Erythrocytosis and Pulmonary Hypertension in a Mouse Model of Human HIF2A Gain-of-Function Mutation*

Qiulin Tan,1 Heddy Kerestes,1 Melanie J. Percy,2 Ralph Pietrofesa,3 Li Chen,4 Tejvir S. Khurana,5 Melpo Christofidou-Solomidou,3 Terence R.J. Lappin,6 and Frank S. Lee1

From the 1Department of Pathology and Laboratory Medicine, 3Divisions of Pulmonary, Allergy and Critical Care, and 4Cardiovascular Medicine, Department of Medicine, and 5Department of Physiology and Pennsylvania Muscle Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, 2Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland, United Kingdom BT9 7AB, and 6Centre for Cancer Research and Cell Biology, Queen’s University, Belfast, Northern Ireland, United Kingdom BT9 7BL

Running Title: Mouse Model of Human HIF2A Mutant Erythrocytosis

To whom correspondence should be addressed: Frank S. Lee, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, 605 Stellar Chance Labs, 422 Curie Blvd., Philadelphia, PA 19104. Tel: 215-898-4701; Fax: 215-573-2272; E-mail: franklee@mail.med.upenn.edu

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**Background:** Missense mutations have been identified in the HIF2A gene in patients with erythrocytosis.

**Results:** A mouse knockin line that models the first described HIF2A mutation exhibits erythrocytosis and pulmonary hypertension.

**Conclusion:** The missense mutation is the cause of erythrocytosis, and is accompanied by pulmonary hypertension.

**Significance:** The study demonstrates sequelae of global Hif-2α gain of function.

**SUMMARY**

The central pathway by which changes in oxygen concentration are transduced to changes in red cell mass is the Prolyl Hydroxylase Domain protein (PHD):Hypoxia Inducible Factor (HIF) pathway. PHD site-specifically prolyl hydroxylates the transcription factor HIF-α, thereby targeting the latter for degradation. Under hypoxia, this modification is attenuated, allowing stabilized HIF-α to activate target genes, including that for Erythropoietin (EPO). Studies employing genetically modified mice point to Hif-2α, one of two main Hif-α isoforms, as being the critical regulator of Epo in the adult mouse. More recently, erythrocytosis patients with heterozygous point mutations in the HIF2A gene have been identified; whether these mutations were polymorphisms unrelated to the phenotype could not be ruled out. In the present report, we characterize a mouse line bearing a G536W missense mutation in the Hif2a gene that corresponds to the first such human mutation identified (G537W). We obtained mice bearing both heterozygous and homozygous mutations at this locus. We find that these mice display, in a mutation dose-dependent manner, erythrocytosis and pulmonary hypertension with a high degree of penetrance. These findings firmly establish missense mutations in HIF-2α as a cause of erythrocytosis, highlight the importance of this HIF-α isoform in erythropoiesis, and point to physiologic consequences of HIF-2α dysregulation.

Red cell mass is highly responsive to changes in and inversely related to oxygen concentration. The central pathway by which changes in oxygen concentration are transduced to changes in red cell mass is the
PHD:HIF pathway\(^7\) (1-3). In this pathway, PHD prolyl hydroxylates HIF-\(\alpha\) in an oxygen-dependent manner (4-6). There are three PHD isoforms, PHD1, PHD2, and PHD3, and there are two main HIF-\(\alpha\) isoforms, HIF-1\(\alpha\) and HIF-2\(\alpha\). The primary site of prolyl hydroxylation is Pro-564 in HIF-1\(\alpha\), Pro-531 in HIF-2\(\alpha\). This distinctive posttranslational modification allows the binding of the von Hippel Lindau protein (VHL). VHL is a component of an E3 ubiquitin ligase that targets hydroxylated HIF-\(\alpha\) for degradation by the proteasome. Under hypoxic conditions, this modification is arrested, allowing the stabilization of HIF-\(\alpha\) and activation of HIF target genes.

The prototypical HIF target gene is the \(EPO\) gene (3,7). Low tissue oxygenation results in activation of the HIF pathway and increased \(EPO\) transcription, leading to increased circulating levels of \(EPO\). \(EPO\) then binds to the \(EPO\) receptor (EPOR) on erythroid precursors, resulting in expansion of red cell mass and producing an increase in the oxygen carrying capacity of the blood. The kidney is the primary organ source of \(EPO\) in adults, although the liver, brain, and bone are other potential sources (8-10).

Erythrocytosis is an uncommon condition characterized by abnormally high red cell mass, and it has provided an opportunity to gain insight into oxygen-sensing pathway in humans (2). The first mutation identified in this pathway was the Chuvash mutation in the \(VHL\) gene (R200W) (11). This hypomorph mutation leads to an impaired ability of VHL to degrade hydroxylated HIF-\(\alpha\). The second mutation identified in this pathway was a missense mutation in the \(PHD2\) gene (12). Subsequent studies in mice have demonstrated a central role for \(Phd2\) in erythropoiesis (13,14).

