

Y.A. Bobetsis¹, S.P. Barros¹, D.M. Lin¹,
J.R. Weidman², D.C. Dolinoy²,
R.L. Jirtle², K.A. Boggess³, J.D. Beck⁴,
and S. Offenbacher^{1*}

¹University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, Department of Periodontology, UNC School of Dentistry, CB #7455, DRC Rm 222, Chapel Hill, NC, USA 27599-7455; ²Duke University Medical Center, Department of Radiation Oncology Environmental Safety, Durham, NC; ³University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, UNC School of Dentistry, and Department of Obstetrics and Gynecology, UNC School of Medicine, Chapel Hill, NC, USA 27599; and ⁴University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, Department of Dental Ecology, UNC School of Dentistry, Chapel Hill, NC, USA 27599; *corresponding author, steve_offenbacher@dentistry.unc.edu

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ABSTRACT

Maternal oral infection, caused by bacteria such as *C. rectus* or *P. gingivalis*, has been implicated as a potential source of placental and fetal infection and inflammatory challenge, which increases the relative risk for pre-term delivery and growth restriction. Intra-uterine growth restriction has also been reported in various animal models infected with oral organisms. Analyzing placental tissues of infected growth-restricted mice, we found down-regulation of the imprinted *Igf2* gene. Epigenetic modification of imprinted genes via changes in DNA methylation plays a critical role in fetal growth and development programming. Here, we assessed whether *C. rectus* infection mediates changes in the murine placenta *Igf2* methylation patterns. We found that infection induced hypermethylation in the promoter region-P0 of the *Igf2* gene. This novel finding, correlating infection with epigenetic alterations, provides a mechanism linking environmental signals to placental phenotype, with consequences for development.

KEY WORDS: epigenetics, chronic inflammation, oral pathogen, fetal growth.

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A supplemental appendix to this article is published electronically only at <http://www.dentalresearch.org>.

Bacterial Infection Promotes DNA Hypermethylation

INTRODUCTION

DNA methylation is an ancestral mechanism for neutralizing potentially damaging DNA elements in the genome (Hendrich and Tweedie, 2003). One fundamental level of epigenetic genome modification involves methylation of cytosine at position C5 in CpG dinucleotides, the most common DNA modification used in eukaryotes. Functionally, DNA methylation regulates many cellular processes, such as embryonic development, transcription, and genomic imprinting. Genomic imprinting is defined as an epigenetic modification of a specific parental chromosome in the gamete or zygote that leads to differential expression of the two alleles of a gene in the somatic cells of the offspring (Robertson, 2005). A substantial proportion of imprinted genes are also involved in the control of fetal growth and placental development (Isles and Holland, 2005). Properly established and maintained, DNA methylation patterns are essential for mammalian development and for the normal functioning of the adult organism. Growing numbers of human diseases, syndromes, and disorders are correlated with a gain or loss of DNA methylation (Robertson, 2005). In development, *Igf2* can affect the growth of the fetus, and has a particularly unique and important action in placental growth (Constancia *et al.*, 2002). The paternally expressed *Igf2* gene (chromosome 7 in the mouse) is part of a cluster of imprinted genes with a homologous cluster on the syntenic region on chromosome 11p15.5 in the human (DeChiara *et al.*, 1991; Paulsen *et al.*, 1998). Deletion of the murine *Igf2* promoter region 0 blocks placental IGF2 expression and leads to reduced placental growth, followed by fetal growth restriction (Constancia *et al.*, 2002). Moreover, *Igf2* knockout mice are 40% smaller at birth (DeChiara *et al.*, 1990). Pre-term births and low birthweight have been associated with an increased risk for life-threatening disabilities, including diabetes, neurodevelopmental anomalies, and coronary heart disease (Christianson *et al.*, 1981; Hattersley and Tooke, 1999). Analysis of emerging data indicates that maternal infections during pregnancy and attendant inflammatory responses may play a role in pre-term births and growth restriction (Goldenberg *et al.*, 2000). Periodontal infections have been associated with an increased risk for pre-term births (Offenbacher *et al.*, 2001), and increasing evidence points to *C. rectus* as a key pathogen (Buduneli *et al.*, 2005). In this study, we analyzed the placental *Igf2* expression in mice and investigated the methylation status of the placental *Igf2* promoter (P0) region following *C. rectus* challenge in pregnant mice.

