ORIGINAL ARTICLE

Characterization of *GDF2* Mutations and Levels of BMP9 and BMP10 in Pulmonary Arterial Hypertension

Joshua Hodgson[1], Emilia M. Swietlik[1,2], Richard M. Salmon[1], Charaka Hadinnapola[1], Ivana Nikolic[3], John Wharton[4], Jingxu Guo[1], James Liley[1], Matthias Haimel[1,5,6], Marta Bleda[1], Laura Southgate[7,8], Rajiv D. Machado[8], Jennifer M. Martin[1,5,6], Carmen M. Treacy[1,2], Katherine Yates[1,5,6], Louise C. Daugherty[5,6], Olga Shamardina[5,6], Deborah Whitehorn[5,6], Simon Holden[9], Harm J. Bogaard[10], Colin Church[11], Gerry Coghlan[12], Robin Condliffe[13], Paul A. Corris[14], Cesare Danesino[15,16], Mélanie Eyries[10], Henning Gall[17], Stefano Ghio[16], Hossein-Ardeschir Ghofrani[4,17], J. Simon R. Gibbs[18], Barbara Girerd[19,20,21], Arjan C. Houweling[22], Luke Howard[4], Marc Humbert[19,20,21], David G. Kiely[13], Gabor Kovacs[23,24], Allan Lawrie[25], Robert V. MacKenzie Ross[26], Shahin Moledina[27], David Montani[19,20,21], Andrea Olschewski[23], Horst Olschewski[23,24], Willem H. Ouwehand[5,6], Andrew J. Peacock[11], Joanna Pepke-Zaba[2], Inga Prokopenko[4], Christopher J. Rhodes[4], Laura Scelsi[16], Werner Seeger[17], Florent Soubrier[10], Jay Suntharalingam[26], Mark R. Toshner[1,2], Richard C. Trembath[7], Anton Vonk Noordegraaf[20], Stephen J. Wort[18,28], Martin R. Wilkins[4], Paul B. Yu[3], Wei Li[1], Stefan Gräf[1,5,6], Paul D. Upton[1]*, and Nicholas W. Morrell[1,6]*

[1]Department of Medicine and [5]Department of Haematology, University of Cambridge, Cambridge, United Kingdom; [2]Royal Papworth Hospital, Papworth, United Kingdom; [3]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; [4]Department of Medicine and [18]National Heart and Lung Institute, Imperial College London, London, United Kingdom; [6]National Institute for Health Research BioResource–Rare Diseases, Cambridge, United Kingdom; [7]Department of Medical and Molecular Genetics, King's College London, London, United Kingdom; [8]Molecular and Clinical Sciences Research Institute, St. George's University of London, London, United Kingdom; [9]Addenbrooke's Hospital, Cambridge, United Kingdom; [10]Département de Génétique, Hôpital Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, and UMR_S 1166-ICAN, INSERM, UPMC Sorbonne Universités, Paris, France; [11]Golden Jubilee National Hospital, Glasgow, United Kingdom; [12]Royal Free Hospital, London, United Kingdom; [13]Sheffield Pulmonary Vascular Disease Unit, Royal Hallamshire Hospital, Sheffield, United Kingdom; [14]University of Newcastle, Newcastle, United Kingdom; [15]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [16]Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [17]University of Giessen and Marburg Lung Center, member of the German Center for Lung Research (DZL) and of the Excellence Cluster Cardio-Pulmonary Institute, Giessen, Germany; [19]Faculté de Médecine, Université Paris-Saclay, Université Paris-Sud, Paris, France; [20]Service de Pneumologie, Centre de Référence de l'Hypertension Pulmonaire, Assistance Publique–Hôpitaux de Paris, Paris, France; [21]Hôpital Bicêtre, Le Kremlin-Bicêtre, INSERM UMR_S 999, Paris, France; [22]Department of Clinical Genetics, Amsterdam Universitair Medische Centra, Vrije Universiteit Amsterdam, Amsterdam, the Netherlands; [23]Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria; [24]Medical University of Graz, Graz, Austria; [25]Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom; [26]Royal United Hospitals Bath NHS Foundation Trust, Bath, United Kingdom; [27]Great Ormond Street Hospital, London, United Kingdom; and [28]Royal Brompton Hospital, London, United Kingdom

Abstract

Rationale: Recently, rare heterozygous mutations in *GDF2* were identified in patients with pulmonary arterial hypertension (PAH). *GDF2* encodes the circulating BMP (bone morphogenetic protein) type 9, which is a ligand for the BMP2 receptor.

Objectives: Here we determined the functional impact of *GDF2* mutations and characterized plasma BMP9 and BMP10 levels in patients with idiopathic PAH.

Methods: Missense BMP9 mutant proteins were expressed *in vitro* and the impact on BMP9 protein processing and secretion, endothelial signaling, and functional activity was assessed. Plasma BMP9 and BMP10 levels and activity were assayed in patients with PAH with *GDF2* variants and in control subjects. Levels were also measured in a larger cohort of control subjects ($n = 120$) and patients with idiopathic PAH ($n = 260$).

Measurements and Main Results: We identified a novel rare variation at the *GDF2* and *BMP10* loci, including copy number variation. *In vitro*, BMP9 missense proteins demonstrated impaired cellular processing and secretion. Patients with PAH who carried these mutations exhibited reduced plasma levels of BMP9 and reduced BMP activity. Unexpectedly, plasma BMP10 levels were also markedly reduced in these individuals. Although overall BMP9 and BMP10 levels did not differ between patients with PAH and control subjects, BMP10 levels were lower in PAH females. A subset of patients with PAH had markedly reduced plasma levels of BMP9 and BMP10 in the absence of *GDF2* mutations.

Conclusions: Our findings demonstrate that *GDF2* mutations result in BMP9 loss of function and are likely causal. These mutations lead to reduced circulating levels of both BMP9 and BMP10. These findings support therapeutic strategies to enhance BMP9 or BMP10 signaling in PAH.

Keywords: BMP9; BMP10; GDF2; pulmonary arterial hypertension

(Received in original form June 7, 2019; accepted in final form October 25, 2019)

Am J Respir Crit Care Med Vol 201, Iss 5, pp 575–585, Mar 1, 2020

Originally Published in Press as DOI: 10.1164/rccm.201906-1141OC on October 29, 2019
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Rare mutations in *GDF2*, the gene encoding BMP9 (bone morphogenetic protein type 9), have been identified in pulmonary arterial hypertension (PAH). Whether BMP9 variants identified specifically in PAH have different functional changes to those variants not exclusively associated with PAH is not known.

What This Study Adds to the Field: This study provides new genetic evidence for the association of *GDF2* mutations with PAH and confirms that these mutations cause impaired BMP9 function and signaling. It shows for the first time that BMP10 levels are altered in PAH and demonstrates biological integration of plasma BMP9 and BMP10 levels. The findings provide further justification for the use of therapeutic approaches that enhance BMP9 signaling in patients with PAH.

Pulmonary arterial hypertension (PAH) is a rare but important life-limiting disease that typically presents with unexplained breathlessness on exertion (1). The lung pathology is characterized by narrowing and obliteration of small pulmonary arteries resulting from proliferation of endothelial cells, smooth muscle cells, and fibroblasts in the vessel wall (2). The resulting elevation in pulmonary vascular resistance leads to severe PAH. The pressure-overloaded right ventricle responds initially by hypertrophy but ultimately dilates and fails (3). Average transplant-free 3-year survival remains only 60% to 70% despite existing therapies (3, 4), demanding a search for more effective treatments based on a more thorough understanding of the pathobiology.

Human genetic studies have revealed important insights into the pathobiology of PAH. Heterozygous mutations in the gene encoding the *BMPR2* (BMP [bone morphogenetic protein]-type 2 receptor) are the most common genetic causes of PAH, accounting for 53% to 86% of familial cases and 14% to 35% of idiopathic cases (5–7). Further cases are accounted for by mutations in *ACVRL1* (activin receptor-like kinase 1 encoding ALK1), *ENG* (endoglin), and by mutations in genes encoding signaling intermediaries downstream of BMP receptors, such as *SMAD9* (7). Additional rare variants have been described in the genes encoding *CAV1* (caveolin-1) (8) and the potassium channel *KCNK3* (9). In a large European cohort study, we provided the first evidence for additional causal mutations in *GDF2* (which encodes BMP9), *ATP13A3* (a P5-type ATPase), *SOX17* (SRY-Box 17), and *AQP1* (aquaporin-1) (10). The identification of *GDF2* mutations has since been independently replicated (11), accounting for 6.7% of idiopathic PAH cases in a Chinese cohort.

