

SUPPLEMENTAL METHODS

Recombinant Proteins, Antibodies and other Reagents: Anti- human sFRP1 (ab126613) and Ki67 (ab16667) antibodies were purchased from Abcam, and anti- β -actin (A5316) antibodies were obtained from Sigma. Anti-human cytokeratin antibodies were obtained from DAKO (M351501-2) and Pharmingen (349205). Extravillous trophoblast-like immortalized HTR-8/SVneo cells (a gift of Dr. Charles Graham) were used.

Tricarbonyldichlororuthenium (II) dimer (CO-RM2) and benzo[a]pyrene were obtained from Sigma. Recombinant sFRP1 and Wnt3A were obtained from R&D systems.

Cigarette smoke extract was generated as previously described (1) by bubbling smoke from 2 Marlboro 100's cigarettes into 10 ml of serum free-RPMI for approximately 5 min and then filtering the media with a 0.45 μ m filter.

mRNA microarray, quantitative RT-PCR and Western blot analysis: Human placental tissue samples were stored in RNAlater at -70°C . Total RNA was extracted from placental tissue using Trizol and reverse transcription-polymerase chain reaction (RT-PCR) were performed as described previously (2). Gene expression profiles were performed at the Genomics core facility at the Beth Israel Deaconess Medical Center using Affymetrix U133 chip containing ~20,000 genes. Quantitative real time-PCR was used to confirm sFRP1 expression in human placentas using commercially available primers for sFRP1 (Hs00610060_m1) and actin (Hs00915253_m1) from Applied Biosystems. In mouse studies, adenoviral expression was confirmed in the maternal liver on E19 by RT-PCR for His-tagged adenoviral sFRP1 gene expression. Sequences of the forward and reverse primers to identify mRNA expression were 5' C ACC ATG

ATG ATG ATG ATG TCC CCC GAC 3' and 5' CTA ATG ATG ATG ATG ATG ATG CAC CGT TGT 3'. Immunoblot analysis for sFRP1 and actin was performed using placental lysates prepared as described previously (2). Monoclonal anti-sFRP1 (1:300) was used at a 1:300 dilution, and monoclonal anti-actin was used at 1:3000 dilution for Western blots.

Ki67 immunostaining: Placentas were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. In addition to hematoxylin and eosin staining, immunohistochemistry for Ki67 was performed on placental tissue sections (6 µm) to detect proliferating cells as described elsewhere(3). ImageJ software was used to quantify Ki67 staining. Total Ki67 positive cells were counted in three separate placentas from each control virus and Ad.sFRP1 virus treated animal (n=3 mothers/group). Ki67 positive cells in human placentas were counted in 5 random areas per control and diseased placenta (n=5 each), and cell count per area was calculated and quantified. sFRP1 (1:50 dilution) and cytokeratin (pre-diluted mix, 1:1) expression were also evaluated by immunohistochemistry in paraffin-embedded human placental tissue according to published protocols (4).

Adenovirus generation: The human sFRP1 cDNA (AA 1-185) was amplified by PCR from sFRP1 cDNA (Origene) in frame with a His tag. The tagged cDNA was then ligated into adenoviral vector (pAd/CMV/V5-DEST) as detailed in Supplemental Figure 2A. Construction of the adenovirus was made using the Invitrogen Virapower™ expression system as per manufacturer instructions. After sequence confirmation, both control

(CMV promoter only) and sFRP1 adenovirus were amplified in a commercial facility (Viraquest).

Mouse studies: Timed pregnant CD1 mice were obtained from Charles River Laboratories. Animals were anesthetized with isoflurane and injected with adenovirus expressing sFRP1 or a control (CMV-null) virus (n=7-9 pregnant mice/group). 2.5×10^9 PFU of sFRP1 virus or control virus was delivered via tail-vein injection on E15. Alternatively, animals were injected with 20 mg/kg of CO-RM2 (n=5 mice) or 1% DMSO vehicle (n=5 mice) via tail-vein injection on E15. Blood was collected by cardiac puncture at time of sacrifice on E19. After a cesarean section was performed, the placentas and fetuses were immediately dissected and weighed. Protein expression of sFRP1 was confirmed in maternal serum on day of delivery (E19) by immunoblot.

