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Differential regulation of a placental Sam68 and sFlt-1 gene pathway and the relevance to maternal vitamin D sufficiency

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Abstract

OBJECTIVE: The goal of this study was to determine if a previously observed axis of placental gene expression associated with early onset and severe preeclampsia (EOSPE) was operative in term pregnancy and correlated with vitamin D sufficiency.

METHODS: qPCR analysis of Nkx2-5, Sam68, sFlt-1 and membrane bound VEGFR1/Flt-1 mRNA expression was conducted in placentas from 43 subjects enrolled in a vitamin D3 pregnancy supplementation trial. Pair-wise rank order correlations between patient-specific gene expression levels were calculated, and their relationship to maternal 25(OH)D status was assessed by a two-sample Wilcoxon test. Additionally, we probed the mechanistic link between Sam68 and sFlt-1 using siRNA depletion in a human trophoblast cell line model.

RESULTS: Positive and highly significant correlations were found between Sam68 vs. sFlt-1 and Sam68 vs. Flt-1 expression levels, as were significant and differential correlations between the expression of these genes and perinatal 25(OH)D status. The variability when stratified by race/ethnicity was qualitatively distinct from those previously observed in EOSPE. Mechanistic studies confirmed a functional role for Sam68 protein in the regulation of sFlt-1 expression. Nkx2-5 expression was not significantly correlated with sFlt-1 or Sam68 expression in these samples, suggesting that its role may be mainly operative at earlier stages of pregnancy or be restricted to pathological settings.

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CONCLUSIONS: These data further support our overarching hypothesis that Sam68 expression is a key determinant of VEGFR1 isoform expression in the placenta, and provide additional insights into how this gene pathway may be differentially deployed or modified in normal and pathological pregnancy.

Keywords

Nkx2-5; sFlt-1; Sam68; pregnancy; preeclampsia

INTRODUCTION

Preeclampsia (PE) remains a significant cause of neonatal and maternal morbidity and mortality, affecting approximately 7% of pregnancies. The onset of PE can lead to severe complications and fatalities in the mother and the child [1, 2]. Currently, the only cure is delivery, and primary mechanisms of pathology are still unclear. To date, the cause of PE is not known, although many agree that PE is a two-stage disease as described by Roberts [3]. The first stage involves poor placental perfusion, usually as a result of impaired placentation and vascular remodeling in early pregnancy. This leads to the second stage, which is the maternal syndrome of hypertension, renal dysfunction and endothelial and leukocyte activation.

Several factors are known to modify the risk for PE. Intercurrent illnesses such as diabetes, obesity, chronic hypertension, and systemic lupus erythematosus are associated with increased risk for PE [4-9]. Racial differences exist for PE, with African Americans (AA) having higher rates and more severe disease as compared to Caucasians Americans (CA) and Hispanic Americans (HA) [10-12]. These observations have led to the hypothesis that several disease entities of variable maternal, fetal and/or environmental etiologies may incite or contribute to PE, but little is known about the cause of these differences [13]. This stems in part from a relative lack of insight into the early mechanisms operative in the genesis of PE.

One of the more recognized and compelling mechanistic links between reduced placental perfusion and the clinical sequelae in PE is sFlt-1. sFlt-1 protein, a soluble truncated isoform of the vascular endothelial growth factor receptor 1 (VEGFR1/Flt-1) has been actively studied as a marker of PE in humans [14-17], along with placental growth factor (PlGF) and the transforming growth factor beta (TGFβ) inhibiting factor endoglin [18]. Maynard et al. demonstrated that sFlt-1, a soluble receptor for VEGF and PlGF, is increased in PE [19]. sFlt-1 binds and inactivates both VEGF and PlGF, and its increase and the corresponding decrease in PlGF correlate with the severity of PE [20-24]. In addition, overexpression of sFlt-1 in pregnant animal models was sufficient to cause hypertension and proteinuria [25-29]. Because of these correlations, recent efforts to develop diagnostic tests for PE risk assessment have focused on the accurate assay of serum PlGF [8, 30], sFlt-1 [25, 31] and their ratio [20-23, 32, 33].