The particular isoform of HIF-\(\alpha\) regulating EPO has been a topic of substantial investigation. In mouse embryo and yolk sac, \(Hif-1\alpha\) is essential for proper signaling through the \(Epo/EpoR\) axis (15). In adult mice, studies employing genetically modified mice have identified Hif-2\(\alpha\) as being the critical isoform (16-18). These studies lead to the question of whether human mutations in \(HIF\) genes might be associated with erythrocytosis. We recently identified a heterozygous gain of function missense mutation in the \(HIF2A\) gene associated with erythrocytosis (19). This mutation, G537W, is in the vicinity of the primary site of hydroxylation. In vitro, the mutation impairs the ability of HIF-2\(\alpha\) to be hydroxylated by PHD2 and to then be recognized by VHL. Multiple, additional mutations in the \(HIF2A\) gene associated with erythrocytosis have subsequently been identified (20-23). These mutations are all heterozygous missense mutations that change amino acids that are in close proximity to the primary site of hydroxylation, Pro-531. It remains conceivable that these mutations are polymorphisms unrelated to the phenotype. Therefore, in the present report, we generated a \(Hif2a\) knockin mouse to model the original \(HIF2A\) mutation. This model provides an opportunity to not only test whether a single amino acid change in Hif-2\(\alpha\) is sufficient to induce erythrocytosis, but also examine whether there are other sequelae from this global dysregulation of Hif-2\(\alpha\) function.

**EXPERIMENTAL PROCEDURES**

*Gene targeting*—The construct for generating a G536W mutation in the mouse \(Hif2a\) gene was prepared by recombineering (24). In brief, a minitargeting vector was constructed in the vector pL452 (25). This minitargeting vector contained genomic DNA encompassing exon 12 of the mouse \(Hif2a\) gene with nucleotide changes encoding for the G536W mutation. A silent, diagnostic Xho I site was introduced upstream of the mutation. The vector contained a neomycin selection cassette flanked by loxP sites, and additional sequences downstream of exon 12. A retrieval plasmid was constructed in the vector pMC1-DTA (26). This retrieval plasmid contained sequences that flank 12 kb of genomic DNA sequence at the mouse \(Hif2a\) locus, as well as a diptheria toxin A negative selection cassette. This retrieval plasmid was used to capture, by
recombineering, 12 kb of mouse Hif2a genomic DNA containing exons 8 to 16 from C57BL/6 bacterial artificial chromosome (BAC) clone RP23-106L19 (Invitrogen). The resulting product was used, in the second recombineering step with the minitargeting vector, to generate the final targeting vector. This targeting vector contains an 8.6 kb 5’ arm containing exon 12 with the G536W knockin mutation, a neomycin selection cassette flanked by loxP sites, and a 3.3 kb 3’ arm (Figure 1). The presence of the knockin mutation as well as the integrity of all exons was confirmed by DNA sequencing.

C57BL/6 ES cells were electroporated with the targeting vector and selected using G418 by Caliper Life Sciences. Screening was performed by Southern blotting. Of 192 clones, 9 potential targeted clones were identified. Sequencing of five of these clones revealed that all five had the G536W heterozygous mutation. Two of these clones were injected into C57BL/6 blastocysts to produce chimeras. Chimeric male mice were then mated with C57BL/6 female mice, and germline transmission (assessed by PCR and Southern blotting) was obtained with chimeras derived from both of the targeted ES clones. Mice with germline transmission of the knockin allele were then mated with C57BL/6-Gt(ROSA)26Sortm16(Cre)Arte mice (Taconic) to delete the neomycin cassette, followed by further crossing with C57BL/6 mice to segregate the Cre allele, thereby creating Hif2aG536W/+ mice. These mice were maintained in a C57BL/6 background.

All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania in compliance with Animal Welfare Assurance.

Southern blotting—Digoxigenin-labeled probes were generated by PCR using a PCR DIG Probe Synthesis Kit (Roche). For the 5’ probe (0.7 kb), the primers were 5’-GTG TGC ATG CGG GAG AGC CT-3’ and 5’-TGA GCT CGA GCT CTC CCG ACT GTA-3’. For the 3’ probe (0.45 kb), the primers were 5’-CTA AAA GCT TAG AAA TGG GAT CTT GC-3’ and 5’-TTA TGA ATT CCC ATT AAA AAC ATT TC-3’. For both probes, DNA derived from BAC clone RP23-106L19 was employed as the template. Southern blotting was performed using DIG Easy Hyb, DIG wash and block buffer set, anti-digoxigenin-AP conjugates, and CDP-Star substrate (Roche).

PCR genotyping—DNA was isolated from mouse tails (27). The following primers were employed for genotyping the G536W knockin mutation: Hex12-2 5’ = 5’-GTC TTC CAT CTT TGA TGC TGG-3’ and Hint12-3 3’ = 5’-ACA CAT CAG CTT ATG GGA CTG CAG-3’. The wild type allele produces a PCR product of 0.60 kb, whereas the knockin mutant allele produces a PCR product of 0.66 kb.