It is now known that BMPR2 and ALK1, together with ENG as an accessory receptor, form a BMP signaling complex largely restricted to endothelial cells (12). Of the 12 different BMPs involved in fundamental and diverse cellular functions, only BMP9 and BMP10 are circulating physiological ligands for the BMPR2/ALK1 receptor complex (13). BMP9 is expressed primarily in the liver (14, 15), whereas BMP10 is highly expressed in the right atrium (15, 16). Recent evidence suggests the presence of circulating BMP9/BMP10 heterodimers (15). These BMP ligands circulate at physiologically active levels and maintain vascular endothelial quiescence (17). Moreover, we previously demonstrated that therapeutic administration of BMP9 prevents and reverses PAH in genetic and nongenetic models of disease (12).

Here we sought to determine whether recently identified and novel heterozygous mutations in *GDF2* cause BMP9 loss of function, based on biochemical characterization and measurements of BMP9 plasma levels in patients. In addition, we assayed BMP9 and BMP10 levels in a large PAH cohort and demonstrated that a subset of patients with PAH, without identified *GDF2* mutations, also exhibit reduced circulating BMP9 and BMP10 levels. Some of these results have been previously reported in the form of abstracts (18, 19).

Methods

A cohort of 1,048 patients with PAH underwent whole genome sequencing, and a case-control rare variant analysis was undertaken, as described previously (10). Variants that occurred at a frequency of more than 1 in 10,000, or were not predicted to disrupt protein structure according to *in silico* prediction analyses, were classified as likely benign (*see* Table E3 in the online supplement).

We selected seven missense PAH-associated potentially pathogenic *GDF2* variants and nine common and/or benign variants for functional comparison. Proteins were expressed as Pro:BMP9 (prodomain-bound BMP9), as previously reported (10), generating three batches per variant.

Plasma levels of BMP9 and pBMP10 (*see* online supplement) were measured in 260 patients with idiopathic or heritable

*Co–senior authors.

Supported by grants from the British Heart Foundation: RG/08/006/25302 (R.C.T.), RG/13/4/30107 (N.W.M.), 4-year Ph.D. program FS/15/62/323032 (J.H.), Intermediate Basic Science Research Fellowship FS/15/59/31839 (C.J.R.), Senior Basic Science Research Fellowships FS/13/48/30453 and FS/18/52/33808 (A.L.), and SP/12/12/29836; Wellcome Trust Institutional Strategic Support Fund awarded to St. George's University of London 204809/Z/16/Z (L.S.); Medical Research Council (MRC) MR/K020919/1; and Département Hospitalo-Universitaire Thorax Innovation: LabEx LERMIT ANR-10-LABX-0033 and RHU BIO-ART LUNG 2020 ANR-15-RHUS-0002. The UK National Cohort of Idiopathic and Heritable PAH is supported by the NIHR, the British Heart Foundation (BHF) (SP/12/12/29836), the BHF Cambridge Centre of Cardiovascular Research Excellence, the UK Medical Research Council (MR/K020919/1), the Dinosaur Trust, NIHR Great Ormond Street Hospital Biomedical Research Centre, and Great Ormond Street Hospital Charity.

Author Contributions: All authors collected data and provided constructive criticism of the study manuscript. J.H., E.M.S., R.M.S., C.H., J.G., W.L., S.G., P.D.U., and N.W.M. undertook the study design and interpretation. J.H., E.M.S., C.H., J.L., S.G., and P.D.U. analyzed data.

Correspondence and requests for reprints should be addressed to Nicholas W. Morrell, M.D., Department of Medicine, University of Cambridge, Level 5 Addenbrooke's Hospital, Box 157, Hills Road, Cambridge CB2 0QQ, UK. E-mail: nwm23@cam.ac.uk.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

PAH and 120 control subjects. Patient samples were made available by the UK PAH Cohort (www.ipahcohort.com, https://www.ukctg.nihr.ac.uk, unique identifier: NCT01907295). All patients provided written informed consent and the study was approved by the ethics review committee (REC Ref: 13/EE/0203). The control samples were described previously (20).

Results

Identification of *GDF2* Mutations

Whole genome sequencing identified seven likely pathogenic missense variants and one frameshift variant in *GDF2* ($n = 8$) (10). In the present study, we searched for additional structural variation at the *GDF2* locus and identified two patients with large deletions, not previously described, encompassing the *GDF2* locus and several neighboring genes (Table 1 and *see* Figure E1A). The p.Y351H variant, found in two unrelated patients, was not previously reported and is predicted to disrupt the hydrophobic core of BMP9 (*see* Figure E1B). With the additional 4 carriers identified in this study, we have now identified a total of 12 *GDF2* mutation carriers. Two patients with *GDF2* missense variants (p.M89V and p.Y351H) had a positive family history for PAH with sufficient information available for the M89V variant carrier to construct a pedigree, although sequencing data were available only for the proband (*see* Figure E1C).

We compared the demographics and clinical parameters of the *GDF2* mutation carriers ($n = 12$) with patients harboring heterozygous *BMPR2* mutations ($n = 159$) and patients with no identified mutation ($n = 750$) (Table 2). *GDF2* mutation carriers were similar to patients with PAH without mutations and showed no features of hereditary hemorrhagic telangiectasia (HHT) or vascular anomaly syndromes. *GDF2* mutation carriers were significantly older and had less severe hemodynamics than *BMPR2* mutation carriers. Transplant-free survival was similar between groups after adjustment for age, sex, and whether cases were incident or prevalent. The two patients with large deletions exhibited earlier onset of disease at 19 and 30 years of age (*see* Table 1). The 19-year-old underwent transplantation after 3 years. Despite the deletions affecting several additional genes (*see* Figure E1), these individuals did not have reported comorbidities. In addition, we searched for potentially deleterious mutations in BMP10. Two rare and deleterious missense variants in *BMP10* (p.A361E and p.R353C) were identified in patients with PAH (*see* Table E4). The individual carrying p.R353C has been reported previously (21).

Biochemical Characterization of BMP9 Variants

BMP9 circulates in a noncovalent complex with its prodomain (Figure 1A) (14). We previously reported that missense mutations predicted to be pathogenic *in silico* cluster at the interface of the growth factor and prodomains (10), leading us to hypothesize that they disrupt the Pro:BMP9 complex. We expressed BMP9 variants *in vitro* that were predicted to be *1*) deleterious in the PAH cohort, *2*) benign in the PAH cohort, *3*) deleterious but not exclusive to PAH, or *4*) benign and not exclusive to PAH (*see* Table E3). Measurement of the concentration of BMP9 in conditioned media by ELISA demonstrated that the secretion of pathogenic variants was markedly reduced (*see* Figure 1B). With the exception of P104L (*see* Discussion), variants predicted to be benign were secreted efficiently. For all detectable variants, the measured absorbances paralleled the standard curve, implying that the ELISA antibodies cross-reacted fully with these variants and reduced detection was not due to epitope change (*see* Figure E2).

We confirmed the ELISA data by western blotting of conditioned media for the growth factor and prodomains of BMP9 (*see* Figure 1C). For pathogenic variants, there was an excess of secreted prodomain compared with growth factor domain, indicating that the Pro:BMP9 complex was indeed disrupted (*see* Figure 1D). There was almost no detectable secreted growth factor domain for Pro:BMP9-R110W, -E143K, -Y351H and -T413N despite quantifiable amounts of secreted prodomain. Western blotting of transfected cell lysates revealed that *ProBMP9* was efficiently expressed within cells, but compared with Pro:BMP9-WT there was a loss of processed species with these pathogenic variants, suggesting reduced stability (*see* Figure 1E).

In addition, we demonstrated a processing defect in the *ProBMP9*-S320C variant, which introduces a substitution immediately adjacent to the furin cleavage site between the growth factor and prodomain (*see* Figure E3A). The *ProBMP9*-S320C mutation resulted in an excess of uncleaved species accumulating in conditioned media (*see* Figure E3B). Within cell lysates, equal amounts of both unprocessed *ProBMP9*-WT and -S320C were present (*see* Figure E3B), indicating a defect during secretion.