While it is known that placental tissue is the primary source of sFlt-1, and that sFlt-1 alone is capable of inducing symptoms of PE in animal models, the mechanism regulating the production of sFlt-1 and thus responsible for high levels of its expression in PE is yet

unclear [25, 34]. It is known that mRNA encoding sFlt-1 is generated by an alternative splicing and premature polyadenylation of VEGFR1 transcripts; in the absence of splicing of VEGFR1 exon 13 to exon 14, transcripts are prematurely polyadenylated (pA) via cryptic sites in the intervening intron, and the resulting transcript encodes a truncated VEGFR1 molecule lacking the transmembrane domain and c-terminal signaling domains. sFlt-1 thus acts as an antagonist for VEGFR signaling, particularly by PlGF [35].

In a prior study, we detected an association between expression levels of the cardiac developmental transcription factor Nkx2-5 and the RNA splicing factor Sam68, with expression levels of sFlt-1 in placentas of women with early onset and severe preeclampsia (EOSPE) [36]. Statistically significant correlations between increased Nkx2-5 and increased Sam68 and sFlt-1 mRNA expression were particularly prominent in CA vs. AA populations with EOSPE. Even stronger positive correlations were observed between Sam68 and sFlt-1 alone, regardless of race/ethnicity. Immunolocalization studies in placenta and in vitro RNA interference experiments in cultured cells supported a mechanistic model whereby abnormally high Nkx2-5 expression levels potentiated high placental sFlt-1 expression and EOSPE through activation of Sam68 and preferential production of sFlt-1. Taken together, these results were consistent with an “axis” of placental gene expression mediating developmental control of angiogenic vs antiangiogenic signaling. Pathological regulation of this axis in EOSPE could result in compromised vascularization, hypoxia and the onset of the PE syndrome.

In an effort to broaden our understanding of the significance of the Nkx2-5/Sam68/sFlt-1 axis to placental development and pregnancy beyond the setting of EOSPE, we assayed an existing set of normal term placental samples that had been collected as part of an investigation of the effects of optimal gestational vitamin D supplementation on pregnancy health. A principal finding of this study had been the detection of a significant correlation between vitamin D sufficiency and a pro-angiogenic trend of the expression of VEGF and sFlt-1 mRNA in the placenta [37]. A subsidiary goal of this current study was thus to test for similar correlations between vitamin D status and expression levels of the genes in the Nkx2-5/Sam68/sFlt-1 axis in term pregnancy.

METHODS

Placental sample collection and mRNA purification.

RNA samples were obtained from a previously described randomized, placebo-controlled clinical trial ([NCT01932788](#)) of high vs. low dose vitamin D and their impact on pregnancy health. In brief, the study population consisted of 43 pregnant women of 18-45 years of age that were randomized to receive placebo or 4000 IU/day vitamin D₃ plus the standard prenatal vitamin (containing 400 IU vitamin D₃). Race/ethnicity and other demographic factors were previously reported, which showed no other significant differences between vitamin D deficient and sufficient groups with regard to insurance, marital status, maternal age, BMI, baseline 25(OH)D, infant gestational age and infant birthweight [37].

Individual placentas were sampled at four separate sites, and mRNA extracted from these biopsies using RNEasy Plus affinity spin columns (Qiagen, Valencia, CA) and standard

methods. Equal ug amounts of RNA from each of the four biopsy sites were pooled and 500 ng of the mixed RNA was used to synthesize cDNA using iScript (BioRad, Hercules, CA) reverse transcriptase and random hexamer priming. For cell culture experiments, RNA was harvested and purified from 12-well plate wells using RNEasy Plus and concentrations determined spectrophotometrically. cDNA were synthesized from 500ng of purified RNA for qPCR analysis. Experiments were performed in triplicate, and results are representative of three independent experiments.

qPCR analysis of placental samples.

cDNA derived from total placental RNA were subject to quantitative real-time PCR to determine transcript levels of human Nkx2-5, sFlt-1, VEGFR1/Flt-1, Sam68, GAPDH and β -actin using iQ SYBR Green master mix (BioRad) and a CFX96 Thermal Cycler (BioRad).

