Jumonji Domain Containing Protein 6 Is Decreased in Human Preeclamptic Placentas and Regulates sFLT-1 Splice Variant Production

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ABSTRACT

The anti-angiogenic protein, soluble fms-like tyrosine kinase-1 (sFLT-1), plays a central role in preeclamptic pathophysiology. A splice variant of FLT-1 (VEGF receptor 1), sFLT-1 is released in excessive amounts from the preeclamptic placenta into the maternal circulation, where it causes endothelial dysfunction manifesting as end-organ disease. However, the mechanisms regulating its production within the placenta remain poorly understood. Recently it was shown in endothelial cells that Jumonji domain containing protein 6 (JMJD6) hydroxylates U2 small nuclear ribonucleoprotein auxiliary factor 65-kDa subunit (U2AF65, a component of the splicesome). The hydroxylation by JMJD6 is oxygen dependent. Under hypoxia, JMJD6 is less able to hydroxylate U2AF65, and this unhydroxylated form of U2AF65 biases splicing of FLT-1 to sFLT-1. We assessed whether oxygen-sensing JMJD6 is differentially expressed in preeclamptic placenta and regulates sFLT-1 splicing in placenta via U2AF65. JMJD6 protein expression was significantly reduced in preterm preeclampsia placenta and regulates sFLT-1 splicing in placenta via U2AF65. The hydroxylation by JMJD6 is oxygen dependent. Under hypoxia, JMJD6 is less able to hydroxylate U2AF65, and this unhydroxylated form of U2AF65 biases splicing of FLT-1 to sFLT-1. We demonstrated that placental JMJD6 colocalized with U2AF65. In turn, we found JMJD6 directly interacts with U2AF65, which in turn produces sFLT-1 mRNA transcripts. Taken together, our findings provide evidence that JMJD6 may play a role in regulating the production of sFLT-1 in the preeclamptic placenta. Decreased placental JMJD6 expression may be an important component to the pathophysiology of preeclampsia.

INTRODUCTION

Preeclampsia affects 5%–8% of all pregnancies, contributing significantly to global maternal and perinatal morbidity and mortality [1]. Central to the pathophysiology of preeclampsia is poor placental implantation, resulting in decreased perfusion and placental hypoxia. The hypoxic placenta releases excessive anti-angiogenic proteins into the maternal circulation, causing widespread endothelial dysfunction that manifests as multisystem maternal end-organ injury [2, 3].

A key anti-angiogenic factor in the pathogenesis of preeclampsia is soluble fms-like tyrosine kinase-1 (sFLT-1), a splice variant of FLT-1 (fms-like tyrosine kinase-1) [2]. FLT-1 belongs to the vascular endothelial growth factor receptor (VEGFR) family, binding a number of ligands that play crucial roles in angiogenesis and vasculogenesis, including vascular endothelial growth factor (VEGF) and placental growth factor (PIGF). The sFLT-1 makes up the ligand-binding domain but lacks the transmembrane and intracellular regions [4]. As such, sFLT-1 antagonizes the actions of VEGF and PIGF by preventing them from activating the full-length cognate receptors.

sFLT-1 is a product of alternative splicing of FLT-1 precursor mRNA [5], and to date, four isoforms of sFLT-1 have been described [6, 7]. Of these, sFLT-1 i13 and sFLT-1 e15a are the two most abundant [8]; sFLT-1 i13 is expressed in many tissues, notably the endothelium but also brain, heart, and kidneys [8], while sFLT-1 e15a is a newly described primate-specific isoform mainly produced in placenta and minimally expressed elsewhere. In placenta, over 80% of all FLT-1 transcripts are spliced to become sFLT-1 e15a, whereas 10%–15% become sFLT-1 i13 [8]. Being highly placental specific, sFLT-1 e15a may be the major sFLT-1 isoform responsible for preeclampsia.

