

1 ***Supplemental Material: Gestational exposure to unmethylated CpG oligonucleotides dysregulates***  
2 **placental molecular clock network and fetoplacental growth dynamics, and disrupts maternal**  
3 **blood pressure circadian rhythms in rats**

4  
5 Jessica L. Bradshaw<sup>1,2</sup>, Spencer C. Cushen<sup>2,3</sup>, Contessa A. Ricci<sup>2</sup>, Selina M. Tucker<sup>2</sup>, Jennifer J.  
6 Gardner<sup>2</sup>, Joel T. Little<sup>1</sup>, Oluwatobiloba Osikoya<sup>2</sup>, Styliani Goulopoulou<sup>4\*</sup>

7  
8 Department of Pharmaceutical Sciences<sup>1</sup>, Department of Physiology and Anatomy<sup>2</sup>, and Texas College  
9 of Osteopathic Medicine<sup>3</sup>, University of North Texas Health Science Center, Fort Worth, Texas, USA;  
10 Lawrence D. Longo, MD Center for Perinatal Biology<sup>4</sup>, Loma Linda University, Loma Linda,  
11 California, USA.

12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23

24 **Table S1. Primer sequences for quantitative real-time PCR analysis of placental cytokine and**  
 25 **clock gene expression.**

<b>Primer</b>	<b>Sequence (5'-3')</b>	<b>Ref</b>
<i>Tnfa</i>	<i>Forward:</i> ACTGAACTTCGGGGTGATTG <i>Reverse:</i> GCTTGGTGGTTTGCTACGAC	(1)
<i>Il6</i>	<i>Forward:</i> TGATGGATGCTTCCAAACTG <i>Reverse:</i> GAGCTTGGAAGTTGGGGTA	(1)
<i>Il1β</i>	<i>Forward:</i> CACCTTCTTTTCCTTCATCTTTG <i>Reverse:</i> GTCGTTGCTTGTCTCTCCTTGTA	(1)
<i>Clock</i>	<i>Forward:</i> ACAGCGCACACACAGGCCTTC <i>Reverse:</i> TGGCGGCGCCCTGTGATCTA	(2)
<i>Bmall</i>	<i>Forward:</i> ACACTGCACCTCGGGAGCGA <i>Reverse:</i> CGCCGAGCTCCAGAGCACAA	(2)
<i>Cry1</i>	<i>Forward:</i> AGCTGGCCACTGAGGCTGGT <i>Reverse:</i> TGCTGGCATCTCCAGGGGCT	(2)
<i>Per1</i>	<i>Forward:</i> CGCACTTCGGGAGCTCAAACCTC <i>Reverse:</i> GTCCATGGCACAGGGCTCACC	(2)
<i>Per2</i>	<i>Forward:</i> TGAGCTCCTTGGCGTTGCCG <i>Reverse:</i> ACTCAGGCCCACTGGCCACA	(2)
<i>Per3</i>	<i>Forward:</i> TTTTCCCCTTCAAGACATGG <i>Reverse:</i> GTCCATGGCACAGGGCTCACC	(2)
<i>Sdha*</i>	<i>Forward:</i> TGGGGCGACTCGTGGCTTTC <i>Reverse:</i> CCCCgcctGCACCTACAACC	(2)

---

*Ppia*\*

*Forward:* AGCATACAGGTCCTGGCATC

(2)

*Reverse:* TTCACCTTCCCAAAGACCAC

---

26 \*, housekeeping genes included in each qPCR plate layout. *Ppia* was found to be more stable in all  
27 samples and was used for determination of comparative gene expression.

28

29

30 **Table S2. Determination of model with best fit for evaluating average fetal weight**

<b>Model parameters</b>	<b>df</b>	<b>AIC31</b>
Treatment, litter size, average placental weight	9	-20.08382
Treatment, average placental weight, number of resorptions	9	<b>-50.29942</b>
Treatment, average placental weight, proportion of resorptions in litter	10	-35.06153

32 **AIC** = Akaike's Information Criterion. Bold text represents best fit (lowest AIC).

