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Postnatal stability, tissue, and timing specific effects of *AHRR* methylation change in response to maternal smoking in pregnancy

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Abbreviations: *AHRR*, aryl hydrocarbon receptor repressor; CBMC, cord blood mononuclear cells; DOHaD, developmental origins of health and disease; HM450, Infinium HumanMethylation450 BeadChip

The intrauterine environment has the potential to "program" the developing fetus in a way that can be potentially deleterious to later health. While in utero environmental/stochastic factors are known to influence DNA methylation profile at birth, it has been difficult to assign specific examples of epigenetic variation to specific environmental exposures. Recently, several studies have linked exposure to smoking with DNA methylation change in the aryl hydrocarbon receptor repressor (*AHRR*) gene in blood. This includes hypomethylation of *AHRR* in neonatal blood in response to maternal smoking in pregnancy. The role of *AHRR* as a negative regulator of pathways involved in pleiotropic responses to environmental contaminants raises the possibility that smoking-induced hypomethylation is an adaptive response to an adverse in utero environmental exposure. However, the tissue specificity of the response to maternal smoking, and the stability of the methylation changes early in life remain to be determined. In this study we analyzed *AHRR* methylation in three cell types—cord blood mononuclear cells (CBMCs), buccal epithelium, and placenta tissue—from newborn twins of mothers who smoked throughout pregnancy and matched controls. Further, we explored the postnatal stability of this change at 18 months. Our results confirm the previous association between maternal smoking and *AHRR* methylation in neonatal blood. In addition, this study expands the region of *AHRR* methylation altered in response to maternal smoking during pregnancy and reveals the tissue-specific nature of epigenetic responses to environmental exposures in utero. Further, the evidence for postnatal stability of smoking-induced epigenetic change supports a role for epigenetics as a mediator of long-term effects of specific in utero exposures in humans. Longitudinal analysis of further specific exposures in larger cohorts is required to examine the extent of this phenomenon in humans.

Introduction

During pregnancy the developing fetus must adapt to its environment in order to optimize growth and to minimize the potential adverse effects of harmful environmental exposures. While beneficial in the womb, such adaptations can also be potentially deleterious to the long-term health of an individual. For example, a fetus in a low nutrient environment may optimize its metabolic status in "anticipation" of a low caloric postnatal world, which would mismatch with a high nutrient postnatal diet. This fetal programming[1] is postulated to explain the higher rates of obesity and diabetes in individuals born small for gestational age.[2,3] The more widely applicable Developmental Origins of Health and Disease (DOHaD) hypothesis states that the intrauterine environment can "program" the fetus through subtle changes in organ structure or function, so as to predispose to disease in adulthood.[4,5] Mounting evidence suggests a key role for epigenetic mechanisms (such as DNA methylation) in mediating this process.[6]

Despite increasing association studies linking DNA methylation change to disease,[7,8] little consistent and reproducible data has emerged linking specific environmental exposures to specific epigenetic change, a missing piece in the hypothesized DOHaD pathway. Epigenome Wide Association Studies (EWAS) have the potential to identify such changes,[9] and one of the largest EWAS performed to date involved the screening of 1062 newborn cord blood samples using the Infinium HumanMethylation450 platform (HM450) in an attempt to identify DNA methylation change in newborns associated with maternal smoking during pregnancy.[10] Methylation changes at four genes were identified:

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hypomethylation of the aryl hydrocarbon receptor repressor *(AHRR)* and growth factor independent 1 transcription repressor *(GFI1),* and hypermethylation of cytochrome P450–1A1 (*CYP1A1)* and myosin IG *(MYO1G*). *AHRR* is involved in the detoxification of chemicals found in tobacco smoke, and lower methylation may be a cellular response to the presence of these chemicals, resulting in higher expression of this gene. Interestingly, other recent studies in adults have also identified hypomethylation in the same region of the *AHRR* gene in association with smoking in lymphoblasts and alveolar macrophages,[11] whole blood,[12] and lymphocytes.[13] Further, a large study of more than 2000 adults, including 498 smokers, identified hypomethylation of this gene in whole blood of smokers.[14] Thus, five independent studies have now linked a decrease in *AHRR* methylation to smoking exposure with an effect size of between 0.075–0.24 (~7.5–24% methylation).