We confirmed that the BMP9 prodomain is glycosylated (*see* Figures E3C–E3E) and the increased molecular weight of the prodomain of the common D218N variant is due to an additional *de novo* glycosylation site, which does not alter function (Figure 2).

Assessment of Signaling and Functional Capacity of BMP9 Variants

We undertook a functional assessment of the signaling activity of BMP9 variants in conditioned media using hALK1-transfected C2C12 cells, which exhibit a concentration-response to 10–100 pg/ml of BMP9 (*see* Figure E4). All secreted growth factor domains had potency equivalent to Pro:BMP9-WT (*see* Figure 2A). There was no activity associated with conditioned media containing pathogenic variants with negligible detectable growth factor domain (*see* Figures 2B and E5). Similarly, for Pro:BMP9-M89V, activity assays suggested a reduced amount of growth factor domain compared with prodomain. The transcriptional activity of BMP9 variants in blood outgrowth endothelial cells (22) was consistent with the ALK1-luciferase experiments (*see* Figures 2C and 2D).

Finally, we assessed the antiapoptotic activity of benign and pathogenic variants on blood outgrowth endothelial cells by flow-cytometry (*see* Figure 2E) (12). Because of the limited throughput of this approach, we screened all the mutants for their capacity to inhibit apoptosis of human pulmonary artery endothelial cells by Caspase-GLO 3/7 (Promega Corporation) assays (*see* Figures 2F and 2G). These also showed most variants possessed antiapoptotic activity, except for the pathogenic variants, which lead to a profound loss of secreted growth factor domain despite the presence of

Table 1. Gene Changes, Demographics, and Pulmonary Hemodynamic Data for Patients with PAH Harboring BMP9 Mutations

ID	E013254	E005056	E006312	E010660	E000837	E010643	E012223	E001147	E004186	W000134	E011142	W000048
Nucleotide change	*c.137_150 delGTGGGCTGCCTGAG*	*c.265A>G*	*c.328C>T*	*c.347A>T*	*c.427G>A*	*c.958A>T*	*c.1040C>T*	*c.1051T>C*	*c.1051T>C*	*c.1238C>A*	*del Chr10:47536678–51819820*	*del Chr10:47535814–51823146*
Protein change	p.Gly46Ala fsTer21	p.Met89Val (M89V)	p.Arg110Trp (R110W)	p.Asp116Val (D116V)	p.Glu143Lys (E143K)	p.Ser320Cys (S320C)	p.Ala347Val (A347V)	p.Tyr351His (Y351H)	p.Tyr351His (Y351H)	p.Thr413Asn (T413N)	N/A	N/A
Consequence	Frameshift variant	Missense variant	Missense variant	Missense variant	Missense variant	Missense variant	Missense variant	Missense variant	Missense variant	Missense variant	Large deletion (4.28 Mbp)	Large deletion (4.29 Mbp)
Ethnicity	European	European	European	European	European	South-Asian	European	European	European	Other	European	South-Asian
Age at diagnosis, yr	45	53	46	52	45	56	30	73	45	54	19	30
Sex	M	F	F	M	M	M	F	F	F	F	M	F
WHO FC	3	3	2	3	n.d.	3	3	3	2	3	4	2
Exercise capacity	470 (6WMT)	432 (6MWT)	588 (6MWT)	395 (6MWT)	n.d.	403 (6MWT)	220 (shuttle)	160 (6MWT)	420 (6MWT)	374 (6MWT)	280 (shuttle)	405 (6MWT)
mPAP, mm Hg	46	34	38	55	52	56	53	59	73	50	n.d.	55
PAWP, mm Hg	16	8	5	9	10	7	n.d.	13	9	7	n.d.	8
PVR, WU	5.8	8.7	6.9	15	8.4	9.4	n.d.	11	15	15	n.d.	4.66
Q, L/min	5	3	4.8	3	5	5.2	4.87	4.1	4.2	2.8	n.d.	10
Family history	No	Yes	No	No	No	No	No	Yes*	No	No	No	No

Definition of abbreviations: 6MWT = 6-minute-walk test; BMP9 = bone morphogenetic protein type 9; mPAP = mean pulmonary artery pressure; N/A = not applicable; n.d. = not done; PAH = pulmonary arterial hypertension; PAWP = pulmonary artery wedge pressure; PVR = pulmonary vascular resistance; WHO FC = World Health Organization Functional Class; WU = Wood units.

*A relative in the parental generation was affected, but there is not enough information to draw a pedigree.

Table 2. Clinical Characteristics of *BMP2* and *GDF2* Mutation Carriers and Patients without These Mutations

	BMPR2 (*N* = 159)	*GDF2* (*N* = 12)	No Known PAH-Causal Mutation (*N* = 750)	*P* Value	*n*
Sex*				0.357	921
F	105 (66)	7 (58)	530 (71)		
Age, yr†	39 (32–51)	46 (41–54)	52 (39–66)	<0.001	921
Diagnosis*				<0.001	921
HPAH	51 (32.1)	2 (16.7)	7 (0.93)		
IPAH	108 (67.9)	10 (83.3)	743 (99.1)		
WHO FC*				—	895
1	2 (1)	0 (0)	15 (2)		
2	31 (20)	3 (27)	140 (19)		
3	95 (60)	7 (64)	482 (66)		
4	30 (19)	1 (9)	89 (12)		
Ethnicity*				0.328	921
African	2 (1.26)	0 (0.00)	20 (2.67)		
East-Asian	1 (0.63)	0 (0.00)	7 (0.93)		
European	136 (85.5)	9 (75.0)	638 (85.1)		
Finnish-European	0 (0.00)	0 (0.00)	1 (0.13)		
Other	14 (8.81)	1 (8.33)	38 (5.07)		
South-Asian	6 (3.77)	2 (16.7)	46 (6.13)		
mPAP, mm Hg†	58 (52–68)	53 (48–56)	52 (43–61)	<0.001	877
mPAWP, mm Hg†	9 (7–12)	8 (7–10)	9 (7–12)	0.931	780
PVR, WU†	14 (11–20)	10 (9–15)	10 (7–14)	<0.001	754
Q, L/min†	3.3 (2.7–4.0)	4.7 (3.5–4.9)	4.0 (3.3–5.1)	<0.001	842
NO challenge*				<0.001	373
Nonresponder	78 (100)	4 (100)	245 (84)		
Vasoresponder	0 (0)	0 (0)	46 (16)		
Kco, pred.†	82 (74–94)	68 (67–71)	69 (49–84)	<0.001	546
Hb, g/L†	162 (151–173)	140 (132–153)	150 (136–163)	<0.001	702
Hct, L/L†	0.5 (0.5–0.5)	0.4 (0.4–0.4)	0.5 (0.4–0.5)	<0.001	575
WBC, $\times 10^9$/L†	9 (7–10)	7 (7–8)	8 (7–10)	0.013	696
Plt, $\times 10^9$/L†	211 (170–251)	225 (208–256)	228 (185–276)	0.079	693
ALP, IU/L†	75 (62–104)	78 (70–90)	87 (69–113)	0.010	675
Bilirubin, μmol/L†	18 (12–26)	8 (8–13)	14 (10–22)	0.001	671

Definition of abbreviations: ALP = alkaline phosphatase; BMP = bone morphogenetic protein; *BMPR2* = bone morphogenetic protein type 2 receptor; *GDF2* = growth differentiation factor 2 gene, which encodes BMP9; Hb = hemoglobin; Hct = hematocrit; HPAH = heritable pulmonary arterial hypertension; IPAH = idiopathic pulmonary arterial hypertension; mPAP = mean pulmonary artery pressure; mPAWP = mean pulmonary artery wedge pressure; NO = nitric oxide; PAH = pulmonary arterial hypertension; Plt = platelet; pred. = predicted; PVR = pulmonary vascular resistance; WBC = white blood cells; WHO FC = World Health Organization Functional Class.
None of the patients showed features of hereditary hemorrhagic telangiectasia. *P* values represent the overall comparison across the three patient groups.
*Data are presented as *n* (%).
†Data are presented as median (interquartile range).

prodomain, which had no antiapoptotic activity.

