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The von Hippel Lindau tumour suppressor gene is a novel target of E2F4-mediated transcriptional repression in preeclampsia

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ABSTRACT

The von Hippel Lindau tumour suppressor (VHL) protein is essential for proper placental development and is downregulated in preeclampsia (PE), a devastating disorder of pregnancy typified by chronic hypoxia. To date, knowledge on *VHL* genetic and epigenetic regulation is restricted to inactivating mutations and loss-of-heterozygosity in renal cell carcinomas. Herein, we sought to examine whether *VHL* DNA is subject to differential methylation, and if so, whether it is altered in early-onset PE (*E*-PE). Sodium bisulfite modification and methylation-specific PCR analysis revealed that *VHL* is subject to extensive methylation in a CpG-rich region within its promoter in the human placenta. Notably, we detected significant differences in methylation in *E*-PE placentae relative to normotensive age-matched controls at key transcription factor binding sites, including that of the transcriptional repressor E2F4. Treatment of JEG3 cells with 5-Aza-2′-deoxycytidine, revealed that loss of DNA methylation promoted *VHL* transcription by attenuating *VHL* association with E2F4. RNAi knockdown of E2F4 *in vitro* confirmed its function on *VHL* repression. Exposure of JEG3 cells to transforming growth factor beta (TGFβ) downregulated *VHL* mRNA. In line with elevated levels of TGFβ3 in *E*-PE, chromatin immunoprecipitation assays revealed that E2F4-*VHL* association was enhanced upon TGFβ3 treatment, indicative of *VHL* transcriptional inhibition. In line with decreased *VHL* expression in *E*-PE, augmented E2F4-*VHL* association was also observed in *E*-PE placental tissue relative to controls. In conclusion, we demonstrate for the first time that hypomethylation of *VHL* DNA at a key transcription factor binding site has significant consequences for its transcriptional repression in early-onset preeclampsia.

1. Introduction

The von Hippel Lindau tumour suppressor gene (*VHL*) was initially identified as the genetic origin of an inherited cancer syndrome, termed VHL disease [1]. Since that discovery, work on the genetic and epigenetic regulation of *VHL* has predominantly been restricted to clear cell renal cell carcinoma (ccRCC), a pathology characterized by early inactivation of the gene [2, 3]. Evidence implicating *VHL* in renal cancer tumourigenesis stems from *in vitro* studies in ccRCC cell lines that demonstrated somatic bi-allelic inactivation of *VHL* in exon 2, including SNPs, frameshift deletions/insertions, missense and nonsense mutations [2, 3]. Importantly, a small but significant proportion of renal carcinoma tumours are also often characterized by epigenetic silencing of *VHL*, involving hypermethylation of a cytosine-phospho-guanine (CpG) island in the 5′ regulatory region of the gene [4]. In addition, a wealth of other studies has established epigenetic inactivation of *VHL* as the driving factor in RCC pathogenesis [5, 6].

The placenta is a transient organ of extraembryonic origin that experiences striking changes in oxygen tension during the course of its development [7], and evidence indicates a role for the transcription factor, Hypoxia Inducible Factor 1alpha (HIF1A) in this process. Canonically, the protein product of the *VHL* gene, pVHL, is a central

Abbreviations: ACOG, The American College of Obstetricians and Gynecologists; ASAH1, Acid Ceramidase enzyme; ccRCC, clear cell Renal Cell Carcinoma; CpG, Cytosine-phospho-Guanine; E2F4, E2 Transcription Factor 4; *E*-PE, Early-onset Preeclampsia; HIF1A, Hypoxia Inducible Factor 1 alpha; IUGR, Intrauterine Growth Restriction; JMJD6, Jumonji domain containing protein 6; L-PE, Late-onset Preeclampsia; PTC, Pre-term Control; SMAD, Sma Mothers Against Decapentaplegic; SNP, Single Nucleotide Polymorphism; TC, Term Control; TGFβ, Transforming Growth Factor Beta; TIE, TGFβ Inhibitory Elements; VHL, von Hippel Lindau tumour suppressor

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Table 1
Clinical parameters of patients.

	PTC $n = 38$	E-PE $n = 39$	TC $n = 14$	L-PE n = 15
Gestational age at delivery	30.15 ± 2.76	28.34 ± 2.73	39.00 ± 1.30	37.20 ± 1.51
Birth weight (g)	1433.33 ± 620.42	980.33 ± 502.23	3189.00 ± 500.00	3055.67 ± 700.12
Blood pressure (mm Hg, S/D)	107.33 ± 5.71/66.67 ± 6.58	168.33 ± 13.63/111.00 ± 9.27	117.50 ± 4.80/70.50 ± 3.95	151.67 ± 19.81/90.67 ± 12.96
Proteinuria (g/day)	Absent	4+	Absent	3+
Mode of delivery (%)	48% CS, 52% VD	90% CS, 10% VD	33% CS, 67% VD	75% CS, 25% VD

Data are presented as mean ± standard deviation.
PTC = Preterm Controls.
E-PE = Early-Onset Preeclampsia.
TC = Term Controls.
L-PE = Late-Onset Preeclampsia.
S/D = Systolic/Diastolic.
CS = Caesarian Section.
VD = Vaginal delivery.