MATERIALS & METHODS

Mouse Infection Model

All mouse experiments were carried out under the approval of the UNC Institutional Animal Care and Use Committee and have been described in detail previously (Lin *et al.*, 2003; Yeo *et al.*, 2005). A stainless steel coil (1 x 0.4 cm) was surgically implanted subcutaneously into female adult BALB/C mice. After healing, mice were mated overnight, and pregnant dams received an



Figure 1. Translocation of *C. rectus* to the placenta at E16.5. Nested PCR for detection of *C. rectus* in placenta at E16.5. Dams were infected by an intrachamber injection with *C. rectus*. Seven out of 15 placentas from fetuses challenged with intra-uterine growth restriction had a positive signal. PC, positive control for *C. rectus*-spiked placenta.

intrachamber injection of 100 μ L of 10^9 CFU/mL live *C. rectus* strain 314 (log growth) or PBS at E7.5. Mice were killed at E16.5, and fetuses ($n = 144$ from 27 unchallenged dams and $n = 193$ from 32 challenged dams) and placental tissues ($n = 106$ from 19 unchallenged dams and $n = 105$ from 19 challenged dams) were collected. We measured the weight and crown-rump length, and performed statistical analysis using a mixed-model method (Little *et al.*, 1996), to account for the fact that effects of challenge on littermates are clustered within dams.

Nested-PCR Analysis for the Detection of *C. rectus*

Placental genomic DNA was extracted with the DNeasy kit (Qiagen, Valencia, CA, USA). Nested PCR was performed with the HotStarTaq DNA Polymerase Kit (Qiagen). For the first reaction, we used 1 μ g of DNA in a final 25- μ L PCR reaction mixture containing 100 μ M of dNTPs, 1 μ M of universal *Campylobacter* rRNA primers, 2.5 units of HotStar Taq polymerase, 10x PCR buffer, and 5x Q-solution. For the second reaction, 1 μ L of the universal reaction was used in 50 μ L of reaction mixture containing 100 μ M of dNTPs, 1 μ M of *C. rectus* primers, 2.5 units of HotStar Taq polymerase, 10x PCR buffer, and 5x Q-solution. Previous experiments with *C. rectus*-spiked samples have demonstrated that the detection limit was at least 50 CFU/placenta. The primer pairs and PCR conditions are provided in the APPENDIX.

Quantitative RT-PCR for *Igf2*

Total RNA was isolated from placental tissues (6 unchallenged and 6 challenged, with intra-uterine growth restriction) with the use of the RNeasy Mini Kit (Qiagen). We then synthesized cDNA from 2 μ g of total RNA using the Omniscript Kit (Qiagen) and oligo-dT primers. Real-time PCR was performed with 1 μ L cDNA, TaqMan Universal PCR mix, and 20X primer (Applied Biosystems, Foster City, CA, USA), in a 7000 Sequence Detection System apparatus (Applied Biosystems). Reactions were performed 2 independent times in triplicate. The results were evaluated with the use of ABI Prism software (Applied Biosystems). Data were normalized to GAPDH expression. Statistical analysis was performed by one-way ANOVA. The *Igf2* primers are provided in the APPENDIX.

DNA Methylation Analysis

Genomic DNA was extracted from control and intra-uterine growth-restricted placentas by means of the DNeasy kit (Qiagen). Following DNA isolation, sodium bisulfite modification of DNA was performed according to a protocol adapted from Grunau *et al.* (2001), as previously described (Waterland and Jirtle, 2003). We divided the placental-specific promoter region included in the differentially methylated region 0 (DMR0) into 3 potential amplicons and conducted a bisulfite-based PCR using the primers listed in the APPENDIX. PCR products were cloned into the

pGEM-T Easy vector (Promega A1380, Madison, WI, USA), and the clones were PCR-amplified with standard Sp6 and T7 primers (APPENDIX). From 50 to 90 clones were finally sequenced for each amplicon region of unchallenged and challenged groups, with the primer Sp6. To test the null hypothesis that there was no difference in the methylation rate between the '*C. rectus*-challenged with intra-uterine growth restriction' group and the unchallenged group, we applied a

logistic regression model for each locus, using empirical sandwich variance estimation and taking into account the correlation of clones within each placenta sample and the overdispersion (Qaqish and Preisser, 2000).