Patients with PAH Carrying Pathogenic *GDF2* Alleles Exhibit Reduced Circulating BMP9 and BMP10 Levels and Plasma BMP Activity

Using a BMP9 ELISA protocol validated for lack of cross-reactivity (*see* Figure E6A), spike recovery (*see* Figures E6B–E6G), and plasma assay diluents (*see* Figures E6B–E6H), we measured circulating BMP9 plasma levels and activity in patients carrying *GDF2* mutations. Plasma levels from female patients with PAH who were heterozygous carriers of either benign (Pro: BMP9-G18V, -G74E, -R82G, -P104L, -I118F, -V154I, -D218N, -G291S, -E297K, -T304M, or -R333W) or pathogenic (Pro: BMP9-M89V, -A347V, -Y351H, or -T413N) alleles, were compared with samples from age-matched healthy females. Patients carrying pathogenic missense *GDF2* variants had significantly lower mean plasma BMP9 levels than control subjects, whereas no significant reduction was evident in patients with PAH who carried benign variants (Figure 3A). Similarly, patients with PAH who carried deletions at the *GDF2* locus, or the frameshift mutation, exhibited reduced levels of plasma BMP9.

To determine whether reduced BMP9 levels led to reduced plasma activity, we exposed HMEC1-BRE luciferase cells, which demonstrate high-affinity responses to BMP9 (*see* Figure E7), to plasma (*see* Figure 3B). The BMP activity of plasma followed a similar pattern to measured BMP9 concentrations, such that patients with pathogenic missense mutations or deletions exhibited reduced activity. Following BMP9 immunoprecipitation in control plasma there was reduced activity, confirming that BMP9 is responsible for the majority of plasma BMP activity in this assay.

In addition, we used a pBMP10 ELISA protocol validated for lack of cross-reactivity (*see* Figure E8A), spike recovery, and plasma diluents (*see* Figures E8B–E8D), to measure circulating pBMP10 plasma levels in patients carrying *GDF2* mutations. Remarkably, plasma BMP10 levels were dramatically reduced in BMP9 mutation carriers (*see* Figure 3C). Moreover, plasma BMP9 and pBMP10 levels correlated closely, both in control subjects and in mutation carriers (*see* Figure 3D).

Plasma BMP9 and pBMP10 Levels in Patients with Idiopathic PAH and Heritable PAH

We next measured BMP9 and pBMP10 levels in 120 control samples and 260 cases of heritable PAH or idiopathic PAH (*see* Table E5). Levels of BMP9 and pBMP10 were significantly higher in females than males in both control and PAH groups (Figure 4A). Levels of ligands were not associated with age (*see* Figure E9). No significant differences were observed in the plasma levels of BMP9 between patients with PAH and control subjects of the same sex (*see* Figure 4A). Although no difference in pBMP10 levels was observed between male control subjects and male patients with PAH, pBMP10 levels in females with PAH were significantly lower than control females (*see* Figure 4B). When the BMP9 or pBMP10 levels in patients with PAH and control subjects of each sex were pooled, patients with PAH were overrepresented by twofold in the lowest quartile of either BMP9 or pBMP10 levels, in both males and females (*see* Table E6). Moreover, a larger proportion of male (14/21 for BMP9 and

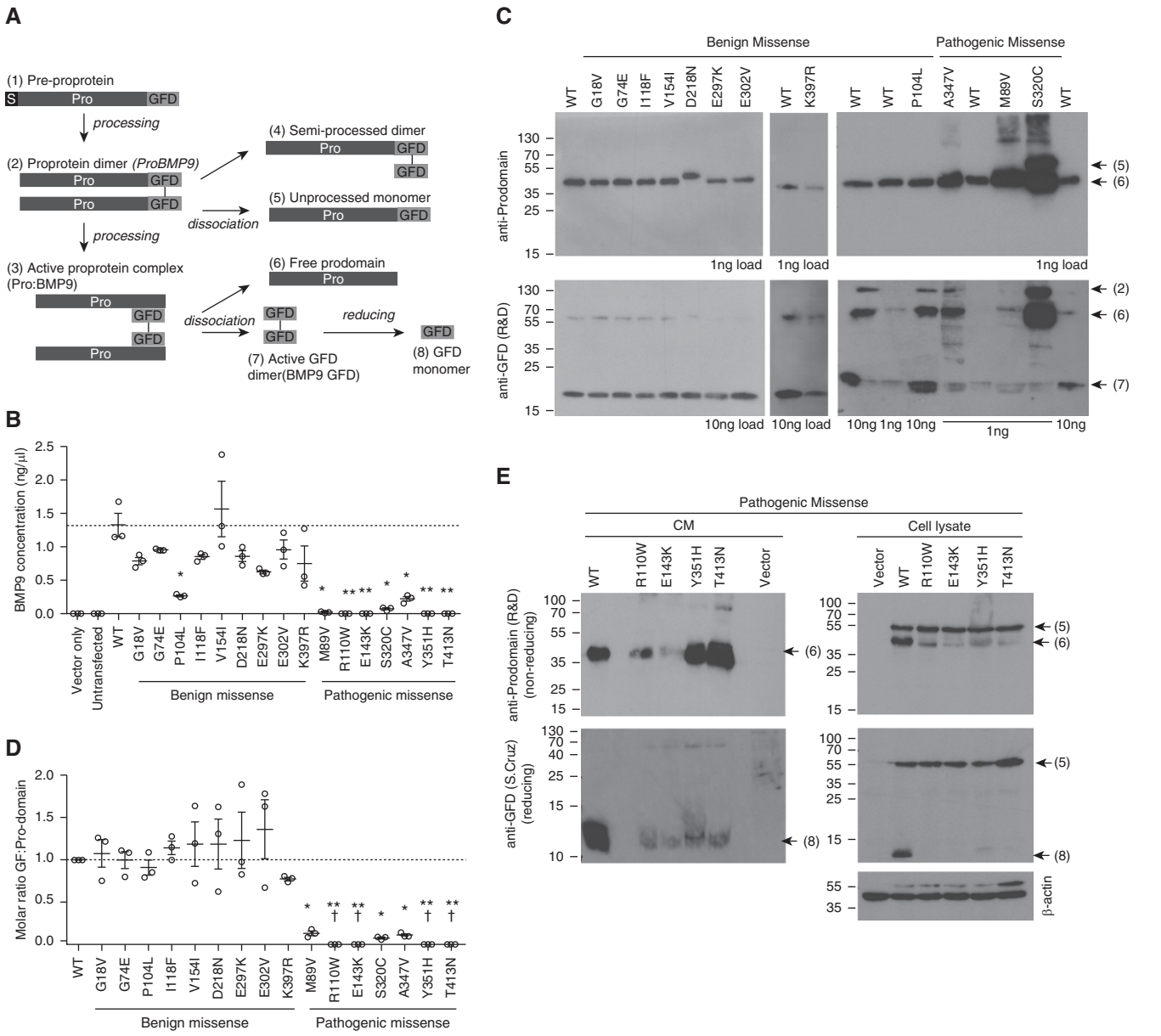

Figure 1. Characterization of expressed BMP9 (bone morphogenetic protein type 9) mutant proteins. (*A*) Schematic of different potential species of BMP9. Numbers in parentheses correspond to labels on western blots. (*B*) Conditioned media from HEK-EBNA cells expressing Pro:BMP9 (prodomain-bound BMP9)–wild-type (WT) or missense Pro:BMP9 variants were serially diluted and assayed for BMP9 GFD (growth factor domain) levels by ELISA. Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test: $^{*}P < 0.05$ and $^{**}P < 0.01$. (*C*) Western blots of variants in conditioned media in nonreducing conditions. The volume loaded was normalized according to concentration based on ELISA. Blots are representative of $n = 3$ separate expression batches. (*D*) The ratio of the band intensities of the growth factor and prodomain for each mutant was normalized to WT. The daggers indicate mutants for which GFD concentration was too low to quantify. Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test: $^{*}P < 0.05$ and $^{**}P < 0.01$. (*E*) Western blotting of those variants not readily detected by ELISA. Cell lysates (30 μg of total protein) and conditioned media (3 μl of Pro:BMP9-WT or 45 μl of Pro:BMP9 mutants and media from the empty vector control) were loaded. CM = conditioned media.