CT thresholds were determined by default thermal cycler settings. Raw fluorescence amplification data were additionally analyzed using the web-based PCR Miner resource [38] ([HTTP:ewindup.info/miner/](http://ewindup.info/miner/)) to correct for relative PCR efficiencies. Expression levels relative to GAPDH or β -actin were then used to determine Spearman rank order correlations between Nkx2-5, Sam68, sFlt1 and VEGFR1/Flt-1. Methods including primer sequences used in these analyses were as previously described [36, 37].

Trophoblast cell culture, siRNA depletion and Western blot analysis.

HTR8/SVneo human trophoblast cells [39] were maintained in RPMI-1640 medium supplemented with pen/strep and 5% FBS in 5% CO₂. Cells were split 1:6 from confluence into 12-well plate wells on the day prior to transfection with 20 pmol scrambled control or anti-huSam68 siRNA oligomers (OriGene, Rockville, MD). Representative wells were harvested by lysis with RIPA buffer (150 mM NaCl/50 mM Tris-HCl (pH 8.0)/1% NP-40/0.5% Na deoxycholate/0.1% SDS/protease and phosphatase inhibitors). Following protein determination by BCA dye binding assay, 3ug total protein samples were separated on 10% SDS-PAGE acrylamide gels in MOPS buffer (Invitrogen, Carlsbad, CA), transferred to nylon membranes (Millipore PVDF-FL) and probed with antibodies to Sam68 (Upstate-Cell Signaling #07-415) and α -tubulin (Sigma-Aldrich T7451) to verify loss of Sam68 expression. Following incubation with chemiluminescent secondary antibodies, blots were scanned and quantitated using an Odyssey CLX imager and associated software. Experiments were performed in triplicate and are expressed as average values and standard error from the mean (SEM). Results shown are representative of three independent experiments.

Measurements of sFlt-1 protein and mRNA expression in cultured trophoblasts.

Following overnight siRNA transfection of HTR-8/SVneo cells, the medium was changed and incubated for another 48 hrs before collection of supernatants and cell pellets. sFlt-1 protein concentrations were measured in non-diluted cell culture supernatants using a high-sensitivity Quantikine ELISA Kit (DVR100C; R&D Systems, Minneapolis, MN), following the manufacturer's instructions. This assay has a dynamic range of 31.25-2000 pg/ml and an intra-assay coefficient of variation (CV) of 2.9%. Each sample was analyzed in duplicate from three independent experiments. Additionally, cell pellets were lysed for RNA

purification using RNEasy affinity purification, cDNA synthesis and qPCR for sFlt-1 and β -actin as above.

Statistical analyses.

Spearman rank order correlations were used to characterize the associations between *CT* expression levels for Sam68, sFlt1, and VEGFR1/Flt-1, where the *CT* expression levels were calculated based on relative expression to GAPDH or β -actin. These correlations were calculated for the entire cohort (n=43), as well as for 3 race/ethnicity groups (AA [n=11], CA [n=14], and HA [n=17]). Wilcoxon rank sum tests were used to assess whether *CT* expression levels were markedly higher for subjects who were vitamin D deficient (25(OH)D < 100 nmol/L) compared to those who were sufficient (> 100 nmol/L), both at visit 1, and at the end of the study (visit 6/7). To determine whether associations between gene expression and vitamin D sufficiency were mitigated by any of 6 covariates (i.e. maternal age, maternal race/ethnicity, gravidity, parity, gestational age, birth weight), a series of multivariable regression models were constructed. In those models, the *CT* expression levels served as the dependent variables, while vitamin D sufficiency was the primary independent variable. Since there were 6 covariates but only n=43 observations, a forward stepwise procedure was used to allow up to 2 covariates to enter the model if they provided even a modest level significance (p<0.20).