Wnt Reporter Assay: HEK293 and HTR cells were transiently co-transfected with luciferase plasmids containing multimeric LEF/TCF cognate sequences (Topflash) and CMV- β -Gal vectors. Briefly, cells cultivated in 96 wells were incubated with 1.5 μ g luciferase reporter plasmid and 0.5 μ g pCMV- β -Gal in 1.5 μ l Lipofectamine LTX (Invitrogen) or 0.8 μ l PolyJet (SignaGen). After 8 hours, medium was changed, and cells were stimulated with DMSO, varying concentrations of CO-RM2 up to 500 μ g/ml concentration and cigarette smoke extract for an additional 24 h. Luciferase activity and β -galactosidase activity were measured on a luminometer using a combined β -galactosidase/luciferase assay system (Promega).

Proliferation: HTR cells were plated onto 24-well plates at 5×10^3 cells/well and attached for 24 hrs in complete DMEM medium. Medium was replaced by DMEM with 0.1% fetal calf serum for 24 hrs prior to CO-RM2 exposure. Fetal calf serum (2.5%), DMSO, CO-RM2, and inactivated (i)CO-RM2 were added alone or in combination to medium. One μCi of [^3H] thymidine (PerkinElmer Life Sciences) was added per well after 18 hrs of exposure. At 24 hrs, cells were trypsinized and harvested with a PhD cell harvester (Brandel), and incorporation of [^3H] thymidine was assayed.

SUPPLEMENTAL REFERENCES

1. Yang GY, Zhang CL, Liu XC, Qian G, and Deng DQ. Effects of cigarette smoke extracts on the growth and senescence of skin fibroblasts in vitro. *Int J Biol Sci.* 2013;9(6):613-23.
2. Rana S, Rajakumar A, Geahchan C, Salahuddin S, Cerdeira AS, Burke SD, George EM, Granger JP, and Karumanchi SA. Ouabain inhibits placental sFlt1 production by repressing HSP27-dependent HIF-1alpha pathway. *FASEB J.* 2014;28(10):4324-34.
3. Gerdes J, Schwab U, Lemke H, and Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer.* 1983;31(1):13-20.
4. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A, Hubel CA, Stillman IE, Parikh SM, and Karumanchi SA. Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension.* 2012;59(2):256-64.

SUPPLEMENTAL TABLE 1

Maternal and infant characteristics

Table 1	Non-smokers	Smokers
<i>N</i>	24	11
Maternal age, years*	32.7	26.8
Preeclampsia, number (%)	7 (29%)	1 (9%)
White Race, number (%)*	13 (54%)	8 (73%)
Gestational Age at Delivery (weeks ± days)	35 5/7	37 5/7
Male Gender, number (%)*	8 (33%)	5 (45%)
FGR <5 th percentile, number (%)	3 (13%)	4 (36%)
Birthweight (grams) ± SEM*	2481 ± 276	2742 ± 299
Cesarean delivery, number (%)	20 (83%)	6 (55%)

*Data missing from three term non-smokers

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

- A.** Heat map of top upregulated and downregulated genes in placentas from maternal smokers with small-for-gestational age infants (S1, S2) compared to non-smokers with appropriate-for-gestational age infants (NS1, NS2).
- B.** Relative quantification (RQ) of sFRP1 mRNA levels measured by qRT-PCR from placentas with smoking-FGR (n=3), placentas from pregnancies with GA- matched, appropriate for gestational age (AGA) fetuses (n=6, + $p<0.05$ versus smoking-FGR by t-test), and to placentas with preeclampsia-FGR (n=3, * $p<0.05$, versus smoking-FGR by t-test). In the comparison of all three groups, $p=0.05$ by ANOVA. Data is presented as mean \pm SEM.
- C.** Representative histology sections of human placentas. Ki67 immunostaining, a marker of proliferation, in maternal smokers (n=5) compared to non-smokers (n=5). Bars in Supplemental **1C**=50 μ m
- D.** Quantification of Ki67 staining by ImageJ for the data in Supplemental Figure 1C is presented as mean \pm SEM (* $p<0.05$ versus nonsmoker by t-test).