14/20 for pBMP10) and female (38/56) patients with PAH shared both BMP9 and pBMP10 levels in their lowest quartiles compared with control males (1/7) and control females (8/77). We confirmed the reduction of BMP9 in these samples by ELISA and confirmed reduced activity for inducing *ID1* and *ID2* transcription in endothelial cells (*see* Figure 4C). Consistent with our observation in *GDF2* mutation carriers, there was a striking correlation between plasma BMP9 and pBMP10 in control subjects (*see* Figure 4D; Spearman $r = 0.72$; 95% confidence interval [CI], 0.61–0.80; $P < 0.0001$) and in patients with PAH (*see* Figure 4E; Spearman $r = 0.75$; 95% CI, 0.68–0.80; $P < 0.0001$).

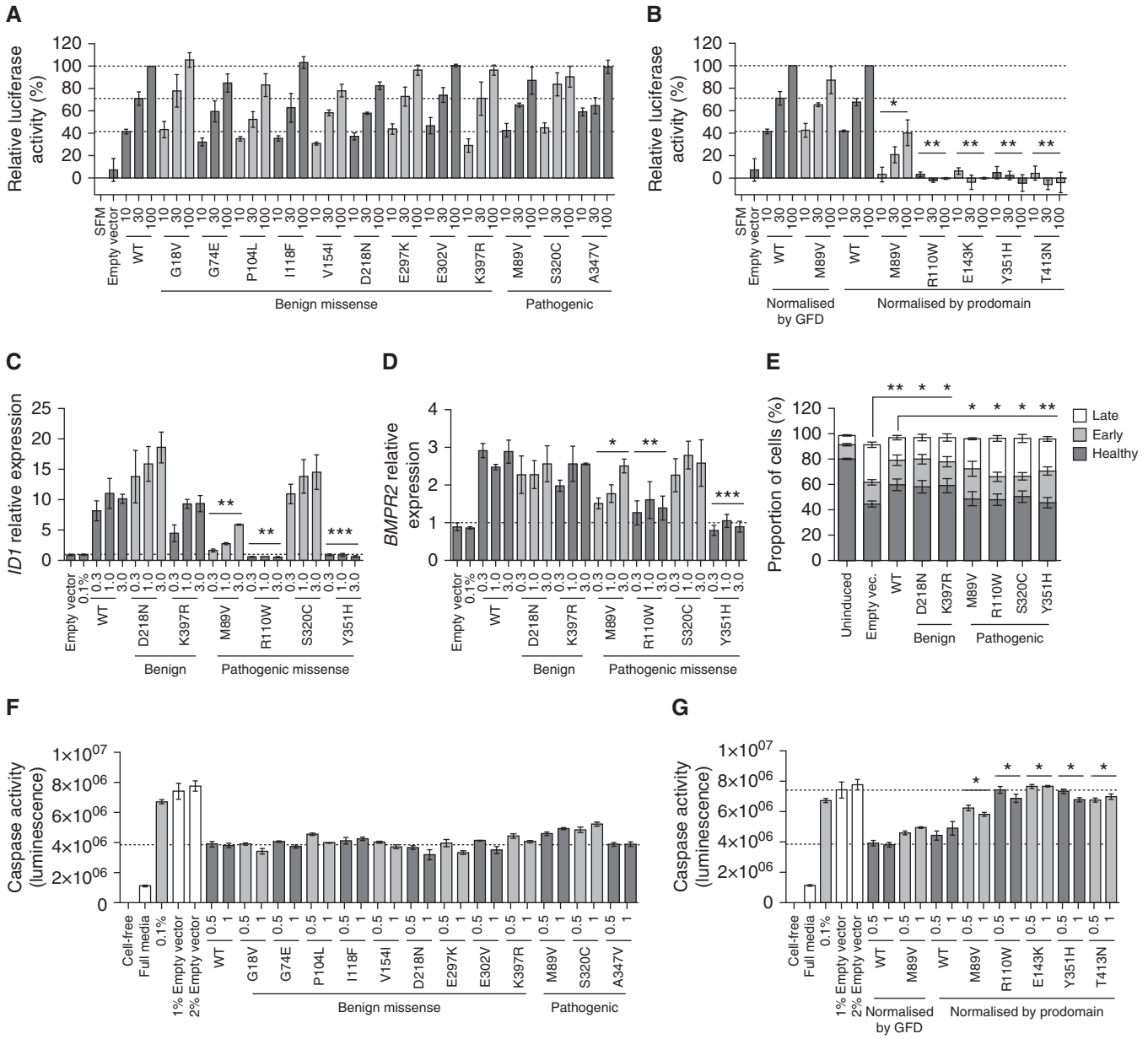

Figure 2. Loss of activity in Pro:BMP9 (prodomain-bound BMP9 [bone morphogenetic protein type 9]) mutants predicted to be pathogenic. (*A* and *B*) C2C12 cells transfected with human ALK1, BRE-*luciferase*, and TK-*Renilla* plasmids were serum-starved, followed by treatment with Pro:BMP9 variants (10, 30, 100 pg/ml) for 6 hours, and luciferase assay. The Firefly:*Renilla* luciferase ratios were calculated for each sample and data normalized, with cells treated with 100 pg Pro:BMP9-WT designated as 100%, and serum-free media–treated cells designated as 0%. Data are mean ± SEM from three independently generated batches of media. van Elteren test: $*P_{adj} < 0.05$ and $**P_{adj} < 0.01$. (*C* and *D*) Transcriptional responses of blood outgrowth endothelial cells to Pro:BMP9-WT and variants based on quantification of the prodomains. Cells were starved in EBM2/0.1% fetal bovine serum (FBS) and then treated with Pro:BMP9-WT and variants (0.5, 1.5, 5 ng/ml) for 4 hours. The expression of (*C*) *ID1* and (*D*) *BMPR2* mRNA were normalized to *B2M* and are expressed as fold change relative to EBM2/0.1% FBS. Data are mean ± SEM from three independently generated batches of media. van Elteren test: $*P_{adj} < 0.05$, $**P_{adj} < 0.01$, and $***P_{adj} < 0.001$. (*E*) Blood outgrowth endothelial cells were treated overnight with 5 ng/ml Pro:BMP9-WT or variants (normalized according to the amount of prodomain) in EBM2/2% FBS followed by induction of apoptosis by addition of TNFα (tumor necrosis factor-α) and cycloheximide for 3.5 hours. Cells were stained with annexin V-FITC and propidium iodide before flow cytometry analysis. Cells were assigned as healthy (AV^-/PI^-), early apoptotic (AV^+/PI^-), or late apoptotic/necrotic (AV^+/PI^+). Data are the mean ± SEM from three independently generated batches of media. Paired Dunnett's multiple comparisons test: $*P < 0.05$ and $**P < 0.01$. (*F* and *G*) Pulmonary artery endothelial cells were starved overnight in EBM2/0.1% FBS to induce apoptosis in the presence of Pro:BMP9-WT or variants (0.5, 1 ng/ml). Each BMP9 treatment was supplemented with conditioned media from HEK-EBNA cells transfected with empty vector to equalize the total volume of conditioned media per well (2% vol/vol for 1 ng/ml, 1% vol/vol for 0.5 ng/ml). Apoptosis was measured by Caspase-GLO 3/7 assays. Data are mean ± SEM from three independent batches of media. van Elteren test: $*P_{adj} < 0.05$. GFD = growth factor domain; SFM = serum-free media; WT = wild type.