Morphometric Analysis

Placental tissue was fixed with 4% paraformaldehyde, bisected sagittally, processed, and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin. The placental zones' measurements (mm^2) were calculated with the use of "Image J" software (<http://rsb.info.nih.gov/ij/>). (AQ) Statistical analysis was performed by one-way ANOVA.

RESULTS

Nested-PCR Analysis for the Detection of *C. rectus*

Intra-uterine growth restriction was observed in 22.8% of the fetuses of challenged litters, compared with 2.7% from unchallenged dams, $p < 0.01$ (data not shown). We could identify the presence of *C. rectus* in placental tissues by nested PCR detected in 7 out of 15 samples analyzed from growth-restricted E16.5 fetuses. Nested PCR analysis of *C. rectus*-16S rDNA from DNA extracted from E16.5 placental samples (Fig. 1). (AQ) Although in our model the infection occurred at a site distant from the uterus, analysis of these data indicates that the bacteria translocated to the placenta being detected in 46.7% of the placentas of intra-uterine growth-restricted fetuses.

Placental Morphometry and *IGF2* Expression

In both mice and humans, maternal blood passes through spiral arteries into a space created by the conceptus that is densely filled with placental villi, termed a 'labyrinth' in the mouse. In the morphometric analysis, placental samples showed structural alterations within the challenged placental layers, with a relative decrease in the labyrinth area (Fig. 2), and a concomitant increase in the decidual layer, also enlarged due to the presence of focal areas of inflammatory infiltrate. In previous experiments using microarrays of *C. rectus*-challenged placentas, we found significant down-regulation of *IGF2* (data not shown). Because of the central role of this gene in regulating fetal growth, real-time PCR assay was used to confirm this finding. Quantitative PCR for *Igf2* expression showed a significant 2.3-fold decrease ($p = 0.0005$) in placental tissue from intra-uterine growth-restricted fetuses (Fig. 3).

Methylation Analysis

To address whether there was an alteration in methylation patterns, we applied the bisulfite sequencing method to

quantify placental DNA methylation in the *Igf2* promoter region 0, which regulates murine placental expression of IGF2 and also contains the differentially methylated region 0. The location of CpG islands and the differentially methylated regions of the *Igf2* gene, as well as the location of the 4 promoters, are shown in Fig. 4a. The positions of the 16 CpG dinucleotides within *Igf2* promoter region 0 are illustrated (Fig. 4b). We sequenced a total of 390 clones, using 3 amplicon regions (Fig. 4b) to evaluate methylation levels for each CpG loci. Since this promoter region is maternally imprinted, there was methylation across this region in unchallenged placental tissues (Figs. 4c, 4d). However, there was an overall 10.3% increase ($p < 0.0001$) in CpG methylation across this entire promoter region in placental DNA from infected intra-uterine growth-restricted placentas. There was a general trend for increased methylation in 11 of the 16 CpG sites following *C. rectus* challenge, with 6 sites demonstrating a CpG hypermethylation greater than 10% relative to unchallenged tissues (Fig. 4c, shown in red). This hypermethylation was statistically significant ($p < 0.05$) at 4 CpG sites (Figs. 4c, 4d, indicated by asterisks). At CpG locus 7845, there was a 1.89-fold increase in methylation relative to control [OR = 1.89 (95% CI, 1.03-3.45)]; at site 7869, a 2.51-fold increase (95% CI, 1.04-6.07); at site 8461, a 1.79-fold increase (95% CI, 1.07-2.98); and at site 8472, a 2.24-fold increase (95% CI, 1.07-4.71). It is interesting to note that the hypermethylated regions are clustered within the *Igf2* promoter region-P0. The concordance of the hypermethylation patterns in each region was very high. For example, analysis of amplicon 1 showed that the overall concordance for site methylation between CpG 7845 and 7869 was 76.7% ($p < 0.0001$). Similarly, analysis of amplicon 3 revealed that the concordance for methylation at CpG sites 8461 and 8472 was 89.4% ($p < 0.0001$). Thus, hypermethylation appears to be a site-directed process, rather