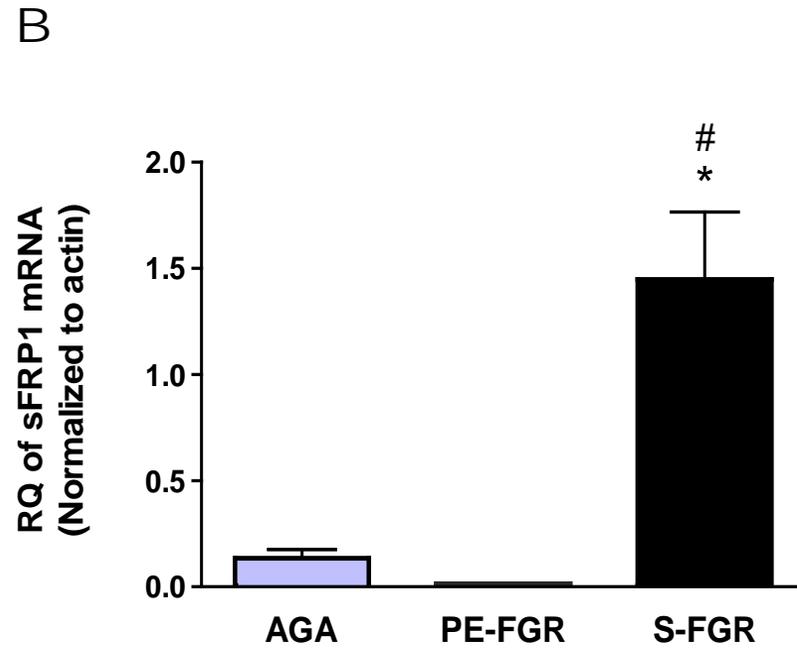
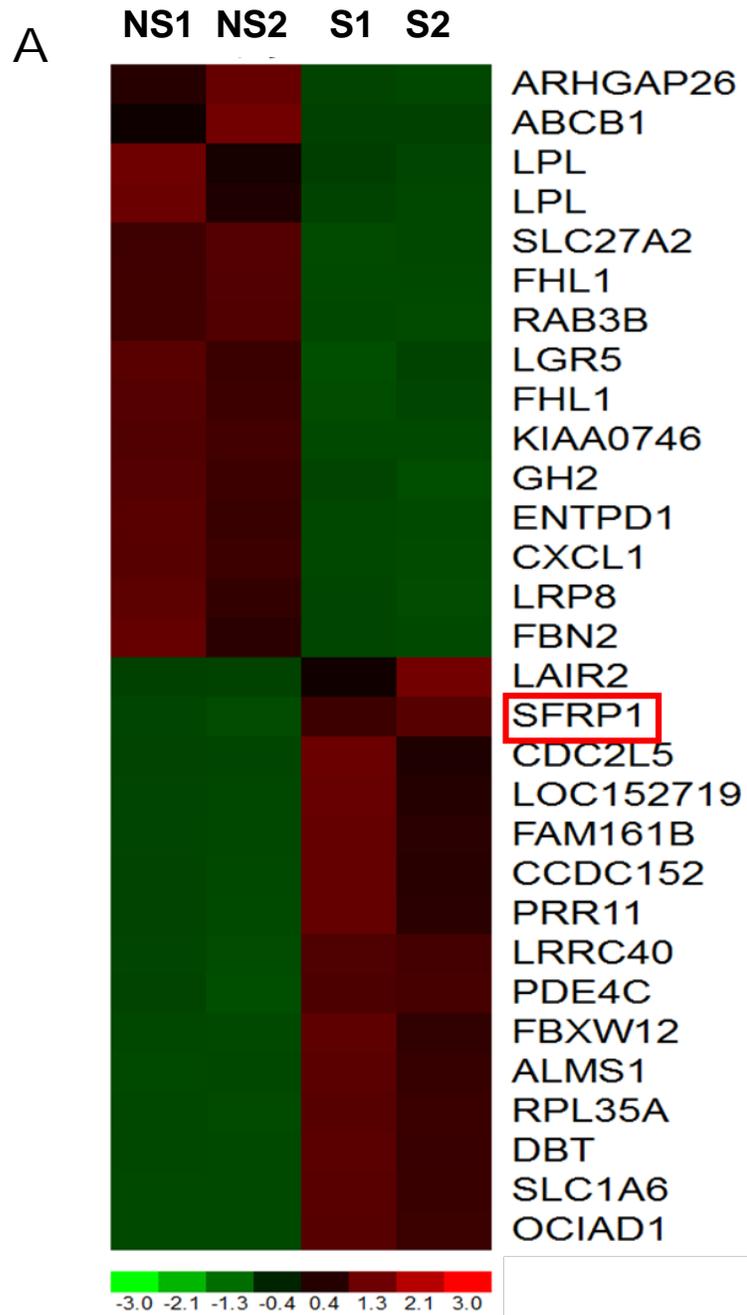
Supplemental Figure 2