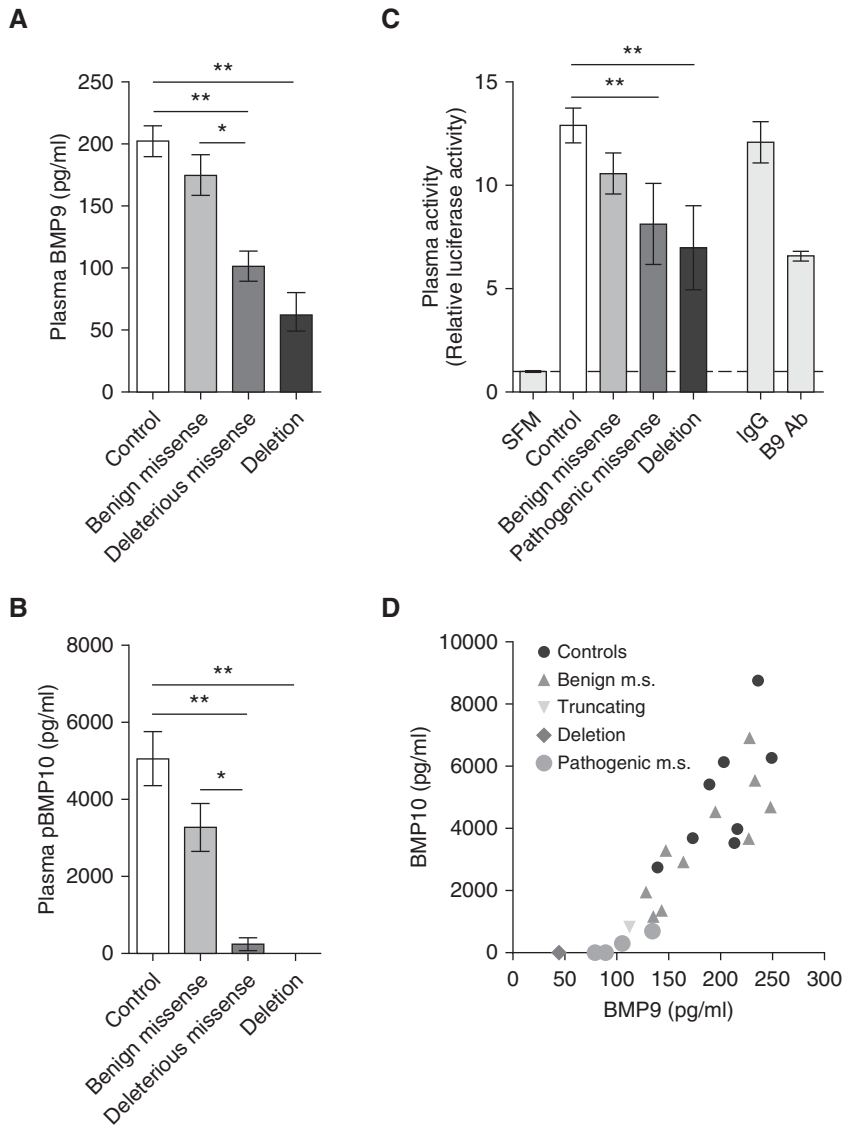

Figure 3. Loss of active BMP9 (bone morphogenetic protein type 9) in patients with pulmonary arterial hypertension who carry putatively pathogenic *GDF2* alleles. (*A*) BMP9 GFD (growth factor domain) concentration in ethylenediaminetetraacetic acid–plasma from diseased, *GDF2* heterozygous females and healthy age-matched females was measured by ELISA. Data are mean ± SEM; one-way ANOVA: $^{*}P < 0.05$ and $^{**}P < 0.01$. (*B*) HMEC1-BRE cells quiesced in serum-free media overnight were treated with 5% plasma for 6 hours before luciferase activity was assessed. Data are mean ± SEM; *t* test, $^{*}P < 0.05$ and $^{**}P < 0.01$. (*C*) Identical to *A* except for pBMP10 concentration. $^{**}P < 0.01$. (*D*) The concentration of pBMP10 plotted against the concentration of BMP9 GFD in ethylenediaminetetraacetic acid–plasma. Data are mean ± SEM; one-way ANOVA: $^{*}P < 0.05$ and $^{**}P < 0.01$. There were 8 healthy control subjects, 11 benign missense (m.s.) carriers, 4 pathogenic m.s. carriers, and 3 deletion carriers (one of which is an early truncation). B9 Ab = anti-BMP9 antibody; SFM = serum-free media.

We hypothesized that a physical association between BMP9 and pBMP10 could explain the correlation in measured levels. Immunoprecipitation of BMP9 and BMP10 from control samples, followed by ELISA and activity measurements, demonstrated that a proportion of circulating BMP9 and pBMP10 are physically associated (*see* Figure E10). Because most circulating activity was associated with BMP9 but measured levels of pBMP10 were much higher than levels of BMP9, we hypothesized that endogenous pBMP10 is predominately unprocessed *ProBMP10*. Therefore, we compared the immunoreactivity of purified *ProBMP10* and Pro:BMP10 in the pBMP10 and BMP10 GFD ELISAs (*see* Figures E11A and E11B). This demonstrated that the pBMP10 ELISA detects unprocessed and processed pBMP10, whereas the BMP10 GFD ELISA only detects processed Pro:BMP10. In control samples, very little endogenous processed Pro:BMP10 was detectable with the BMP10 GFD ELISA, despite efficient spike recovery, which suggests that endogenous pBMP10 is indeed unprocessed (*see* Figure E11C).

Associations between BMP9 and BMP10 Levels and Clinical Parameters

We compared clinical characteristics between BMP9 (*see* Table E7) and pBMP10 (*see* Table E8) tertiles, and also undertook correlation analysis for relationships (continuous variables) between ligand concentrations and clinical parameters. In patients with PAH, BMP9 and pBMP10 levels were not associated with exercise capacity measured by the 6-minute-walk test ($r = -0.045$; 95% CI, −0.22 to 0.13; $P = 0.634$ and $r = 0.15$; 95% CI, −0.032 to 0.32; $P = 0.106$, respectively). Hemodynamics contemporary to the time of sampling were available for 38 patients but did not show any correlation with BMPs. Of note, BMP9 and pBMP10 levels negatively correlated with body mass index (BMI) in PAH cases but not in control subjects (*see* Figure E12).

Analysis of clinical blood tests in the PAH cohort revealed that pBMP10 negatively correlated with red cell distribution width ($r = -0.21$; 95% CI, −0.35 to −0.06; $P = 0.006$) and alkaline phosphatase activity ($r = -0.16$; 95% CI, −0.28 to −0.03; $P = 0.014$) and positively with albumin concentration ($r = 0.32$; 95% CI, 0.20 to 0.43; $P < 0.001$), whereas BMP9 levels correlated positively with platelet count ($r = 0.18$; 95% CI, 0.05 to 0.30; $P = 0.006$). Both BMP9 and pBMP10 correlated negatively with CRP (C-reactive protein) ($r = -0.32$; 95% CI, −0.45 to −0.18; $P < 0.001$ and $r = -0.35$; 95% CI, −0.47 to −0.20; $P < 0.001$, respectively). Binary logistic regression showed that plasma pBMP10 concentrations were significantly negatively associated with systemic hypertension, even after controlling for BMI and sex in the PAH cohort. When pBMP10 levels were in the lower tertile, the log odds ratios of suffering from systemic hypertension were −1.30 (95% CI, −2.21 to −0.49). Low pBMP10 concentrations were also predictive of