The molecular mechanism by which placental hypoxia regulates sFLT-1 splicing in preeclampsia has been avidly sought, but remains unknown. Recently, oxygen-sensing jumonji domain containing protein 6 (JMJD6) was shown to influence the splicing pattern of FLT-1 in endothelial cells [9]. Under normoxic conditions JMJD6 was able to exert its normal enzymatic functions and hydroxylates a component of the splicing machinery, U2 small nuclear ribonucleoprotein auxiliary factor 65-kDa subunit (U2AF65). This leads to the splicing machinery producing the full-length membrane-bound FLT-1 transcript [9]. However, under hypoxic conditions, there was decreased JMJD6 activity and JMJD6 was less able to hydroxylate U2AF65. As a result, the splicing machinery produced shorter alternatively spliced sFLT-1 transcripts from the FLT-1 transcript.

We hypothesized this may be the molecular mechanism through which placental hypoxia, observed in preeclampsia, results in the production of excess sFLT-1 secretion. We...
therefore measured JMJD6 mRNA and protein expression in a cohort of preterm preeclamptic placenta and functionally explored its association with U2AF65 and sFLT-1 expression.

MATERIALS AND METHODS

Reagents

Primary antibodies used were against Jmjd6 (sc-28348; Santa Cruz Biotechnology), U2AF65 (ab37530; Abcam), and GAPDH (3683s; Cell Signaling Technology; details are found in Table 1.

Tissue Collection

Placental tissue was obtained following informed written consent from women with either early-onset preeclampsia, defined as delivery at <34 wk gestation and in accordance with American College of Obstetrics and Gynecology [10] (n = 21), or gestationally matched normotensive control placentas were also utilized for immunohistochemical analysis (n = 6). Approval was obtained for this study by the Mercy Health Human Research Ethics Committee.

Primary Cell Isolation

All cells were maintained at 37°C in 5% CO₂. Human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco-modified Eagle medium (Invitrogen) with 10% fetal calf serum (Gibco, Invitrogen) and 1% penicillin-streptomycin (Gibco). Primary trophoblasts were cultured in Dulbecco-modified Eagle medium with 10% fetal calf serum and 1% antibiotic-antimycotic (Gibco). Primary trophoblasts were isolated from term placenta as described previously [11]. Briefly, term placentas were washed to remove excess blood before villous tissue was scraped from placental vasculature and digested using trypsin/DNase. Trophoblast cells were isolated using a discontinuous Percoll gradient followed by negative selection with CD9 that produced a population highly enriched (>95% pure) for trophoblast cells with very little fibroblast contamination [11]. Primary trophoblasts were maintained at 8% O₂, whilst HUVECs were maintained at 20% O₂.

RT-PCR

RNA was extracted using the RNeasy mini kit (Qiagen) before cDNA conversion using the SuperScript VILO cDNA synthesis kit (Life Technologies), both in accordance with manufacturer’s instructions. Jmjd6 and YWHAZ were assessed by Taqman gene expression assays (Applied Biosystems) as per the manufacturer’s instructions. The sFlt-1 splice variant expression was determined using variant-specific primer sets: sFlt-1 i13 forward 5′-ACAATCAGAGGTGAGCACTGCAA-3′ and reverse 5′-TCCAGGCTCTTAAAGTTAGCAA-3′; and sFlt-1 e15a forward 5′-CTCTCGGAAACCTCAGTG-3′ and reverse 5′-GAGCATGTCCTGTTAGTG-3′. GAPDH was used for housekeeping. Run conditions were 95°C for 20 min, 95°C for 1 sec, 60°C for 20 sec repeated for 40 cycles. All RT-PCR was performed on the CFX 384 (Bio-Rad) with the results analyzed using the comparative Ct method (ΔΔCt, whereby gene expression was normalized to both housekeeping genes and the control group).

Protein Analysis: Western Blot Analysis, Enzyme-Linked Immunosorbent Assay, Immunohistochemistry, and Immunofluorescence

Western blot analysis involved the separation of 20 μg of placental lysate on 10% polyacrylamide gels prior to wet transfer to polyvinylidene difluoride membranes (Millipore). Following blocking, membranes were incubated overnight at 4°C with primary antibodies against either Jmjd6 (1:250) or GAPDH (1:5000). Following exposure to horseradish peroxidase-conjugated secondary antibodies, bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare) and the ChemiDoc XRS (Bio-Rad). Total sFlt-1 protein levels in culture media were measured using an sFlt-1 enzyme-linked immunosorbent assay (ELISA) (R&D Systems) in accordance with the manufacturer’s instructions.