Despite these findings, the full domain of methylation change across the *AHRR* gene in response to maternal smoking and the extent across different tissues remains to be determined. Similarly, the postnatal stability of the altered DNA methylation profile remains unclear. The aim of this study was to (1) replicate previous findings of an effect of maternal smoking on *AHRR* methylation in particular, as well as *GFI1* and *MYO1G* methylation, (2) to expand the region of the gene assayed for methylation change, (3) to assess methylation change across multiple tissues and, (4) to assess postnatal stability of any methylation difference in the first years of life. Through the use of twin samples collected as part of the Peri/Postnatal Epigenetics Twin Study,[15,16] we also sought to (5) explore the association between the underlying genetics and methylation at this locus.

Results

Characterization of tissue-specific DNA methylation patterns within the AHRR gene body

DNA methylation within intron 3 of the *AHRR* gene was measured using three overlapping assays covering 32 individual CpG sites contained in 18 measurable CpG units (**Fig. 1A**; **Fig. S1**). This region contains the previously identified smoking-associated CpG site (HM450 probe cg05575921). DNA methylation was measured in three tissues from newborn twins: CBMCs (n = 46 pregnancies), buccal epithelium (n = 15 pregnancies), and placenta (n = 24 pregnancies). CBMCs and buccal epithelium showed intermediate to high methylation within the CpG island shore (flanking the CpG island), with an almost completely unmethylated pattern within the CpG island (CGI) (**Fig. 1B**). Placenta on the other hand showed an intermediate methylation pattern throughout the CpG island shore and the island itself. This supports a previous finding that a CpG island within *AHRR* is monoallelically methylated in human first and third trimester placenta.[17] Of particular interest is the lower methylation at CpG_A7 (cg05575921) in buccal epithelium and placenta (average ~35% methylation) compared with CBMCs, which showed consistently high methylation at this site (average ~80% methylation) (**Fig. 1B**). Coupled with the previously demonstrated enrichment for active histone marks at this region (**Fig. S1A**), this tissue specific methylation pattern supports a functional role for this region of the *AHRR* gene.

Maternal smoking throughout pregnancy is associated with AHRR hypomethylation specifically in cord blood mononuclear cells

Methylation data were separated into three groups for analysis: "smoked throughout," "smoked early," and "never smoked." The "smoked throughout" group reported smoking prior to pregnancy, at the time they found out about the pregnancy and at each trimester (12, 24, and 36 wk). The "smoked early" group reported smoking up to the time of finding out about the pregnancy, but not thereafter (**Table 1**). Plasma cotinine levels were measured in a selection of maternal and infant samples and a strong correlation with questionnaire data was obtained at both time points (**Table S2**). The average level of methylation at CpG_A7 (corresponding to cg05575921) in CBMCs was 0.10 (10%) lower in the "smoked throughout" (0.73) compared with the "never smoked" (0.83) group (**Fig. 2A**; **Table S3**), while the corresponding range of methylation within the groups was 0.62–0.89 and 0.71–0.91, respectively. Across the region assayed, 6 contiguous CpG analytical units (spanning 8 CpG sites) over 275 bp showed lower methylation in the smoking group (delta $\beta > 0.05$, $P < 0.05$; **Table 2**). Interestingly, there were no differences in mean methylation between the "never smoked" and "smoked early" groups across assay A and B (**Fig. 2B and C**) and no difference in methylation at the CpG island (assay C), which was hypomethylated in all groups (**Fig. 2D**). Of particular interest, we found no evidence for an association between maternal smoking and methylation at this region in buccal epithelium or the placenta of the same pregnancies (**Fig. S2**). However, it should be noted that due to lack of available tissue, buccal, and placenta analysis was limited to 7 and 8 smoking exposed pregnancies, respectively, and thus our study would have been unpowered to detect smaller methylation differences (~<6%) between smokers and non-smokers. Furthermore, there was no difference in methylation level between CBMCs from males and females (**Fig. S3**). These findings suggest that the effect of maternal smoking on *AHRR* methylation is tissue and timing specific, possibly requiring prolonged exposure in utero. However, in contrast to the widely held view that early pregnancy is an especially sensitive time point for environmentally induced epigenetic change, we found no evidence that first trimester smoking exposure in isolation has any effect on AHRR methylation levels in progeny.