player in the cellular oxygen sensing pathway that maintains HIF1A protein stability under tight control [8]. In the presence of molecular oxygen, pVHL forms a multi-protein complex (VHL^{CBC}) that recognizes two hydroxylated proline residues on HIF1A to target it for proteasomal degradation [8]. In humans, during placental development, pVHL has been shown to exhibit a spatial and temporal pattern of expression that is critical to maintain proper HIF1A homeostasis, thereby controlling trophoblast differentiation events [9]. The importance of VHL in the developing placenta is further exemplified by mice lacking pVHL ($Vhl^{-/-}$) that display lethality between E10.5 to E12.5 days of gestation, primarily due to disruption of placental angiogenesis and vasculogenesis [10]. In particular, during this critical time period for murine placental development, loss of *Vhl* resulted in placental lesions and profound impairments in blood vessel formation within the labyrinthine layer that is responsible for feto-maternal nutrient and gas exchange [10]. Taken together, these studies highlight the importance of VHL to normal placental function in human and mouse, which in turn is necessary for maintaining a healthy pregnancy.

Improper placental development underlies the pathogenesis of serious disorders of pregnancy such as preeclampsia (PE), which can have long-term consequences for both fetal and maternal health. PE is a multi-factorial disease, estimated to affect between 5 and 8% of all pregnancies and clinically manifests as maternal hypertension [11, 12]. PE has long been described as two distinct disorders based on unique biological profiles, feto-maternal outcomes and etiopathogenesis [13, 14]. Early-onset preeclampsia (*E*-PE), manifesting prior to 34 weeks of gestation, is predominantly of placental origin, while late-onset preeclampsia (L-PE) occurs after 34 weeks of gestation and represents an unusual maternal vascular response to normal placentation [13, 15]. At the core of *E*-PE is chronic placental hypoxia, and work from our laboratory has shown aberrant oxygen sensing as an important contributing factor to disease pathogenesis [16]. In particular, *E*-PE placentae are characterized by elevated levels of HIF1A [17, 18] that is responsible for increased transforming growth factor beta 3 (TGFβ3) expression leading to disrupted trophoblast differentiation and invasion [19]. TGFβ3 belongs to the TGFβ superfamily of growth factors that have pleiotropic functions on cell fate. We have previously demonstrated that in the human placenta, TGFβ3, but not TGFβ1 or TGFβ2, is an inhibitor of trophoblast cell differentiation towards an invasive phenotype [20]. TGFβ3 signalling is intimately linked to cellular oxygen status and is a potent activator of the Sma mothers against decapentaplegic (SMAD) family of transcription factors that regulate gene transcription [21]. Notably, TGFβ-mediated SMAD signalling contributes to the pathogenesis of a variety of cancers [22, 23] and it is known to be dysregulated in pregnancy-related disorders including PE and IUGR [23, 24].

We recently reported that Jumonji domain containing protein 6 (JMJD6) regulates VHL expression [25], and that decreased *VHL* expression in *E*-PE is in part dependent on impaired JMJD6 histone demethylase activity due to hypoxia [26]. However, despite the wealth of knowledge on epigenetic mechanisms underlying loss of *VHL* in RCC disease and the discovery of the relevance of histone modification events in controlling *VHL* expression, there is no information on *VHL* DNA methylation status or its implications for transcription in the human placenta. Hence, we examined the involvement of TGFβ3 in the epigenetic regulation of *VHL* in the human placenta in physiological and pathological conditions.

2. Material and methods

2.1. Placental tissue collection

Written, informed consent was obtained from all study participants. Collection procedures were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and Ethics Guidelines outlined by the Mount Sinai Hospital Research Ethics Board. Human placental tissues were collected by the Research Centre for Women's and Infants' Health (RCWIH) Biobank. Placentae were obtained from pregnancies complicated by early (*E*-PE; $n = 39$ and late-onset preeclampsia (L-PE, $n = 15$) diagnosed according to The American College of Obstetricians and Gynecologists (ACOG) guidelines [11]. Control placentae were obtained from preterm (PTC, $n = 38$) and term (TC, $n = 14$) normotensive age-matched deliveries. Table 1 summarizes clinical parameters of the patient population.

2.2. Cell culture conditions

JEG3 choriocarcinoma cells (ATCC) or alternatively, HEK293 cells (ATCC) were authenticated by Short Tandem Repeat genotyping. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C. Cells were plated at a density of 2×10^5 cells/well in 6-well plates at 60–70% confluency. For RNAi knockdown studies, JEG3 cells were cultured to 50–60% confluency in 6-well plates and 30 nM of *E2F4 and E2F4/5* Silencer® siRNA duplexes and control Silencer® Negative Control siRNA (Ambion Inc., Austin, TX, USA) were transfected using jetPRIME® buffer (Polyplus transfection™, Illkirch, France). Cells were harvested for Trizol RNA (Life Technologies Inc., Burlington, ON) extraction and protein isolation in RIPA buffer after 24 h. For TGFβ treatments, JEG3 cells were cultured to 60–70% confluency and treated with 5 ng/mL TGFβ3 (R&D Systems, Minneapolis, MN, USA) or control vehicle (4 mM HCl/1 mg/mL BSA solution) for 3 or 24 h. Cells were exposed to 5 μM 5-Aza-2′-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) or control vehicle (equivalent volume of DMSO) for 48 h. Following the respective