RESULTS

As shown in Figure 1A, and consistent with our previous findings, a rank order correlation analysis found that sFlt-1 and Sam68 mRNA levels were positively correlated ($\rho = +0.696$) with high significance ($p = 2.2 \times 10^{-7}$; n = 43). We had previously found evidence for racial disparity in the correlation between sFlt-1 and Sam68 expression in the EOSPE placenta, where correlations between Nkx2-5, Sam68 and sFlt-1 were positive and significant to highly significant for CA, but not significant for AA [36]. In contrast, in these term placental samples, we found that positive and highly significant correlations between sFlt-1 and Sam68 mRNA extended to all racial/ethnic groups, including in HA that were not included in our prior EOSPE study (AA [n= 11]: $\rho = +0.769$, $p = 0.003$; CA [n = 14]: $\rho = +0.701$, $p = 0.005$; HA [n = 17]: $\rho = +0.662$, $p = 0.004$) (Figure 1B-D).

Our prior study had found that correlations with Sam68 expression levels were unique to the sFlt-1 encoding alternatively spliced isoform of VEGFR1 and were not found with Flt-1 transcripts where continued splicing from the Exon 13-14 boundary resulted in mRNAs encoding membrane-bound VEGFR1/Flt-1 receptor. However, in the term population, we were able to detect a highly significant and positive correlation in placenta between Flt-1 and Sam68 mRNA expression ($\rho = +0.553$, $p = 1.2 \times 10^{-4}$) (Figure 2A). A degree of racial/ethnic disparity in this correlation that was not present in the sFlt-1 – Sam68 analysis was found when the analysis was divided into subgroups: while Flt-1 and Sam68 expression levels continued to show positive correlations with one another, these correlations were highly significant only in AA ($\rho = +0.940$, $p = 1.6 \times 10^{-4}$) while remaining significant in CA ($\rho = +0.569$, $p = 0.03$), but less positive and not significant in HA ($\rho = +0.275$, $p = 0.29$) (Figure 2B -D).

These disparities were also reflected in an analysis of the correlation between expression levels of sFlt-1 vs. Flt-1 isoforms. The overall correlation in the total population again appeared positive and highly significant ($\rho = +0.546$, $p = 1.5 \times 10^{-4}$) (Figure 3A). The correlation was very strong in AA ($\rho = +0.713$, $p = 0.01$) (Figure 3B) but only moderately strong among the CA ($\rho = +0.402$, $p = 0.15$) (Figure 3C) and HA populations ($\rho = +0.453$, $p = 0.07$) (Figure 3D). While the loss of significance of rank order correlations in some ethnic subpopulations may have been due in part to smaller population sizes, the Flt-1 – Sam68 correlation remained most significant in AA, which contained the smallest number of patients ($n = 11$), while being less positive and not significant in the HA subgroup which contained the largest number of patients ($n = 17$).

By contrast, and as shown in Figure 4A, a contemporaneous re-interrogation of mRNA samples from our previous EOSPE placental sample population [36] did not detect a significant correlation between Sam68 and Flt-1 mRNA expression, either in the population as a whole [$n = 33$] ($\rho = +0.231$, $p = 0.196$), or when divided into CA [$n = 14$] ($\rho = -0.130$, $p = 0.659$) and AA [$n = 19$] ($\rho = +0.009$, $p = 0.972$) subpopulations (Figure 4B, C).

In contrast to results in our prior study of EOSPE placentas, we were unable to detect significant levels of Nkx2-5 expression in these term placental samples (data not shown). These differences in Nkx2-5 expression may due to the specific disease pathology driving EOSPE in our previous study cohort. Alternatively, this may reflect differences in gestational age, as the EOSPE samples were predominantly from preterm pregnancies [36].

In their previous analysis of placental mRNA expression from the term vitamin D study cohort, Schulz et al. detected a significant correlation between differential VEGF axis mRNA expression levels and vitamin D levels post-supplementation [37]. Specifically, they detected significantly higher placental VEGF and lower sFlt-1 mRNA levels when comparing samples from women who were vitamin D deficient (vitamin D <100 nmol/L) vs. those that were vitamin D sufficient (vitamin D ≥ 100 nmol/L). In order to determine whether correlations between vitamin D deficiency/sufficiency would extend to Sam68 expression and our hypothesized mechanistic relationship to sFlt-1 and VEGFR1 mRNA regulation, we conducted a similar analysis comparing sFlt-1, Sam68 and Flt-1 mRNA expression levels in the two vitamin D groups.