Hematologic, Epo, and Vegf measurements—Hematocrit, hemoglobin, and complete blood counts were measured on retroorbital blood samples as previously described (28). Serum was obtained by centrifugation at 2,000 x g for 20 minutes. Serum Epo and Vegf-a levels were measured using ELISA kits (R & D Systems).

Real Time PCR analysis—Total RNA was isolated from kidney, liver, lung, bone, and brain tissues by using Trizol reagent. One µg of total RNA was used for reverse transcription. Twenty ng equivalents of cDNA was used for SYBR Green Real Time PCR analysis. The sequences of the primers employed for Real Time PCR analysis are provided in the Table 1. Relative quantification was performed employing the ΔΔCt method and 18S RNA as the endogenous control.

Histologic analysis—Lungs were inflated by injection of 10% buffered formalin through the trachea and placed in 10% buffered formalin for fixation, and then embedded in paraffin. Immunohistochemistry was performed by standard protocols. After deparaffinization/rehydration and antigen retrieval, the slides were stained with rabbit anti-smooth muscle α-actin (α-SMA) (1:500, Genetex cat no. 100034) or anti-Proliferating Cell Nuclear Antigen (PCNA) (1:200,
Genetex cat no. 100539), biotinylated goat anti-rabbit antibody (1:200, BD Pharmingen), and streptavidin-horseradish peroxidase (BD Pharmingen).

Vascular changes in pulmonary arteries identified in α-SMA–stained lung tissues were assessed by percent medial thickness (%MT) as previously described (29). The medial thickness and the external diameter were measured by using NIH ImageJ software (http://imagej.nih.gov/ij/). The %MT was calculated for at least 40 small pulmonary arteries (from 20 µM to 80 µM) for each mouse. Circular or near circular vessels were chosen for analysis.

Vessel muscularity was determined using α-SMA–stained lung sections, with at least 60 small pulmonary arteries scored per animal. The number of fully muscular (completely circumferential α-SMA staining), partially muscular (partially circumferential α-SMA staining) or non-muscular (no α-SMA staining) vessels were counted and the percentage of each category calculated. For PCNA immunostaining, 12 - 20 small pulmonary arteries were analyzed per mouse.

Pulmonary function tests—Pulmonary function was assessed by plethysmography. The mice were placed into a 150 ml whole body plethysmograph connected to a pneumotachometer and the difference of pressure integrated in a Spirometer (ADInstruments). The O₂ concentration in the chamber was controlled by Pegas-400 MF Gas Mixer (Columbus Instruments). Respiration data for mice under normoxia (21% O₂) or hypoxia (12% O₂) were recorded using a PowerLab 8/SP instrument (ADInstruments) and analyzed with LabChart 6 software (ADInstruments). Ventilatory frequency (f), tidal volume V₆ (µl), and minute ventilation Vₑ (µl/min) were measured. Calibration was performed according to the manufacturer’s manual.

Arterial blood gas measurements. Blood from the carotid artery was drawn into a heparin-lined syringe and analyzed using an i-STAT instrument equipped with G3+ cartridges (Abbott Laboratories). Blood oxygen saturation (SaO₂) measurements were obtained on awake (unanesthetized) animals using a non-invasive pulse oximeter collar (Starr Life Sciences) placed over the carotid arteries of the mice. Mice were allowed to walk freely in a small chamber while measurements were taken. A minimum of three minutes of continuous recording was obtained.

Core body temperature measurements—Rectal temperature was measured at room temperature using a thermistor (YSI Model 4600).

Statistical analysis—Data were analyzed by ANOVA or by unpaired student’s t test. p values below 0.05 were considered significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

We generated a Hif2a G536W knockin mutation in a C57BL/6 background. The targeting strategy is shown in Figure 1A. The targeting vector contains 5’ and 3’ incision was made to expose the right jugular vein by microsurgical techniques. A 1.4 French miniaturized pressure catheter (Millar instruments) was inserted via the right jugular vein and advanced to the right ventricle chamber for the measurement of right ventricle pressure and heart rate. Data were recorded and analyzed using PowerLab equipment (ADInstruments) and LabChart 6 software.

For echocardiography, an ultra-high-frequency small animal ultrasound system was used. This consisted of a Vevo 770 ultrasound system (VisualSonics Inc., Toronto, ON, Canada) equipped with a 30-MHz probe (RMV-707B), with an attached Integrated Rail System III for image acquisition. Mice were anesthetized with isoflurane. All measurements were analyzed using the Vevo 770 Standard Measurement Package.