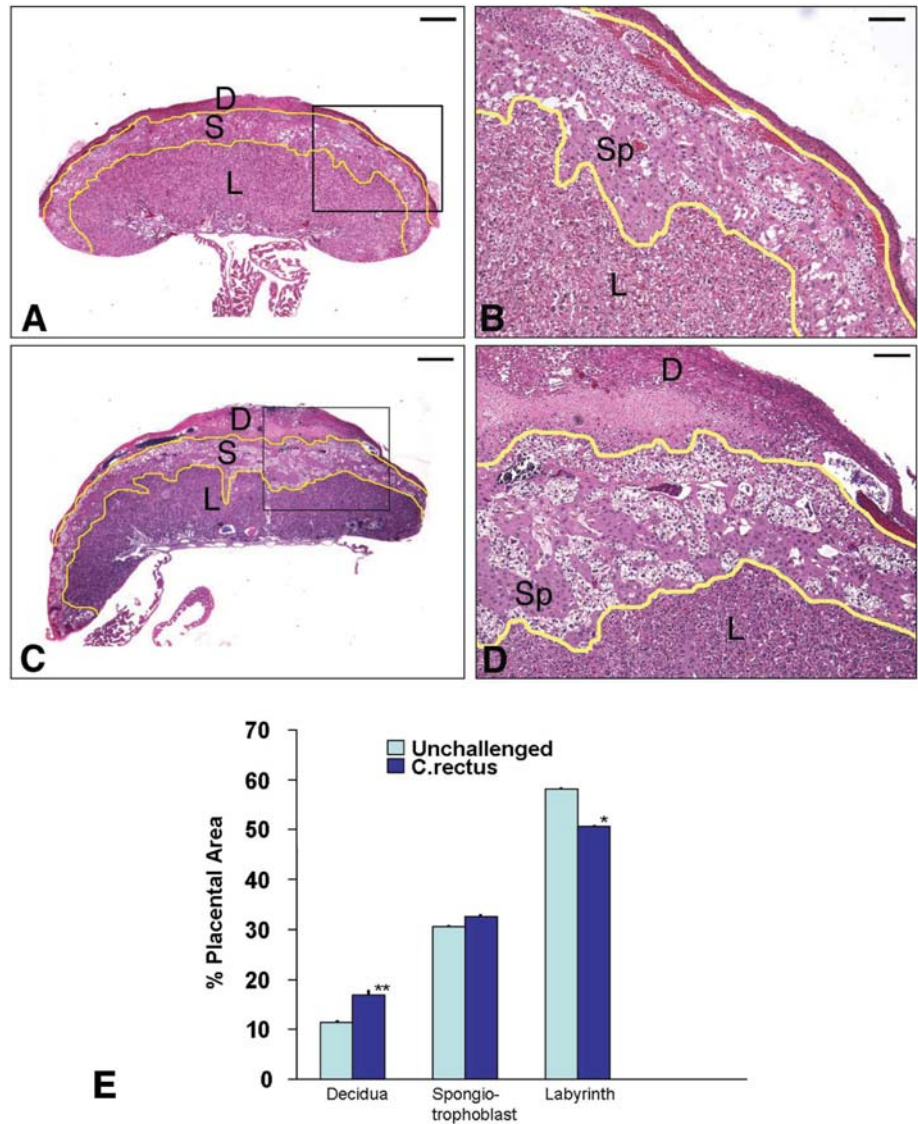
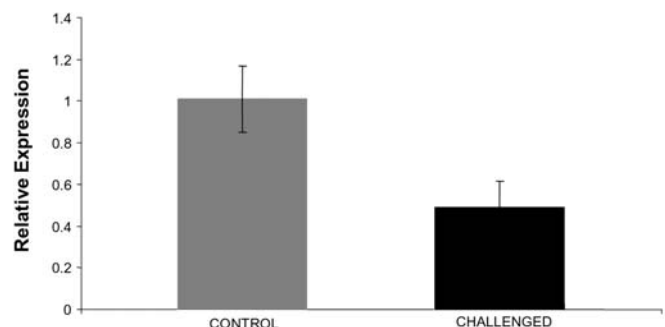


Figure 2. *C. rectus* infection induced structural abnormalities in the placenta. H&E staining from unchallenged (a,b) and growth-restricted challenged (c,d) placentas. In the intra-uterine growth-restricted challenged samples, there is a significant relative decrease in the labyrinth area, with a concomitant increase in the decidua. The spongio-trophoblast layer (S) is not altered. (e) Statistical analysis was performed with one-way ANOVA. * $p < 0.001$, ** $p = 0.04$, n = number of samples(AQ). (b,d) Histological sections indicated by rectangles in (a) and (c) with high-power magnification. Scale bar in (a) and (c), 500 μ m, and in (b) and (d), 125 μ m. (L) labyrinth, (S) spongio-trophoblast layer, (Sp) spongio-trophoblast cells, (D) decidua.