As shown in Table 1, median CT values for sFlt-1, Sam68 and Flt-1 showed no significant differences in expression between vitamin D deficient ($n = 36$) vs. vitamin D sufficient ($n = 7$) patients as assessed at baseline (visit 1 or V1). The lack of association was confirmed in our multivariable regression models that included covariate adjustments for maternal age and race/ethnicity. However, when compared according to the final vitamin D status proximal to delivery (visit 6/7 or V6/7), highly significant differences were apparent in median CT values for both Flt-1 ($p = 0.016$) and Sam68 ($p = 0.005$). A comparison of median fold expression levels calculated as 2^{-CT} found a 2.6-fold decreased level of Flt-1 receptor expression in placentas from vitamin D sufficient pregnancies as compared to vitamin D deficient pregnancies, while median Sam68 expression levels were 6.3-fold lower in vitamin D sufficient pregnancies. Not surprisingly, given the high degree of correlation detected between Flt-1, sFlt-1 and Sam68 expression levels we detected, we similarly found that

median sFlt-1 mRNA levels were 3.3-fold decreased in vitamin D sufficient placentae when considering V6/7 vitamin D status, and 1.8-fold decreased when considering V1 status, confirming findings by Schulz et al. in their prior study [37]. Due perhaps to minor variations in sample preparation and qPCR analysis regime and associated scatter, these differences did not however reach the threshold of significance in our analysis (V1: $p = 0.38$; V6/7: $p = 0.12$). In multivariable regression models that included covariate adjustments for maternal infant characteristics, associations between CT values and mother's V6/7 vitamin D status remained unchanged for sFlt-1 and Sam68, since no covariates met the threshold for inclusion into the models. However, for Flt-1, the significant association between it and mother's V6/7 vitamin D status was no longer significant ($p = 0.51$), primarily due to an association noted between Flt-1 CT values and race/ethnicity (AA: median[IQR] $CT = 1.31 [-0.29, 4.93]$; CA: $0.33 [-1.11, 0.69]$; HA: $-0.47 [-1.76, 1.02]$; $p = 0.016$).

Given the positive correlations between VEGFR1 mRNA isoform expression and the expression of a putative splicing regulator Sam68, we tested the effect of altered Sam68 expression on sFlt-1 mRNA and protein expression in a trophoblast cell line model. Previously, we found that partial depletion of Sam68 protein levels in human kidney epithelial cells resulted in significantly decreased levels of sFlt-1 vs exon 13-14 spliced VEGFR1/Flt-1 mRNA isoforms, supporting the hypothesis that Sam68 mediated preferential splicing of sFlt-1 encoding mRNA [36]. In order to test whether Sam68 similarly regulated sFlt-1 splicing and protein expression in placental trophoblasts, we performed antisense knockdown or protein depletion experiments in HTR8/SVneo cells, which model human extravillous trophoblasts. Using two distinct silencer antisense RNA molecules targeting Sam68, we reproducibly achieved an approximately 60-80% depletion of Sam68 protein expression as measured by Western blot (Figure 5A, B). qPCR for specific VEGFR1 isoforms showed variable changes in both sFlt-1 and Flt-1 expression levels, with a trend toward increasing levels of both mRNAs and a stable sFlt-1/Flt-1 ratio. ELISA assay for sFlt-1 in the culture medium detected an approximately 50% increase in sFlt-1 protein secretion. These results were at variance with our previous results in HEK293 cells [36], and indicated the potential for cell type-specific differences in the functional relationship of Sam68 and sFlt-1 splicing and expression.

DISCUSSION

In this study, we have found additional evidence corroborating an association between maternal vitamin D sufficiency and the overall regulation of angiogenic signaling relevant to pregnancy. Specifically, we extended the previously reported association between vitamin D sufficiency and sFlt-1 mRNA levels to now include differential expression of a known RNA binding protein and alternative splicing factor, Sam68, that we have implicated in the regulation of sFlt-1 in the setting of pathological pregnancy with EOSPE, and related mRNA species encoding the membrane-bound alternatively spliced VEGFR1/Flt-1 isoform.