Echocardiography and right ventricular pressure measurements—For right ventricular pressure measurements, mice were anesthetized by isofluorane inhalation, placed in the supine position, and a midline cervical
homology arms, as well as neomycin positive selection and diphtheria toxin A negative selection cassettes. Following electroporation into C57BL/6 ES cells and selection using G418, clones with the correct recombination event and mutation were identified. Southern blotting with a 5’ probe revealed the expected 9.5 kb band upon hybridization with Sph I digested DNA in addition to the wild type 12 kb band (Figure 1B, left panel, lane 2). Southern blotting with a 3’ probe revealed the expected 7.8 kb band upon hybridization with Xho I digested DNA in addition to the wild type 11.1 kb band (Figure 1B, right panel, lane 2). Sequencing confirmed the presence of a heterozygous G536W mutation in the Hif2a gene (Figure 1C). Chimeras were derived and germline transmission was obtained, as assessed by Southern blotting (Figure 1B, left and right panels, lane 3). The neomycin cassette was deleted and confirmed by Southern blotting (Figure 1B, left panel, compare lanes 3 and 5). A PCR genotyping strategy was developed (Figure 1D).

We mated Hif2a<sup>G536W/+</sup> mice and were able to obtain both Hif2a<sup>G536W/+</sup> and Hif2a<sup>G536W/G536W</sup> mice, with the latter at a frequency (13%) lower than the expected (25%) Mendelian frequency (Table 2), suggesting that homozygosity of the mutation confers a survival disadvantage during embryogenesis. Notably, the homozygous mouse survival at 18 months (6/6 mice) was the same as that of either heterozygote (10/10) or wild type mice (10/10). We also measured body weights and did not observe any significant differences between the three groups over a 5 month period of observation (data not shown).

We measured hemoglobin levels in these mice and observed increased levels of both parameters in both the Hif2a<sup>G536W/+</sup> and Hif2a<sup>G536W/G536W</sup> mice as compared to wild type mice (Figure 2A and B). For hemoglobin, the values (means ± SEM) were 15.2 ± 0.16, 16.8 ± 0.17, and 17.7 ± 0.41 g/dl for Hif2a<sup>+/+</sup>, Hif2a<sup>G536W/+</sup>, and Hif2a<sup>G536W/G536W</sup> mice, respectively. The red blood cell counts were also increased in a mutation dose-dependent manner (Figure 2C).

We measured platelet and white blood cell counts (Figure 2D and E, respectively). There was no significant difference in platelet counts between the three groups. We did observe changes in white blood cell counts in Hif2a<sup>G536W/+</sup> as compared to wild type mice. The white blood cells were 12.5 ± 0.63, 16.3 ± 1.2, and 15.9 ± 1.4 K/ul for Hif2a<sup>+/+</sup>, Hif2a<sup>G536W/+</sup>, and Hif2a<sup>G536W/G536W</sup> mice, respectively. When individual components of the white blood cell population were examined, we observed increases in lymphocytes and monocytes in Hif2a<sup>G536W/+</sup> mice, and in monocytes in Hif2a<sup>G536W/G536W</sup> mice (Figure 2F). Others have noted functional effects of Epo on macrophages (30). We measured serum Epo levels and did not observe a statistically significant increase in Epo levels in heterozygous compared to wild type mice (Figure 3A). However, Epo levels were increased in homozygous mice as compared to wild type mice or heterozygous mice. For the Hif2a<sup>+/+</sup> and Hif2a<sup>G536W/G536W</sup> mice, the values were 158 ± 14 and 282 ± 22 pg/ml, respectively.

We measured Epo mRNA levels in the kidneys by Real Time PCR (Figure 3B). While there was a trend towards an increase in Epo message with increasing mutation dose, the results were not statistically significant. We also examined other tissues that are potential sources of Epo (8-10). Compared with kidney, substantially lower levels of Epo message were detected in the brain. In this organ, levels of Epo message were increased in the Hif2a<sup>G536W/+</sup> and Hif2a<sup>G536W/G536W</sup> mice (Figure 3B). We did not observe significant differences in levels of Epo message in the liver, which in all cases were substantially lower than that seen in the kidney (Figure 3B). Furthermore, we could not detect Epo message in the bone (data not shown).

Serum Vegf levels were also measured (Figure 3C). No significant differences were seen among the three
genotypes. We measured Vegfa mRNA levels and did not observe any significant differences in kidney, liver, or lung (data not shown). Spleen weights of Hif2a G536W/G536W mice were slightly increased (Figure 3D). We did not observe changes in the weights of livers, kidneys, or lungs (data not shown).

Mice with constitutive activation of the Hif pathway due to liver specific knockout of Vhl display steatosis and hemangiomas that are Hif-2α dependent (31,32), while other mice with renal proximal tubule specific knockout of Vhl display renal cysts that are Hif-2α dependent (33). We therefore conducted histologic examinations of the livers and kidneys of these mice. While there were focal areas of microsteatosis in some of the heterozygous or homozygous mice, these areas were not increased in incidence compared to wild type mice (data not shown).

Abnormalities in pulmonary function have been observed in HIF dysregulation due to the Chuvash mutation in the VHL gene (34,35). We therefore measured the respiratory frequency (f), tidal volume (VT), and minute ventilation (VE) of these mice under normoxic and hypoxic (12% O2) conditions (Figure 4). Under normoxic conditions, no differences were seen in any of the parameters. Under hypoxic conditions, we find that Hif2a G536W/+ or Hif2a G536W/G536W mice display an increase in respiratory frequency when compared to either heterozygote or wild type mice (Figure 4A). Of note, studies of human patients with gain of function HIF2A mutations reveal no changes in minute ventilation under either normoxic or hypoxic conditions; however, they do display increased ventilation relative to metabolism (as reflected by the ratio of arterial to end tidal PCO2) (36).