33

34

35 **Table S3. Pre-pregnancy blood pressure and heart rate averages during 12-hr sleep and wake**  
 36 **cycles.**

		<b>Saline</b>	<b>ODN2395</b>	<b>P-value</b>
<b>SBP</b>	<b>Sleep</b>	132.9 ± 10.77	126.4 ± 5.09	0.21
	<b>Wake</b>	135.2 ± 10.03	126.9 ± 4.47	0.09
<b>DBP</b>	<b>Sleep</b>	98.95 ± 3.83	96.99 ± 6.50	0.52
	<b>Wake</b>	100.9 ± 4.45	97.48 ± 8.34	0.95
<b>MAP</b>	<b>Sleep</b>	115.8 ± 5.81	110.9 ± 4.87	0.11
	<b>Wake</b>	114.5 ± 10.13	111.4 ± 5.73	0.49
<b>HR</b>	<b>Sleep</b>	212.5 ± 11.85	208.4 ± 5.33	0.54
	<b>Wake</b>	210.0 ± 5.03	208.1 ± 17.64	0.78

37 Values presented as mean ± SD and analyzed by unpaired t-test or Mann-Whitney test (n = 6-8/group).  
 38 Each 12-hour sleep or wake cycle was averaged over three days prior to mating to obtain pre-pregnancy  
 39 baseline measurements. SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean  
 40 arterial pressure, HR = heart rate.

41

42 **Table S4. Weighted multiple linear regression model assessing effects of covariates on fetal weight**  
 43 **on gestational day 20.**

Variable	Estimate	SE	<i>t</i> -value	<i>p</i> -value
(Intercept)	1.32	0.63	2.09	0.09
Treatment	0.93	0.64	1.45	0.21
Placental weight (g)	2.08	1.19	1.74	0.14
Resorptions	0.42	0.20	2.09	0.09
Treatment x Placental weight (g)	-1.87	1.21	-1.54	0.18
<b>Treatment x Resorptions</b>	<b>-0.86</b>	<b>0.25</b>	<b>-3.48</b>	<b>0.02</b>
Placental weight (g) x Resorptions	-0.89	0.38	-2.35	0.07
<b>Treatment x Placental weight (g) x Resorptions</b>	<b>1.76</b>	<b>0.51</b>	<b>3.42</b>	<b>0.02</b>

44 SE = standard error. Multiple R<sup>2</sup>: 0.9598; Adjusted R<sup>2</sup>: 0.9036; model *p*-value: 0.003; bolded text  
 45 indicates statistical significance.

46  
 47  
 48  
 49  
 50  
 51  
 52  
 53  
 54  
 55

56 **Table S5. Cytokine and chemokine protein expression across various reproductive status.**

Cytokine/ Chemokine	Cytokine/Chemokine Function	Pregnancy Status	Treatment	Mean $\pm$ SE	p- value
TNF- $\alpha$	Proinflammatory	NP	Saline	194.3 $\pm$ 25.24	0.78
			ODN2395	181.4 $\pm$ 35.5	
		GD14	Saline	139.1 $\pm$ 19.77	<b>0.02</b>
			ODN2395	75.16 $\pm$ 11.83	
		GD20	Saline	349.3 $\pm$ 38.58	0.12
			ODN2395	267.4 $\pm$ 27.15	
IL-6	Proinflammatory	NP	Saline	20.91 $\pm$ 11.74	0.53
			ODN2395	13.93 $\pm$ 10.79	
		GD14	Saline	3.14 $\pm$ 0.0	1.0
			ODN2395	3.14 $\pm$ 0.0	
		GD20	Saline	310.2 $\pm$ 47.93	0.36
			ODN2395	188.2 $\pm$ 56.47	
IL-1 $\beta$	Proinflammatory	NP	Saline	13.31 $\pm$ 10.40	0.92
			ODN2395	9.23 $\pm$ 5.361	
		GD14	Saline	61.28 $\pm$ 23.16	<b>0.02</b>
			ODN2395	1.463 $\pm$ 0.0	
		GD20	Saline	8.56 $\pm$ 7.10	0.37
			ODN2395	1.46 $\pm$ 0.0	
IL-1 $\alpha$	Proinflammatory	NP	Saline	53.38 $\pm$ 18.22	0.59
			ODN2395	69.67 $\pm$ 23.38	