A limitation of our prior study was the inclusion of only a small number of non-pathological placentas; e.g., from uncomplicated term pregnancies. It was thus unable to address the question of whether these observations reflected an exclusively pathological state, or whether aspects of the Nkx2-5/Sam68/sFlt-1/Flt-1 gene regulation were operative during

normal gestation and placental development but were perturbed in the setting of PE. Additionally, the population study included only CA and AA, and so was unable to examine possible correlations to other groups. In the current analysis of predominantly uncomplicated term pregnancies, we have found highly significant correlations of the expression of core elements of the axis pertaining to sFlt-1 regulation, i.e. of Sam68, sFlt1 and Flt-1 mRNA levels that extended across the population as a whole, and to a large part within AA, CA and HA groups. As in the case of our prior study, differences in the significance of correlation were found for select gene associations when results were stratified by race/ethnicity. Specifically, while sFlt-1 – Sam68 associations remained strong in all groups, the Flt-1 – sFlt-1 correlations were somewhat attenuated in CA and HA groups; in the HA group the correlation between Flt-1 and Sam68 ($p = 0.275$) was markedly lower than that of the AA ($p = 0.940$) or CA group ($p = 0.569$), indicating that regulation of membrane-bound VEGFR1/Flt-1 isoform may be differentially responsive to additional modifiers beyond Sam68 expression and activity in specific groups. Indeed, significant differences in Flt-1 levels between groups, particularly HA, appeared to contribute to the loss of significant correlation of Flt-1 levels to vitamin D sufficiency.

Mechanistically, Sam68 protein knockdown experiments in HTR8/SVneo trophoblast cells revealed additional complexity in the relationship between Sam68 and sFlt-1 expression. In HEK293 kidney epithelial cells, siRNA depletion of Sam68 resulted in significant modulation of sFlt-1 mRNA levels, with a significant decrease in both the absolute level of sFlt-1 mRNA expression, and in the ratio of sFlt-1/Flt-1 spliced [36]. In HTR8/SVneo cells, however, the primary effect of Sam68 siRNA depletion was an apparent increase in the level of sFlt-1 protein secretion in the absence of consistent downregulation of sFlt-1 vs. Flt-1 mRNA alternative splicing or expression levels. Beyond the regulation of alternative splicing, Sam68 RNA binding has also been implicated in the post-transcriptional regulation of protein translation rates, in large part due to its association with RNA and protein-rich stress granules, whose accumulation and stability are modulated by inputs like oxidative stress and other signaling pathways [40-42]. Cell-type specific modulation of Sam68 activity, principally through kinase signaling pathways, potentially add additional layers of complexity to the relationship between Sam68 expression and regulation of sFlt-1 mRNA and protein levels [43-45]. Although our present finding of the relationship of Sam68 and sFlt-1 protein in cultured trophoblasts appears to be in the opposite direction to that from human placental tissues, it nevertheless supports a dynamic regulatory relationship between the two. A an additional possible explanation for this seeming discrepancy is that HTR8/SVneo cells represent primarily extravillous invasive trophoblasts, which were likely not reflected by the placental tissues examined in this study.

Interestingly, we found little evidence of significant Nkx2-5 mRNA expression in these samples, while we had previously observed pronounced elevations in the Nkx2-5 expression in EOSPE. In mice, we have observed that total placental Nkx2-5 protein levels vary during gestation, reaching a peak in late gestation, and falling to low levels toward term (unpublished), so this difference may be due to the differences in gestational ages, as the majority of EOSPE placentas were obtained coincident with preterm delivery [36, 46] while this current study contained samples from term pregnancy. Alternatively, elevated Nkx2-5 mRNA levels in EOSPE may be an expression of disease-specific pathology, either in and of

itself, or as a modifier of developmentally-regulated expression. Indeed, in other studies we have detected substantial placental Nkx2-5 mRNA expression in the setting of diabetes and late onset PE (manuscript in preparation).