Dysregulation of the HIF pathway has also been reported to result in pulmonary hypertension both in mouse models and in some human patients with HIF2A mutations (21,34,35). We therefore measured right ventricular heart pressure by cardiac catheterization, and right ventricular wall thickness by echocardiography (Figure 5). We find a significant increase in right ventricular pressure in heterozygotes, with an even more dramatic increase in homozygotes. The right ventricular pressures were 28.3 ± 2.3, 45.9 ± 1.1, and 65.7 ± 10.1 mm Hg for Hif2a +/+, Hif2a G536W/+, and Hif2a G536W/G536W mice, respectively (Figure 5A). These measurements were made in mice that were 16 to 19 months of age. We also examined Hif2a G536W/G536W mice at 4 to 6 months of age, and observed pulmonary hypertension at this age as well (25.6 ± 1.2 and 41.8 ± 1.4 mm Hg for Hif2a +/+ and Hif2a G536W/G536W mice, respectively; Figure 5B). The degree of pulmonary hypertension in the younger mice was less than that seen in the older ones, suggesting an age-dependent effect on this phenotype.

Measurements of right ventricular wall thickness by echocardiography show increases in Hif2a G536W/G536W mice under either systole or diastole (Figure 5C and D). For example, under systole, the right ventricular thickness was 0.40 ± 0.02 and 0.61 ± 0.03 mm for Hif2a +/+ and Hif2a G536W/G536W mice, respectively. No changes were seen in right ventricular inner diameter, main pulmonary artery inner diameter, or in heart rate (Figure 5E-F, and data not shown). The heart/body weight ratio was increased in Hif2a G536W/G536W mice. It was 0.498% ± 0.020% and 0.654% ± 0.023% for Hif2a +/+ and Hif2a G536W/G536W mice, respectively (Figure 5G).

Patients with gain of function mutations in the HIF2A gene display decreased PaCO2 but not PaO2 or pH compared to controls (36). We measured arterial blood gases in Hif2a +/+ and Hif2a G536W/G536W mice, and did not observe any significant differences in these or other parameters (Table 3). There was no difference in oxygen saturation between these two genotypes. We also measured core body temperature, and did not observe any difference between Hif2a +/+ and Hif2a
G536W/G536W mice (37.72 °C ± 0.31 and 38.25 °C ± 0.11, respectively, n = 4).

Histologic examinations of the lungs revealed a slight increase in the thickness of small pulmonary arteries in Hif2a G536W/G536W mice as compared to wild type mice (Figure 6A and B). We also determined the proportions of fully, partially, and non-muscularized small pulmonary arteries, and observed a decrease in the non-muscularized arteries in Hif2a G536W/G536W mice as compared to wild type mice (Figure 6C).

Pulmonary hypertension, in general terms, can be due to number of different pathophysiologic processes, including imbalances between pulmonary arterial vasoconstrictors and vasodilators, and inappropriate proliferation of smooth muscle cells (37-39). Immunostaining for PCNA revealed occasional PCNA-positive smooth muscle cells in small pulmonary arteries of Hif2a G536W/G536W mice (Figure 6D). However, these were not present in significantly different numbers compared to Hif2a +/- controls (Figure 6E), suggesting that changes in the mitotic rate of smooth muscle cells alone cannot account for the increased thickness.

Mediators of pulmonary hypertension that have been identified include the vasoconstrictor Endothelin-1 (Et-1) and the smooth muscle mitogen Pdgf-b. Other genes have been implicated as well, including those encoding for the chemokine Sdf-1 (also known as Cxcl12), the prothrombotic protein Serpine (also known as Pai), Arginase II, Retnla (also known as fizz1), and Fhl-1 (40-44). A number of these genes are Hif target genes (35). We examined these genes by Real Time PCR (Figure 6F). We observe substantial increases in Et-1, Pdgfb, and Sdf-1 mRNA in lungs from Hif2a G536W/G536W mice compared to wild type. No significant differences were observed in the levels of expression of the other target genes, nor in the canonical Hif-1α target gene Pgk-1 (Figure 6F), which was not elevated in either liver or kidney (data not shown).

DISCUSSION

The HIF pathway is the central pathway governing the transcriptional response to hypoxia (6). The present mouse model provides an opportunity to examine the consequences of global expression of a hypermorphic Hif2a allele both in a heterozygous state that models a human disease, as well as in a homozygous state that is not known to occur in humans. Notable features of this mouse model are (1) it introduces a gain of function allele, as opposed to a hypomorphic or knockout allele, (2) it is globally expressed from its endogenous locus, and (3) it is selective for one, as opposed to both, Hif-α isoforms. The two most striking findings are the marked erythrocytosis and dramatic pulmonary hypertension--both of which were observed with high penetrance, and both of which occur in a mutation dose-dependent manner.