Jmjd6 localization was determined using 5 μm sections of paraffin-embedded formalin-fixed placenta from preeclamptic (n = 6), preterm (n = 6), and term (n = 6) pregnancies. Sections were dewaxed and rehydrated prior to antigen retrieval using sodium citrate buffer for 20 min, then incubated in the hot buffer for a further 20 min. Sections were then permeabilized in 0.5% 

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### TABLE 1. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Peptide/protein target</th>
<th>Name of antibody</th>
<th>Manufacturer (catalog no.)</th>
<th>Species raised; monoclonal or polyclonal</th>
<th>Dilution used</th>
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<tbody>
<tr>
<td>JMJD6</td>
<td>PSR (H-7)</td>
<td>Santa Cruz (sc-28348)</td>
<td>mouse monoclonal</td>
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<td>U2AF65</td>
<td>Anti-U2AF65</td>
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<td>Sigma (U4758)</td>
<td>mouse monoclonal</td>
<td>5 μg</td>
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<tr>
<td>Secondary</td>
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<td>GE Healthcare (NA931)</td>
<td>rabbit polyclonal</td>
<td>1:5000</td>
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<td>GE Healthcare (NA934V)</td>
<td>sheep whole antibody</td>
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### TABLE 2. Baseline clinical characteristics of preterm and preeclamptic women.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Preterm (n = 10)</th>
<th>Preeclampsia (n = 21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (yr)</td>
<td>35.0 ± 1.82</td>
<td>30.1 ± 1.34</td>
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</tr>
<tr>
<td>GA at delivery (wk)</td>
<td>31.2 ± 1.10</td>
<td>31.8 ± 0.66</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>32.3 ± 3.37</td>
<td>29.0 ± 2.26</td>
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<td>Birthweight (g)</td>
<td>1898 ± 260.8</td>
<td>1593 ± 147.4</td>
<td>0.28</td>
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<tr>
<td>Primiparous (n) (30%)</td>
<td>3 (30%)</td>
<td>11 (52.4%)</td>
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<tr>
<td>Ethnicity</td>
<td></td>
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<tr>
<td>White</td>
<td>9 (90%)</td>
<td>18 (84%)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>1 (10%)</td>
<td>2 (11%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (5%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>120.6 ± 6.37</td>
<td>163.9 ± 6.51</td>
<td>0.0003</td>
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<tr>
<td>Diastolic BP (mm Hg)</td>
<td>68.1 ± 4.95</td>
<td>103.8 ± 3.42</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

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a GA, gestational age; BMI, body mass index; and BP, blood pressure.

b Data is presented as mean ± SEM or number (%).
TABLE 3. Baseline clinical characteristics of term women.

| Characteristic     | Term (n = 6)
|-------------------|------------
| Maternal age (yr) | 37.2 ± 1.07
| GA at delivery (wk) | 38.9 ± 0.30
| BMI (kg/m²)       | 25.7 ± 1.6
| Birthweight (g)   | 3631.7 ± 137.4
| Primiparous (n)   | 1 (16.6%)
| Ethnicity         |            
| White             | 5 (84.4%)
| Asian             | 1 (16.6%)
| Other             |            
| Systolic BP (mm Hg) | 114.17 ± 3.85
| Diastolic BP (mm Hg) | 66.7 ± 5.23

* GA, gestational age; BMI, body mass index; and BP, blood pressure.

Jmjd6 Small Interfering RNA Treatment of Cell Culture and Primary Cells

HUVECs were transfected with Jmjd6 mission small interfering RNA (siRNA) (Sigma), negative siRNA (Qiagen), or transfection reagent alone (Lipofectamine RNAiMAX; Invitrogen). Primary trophoblast cells were transfected with SMARTpool siRNA (Thermo Scientific). The transfection was performed in accordance with Invitrogen’s RNAiMAX recommended protocol.