As with the parent vitamin D pregnancy study, limitations included variation of patient compliance with treatment and the exclusion of study individuals less than 75% compliant with supplementation, and potential variations in placental tissue sampling [37]. As in the previous study, gene expression analysis was limited to a few candidates of interest that were selected due to their potential functional linkage to the angiogenic genes previously identified as being associated with vitamin D sufficiency vs. deficiency. As with the prior study, analysis focused on mRNA expression and thus did not measure correlation with protein levels. While these findings extend our insight into possible mechanisms by which vitamin D sufficiency might benefit pregnancy health through VEGF/PlGF-related signaling pathways, further study is required to appropriately delineate a broader context of overall placental development. As well, future studies of other patient populations are needed to more firmly establish associations between the Nkx2-5/Sam68/sFlt-1 axis and clinical outcomes. Particularly with regard to the association with Nkx2-5 expression per se, the absence of patients with diagnoses of PE patients and non-PE preterm delivery in this and the earlier EOSPE cohort [36] prevents us from distinguishing between disease-related vs. gestational age-related mechanisms as a cause for varying Nkx2-5 mRNA expression. Finally, since the sample sizes were relatively low within specific racial/ethnic subgroups, it should be noted that this study was not powered to detect differences between these subgroups; while some potential racial/ethnic differences were noted, these should be considered preliminary and warrant further investigation in larger cohorts.

Given these limitations, the findings of this study still provide a broader perspective for our emerging hypotheses regarding the role of specific developmentally-related gene pathways to the progression of pregnancy, the risk of pregnancy-related disease, and their potential to be modified by nutritional and metabolic factors. Going forward, future studies must be geared both toward investigating the molecular details modulating gene expression, e.g. the effect of vitamin D signaling on transcriptional regulation of the Sam68/sFlt-1/Flt-1 axis, and toward testing the effect of other known pregnancy disease modifiers on the modulation of these molecular markers. This combination of mechanistic and populational studies will be important for defining differential risk for pregnancy complications like PE, and for fostering the development of rational therapies targeting modifiable factors to reduce such risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Relative gene expression levels of sFlt-1, Sam68 and Flt-1 are significantly associated in human placenta from term pregnancies, similar to findings from early onset and severe preeclampsia.
- Consistent with previous findings, expression levels of these genes are significantly different in placenta from vitamin D sufficient vs. deficient mothers.
- In contrast to preeclampsia placentas, term placentas show negligible expression of the Nkx2-5 transcription factor.
- In model trophoblast cells, the RNA binding protein Sam68 appears to modulate sFlt-1 secretion at the level of translational regulation.
- sFlt-1 levels and angiogenic signaling during pregnancy may be subject to a coordinated axis of regulation subject to epigenetic inputs.

Figure 1. Correlated expression of sFlt-1 and Sam68 mRNA in term placentae.

A. Scatter diagram comparing ranked expression levels of sFlt-1 (x-axis) vs. Sam68 (y-axis) in placentae from the overall study population (n = 43). Pearson rank order analysis revealed a positive ($\rho = +0.696$) and highly significant ($p \ll 0.01$) correlation between sFlt-1 and Sam68 in these samples. Rank order values and correlations were calculated separately for samples as categorized by race/ethnicity: **B.** AA (diamonds) (n = 14; $r = +0.769$); **C.** CA (squares) (n = 11; $r = +0.701$), and, **D.** HA (triangles) (n = 17; $r = +0.662$). Positive and significant or highly significant correlations were detected in all subpopulations. *significant ($p \leq 0.05$); **highly significant ($p \leq 0.01$); ns: not significant.

Figure 2. Correlated expression of Flt-1 and Sam68 mRNA in term placentae.

A. Scatter diagram comparing ranked expression levels of Flt-1 (x-axis) vs. Sam68 (y-axis) in placentae from the overall study population (n = 43). Pearson rank order analysis revealed a positive ($\rho = +0.553$) and highly significant ($p = 1.2 \times 10^{-4}$) correlation between Flt-1 and Sam68 in these samples. Rank order values and correlations were calculated separately for samples as categorized by race/ethnicity: **B.** AA (diamonds) (n = 14; $r = +0.940$); **C.** CA (squares) (n = 11; $r = +0.569$); and, **D.** HA (n = 17; $r = +0.275$). Positive and significant or highly significant correlations were detected in AA and CA populations, but not in the HA population. * significant ($p \leq 0.05$); **highly significant ($p \leq 0.01$); ns: not significant.