The pulmonary hypertension in Hif2a knockin mice could, in principle, be due to the erythrocytosis. However, the following observations argue against this. In other studies, mice with a heterozygous loss of function mutation in Phd2 (P294R), which models a human erythrocytosis-associated PHD2 mutation (P 317R), exhibited increased hematocrit and hemoglobin levels (P. Arsenault, personal communication) that are comparable to that seen in the Hif2a G536W/+ mice. However, in contrast to Hif2a G536W/+ mice, these Phd2 knockin mice did not develop pulmonary hypertension. In addition, mice with transgenic overexpression of EPO develop erythrocytosis (Hct > 80%) that is even more severe than that observed with the Hif2a G536W/G536W mice, yet they do not develop pulmonary hypertension (45).

Pulmonary hypertension has been observed in patients with Chuvash polycythemia, in a mouse model of Chuvash polycythemia, and in some patients with HIF2A erythrocytosis-associated mutations (21,34,35,46). Heterozygosity of Hif2a deletion reverses the pulmonary hypertension seen in the mice bearing the Chuvash mutation as well as that induced by chronic hypoxia,
indicating that the pulmonary hypertension is at least, in part, Hif2α-dependent (35,47). Those mouse studies provided compelling evidence that Hif2α is necessary, but left open the question of whether it alone is sufficient for the development of pulmonary hypertension. The present findings show that in a mouse model, a single missense mutation in Hif-2α is indeed sufficient to induce pulmonary hypertension. This observation is all the more notable given the fact that mutation is a partial, as opposed to complete, gain of function. For example, in vitro studies indicate that the G537W mutation stabilizes HIF-2α, but it does so less effectively than mutation of the primary hydroxylacceptor proline, Pro-531 (48). Real Time PCR analysis identifies genes, Et-1, Pdgfb, and Sdf-1, that are upregulated in these mice. It is plausible that Hif-2α upregulates a panel of genes, including those just mentioned, that in turn mediate the development of pulmonary hypertension.

Epo protein levels were elevated in the serum obtained from the Hif2α G536W/G536W homozygotes, but not Hif2α G536W/+ mice. Most patients with erythrocytosis-associated HIF2A mutations display elevated EPO levels, although it might be noted that some display normal EPO levels (23,49). It is conceivable that Hif-2α may have effects on either the bone marrow microenvironment (50) or on erythroid precursors (11), possibilities that will require further investigation. Interestingly, the Hif2α G536W/G536W homozygous mice do not display a statistically significant increase in their Epo mRNA levels in their kidneys. It is reasonable to speculate that elevated serum Epo in Hif2α G536W/G536W mice may be from the contribution of several organs in these mice with this global hypermorphic Hif2α mutation. For example, there may be a contribution to circulating Epo from the brain, which does display increased Epo mRNA levels in Hif2α G536W/G536W mice (Figure 3B). The liver might be considered another potential source; however, we were not able to detect any significant changes in Epo mRNA level in this tissue, which was low in comparison to the levels seen in the kidney.

It is noteworthy that the knockin mice did not display certain stigmata of dysregulation of the HIF pathway seen in other contexts. For example, conditional knockout of Vhl in the mouse liver results in hemangiomas and steatosis that are reversed by concurrent deletion of Hif2α, while conditional knockout of Vhl in the kidney results in cysts that are absent when Hif2α is concurrently deleted (31-33). Patients with Chuvash polycythemia display an increased incidence of vertebral hemangiomas (51). Moreover, conditional expression of a strongly constitutively active HIF-2α (P405A/P531A) in the mouse liver results in hemangiomas (52). Possible explanations for our present results in comparison to previous ones are as follows: (1) Hif-2α expression may be necessary but not sufficient for the development of these lesions, (2) there may be Hif-independent functions of Vhl, and (3) the G536W gain of function mutation in Hif-2α, even in its homozygous state, may not be as strong as that induced by Vhl ablation or the P405A/P531A mutation noted above. As previously mentioned, in vitro studies indicate that the G537W mutation in HIF-2α stabilizes HIF-2α to a lesser extent than a P531A mutation (48).

Heterozygous HIF2A mutations exist in humans (1), and it is formally possible that these individuals may have modifier genes or compensatory mutations at other gene loci that allow these mutations, which might otherwise be incompatible with viability, to exist in nature. The present studies, which were conducted in uniform strain background (C57BL/6) argue against this possibility. Specifically, they indicate that constitutive global activation of Hif-2α due to a heterozygous gain of function mutation is compatible with viability in mice. Indeed, mice that are homozygous for the Hif2α knockin mutation are viable as well.