HUVECs were transfected with 10 nM siRNA and 0.5 μl RNAiMAX, cultured for 48 h before a media change with cell lysates and culture media collected 24 h later. Primary trophoblasts were similarly transfected, and cell lysates and culture media were harvested 72 h later. Experiments were repeated three to five times with triplicates performed for each experiment.

Statistical Analysis

All in vitro experiments were performed with technical triplicates, and all experiments were repeated a minimum of three times. Statistical analysis was performed using GraphPad prism, with an unpaired Student t-test for parametric data or Mann Whitney U-test for nonparametric data being used; P ≤ 0.05 was considered statistically significant.

RESULTS

Jmjd6 Is Expressed in Placenta and Significantly Reduced in Severe Preeclampsia

We first set out to localize Jmjd6 in placenta by immunohistochemistry. As expected, Jmjd6 was expressed within the placental syncytiotrophoblast nuclei, the cell layer that forms the maternal-fetal interface and site of sFLT-1 production [12, 13]. Jmjd6 was also present in the nuclei of the underlying cytotrophoblast and cells within the villous tip (Fig. 1A). We next measured Jmjd6 protein expression in a cohort of severe preterm preeclamptic (n = 21) and normotensive preterm control (n = 10) placentas. Western blot (Fig. 1B) with densitometric analysis (Fig. 1C) confirmed Jmjd6 protein expression in pre-eclamptic placenta was significantly reduced relative to preterm controls (P < 0.0001; Supplemental Fig. S1 shows all the Western blots; all Supplemental Data are available online at www.biolreprod.org). Hence, we conclude Jmjd6 is expressed in the nuclei of cells within the villous tip (predominantly syncytiotrophoblast) and is reduced in preeclamptic placenta.

Hypoxia Downregulates Jmjd6 Expression and Upregulates the sFLT-1 Variants

We next examined whether low oxygen tension regulates placental Jmjd6. Given previous findings of increased Jmjd6 expression in 3% O₂ relative to 20% O₂ [14], we cultured primary trophoblast at 1%, 3%, 8%, and 20% O₂ (Fig. 2 and Supplemental Fig. S2) We observed a significant reduction in Jmjd6 mRNA expression under hypoxia (both 1% and 3% O₂) relative to 8% O₂ (P < 0.05; Fig. 2A and Supplemental Fig. S2, A and D). In addition, 20% O₂ resulted in significantly decreased (P < 0.01) Jmjd6 expression compared to 8% O₂, but was not significantly different to hypoxia (1% or 3% O₂; Supplemental Fig. S2, A and D). Jmjd6 expression was also decreased when HUVECs were exposed to hypoxia (Fig. 2E; P < 0.0001). As expected, hypoxia also increased sFLT-1 expression in both primary trophoblast (relative to 8% O₂) as well as endothelial cells (Fig. 2, B, C, F, and G, and Supplemental Fig. S2, B and C). We also found significantly increased sFLT-1 secretion from primary trophoblast (Fig. 2D).

Thus, consistent with our hypothesis and previous observations in other cell types [9, 15], we found hypoxia (1% or 3% O₂)
decreased JMJD6 expression in primary trophoblast and endothelial cells.

**JMJD6 Knockdown Increases sFLT-1 Production**

Having shown that JMJD6 expression is decreased in early preeclamptic placenta and reduced by hypoxia, we next assessed whether decreasing JMJD6 levels would increase sFLT-1 production. We administered siRNAs to silence JMJD6 in primary trophoblast cells. We obtained a >70% knockdown of JMJD6 expression (Fig. 3A; \( P < 0.0001 \)) and this significantly increased sFLT-1 protein secretion (Fig. 3B; \( P = 0.008 \)). Interestingly, this increase appeared to be principally attributable to increased sFLT-1 e15a mRNA expression (Fig. 3C; \( P = 0.02 \)) because there was no significant change in sFLT-1 i13 mRNA levels (Fig. 3D; \( P = 0.96 \)).