A. Schematic diagram of the adenovirus Ad-CMV-sFRP1, which was generated by inserting a truncated His-tagged human sFRP1 cDNA (AA 1-185) that maintains the Wnt-binding cysteine rich domain (CRD) into an adenoviral vector (pAd/CMV/V5-DEST).

B. Western blot confirmation of the expression levels of the soluble truncated sFRP1 (~25 kDa) in the serum of the sFRP1 adenovirus-treated mice (T) compared to mice treated with control virus (C) 4 days post-injection. Purified viral lysates of sFRP1 isolated from HEK293 cells are shown (P2 and P1) as the positive controls in two concentrations.

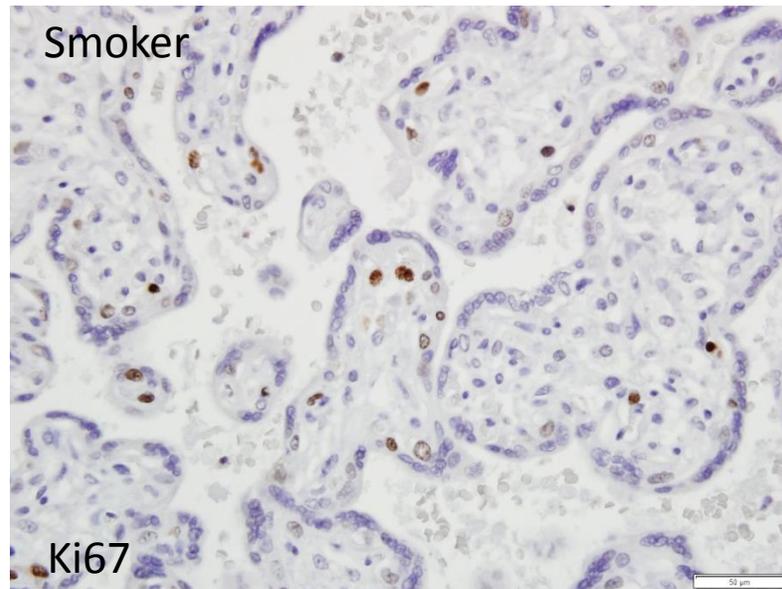
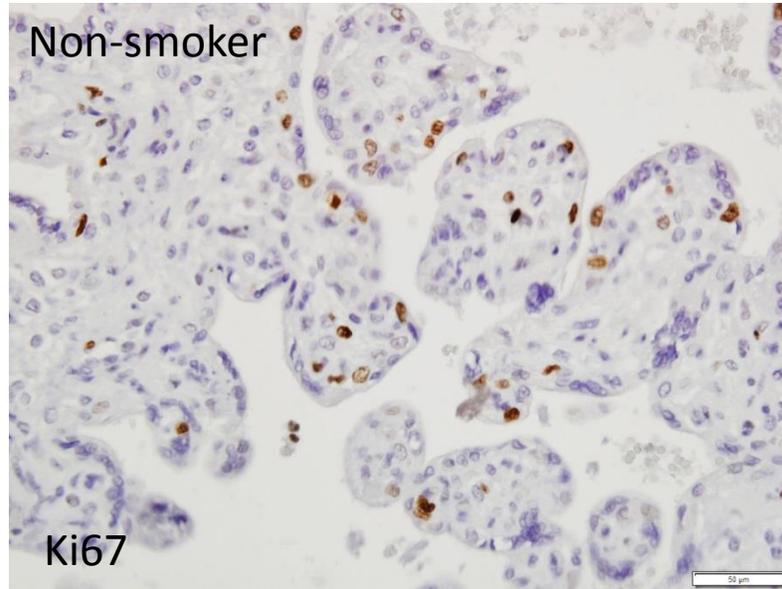
Supplemental Figure 3