These studies, taken together, indicate that a missense mutation in one Hif-α isoform is sufficient to produce significant
hematopoietic and cardiopulmonary phenotypes, although interestingly, it does not recapitulate all of the sequelae that have been observed due to HIF-α dysregulation in other contexts. There is currently great interest in manipulating the HIF pathway for therapeutic benefit in ischemic diseases and anemia (4). The current studies provide valuable information for strategies that seek to selectively upregulate the HIF-2α isoform. Conversely, these studies also highlight HIF-2α as a potential therapeutic target for the treatment of pulmonary hypertension (35,47).
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FOOTNOTES

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The abbreviations used are: HIF, Hypoxia Inducible Factor; PCR, polymerase chain reaction; PHD, Prolyl Hydroxylase Domain protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VHL, von Hippel Lindau protein.
FIGURE LEGENDS

FIGURE 1. **Generation of Hif2a G536W knockin mice.** (A) Gene targeting strategy for introduction of Hif2a G536W mutation. * indicates G536W mutation. Black boxes indicate exons, with exon number indicated beneath box. Open box indicates 3' untranslated region. DTA and neo denote diphtheria toxin A and neomycin cassettes, respectively. Positions of Hex12-2 5' and Hint 12-3 3' PCR primers are shown. (B) Southern blots employing 5' (left) and 3' (right) probes of Sph I or Xho I digested DNA, respectively, of ES cell or mouse tail DNA. Hif2a genotypes are provided at top. The presence of the neomycin cassette is denoted by neo. (C) DNA sequencing chromatogram of targeted ES cell DNA. Sequence is from 3' to 5' (reverse complement). Codon at residue 536 is in the middle and is heterozygous for Gly (GGC) and Trp (TGG). (D) PCR genotyping of Hif2a knockin mice by using Hex12-2 5' and Hint 12-3 3' primers. The 0.60 kb product is derived from the wild type allele, while the 0.66 kb product is derived from the G536W mutant allele. Hif2a genotypes are shown at top.

FIGURE 2. **Hif2a G536W/+ and Hif2a G536W/G536W mice display erythrocytosis.** (A) Hematocrit, (B) hemoglobin, (C) red blood cell, (D) platelet, and (E) white blood cell counts were measured in blood obtained from mice with indicated Hif2a genotypes (age = 1-3 months; n = 6 - 10 per group). (F) The neutrophil (NE), lymphocyte (LY), monocyte (M), eosinophil (EO), and basophil (BA) concentrations were measured and normalized to that of wild type mice. The data are presented as means ± SEM. * indicates p < 0.05, and ** indicates p < 0.01.

FIGURE 3. **Hif2a G536W/G536W mice display increased serum Epo.** (A) Epo concentrations in serum were measured by ELISA (age = 3 - 8 months; n = 4 - 10 per group). (B) Epo mRNA levels in the indicated organs at 6 - 8 months of age were determined by Real Time PCR, and normalized to that of 18S rRNA (n = 4). The data are presented as the fold change in reference to mRNA levels in kidneys of wild type mice. In (B), there was a trend towards an increase of renal Epo mRNA in Hif-2α G536W/G536W mice as compared to wild type mice, but it was not statistically significant (p = 0.13). The data are presented as means ± SEM. * indicates p < 0.05, and ** indicates p < 0.01. (C) Vegf concentrations in serum were measured by ELISA (age = 3 - 8 months; n = 4 - 10 per group). (D) Spleen weights were measured in mice with indicated Hif2a genotypes (age = 16 - 19 months; n = 3 - 6 per group). * indicates p < 0.05.

FIGURE 4. **Pulmonary function parameters in Hif2a G536W/+ and Hif2a G536W/G536W mice.** The (A) respiratory frequency f, (B) tidal volume V_T, and (C) minute ventilation V_E of wild type, Hif2a G536W/+ and Hif2a G536W/G536W mice (age = 10 - 17 months; n = 3 -5 per group) under normoxic (21% O_2) and hypoxic (12% O_2) conditions were measured. V_T and V_E were normalized to body weight. * indicates p < 0.05 in comparison to either wild type or Hif-2α G536W/+ mice. (D) Representative plethysmography traces for wild type and Hif2a G536W/G536W mice under normoxic and hypoxic conditions.

FIGURE 5. **Hif2a G536W/+ and Hif2a G536W/G536W mice develop pulmonary hypertension and right ventricular hypertrophy.** (A and B) Right ventricular systolic pressure, (D) right ventricular wall thickness in diastole or systole, (E) right ventricular inner diameter (RVID), (F) heart rate, and (G) heart weight (as a percentage of total body weight) were measured in mice with indicated Hif2a genotypes (age = 16 -19 months, n = 4 - 7 per group for panels A and D-G; age = 4 - 6 months, n = 4 - 5 per group for panel B). (C) Representative M-mode echocardiographs for mice with indicated Hif2a genotypes. Right ventricular wall thickness in diastole and systole are indicated by d and s, respectively. In (A), due to the variability in data for
the homozygous mice, data were subjected to log transformation prior to ANOVA. * indicates $p < 0.05$, and ** indicates $p < 0.01$.

