect the secretion of a second, according with findings from GDF9 and BMP15 (30), ligands that also lack the fourth of seven conserved cysteine residues. Our results are supported by luciferase analysis, demonstrating that co-transfection of GDF3–R266C and GDF6–A199T resulted in significantly less activation than either mutation alone, and by co-inhibition of gdf6a and dvr1 resulting in a greater prevalence of zebrafish phenotypes than inhibition of either gdf6a or dvr1 alone. This close correlation between patient phenotypic, western blot, reporter assay and zebrafish data supports the hypothesis that sequence variation in different BMP ligands may contribute additively to a phenotype. Such findings are consistent with synergistic interactions noted between TGF-β ligands generating novel phenotypes not evident in a single murine genotype (31), thus implicating the overall level of TGF-β signaling in specific developmental roles.

In summary, the combined human, biochemical and model organism data presented provide compelling evidence for GDF3’s involvement in ocular and skeletal development, and illustrate the contribution that perturbed GDF3 function makes to a spectrum of disorders. By defining the human phenotypes attributable to mutation of a third BMP ligand, this study extends the contribution of altered BMP signaling in disease and implicates other members of this large gene family in related disorders. Our findings concur with earlier results regarding the breadth and inheritance of GDF6-attributable phenotypes, with the combined results from Western, luciferase and morphant analyses suggesting that GDF3 and GDF6 have independent yet complementary developmental roles.

MATERIALS AND METHODS

Patient samples and mutational analysis

DNA samples from 472 probands with either ocular anomalies (microphthalmia, anophthalmia and/or colobomata) or skeletal phenotypes (Kippel–Feil type vertebral fusions or scoliosis) were screened for GDF3 mutations. PCR were performed using three sets primers (Table 1) and the following conditions (94°C 30 s, 57°C 30 s, 72°C 30 s, 35 cycles). The amplicons were sequenced on an ABI Prism 3100 capillary sequencer (Applied Biosystems), analyzed using Sequencher 4.5 (GeneCodes, Madison, WI, USA) and ClustalW, with mutations confirmed by bi-directional sequencing and restriction enzyme digestion of PCR products. The prevalence of mutations in normal individuals was determined for GDF3 by screening: 480 control DNA samples (380 North American and European, 100 Asian), 200 by restriction enzyme digest (TspRI, MspRI, BsaXI and MspI for R266C, R274W, L305P and R195Q, respectively) and a further 280 by direct sequencing; and for GDF6–A199T by screening 200 North American and European control DNA samples. In silico modeling of GDF3 mutations was performed with Swiss-PdbViewer (32) and POV-RayTM v3.6, and the models evaluated with ANOLEA, as described previously (32).
Gene cloning and mutagenesis

Full length wild-type GDF3 and GDF6 generated by PCR of genomic DNA, were cloned into pCR®4-TOPO (Invitrogen™, ON, Canada). Point mutations were introduced by site-directed mutagenesis. Both wild-type and mutant genes were subsequently subcloned into pcDNA™3.2/V5-DEST vector using Gateway® System (Invitrogen™, ON, Canada).

Cell transfection and western blot analysis

Monkey kidney cells COS-7 were cultured to 80% confluency in DMEM plus 10% FBS at 37°C. For each V5-tagged-GDF3 plasmid, 4 µg of DNA was transfected and transiently expressed in COS-7 cells by FuGENE (Roche Diagnosis, IN, USA) according to the manufacturer’s instructions. Both cell lysate and culturing medium were collected at 48 h post-transfection as previously described (3). Proteins from cell lysate and medium were separated by 15% SDS-PAGE, transferred to 0.45 µm nitrocellulose membranes (BioRad, CA, USA), and blotted by mouse anti-V5 antibody (1:10 000, Invitrogen, ON, Canada) and subsequently by HRP conjugated anti-mouse IgG (1:5000, Jackson Labs). Western blot analyses were resolved by ECL reactions (efficient chemiluminescence, ThermoScientific, IL, USA). Alpha-tubulin and secreted alkaline phosphatase were used as loading controls for cytosol GDF3 and secreted GDF3, respectively.

Reporter gene assay

PLM cells were harvested from embryonic age E11.5 CD-1 mouse embryos as previously described (23). In 384-well plates, GDF3 wild-type or its mutants in pcDNA™3.2/V5-DEST vectors were transfected and transiently expressed in PLM cells by Effectene (Qiagen, ON, Canada) according to the manufacturer’s instructions. Briefly, DNA-Effectene mixtures were aliquoted into wells followed by the addition of approximately 100 000 PLM cells, and wells were topped-up to a total volume of 100 µl. Media was replenished 24 h post-transfection, and cell lysates were collected 48 h post-transformation. Luciferase activity was measured using the Dual Luciferase Kit (Promega, CA, USA) and normalized to an internal renilla luciferase control. For each transfection, luciferase assays were performed in triplicate and repeated three times.

In situ hybridization and zebrafish eye histology sectioning

An RNA probe to the second exon of dvr1, the zebrafish co-orthologue of humane GDF3 and GDF1, was synthesized and labeled with digoxigenin by T7 RNA polymerase (Invitrogen™, ON, Canada). RNA in situ hybridization of dvr1 was performed as previously described (24). NBT/BCIP stained embryos were mounted in 70% glycerol and the expression of dvr1 in zebrafish embryos was photographed with a dissecting microscope (Carl Zeiss, ON, Canada) and QImaging microublisher digital CCD camera (QIMAGING, BC, Canada). For in situ hybridization of lens and retinal patterning markers, 28 hpf p53MO and dvr1MO1 zebrafish embryos were used, following the same protocol as for dvr1 (above). A minimum of 20 embryos were examined for each hybridization (n = 40 embryos for foxgly and vax2), and replicate experiments were performed to confirm the findings. For zebrafish eye sectioning, 4 dpf larvae were fixed for one hour in 4% paraformaldehyde, washed with 1 × PBS, and then dehydrated through a series of dehydration steps ranging from 50 to 100% ethanol. For pre-infiltration, larvae were incubated in 1:1 100% ethanol and Solution A [25 ml Technovit 7100 (Heraeus Kulzer, Germany) plus 0.25 g Hardener I] for 2 times 30 min. For infiltration, larvae were incubated in 100% Solution A at 4°C overnight. For polymerization, Solution A was mixed with Hardener II (15:1), and larvae were embedded in the polymerization solution. Three to 5 µm sections were cut using a rotation microtome. Slides were dried, stained with Richardson (Bromeis) solution (1% Methylene Blue, 1% Borax, 1% Azur II, in a ratio of 1:1:2) and covered in BTX mounting medium (VWR International, England).

Morpholio inhibition

Two overlapping morpholio oligonucleotides were designed targeting the translation start site of dvr1 [dvr1MO1 AGGCTCTGAGGAGGACTAAGAACAT; dvr1MO2 GCTCTGAGGAGGACCAAGAACATTA] and knockdown assays were performed in AB strain zebrafish as previously described (24). Controls were provided by a dvr1 mis-match morpholio containing five base substitutions (dvr1MM UCUGGCTUATGAGUAAGGAAUCATTA) and knockdown assays were performed in AB strain zebrafish as previously described (24). Controls were provided by: a dvr1MO1 morpholio containing five base substitutions (dvr1MO-MM UCUGGCTUATGAGUAAGGAAUCATTA); un-injected zebrafish embryos; and those injected with just 2 ng of a morpholio to p53 (p53MO). The gdf6a and dvr-1 double-knockdown assay used the identical protocol (24), and the previously reported splice-blocking gdf6a morpholio (2).

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Conflicts of Interest statement. None.

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REFERENCES