		GD14	Saline	$47.25 \pm 20.73$	<b>0.03</b>
			ODN2395	$1.89 \pm 0.0$	
		GD20	Saline	$108.2 \pm 14.87$	<b>0.03</b>
			ODN2395	$63.22 \pm 10.38$	
IL-18	Proinflammatory	NP	Saline	$428.6 \pm 84.88$	0.10
			ODN2395	$271.6 \pm 26.66$	
		GD14	Saline	$445.1 \pm 136.6$	0.60
			ODN2395	$352.2 \pm 104.2$	
		GD20	Saline	$2220.0 \pm 348.1$	0.40
			ODN2395	$1716.0 \pm 476.6$	
IL-12p70	Proinflammatory	NP	Saline	$74.58 \pm 12.25$	0.91
			ODN2395	$76.92 \pm 15.74$	
		GD14	Saline	$53.33 \pm 8.95$	<b>0.03</b>
			ODN2395	$30.15 \pm 3.70$	
		GD20	Saline	$148.2 \pm 32.91$	0.06
			ODN2395	$63.44 \pm 21.70$	
IL-17A	Proinflammatory	NP	Saline	$29.18 \pm 8.34$	0.35
			ODN2395	$19.70 \pm 2.82$	
		GD14	Saline	$21.55 \pm 3.38$	<b>0.01</b>
			ODN2395	$10.64 \pm 1.29$	
		GD20	Saline	$25.47 \pm 4.18$	<b>0.03</b>
			ODN2395	$10.95 \pm 4.11$	
IFN $\gamma$	Proinflammatory	NP	Saline	$165.1 \pm 40.04$	0.50



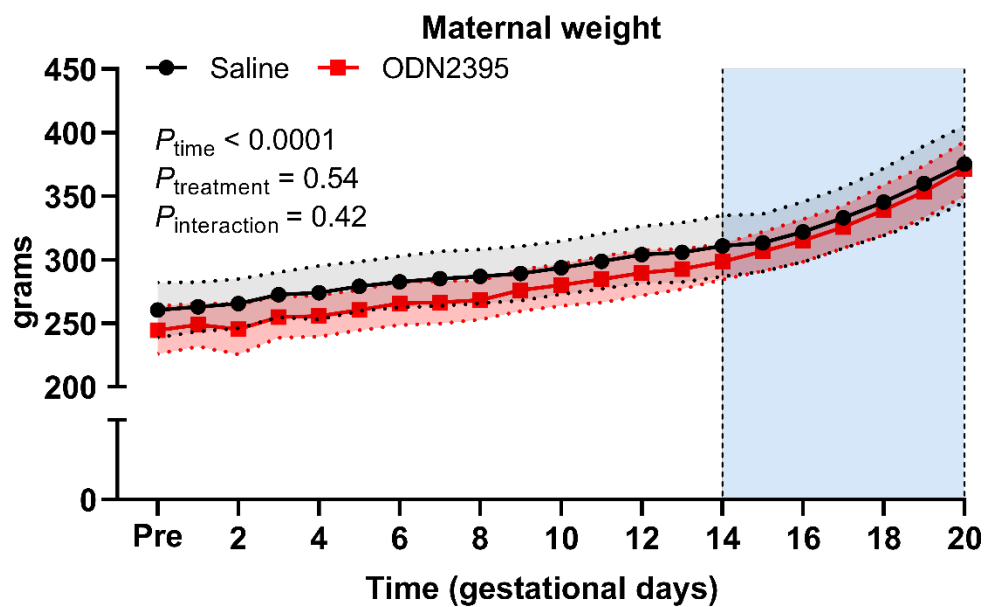
			ODN2395	124.9 ± 42.30	
		GD14	Saline	12.66 ± 7.14	0.47
			ODN2395	6.32 ± 4.34	
		GD20	Saline	174.8 ± 47.64	0.15
			ODN2395	87.12 ± 27.75	
IL-7	Proinflammatory	NP	Saline	19.69 ± 3.78	0.98
			ODN2395	19.56 ± 3.21	
		GD14	Saline	23.18 ± 4.84	<b>0.03</b>
			ODN2395	10.46 ± 1.84	
		GD20	Saline	27.36 ± 5.54	0.12
			ODN2395	17.19 ± 5.52	
IL-4	Anti-inflammatory	NP	Saline	141.9 ± 17.77	0.58
			ODN2395	124.3 ± 24.10	
		GD14	Saline	84.85 ± 13.94	0.08
			ODN2395	59.22 ± 5.85	
		GD20	Saline	138.0 ± 25.44	0.20
			ODN2395	97.51 ± 29.12	
IL-5	Anti-inflammatory	NP	Saline	410.5 ± 32.85	0.37
			ODN2395	360.6 ± 41.64	
		GD14	Saline	309.0 ± 24.22	<b>0.04</b>
			ODN2395	251.7 ± 12.07	
		GD20	Saline	496.7 ± 46.34	0.38
			ODN2395	414.8 ± 82.07	