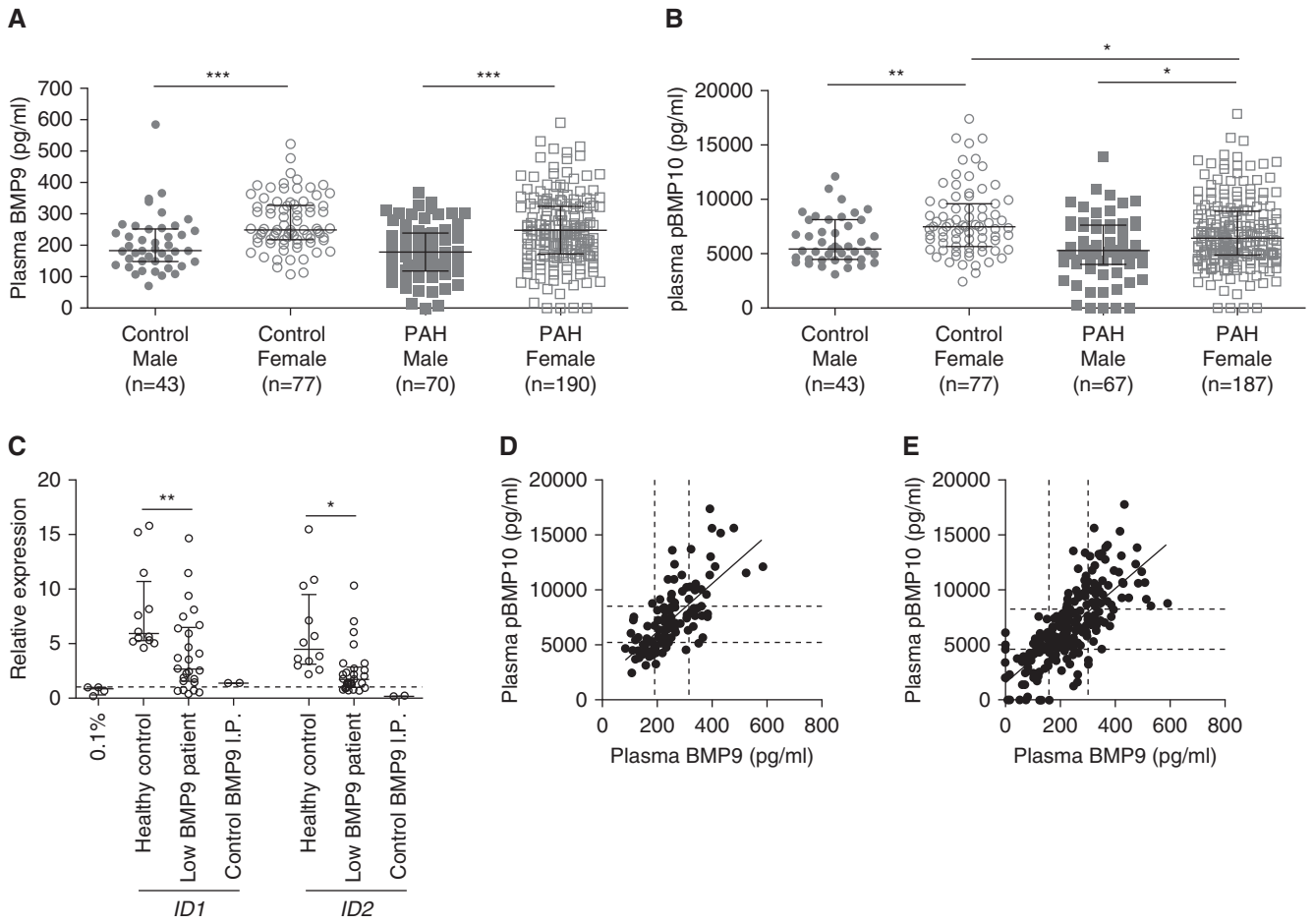

Figure 4. Plasma BMP9 (bone morphogenetic protein type 9) and pBMP10 levels are not reduced in pulmonary arterial hypertension (PAH), but a subset of patients with PAH exhibit reduced plasma BMP9 and pBMP10 levels. Plasma samples collected from control subjects and patients with PAH were assayed for (*A*) BMP9 and (*B*) pBMP10. Data are presented as median ± interquartile ranges (Kruskal-Wallis test: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (*C*) Human aortic endothelial cells quiesced in 0.1% basal media were incubated with 3% ethylenediaminetetraacetic acid–plasma for 1 hour. Expression of *ID* mRNA was measured by quantitative PCR and normalized to *B2M* and data expressed as fold change relative to the EBM2/0.1% fetal bovine serum control (mean ± SEM; *t* test, $^{*}P < 0.05$ and $^{**}P < 0.01$). (*D* and *E*) Plasma BMP9 levels were plotted against plasma pBMP10 levels in control individuals ($n = 120$) (*D*) and patients with PAH ($n = 187$) (*E*). Dashed lines represent 25th and 75th percentiles. Spearman correlation is shown by a solid regression line.

diabetes mellitus type 2, but this association disappeared after controlling for BMI and sex. Receiver operating characteristics curves (*see* Figure E13) suggested that the cut-off values of 6,784.7 pg/ml (specificity, 46.3%; sensitivity, 79.2%; area under the curve [AUC], 63.8%) and 6,180.1 pg/ml (specificity, 51.9%; sensitivity, 78.9%; AUC, 66%) (data for PAH) were the most discriminative to predict systemic hypertension and diabetes mellitus, respectively. However, the AUC was low for both diseases, indicating a modest predictive capacity. Circulating BMP9 levels were not associated with either diabetes mellitus or systemic hypertension.

During the median follow-up of 4.4 years since sampling, 48 (19%) patients with PAH died, resulting in a 1 and 3 years overall survival of 95% and 86%, respectively. Neither BMP9 nor pBMP10 concentrations were predictive of mortality (*see* Figure E14).

Discussion

In this study we provide a detailed functional analysis of rare potentially damaging mutations in *GDF2* identified in a large European cohort of patients with PAH. The majority of mutations led to altered cellular processing of the mature protein and reduced cellular secretion. The mutations were associated with reduced circulating levels of BMP9 in these individuals and reduced plasma BMP9 activity. These findings support a causal role for these mutations in the pathobiology of PAH and provide strong evidence in humans that reduced BMP9 levels and activity promote the development of PAH. In addition, the finding of two patients with deletions at the *GDF2* locus provides new genetic evidence supporting a causal role in PAH.

Having identified several likely causal missense *GDF2* variants based on *in silico* analyses, we went on to functionally characterize these variants. Our observations confirm that the Pro:BMP9-M89V, -R110W, -E143K, -S320C, -A347V, -Y351H, and -T413N mutations exhibit impaired BMP9 processing, secretion, or stability. Those mutants predicted to be benign functioned normally *in vitro*, but the common Pro:BMP9-D218N variant,

predicted to be deleterious *in silico*, was functionally normal. One exception was Pro:BMP9-P104L, which was predicted to be deleterious but functional analysis suggested it is likely to be benign. This variant was secreted less efficiently from transfected cells but was not disrupted to the same extent as the pathogenic mutants, such that it exhibited normal processing and signaling, and the individual carrying this variant had normal plasma levels of BMP9. The clustering of pathogenic mutations around the interface between the BMP9 growth factor and prodomains suggests that destabilization of the BMP9 protein, exemplified by the Pro:BMP9-M89V variant, is an important mechanism. The S320C variant, which is altered at the prohormone cleavage site, exhibits a processing cleavage defect, confirming a recent study of the same mutation (11). These results highlight the importance of careful characterization of missense variants before pathogenicity can be confirmed.

Two recent studies independently validated the original finding of heterozygous *GDF2* mutations in PAH cohorts (11, 21). A single previous case report had identified a case of childhood-onset PAH harboring a homozygous *GDF2* truncating mutation (p.Q26X) (23). In a Chinese PAH cohort, the authors measured plasma BMP9 by ELISA in 19 *GDF2* mutation carriers compared with age-matched and sex-matched patients with idiopathic PAH and healthy control subjects (11). Median plasma BMP9 levels were significantly reduced in patients with idiopathic PAH and were lowest in patients carrying *GDF2* mutations. In our larger analysis of a cohort of control subjects and patients with idiopathic PAH, we did not observe a significant difference in overall BMP9 levels between control subjects and patients with idiopathic PAH. However, we observed that some patients with idiopathic PAH (approx. 5.4%) have very low BMP9 levels, unexplained by the presence of mutations, with corresponding reduced plasma BMP activity on endothelial cells. A recent study found that patients with portopulmonary hypertension have profound reductions in plasma BMP9 and that low levels predict the presence of PAH in patients with cirrhosis (24). Liver disease was specifically excluded in the patients with idiopathic PAH recruited to our cohort. Taken together, the genetic and nongenetic evidence in humans suggests that loss of BMP9 levels, or BMP9 signaling, is an important driver for the development of PAH. The observed difference between plasma levels of BMP9 (and pBMP10) in males and females is of interest, and the underlying mechanism for this difference warrants further study. Although higher levels in females superficially conflicts with the observation that idiopathic and heritable PAH more commonly affects females, the prognosis of PAH is usually worse in male patients.

To our knowledge, ours is the first study to measure plasma levels of BMP10 in patients with PAH, or indeed in a large cohort of control subjects. Overall, levels of pBMP10 were significantly lower in female patients with PAH compared with control subjects. Remarkably, we observed a close correlation between the levels of BMP9 and BMP10 in human plasma, suggesting a degree of coregulation that might be explained, at least in part, by the presence of circulating heterodimers of these ligands (15). This is important because *GDF2* mutations leading to reduced secretion of ligand would be predicted to impact the circulating levels of BMP10, as well as BMP9, as observed in this study. We confirmed by immunoprecipitation that a proportion of BMP9 and pBMP10 are physically associated in the same complex. However, we measured levels of pBMP10 by ELISA that were much higher than BMP9, which suggests the association is not 1:1. Additionally, only about half of the plasma BMP9 and pBMP10 were in complex, implying that BMP9:10 heterodimers cannot represent the only active form. Intriguingly, we detected appreciable levels of circulating processed Pro:BMP9 by ELISA, associated with high activity on cells but minimal circulating processed Pro:BMP10. This suggests that the majority of circulating pBMP10 is unprocessed. The potentially important role of BMP10 in PAH is further supported by the finding of two individuals with likely pathogenic missense mutations, providing independent validation of the previous report of *BMP10* mutations from France (21). Unfortunately, plasma was not available from these individuals for further analysis.