FIGURE 6. Upregulation of Et-1 and Pdgfb mRNA in Hif2a $^{G536W/G536W}$ mice. (A) Photomicrographs of immunoperoxidase-stained sections of lungs from wild type and Hif2a $^{G536W/G536W}$ mice (magnification = 400x) using antibodies against smooth muscle actin. Small pulmonary arteries are in the center of each photomicrograph. Bars indicate 25 $\mu$M. (B) The medial thickness of small pulmonary arteries (expressed as a percentage of the external diameter), and (C) the proportions of (N) non-muscularized, (P) partially muscularized, and (F) fully muscularized pulmonary arteries were measured in lungs obtained from mice with the indicated genotypes (age = 10 - 17 months; $n = 3$ per group). (D) Photomicrograph of immunoperoxidase-stained section of lung from Hif2a $^{G536W/G536W}$ mice using antibodies against PCNA. A PCNA-positive smooth muscle cell is indicated by the arrow. (E) Numbers of PCNA-positive smooth muscle cells in small pulmonary arteries of wild type and Hif2a $^{G536W/G536W}$ mice (age = 10 - 17 months; $n = 3$ per group). (F) The levels of the indicated mRNAs in lung were determined by Real Time PCR ($n = 4$). Mice were 6 - 8 months. The data are presented as the fold change in reference to mRNA levels in wild type mice. * indicates $p < 0.05$ in comparison to wild type mice. In (C), # indicates $p = 0.08$ in comparison to wild type mice.
| Gene  | Forward primer            | Reverse primer            |
|-------|---------------------------|---------------------------|
| Epo   | CATCTGCGACAGTGGAGTTCTG    | CACAACCATCGTGACATTTTC     |
| Vegfa | TACCTCCACCATGCAAAGTG      | TGGGACTTCTGCTCTCCTCTG     |
| 18S   | TCAGGACTGAGGCATGATT       | TACCGGCCTACGAATGGC        |
| Pgk-1 | GGAACGCGTGCGTGATGA        | GCCTTGATCCTTGGTTGTTTG     |
| Er-1  | GCTGGTGAAAGGAAGAACTAC     | AAAAGATGCTCTGATGCTATTGC   |
| Pdgfb | CGCACAGAGGTGTGGCTAGATC    | CAGGAAGTGGCGTTGG          |
| Sdf-1 | CCCTGCCGGTTCTTCCA         | GAGGATTTTCAGATGCGTTGAGTT |
| Serpine | CGACACCCTCAGCATGTTCA   | CGGAGAGGGTGCACATCTCTCT    |
| Arginase II | GATTGATCGACTGTTGGGATA   | TGCCAATCGCCGATCAAA        |
| RetnlA | TCGTGGAGAAATAGGGCTAAGGAA | TCTTCGTTACAGTGGAGGGATAGTT |
| Fhl-1 | GCCACCGTGCTGGCATAGAAGA    | CCGGTCCTCATAGCTTGC        |
| Genotype       | % expected | % observed  |
|----------------|------------|------------|
| Hif2a<sup>G536W/G536W</sup> | 25         | 13 (31/240) |
| Hif2a<sup>G536W/+</sup>     | 50         | 50 (120/240) |
| Hif2a<sup>+/+</sup>          | 25         | 37 (89/240)  |

In parentheses, the number observed with the indicated genotype / total number is shown.
TABLE 3
Arterial blood gas analysis of *Hif2a*^{+/+} and *Hif2a*^{G536W/G536W} mice

|                | *Hif2a*^{+/+}       | *Hif2a*^{G536W/G536W} |
|----------------|--------------------|-----------------------|
| pH             | 7.28 ± 0.023       | 7.29 ± 0.061          |
| PaCO₂          | 36.7 ± 2.0         | 29.8 ± 3.7            |
| PaO₂           | 85.3 ± 3.4         | 91.8 ± 2.2            |
| HCO₃⁻          | 18.2 ± 0.6         | 14.9 ± 2.1            |
| TCO₂           | 19.3 ± 0.7         | 16.0 ± 2.3            |
| BE_{ecf}       | -9.0 ± 1.0         | -12.5 ± 2.5           |
| SaO₂           | 96.5 ± 0.59        | 96.4 ± 0.81           |

Shown are mean ± SEM. *n* = 4 - 6 per group (4 to 6 months of age). No significant differences were seen between the two groups (*p* > 0.05 for all parameters).
Tan et al. Fig 2
Serum Epo (pg/ml)

Kidney | Liver | Brain
---|---|---
0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5

Relative Epo mRNA level

A

Serum Vegf (pg/ml)

0 | 40 | 80 | 120 | 160

B

C

D

Spleen weight (% of body weight)

A

Tan et al. Fig 3
A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)

Tan et al. Fig 4
Tan et al. Fig 6

A

B

C

D

E

F
Erythrocytosis and Pulmonary Hypertension in a Mouse Model of Human HIF2A Gain-of-Function Mutation
Qiulin Tan, Heddy Kerestes, Melanie J. Percy, Ralph Pietrofesa, Li Chen, Tejvir S. Khurana, Melpo Christofidou-Solomidou, Terence R.J. Lappin and Frank S. Lee

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