Figure 3. Quantitative RT-PCR analysis for Igf2. The relative expression of *Igf2* from unchallenged placenta (n = 6) and from growth-restricted challenged placenta (n = 6) was compared. Reactions were performed 2 independent times in triplicate. Mean values and error bars are presented. Statistical analysis was performed with one-way ANOVA. There was a significant ($p = 0.0005$) 2.3-fold decrease in the relative expression of *Igf2* in the challenged placenta at E16.5.



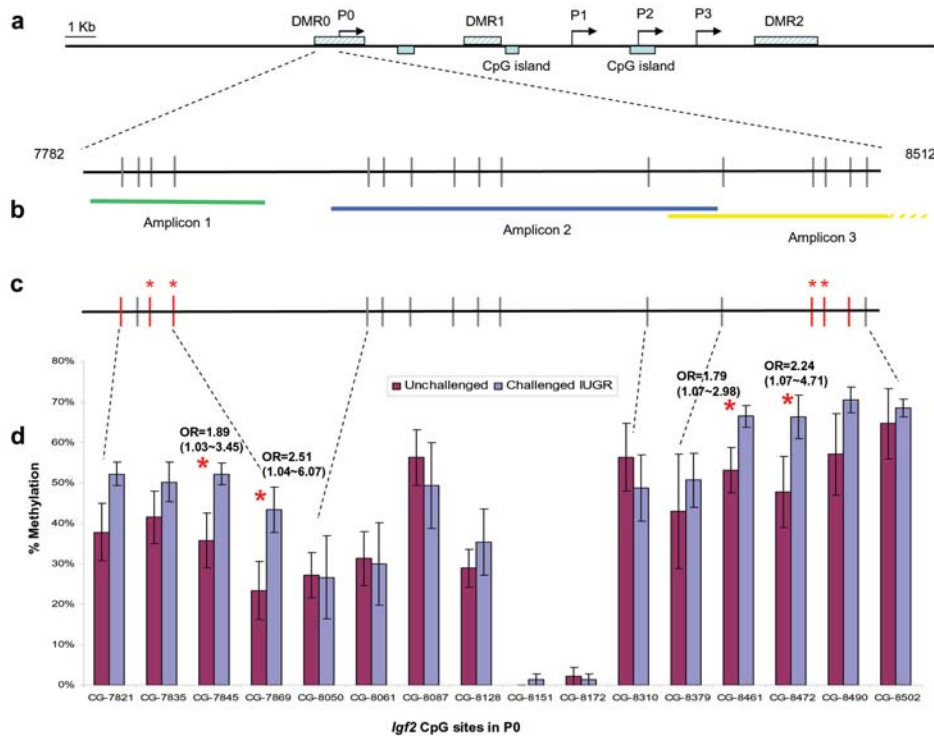


Figure 4. *C. rectus* infection induces hypermethylation of the placental-specific P0 promoter region of *Igf2* in the placenta. DNA from unchallenged and from intra-uterine growth-restricted placentas were bisulfite-treated and sequenced (details in MATERIALS & METHODS). (a) The location of the CpG islands and the differentially methylated regions (DMR) of the *Igf2* gene, as well as the location of the 4 promoters (P0, P1, P2, P3). (b) The 16 CpG dinucleotides (vertical gray lines) within the P0, which are also part of the DMR0. This DNA fragment extends from sites 7782 to 8512 (GenBank Accession #U71085). To facilitate the sequencing process, we divided this region into 3 areas (amplicons 1-3). (c) For each CpG locus, from 50 to 90 clones were sequenced *per* experimental group, giving a total of 390 clones. The 6 red vertical lines represent the CpG sites that were hypermethylated by more than 10% after *C. rectus* infection. *The CpG sites where hypermethylation was statistically significant ($p < 0.05$). (d) Depicts the percentage of methylation at each of the CpG sites from the unchallenged (red) and challenged with Intra-uterine Growth Restriction (blue) placentas. Mean % methylation and error bars are presented. Statistical analysis can be found in MATERIALS & METHODS. *CpG sites where hypermethylation was statistically significant ($p < 0.05$). OR, odds ratio; parentheses include the confidence interval. Four CpG sites were significantly hypermethylated (7845, 7869, 8461, and 8472), and these sites appear to be clustered.

than a non-specific increased methylation across the entire *Igf2* promoter region P0.