IL-13	Anti-inflammatory	NP	Saline	102.2 ± 43.33	0.51
			ODN2395	57.74 ± 25.69	
		GD14	Saline	28.76 ± 20.08	0.15
			ODN2395	1.14 ± 0.00	
		GD20	Saline	76.02 ± 38.72	0.20
			ODN2395	64.43 ± 63.29	
IL-10	Anti-inflammatory	NP	Saline	104.6 ± 15.57	0.97
			ODN2395	106.0 ± 26.29	
		GD14	Saline	88.49 ± 24.24	0.06
			ODN2395	20.87 ± 11.25	
		GD20	Saline	14.28 ± 6.08	0.84
			ODN2395	25.67 ± 14.73	
IL-2	Anti-inflammatory	NP	Saline	762.4 ± 174.5	0.34
			ODN2395	554.8 ± 121.6	
		GD14	Saline	422.4 ± 108.7	<b>0.01</b>
			ODN2395	85.22 ± 49.52	
		GD20	Saline	707.2 ± 168.9	0.47
			ODN2395	683.1 ± 406.0	
GRO/KC	Chemoattractant	NP	Saline	21.88 ± 6.68	0.55
			ODN2395	54.03 ± 16.75	
		GD14	Saline	20.49 ± 9.27	0.61
			ODN2395	7.19 ± 1.96	
		GD20	Saline	8.72 ± 2.50	0.42

			ODN2395	9.37 ± 4.09	
MCP-1	Chemoattractant	NP	Saline	269.2 ± 16.04	0.52
			ODN2395	285.8 ± 15.90	
		GD14	Saline	396.4 ± 37.94	<b>0.03</b>
			ODN2395	286.0 ± 21.42	
		GD20	Saline	150.0 ± 8.74	<b>0.01</b>
			ODN2395	93.74 ± 17.41	
MIP-1 $\alpha$	Chemoattractant	NP	Saline	9.58 ± 1.78	0.63
			ODN2395	10.62 ± 1.16	
		GD14	Saline	14.53 ± 2.06	0.71
			ODN2395	13.12 ± 3.04	
		GD20	Saline	9.93 ± 3.21	0.39
			ODN2395	6.62 ± 2.98	
MIP-3 $\alpha$	Chemoattractant	NP	Saline	13.28 ± 0.98	0.62
			ODN2395	14.25 ± 1.57	
		GD14	ODN2395	18.98 ± 2.09	<b>0.03</b>
			Saline	12.53 ± 1.45	
		GD20	ODN2395	17.81 ± 1.91	<b>0.05</b>
			Saline	12.82 ± 1.18	
RANTES	Chemoattractant	NP	Saline	116.6 ± 17.90	0.54
			ODN2395	132.0 ± 16.38	
		GD14	Saline	265.4 ± 35.23	<b>0.01</b>
			ODN2395	135.0 ± 23.63	

		GD20	Saline	250.3 ± 31.66	0.40
			ODN2395	196.1 ± 55.76	

57 Values presented as mean ± SE and analyzed by unpaired t-tests (n = 6-9/group). Data that were not  
58 normally distributed were log transformed prior to statistical comparisons. Significant *p*-values are  
59 bolded.  
60



61  
62 **Figure S1. Maternal weight gain throughout gestation.** ODN2395 administration had no effect on  
63 maternal body weight gain during pregnancy. Analyzed using repeated measures Two-Way ANOVA  
64 with Sidak's multiple comparisons post-hoc analysis. All values are presented as mean ± SD. N = 7-  
65 9/group. PRE, average of 3 days prior to mating. Shaded vertical region represents treatment window.

66  
67  
68  
69

70 **REFERENCES**

- 71 1. **Khan HA, Abdelhalim MA, Alhomida AS, and Al Ayed MS.** Transient increase in IL-1beta,  
72 IL-6 and TNF-alpha gene expression in rat liver exposed to gold nanoparticles. *Genet Mol Res* 12: 5851-  
73 5857, 2013.
- 74 2. **Wharfe MD, Mark PJ, and Waddell BJ.** Circadian variation in placental and hepatic clock  
75 genes in rat pregnancy. *Endocrinology* 152: 3552-3560, 2011.

76