Luciferase activity of the canonical Wnt reporter in HEK293 cells transfected with the TOPFLASH TCF reporter plasmid after exposure to varying concentrations of cigarette smoke extract (CSE), carbon monoxide releasing molecule (CO-RM2) for a period of 24 hours. Normalized value of unstimulated control was arbitrarily set at 100%. Bars represent mean values of four different experiments, error bars indicate SEM, # $p < 0.05$ for 100 μ M CO-RM2 versus DMSO control, * $p < 0.05$ for 500 μ M CO-RM2 versus DMSO control.

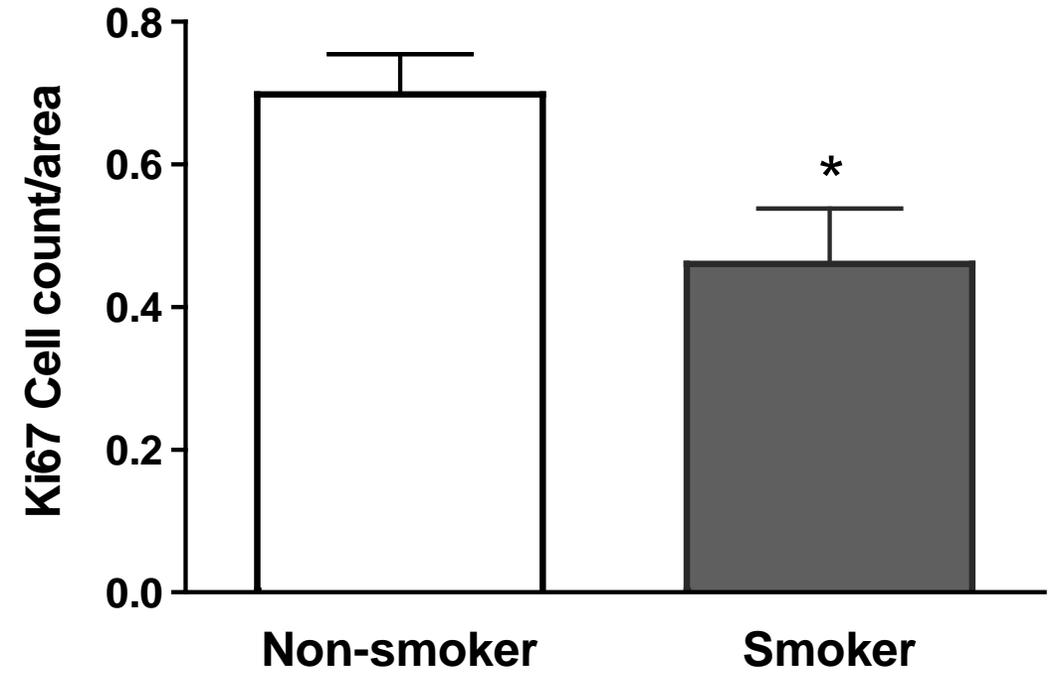


Supplemental Figure 1

C

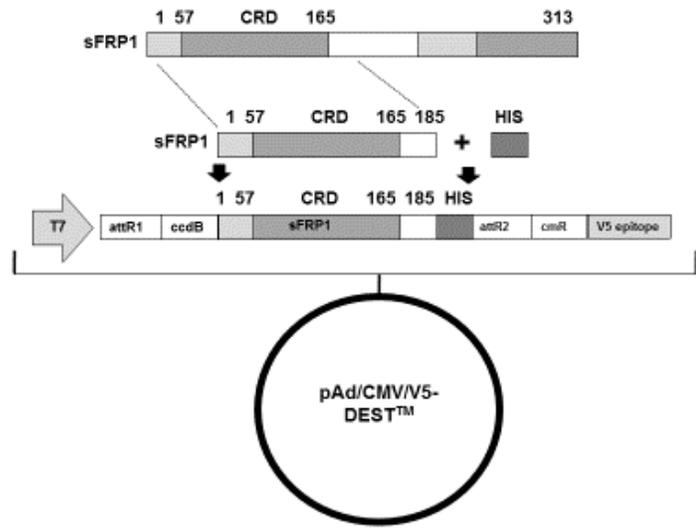


D

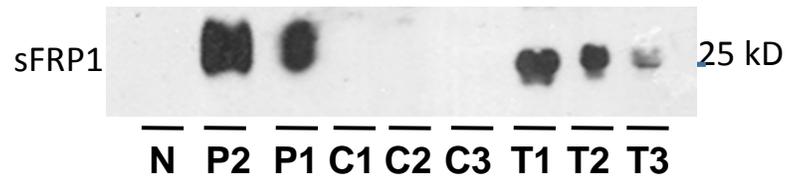


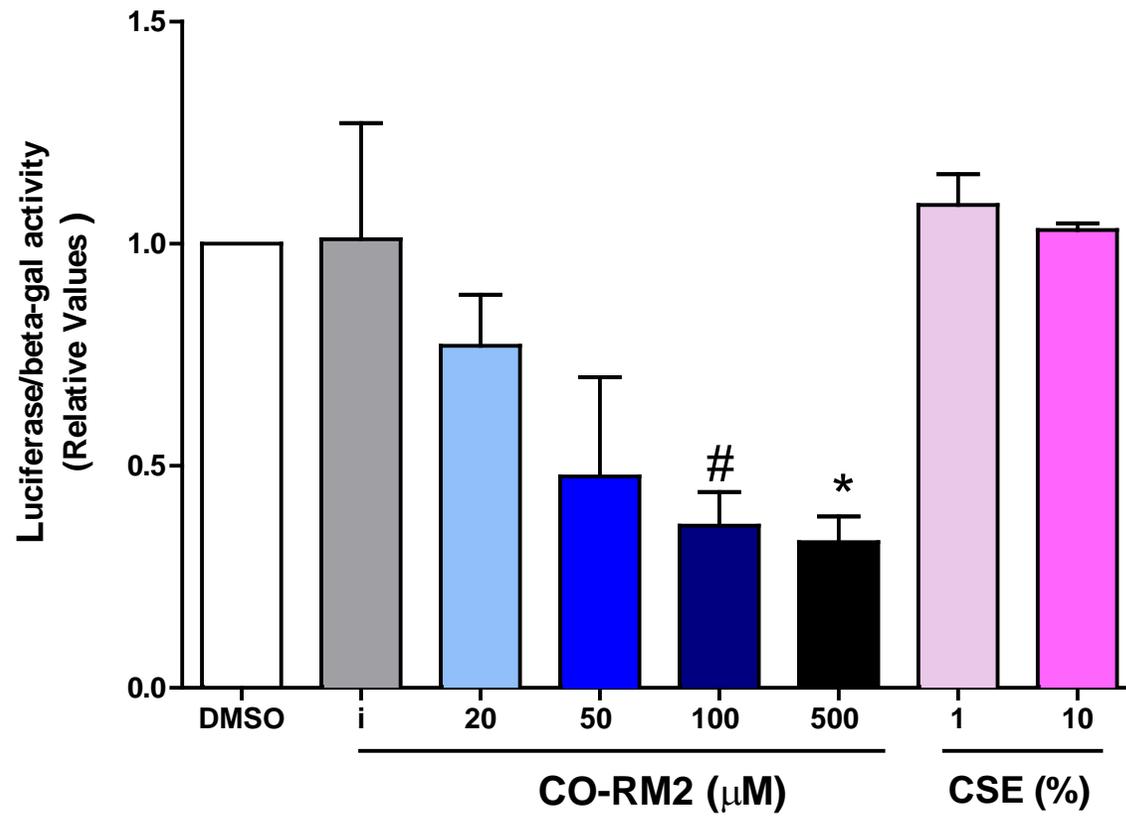
Supplemental Figure 1

A



B





Supplemental Figure 3