DISCUSSION

To address the effects of maternal infection on fetal growth restriction and possible alteration of the methylation status of placental *Igf2*, we utilized an infection/pregnancy mouse model (Offenbacher *et al.*, 2005), challenging the pregnant mice with *C. rectus*. In our model, although the infection occurred at a distant site from the uterus, the bacteria translocated to the placenta, as reflected by the nested PCR detection in 46.7% of the placentas of intra-uterine growth-restricted fetuses. There were also structural alterations in the placental layers, with a decrease in the proportion of the labyrinth. We also observed a 27% reduction in weight at E16.5 (data not shown) that is similar to the decrease in birth weight detected in mice in which the *Igf2* gene has been completely deleted (De Chiara *et al.*, 1990; Constancia *et al.*, 2002), and is also comparable with our previous findings (Offenbacher *et al.*, 2005; Yeo *et al.*,

2005). Analysis of our data from real-time PCR also showed a significant 2.3-fold decrease ($p = 0.0005$) in IGF2 mRNA levels in placentas from intra-uterine growth-restricted fetuses. Since IGF2 synthesis is trophoblast-dependent, a two-fold decrease at a tissue level may have significant effects on fetal growth. This is supported by McMinn and colleagues, who demonstrated that, in humans with intra-uterine growth restriction, placental tissues displayed a significant 2.1- and 2.6-fold decreased expression of *IGF1* and *IGF2*, respectively (McMinn *et al.*, 2006). Thus, *C. rectus* infection suppresses *Igf2* expression, and this finding provides the first report associating bacterial infection mediating placental suppression of a growth factor known to be critical to both placental and somatic fetal growth.

Since the P0 promoter region of *Igf2* controls the expression of this gene in the placenta (Moore *et al.*, 1997), we tested our hypothesis, that the observed suppression of *Igf2* expression could be the result of epigenetic hypermethylation of CpG sites in the promoter region. The findings indicated that bacterial infection is associated with hypermethylation of the *Igf2* promoter region-P0 at 4 loci, with a site-specific odds ratio for hypermethylation ranging from 1.79 to 2.51. Previous investigations have showed that methylation of the

Igf2 promoter is associated with gene silencing, and that *trans* acting H19 locus (Feil *et al.*, 1994; Yang *et al.*, 2003) is also important in controlling IGF2 gene expression. Although promoter methylation is associated with altered gene expression, it remains to be seen whether infection-mediated methylation on *Igf2* promoter region-P0 is functionally related to the observed *Igf2* down-regulation. However, it is interesting to note that the region of hypermethylation at CpG sites 8461 and 8472 contains an Sp1 binding element. The inability of transcription factors to bind to their DNA binding sites when methylated has been proposed as a possible mechanism of gene silencing, and may provide one possible explanation for the transcriptional down-regulation of this gene.

This investigation provides the first report that infection can lead to host epigenetic modification of an imprinted gene. It is not known whether this hypermethylation occurs due to direct bacterial interaction with the tissue, or as a consequence of a host inflammatory response. For example, *H. pylori*, an organism that is phylogenetically closely related to *C. rectus*,

has been reported to be associated with altered oncogene methylation patterns in the gastric mucosa at local sites of infection, a process which is strongly linked with an increased risk for cancer (Maekita *et al.*, 2006). The potential for oral bacteria such as *C. rectus* to modify DNA methylation patterns locally within the oral mucosa, and thereby alter local gene expression patterns, is currently under investigation.

In our experiments, the alteration in DNA methylation patterns was evaluated in the placental tissues; however, it may also occur in other fetal tissues or organs. This raises the possibility that somatic epigenetic modification may occur *in utero* as a consequence of infection, and also provides a potential link for exploring DNA methylation in the offspring in response to infection as it modifies development and potentially mediates long-term disabilities in various organ systems. Infection-mediated alterations in intra-uterine imprinting may underlie the linkages seen between pre-term delivery and diseases of the offspring that include neurological impairments and adult-onset conditions, such as diabetes and cardiovascular disease (Allin *et al.*, 2006; Mericq, 2006). In humans, abnormalities in IGF regulation have been linked to mental retardation, diabetes, and atherosclerosis (Zaina and Nilsson, 2006). (AQ) We have recently reported that, in pregnant mice, *C. rectus* challenge may lead to white matter damage and hypomyelination (Offenbacher *et al.*, 2005). Our data showing infection-mediated hypermethylation of the placental Igf2 promoter, coupled with the knowledge that changes in expression of imprinted genes have major implications for developmental programming, may, in part, explain the poor prognosis of the infant born small for gestational age, or with pre-term birth. The role of maternal infection as a possible source of abnormal epigenetic imprinting opens up the consideration of not only the potential somatic effects of IGF abnormalities, but also the potential effects on other key imprinted genes that control growth and development.