The human data presented here are important because a recent study in rodents suggested that inhibition of BMP9 signaling might be protective against the development of pulmonary hypertension (25). *Gdf2* knockout mice do not develop spontaneous pulmonary hypertension, but the authors found that *Gdf2* knockout mice were protected from the development of hypoxia-induced pulmonary hypertension. They went on to show that inhibition of Bmp9 signaling with a neutralizing anti-BMP9 antibody could also slightly prevent the development of pulmonary hypertension during chronic hypoxia. Their results directly conflict with another study showing that inhibition of BMP9 and BMP10 with Fc-ALK1 exacerbated pulmonary hypertension during chronic hypoxia (24). Taken together, the human genetic evidence (10, 11, 21, 23) and data from patients with cirrhosis (24) support the view that BMP9 and BMP10 are protective to the pulmonary vascular endothelium, consistent with the beneficial effects of therapeutic supplementation of BMP9 in preclinical models (12).

Heterozygous *GDF2* mutations have been identified previously in a small number of patients with a vascular anomaly syndrome resembling HHT (26, 27). The mutations in patients with HHT were distinct from those reported in PAH but were also predicted to lead to altered cellular processing. The finding of mutations in *GDF2* in both HHT-like syndromes and PAH is perhaps not surprising given that mutations in the endothelial receptor for BMP9, ALK1, can also cause either HHT or PAH (28). Of note, none of the patients carrying *GDF2* mutations in the present study had clinical features of HHT.

Conclusions

The present study demonstrates that rare heterozygous mutations in *GDF2* are loss of function and likely causal in the pathobiology of PAH. Furthermore, *GDF2* mutations lead to reduced circulating levels of BMP10, as well as BMP9, supporting a degree of coregulation of these ligands. Taken together, these findings further support the central role of the BMP9 / BMPR2 /ALK1 axis in PAH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank all the patients and their families who contributed to this research. They also thank the National Institute for Health Research BioResource–Rare Diseases staff and coordination teams at the University of Cambridge. They also thank the research nurses and coordinators at the specialist pulmonary hypertension centers involved in this study.

References

1. Farber HW, Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med* 2004;351:1655–1665.
2. Tuder RM, Archer SL, Dorfmüller P, Erzurum SC, Guignabert C, Michelakis E, *et al*. Relevant issues in the pathology and pathobiology of pulmonary hypertension. *J Am Coll Cardiol* 2013;62(Suppl):D4–D12.
3. Thenappan T, Ormiston ML, Ryan JJ, Archer SL. Pulmonary arterial hypertension: pathogenesis and clinical management. *BMJ* 2018;360: j5492.
4. Evans JDW, Girerd B, Montani D, Wang X-J, Galiè N, Austin ED, *et al*. *BMPR2* mutations and survival in pulmonary arterial hypertension: an individual participant data meta-analysis. *Lancet Respir Med* 2016;4: 129–137.
5. Morrell NW, Aldred MA, Chung WK, Elliott CG, Nichols WC, Soubrier F, *et al*. Genetics and genomics of pulmonary arterial hypertension. *Eur Respir J* 2019;53:1801899.
6. Soubrier F, Chung WK, Machado R, Grünig E, Aldred M, Geraci M, *et al*. Genetics and genomics of pulmonary arterial hypertension. *J Am Coll Cardiol* 2013;62(Suppl):D13–D21.
7. Machado RD, Southgate L, Eichstaedt CA, Aldred MA, Austin ED, Best DH, *et al*. Pulmonary arterial hypertension: a current perspective on established and emerging molecular genetic defects. *Hum Mutat* 2015;36:1113–1127.
8. Austin ED, Ma L, LeDuc C, Berman Rosenzweig E, Borczuk A, Phillips JA III, *et al*. Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. *Circ Cardiovasc Genet* 2012;5:336–343.
9. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, *et al*. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med* 2013;369:351–361.
10. Gräf S, Haimel M, Bleda M, Hadinnapola C, Southgate L, Li W, *et al*. Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat Commun* 2018;9:1416.
11. Wang XJ, Lian TY, Jiang X, Liu SF, Li SQ, Jiang R, *et al*. Germline *BMP9* mutation causes idiopathic pulmonary arterial hypertension. *Eur Respir J* 2019;53:1–10.
12. Long L, Ormiston ML, Yang X, Southwood M, Gräf S, Machado RD, *et al*. Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nat Med* 2015;21:777–785.
13. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* 2007;109:1953–1961.
14. Bidart M, Ricard N, Levet S, Samson M, Mallet C, David L, *et al*. BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. *Cell Mol Life Sci* 2012;69:313–324.
15. Tillet E, Ouarné M, Desroches-Castan A, Mallet C, Subileau M, Didier R, *et al*. A heterodimer formed by bone morphogenetic protein 9 (BMP9) and BMP10 provides most BMP biological activity in plasma. *J Biol Chem* 2018;293: 10963–10974.
16. Jiang H, Salmon RM, Upton PD, Wei Z, Lawera A, Davenport AP, *et al*. The prodomain-bound form of bone morphogenetic protein 10 is biologically active on endothelial cells. *J Biol Chem* 2016; 291:2954–2966.
17. David L, Mallet C, Keramidas M, Lamandé N, Gasc JM, Dupuis-Girod S, *et al*. Bone morphogenetic protein-9 is a circulating vascular quiescence factor. *Circ Res* 2008;102:914–922.
18. Swietlik E, Hodgson J, Hadinnapola C, Bleda M, Haimel M, Church C, *et al*. S40 phenotypic characterisation of *GDF2* mutation carriers in a large cohort of patients with pulmonary arterial hypertension [abstract]. *Thorax* 2018;73:A24–A26.
19. Hodgson J, Swietlik E, Salmon R, Hadinnapola C, Wharton J, Haimel M, *et al*. S41 characterisation of mutations in the gene encoding growth and differentiation factor 2 (*GDF2*) in patients with pulmonary arterial hypertension [abstract]. *Thorax* 2018; 73:A26.
20. Rhodes CJ, Ghataorhe P, Wharton J, Rue-Albrecht KC, Hadinnapola C, Watson G, *et al*. Plasma metabolomics implicates modified transfer RNAs and altered bioenergetics in the outcomes of pulmonary arterial hypertension. *Circulation* 2017;135:460–475.
21. Eyries M, Montani D, Nadaud S, Girerd B, Levy M, Bourdin A, *et al*. Widening the landscape of heritable pulmonary hypertension mutations in paediatric and adult cases. *Eur Respir J* 2019;53: 1801371.
22. Toshner M, Dunmore BJ, McKinney EF, Southwood M, Caruso P, Upton PD, *et al*. Transcript analysis reveals a specific HOX signature associated with positional identity of human endothelial cells. *PLoS One* 2014;9:e91334.
23. Wang G, Fan R, Ji R, Zou W, Penny DJ, Varghese NP, *et al*. Novel homozygous BMP9 nonsense mutation causes pulmonary arterial hypertension: a case report. *BMC Pulm Med* 2016;16:17.
24. Nikolic I, Yung L-M, Yang P, Malhotra R, Paskin-Flerlage SD, Dinter T, *et al*. Bone morphogenetic protein 9 is a mechanistic biomarker of portopulmonary hypertension. *Am J Respir Crit Care Med* 2019;199: 891–902.
25. Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C, *et al*. Selective BMP-9 inhibition partially protects against experimental pulmonary hypertension. *Circ Res* 2019;124:846–855.
26. Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL, *et al*. BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 2013;93: 530–537.
27. Hernandez F, Huether R, Carter L, Johnston T, Thompson J, Gossage JR, *et al*. Mutations in *RASA1* and *GDF2* identified in patients with clinical features of hereditary hemorrhagic telangiectasia. *Hum Genome Var* 2015;2:15040.
28. Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, *et al*. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *N Engl J Med* 2001;345:325–334.