We administered siRNAs targeting Jmjd6 in HUVECs, and this resulted in an 80% knockdown of Jmjd6 (Fig. 4A; \( P < 0.0001 \)). Silencing JMJD6 in HUVECs increased sFLT-1 protein secretion (Fig. 4B; \( P < 0.0001 \)) and sFLT-1 i13 and sFLT-1 e15a mRNA expression (Fig. 4, C and D; \( P < 0.0001 \)).

**U2AF65 Interacts with Both JMJD6 and sFLT-1 Transcripts Within the Placenta**

Finally, we sought evidence that there may be direct interactions between JMJD6 and U2AF65 as well as between U2AF65 and sFLT-1 mRNA transcripts. Using immunofluorescence, we confirmed that JMJD6 and U2AF65 colocalize within the nuclei of the syncytiotrophoblast and the underlying cytotrophoblast cells in placenta (Fig. 5A). We also immunoprecipitated U2AF65 and probed for JMJD6 by Western blots (Fig. 5B) and were able to show the presence of a band consistent with JMJD6 that was absent in the rabbit IgG control (the large band at 50 kDa in both the U2AF65 immunoprecipitation and rabbit IgG immunoprecipitation lanes likely represents heavy chain IgG). Finally we immunoprecipitated U2AF65 in placenta and performed quantitative RT-PCR (qPCR) to see whether sFLT-1 mRNA transcripts were present. Compared with immunoprecipitation using antibodies against IgG, U2AF65 immunoprecipitation was associated with a significant enrichment for sFLT-1 i13 and sFLT-1 e15a (40.3- and 329.1-fold enrichment, respectively; Fig. 5C). Therefore in total, we have demonstrated interaction between JMJD6 and
U2AF65, and U2AF65 and the sFLT-1 mRNA transcripts within placenta.

**DISCUSSION**

Preeclampsia remains one of the most serious complications of pregnancy with no treatment other than delivery of the placenta and fetus. The discovery of anti-angiogenic factor sFLT-1 as a key contributor to the pathogenesis of preeclampsia was an enormous leap forward for the field. However, the molecular steps leading to the splicing of FLT-1 to produce sFLT-1 in the placenta are yet to be described. This work demonstrates the possible mechanism regulating production of sFLT-1 from the placenta, mapped from low oxygen to molecules in the splicing machinery, and to direct interactions with sFLT-1 transcripts (Fig. 6). We demonstrate that there is reduced expression of oxygen-sensing protein JMJD6 with hypoxia in placenta and that silencing JMJD6 in

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**FIG. 2.** Jmjd6, sFlt-1 e15a, and sFlt-1 i13 mRNA expression was assessed using qPCR in primary trophoblast and HUVECs exposed to hypoxia. Quantitative RT-PCR showed a significant decrease in Jmjd6 mRNA (A, E) expression. This was accompanied by significant increases in sFlt-1 e15a (B, F) and sFlt-1 i13 (C, G) mRNA expression and sFLT-1 secretion (D). Mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, Experiments repeated a minimum of three times.
primary trophoblast increases sFLT-1 secretion. Finally, we provide evidence of interaction between JMJD6 and U2AF65 (a component of the splicing machinery) as well as between U2AF65 and sFLT-1 mRNA variant transcripts within the placenta.

FIG. 3. Effect of Jmjd6 siRNA on sFlt1 production by primary trophoblasts was assessed using ELISA and qPCR. Jmjd6 siRNA induced a significant decrease in Jmjd6 mRNA expression (A), significantly increased sFLT-1 protein secretion (B) and sFLT-1 e15a mRNA expression (C), but did not affect sFlt1-i13 mRNA expression (D). Mean ± SEM; *P < 0.05, ****P < 0.0001. Experiments were repeated five times using separate trophoblast isolations.

FIG. 4. Effect of Jmjd6 siRNA on sFLT-1 production by HUVECs was assessed using ELISA and qPCR. Jmjd6 siRNA induced a significant decrease in Jmjd6 mRNA expression (A) and significantly increased sFLT-1 protein secretion (B). This was likely a result of significantly increased sFLT-1 e15a (D) and sFLT-1 i13 (C) mRNA expression. Mean ± SEM; ***P < 0.0001. Experiments were repeated three times.