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Biological

Y.A. Bobetsis¹, S.P. Barros¹, D.M. Lin¹,
J.R. Weidman², D.C. Dolinoy²,
R.L. Jirtle², K.A. Boggess³, J.D. Beck⁴,
and S. Offenbacher^{1*}

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¹University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, Department of Periodontology, UNC School of Dentistry, CB #7455, DRC Rm 222, Chapel Hill, NC, USA 27599-7455; ²Duke University Medical Center, Department of Radiation Oncology Environmental Safety, Durham, NC; ³University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, UNC School of Dentistry, and Department of Obstetrics and Gynecology, UNC School of Medicine, Chapel Hill, NC, USA 27599; and ⁴University of North Carolina at Chapel Hill, Center for Oral and Systemic Diseases, Department of Dental Ecology, UNC School of Dentistry, Chapel Hill, NC, USA 27599; *corresponding author, steve_offenbacher@dentistry.unc.edu

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APPENDICES

Appendix Table 1. (AQ)

Technique	Primers	Primer Sequences
Nested PCR for <i>C. rectus</i> , Round 1	Universal <i>Campylobacter</i> 16S rRNA Position: 405-1,128	Forward 5'-GGATGACACTTTTCGGAGC-3' Reverse 5'-CATTGTAGCACGTGTGTC-3'
	<i>C. rectus</i> -specific (16S rRNA) Position: 415-1,1012	Forward 5'-TTTCGGAGCGTAAACTCCTTTTC-3' Reverse 5'-TTTCTGCAAGCAGACACTCTT-3'
Quantitative PCR for IGF2	Mouse IGF2	Mmoo439563_m1 (Taqman, Applied Biosystems)

Appendix Table 2.(AQ)

Technique	Primers	Primer Sequences
Amplification of bisulfite-treated promoter, Po region of IGF2	Region 1: position 7,790-7,950	Forward 5'-CTAATACAAATAACATATATAA-3'
		Reverse 5'-GTAGTTGTTTTTAAGAAGG-3'
	Region 2: position 8,017-8,374	Forward 5'-GAATTTTAAATTGAGTGTTTATG-3'
		Reverse 5'-CCCACACTAAAATATAAAAAAC-3'
	Region 3: position 8,319-8,560	Forward 5'-GTTTGTTAGGTAGGGGTTAAAG-3'
		Reverse 5'-CCCCTAAAAATAACTTCTACTC-3'
Clone amplification	T7	Forward 5'-TAATACGACTCACTATA-3'
	Sp6	Reverse 5'-TATTTTAGGTGACACTATAG-3'

Appendix Table 3. PCR Conditions

Technique	Denaturation Step Temperature, Time	Cycles			Elongation Step Temperature, Time	
		Denaturation Temperature, Time	Annealing Temperature, Time	Elongation Temperature, Time		
Nested PCR for <i>C. rectus</i> , Round 1	95°C, 15 min	94°C, 1 min	50°C, 1 min	72°C, 2 min	72°C, 10 min	
Nested PCR for <i>C. rectus</i> , Round 2	95°C, 15 min	94°C, 30 sec	60°C, 1 min	72°C, 1 min	72°C, 10 min	
Quantitative RT-PCR for <i>Igf2</i>	95°C, 10 min	95°C, 15 sec	60°C, 2 min			
Amplification of bisulfite- treated promoter, Po region of <i>Igf2</i>	Amplicon 1	94°C, 2 min	94°C, 30 sec	50°C, 30 sec	72°C, 1 min	72°C, 10 min
	Amplicon 2	94°C, 2 min	94°C, 30 sec	50°C, 30 sec	72°C, 1 min	72°C, 10 min
	Amplicon 3	94°C, 2 min	94°C, 30 sec	56°C, 30 sec	72°C, 1 min	72°C, 10 min
Clone amplification	94°C, 4 min	94°C, 30 sec	55°C, 30 sec	72°C, 30 min	72°C, 10 min	