Figure 3. Correlated expression of sFlt-1 and Flt-1 mRNA in term placentae.

A. Scatter diagram comparing ranked expression levels of Flt-1 (x-axis) vs. sFlt-1 (y-axis) in placentae from the overall study population ($n = 43$). Pearson rank order analysis revealed a positive ($\rho = +0.546$) and highly significant ($p \ll 0.01$) correlation between Flt-1 and Sam68 in these samples. Rank order values and correlations were calculated separately for samples as categorized by race/ethnicity: **B.** AA (diamonds) ($n = 14$; $r = +0.713$); **C.** CA (squares) ($n = 11$; $r = +0.402$); and, **D.** HA ($n = 17$; $r = +0.453$). Positive and significant or highly significant correlations were detected in AA and CA, but not in the HA population.

*significant ($p \leq 0.05$); **highly significant ($p \leq 0.01$); ns: not significant.

Figure 4. Expression of Flt-1 vs. Sam68 mRNA is not correlated in EOSPE placentae.

A. Scatter diagram comparing ranked expression levels of Flt-1 (x-axis) vs. Sam68 (y-axis) in placentae from an EOSPE population ($n = 33$) [36]. Pearson rank order analysis failed to reveal a significant correlation between Flt-1 and Sam68 in these samples. Rank order values and correlations were calculated separately for samples as categorized by race/ethnicity: **B.** AA (squares) ($n = 14$; $r = -0.130$); and **C.** CA (diamonds) ($n = 19$; $r = +0.009$). Significant correlations between ranked Flt-1 and Sam68 were not detected in these subpopulations. *significant ($p \leq 0.05$); **highly significant ($p \leq 0.01$); ns: not significant.

Figure 5. siRNA depletion of Sam68 modulates sFlt-1 protein secretion from HTR8/SVneo trophoblast cells.

A. Western blot; and, **B.** Immunofluorescent quantitation of Sam68 vs α -tubulin protein levels following transfection of HTR8/SVneo trophoblast cells with control scrambled (Scr) and two independent siRNA species (Si1, Si2) targeting Sam68 mRNA. siRNA knockdown resulted in approximately 60-75% reduction in detectable Sam68 protein levels 48 hours post-transfection. **C.** qPCR for sFlt-1 mRNA in control and depleted HTR8/SVneo cells following 48 hrs depletion. **D.** sFlt-1/Flt-1 mRNA ratios as determined by qPCR. **E.** ELISA assay for sFlt-1 protein in conditioned medium from siRNA-depleted HTR8/SVneo cells. Sam68 reduction resulted in an approximately 40-45% increase in sFlt-1 secretion as compared to controls. ** highly significant ($p < 0.01$); ns: not significant.

Table 1.

The effect of vitamin D deficiency* on placental gene expression (CT)

Gene	Vitamin D deficient at V1 (n=36) Median (IQR)	Vitamin D sufficient at V1 (n=7) Median (IQR)	<i>p</i> -value	Vitamin D deficient at V6/7 (n=13) Median (IQR)	Vitamin D sufficient at V6/7 (n=30) Median (IQR)	<i>p</i> -value
sFlt-1	-2.13 (-3.51, -0.82)	-2.99 (-4.06, -1.18)	0.38	-1.07 (-2.31, -0.13)	-2.80 (-3.73, -1.34)	0.12
Flt-1	0.15 (-1.24, 1.28)	0.13 (-1.11, 0.91)	0.75	1.02 (0.05, 1.82)	-0.37 (-1.76, 0.69)	0.016
Sam68	-2.30 (-3.64, 0.30)	-1.74 (-3.37, -0.47)	1.00	0.13 (-1.03, 0.64)	-2.53 (-4.25, -1.41)	0.005

*
25(OH)D < 100 nmol/L