JMJD6 is an oxygen-sensing protein belonging to the 2-oxoglutarate-dependent oxygenase superfamily [16]. These proteins play diverse roles in cellular processes, including collagen biosynthesis, intracellular signaling, and histone modification [16]. Like other family members, JMJD6 appears...
dependent on oxygen availability for its catalytic activity [17, 18]; however, there has been significant conflict regarding the precise role of JMJD6. Importantly, recent work demonstrated that JMJD6 was directly involved in regulating the splicing of FLT-1 to produce sFLT-1 within endothelial cells [9]. Given that the activity of JMJD6 is thought to be impaired in the presence of cellular hypoxia, a posttranslational event, our finding that JMJD6 expression is decreased in preterm preeclamptic placenta was unexpected. However, it is believed that the preeclamptic placenta is under chronic hypoxia. Thus, the fact that rendering endothelial and trophoblast cells hypoxic also decreased JMJD6 expression provides further support to the interesting hypothesis that one of the potential mechanisms involved in the elevated sFLT-1 secretion in preeclampsia is low JMJD6 expression due to hypoxia.

Indeed, we confirmed that silencing of JMJD6 expression increased sFLT-1 expression and secretion in primary trophoblast and extended our research to examine the relationship between JMJD6 and the multiple sFLT-1 splice variants. We demonstrated that the mechanism behind this alteration in sFLT-1 variants is likely to be via interaction of JMJD6 and U2AF65, which we showed is enriched for sFLT-1 i13 and sFLT-1 e15a. One limitation of our data is that we have not assessed hydroxylation of U2AF65 in primary trophoblast. Nevertheless, our findings are consistent with the findings of Boeckel et al. [9] who demonstrated that the effects of hypoxia would minimize the catalytic activity of JMJD6, leading to a reduction in the hydroxylation of U2AF65 and in turn causing alternative splicing of FLT-1 to produce sFLT-1. Therefore, our data indicates that JMJD6 is involved in the regulation of both sFLT-1 i13 and sFLT-1 e15a splicing, whereby a loss of JMJD6 (either under hypoxic conditions or via siRNA knockdown) produced preferential splicing leading to sFLT-1 variant production.

Our findings are opposite to a recent publication by Alahari et al. [14] who found that JMJD6 expression was increased under hypoxic conditions in cytotrophoblast cell

FIG. 5. U2AF65 interacts with Jmjd6 and sFlt-1 transcripts within the placenta. A) Representative immunofluorescence showing Jmjd6 (red) and U2AF65 (green) colocalized in placenta (white arrows). 4',6-Diamidino-2-phenylindole nuclei stain is shown in blue. Images are shown at ×200 magnification. B) Representative immunoprecipitation of U2AF65 from placenta probed with Jmjd6 indicates interaction at the protein level (band indicated by blue arrow), whilst rabbit IgG immunoprecipitation only shows the presence of heavy chain IgG. C) Immunoprecipitation of U2AF65 from placenta also produced a highly significant enrichment for sFlt-1 i13 and sFlt-1 e15a compared with IgG controls.
FIG. 6. The potential mechanism through which oxygen-dependent Jmjd6 regulates FLT-1 splicing. Under normoxic conditions, JMJD6 is active and hydroxylates U2AF65, resulting in production of FLT-1. In contrast, under hypoxia, the catalytic activity of JMJD6 is minimized, resulting in reduced hydroxylation of U2AF65 and in turn alternate splicing of FLT-1 pre-mRNA that leads to the production of sFLT-1 variants.

ACKNOWLEDGMENT

The authors acknowledge Clinical Research midwives Gabrielle Pell, Debra Jinks, Rachel Murdoch, and Genevieve Christophers and the Obstetrics midwifery staff and patients at the Mercy Hospital for Women (Heidelberg) for their provision of placental tissue.

REFERENCES