Furthermore, we measured methylation at the *GFI1* and *MYO1G* genes, which showed significant differences in response to smoking in the Joubert study,[10] but was not identified as significant in adult studies.[11-14] Our assay targeted the region reported to be the most differentially methylated in both genes and several surrounding CpG sites.We found no evidence for an effect of maternal methylation in either case (**Figs. S4 and S5**). This is in agreement with other HM450-based smoking studies in adults,[11-14] though it should be noted that two different CpG sites within *GFI1* were associated with smoking by Zeilinger et al.[14]

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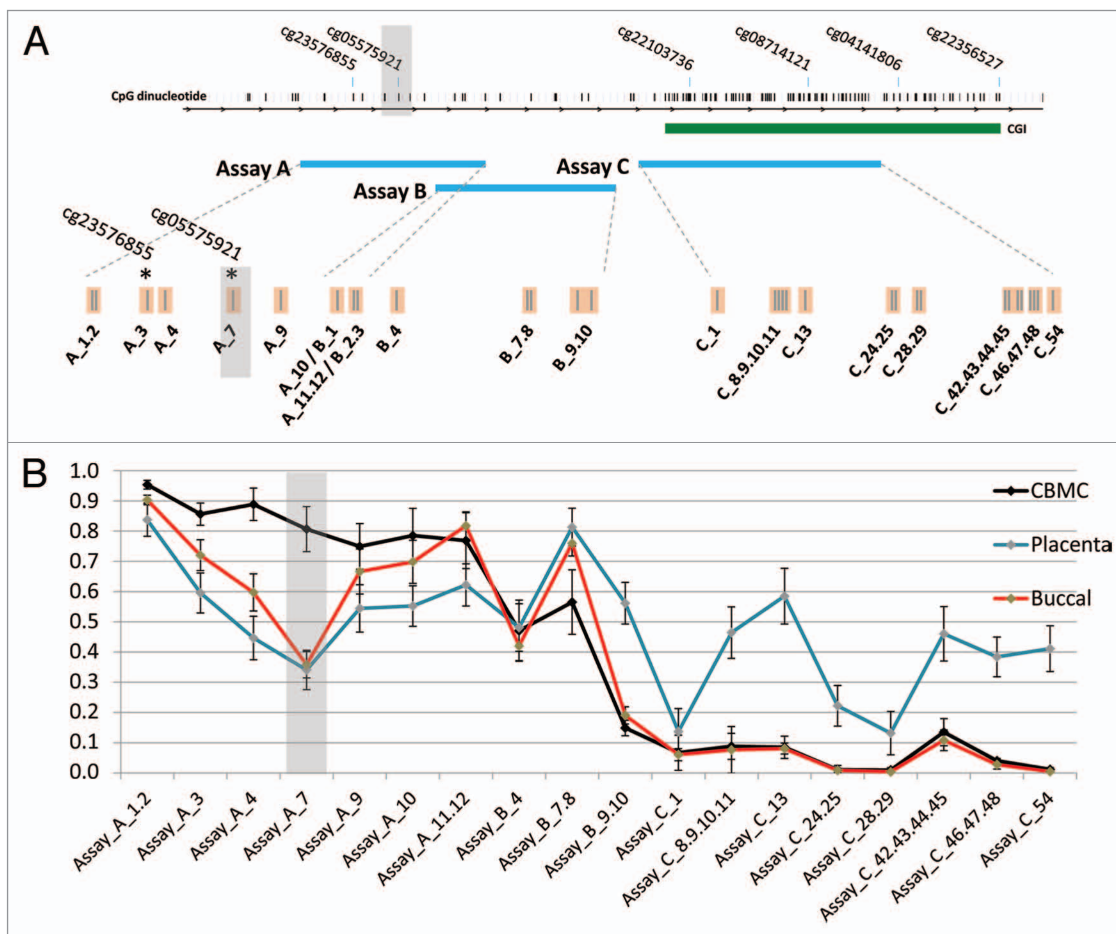

Figure 1. Tissue specific DNA methylation patterns within *AHRR*. (**A**) Map of the EpiTYPER assays (blue rectangles) covering the *AHRR* region of interest, showing analyzable CpG sites. Assay A and B cover the CpG site of interest (CpG_A7 - cg05575921) and surrounding CpG sites. The CGI is represented as a green rectangle. (**B**) DNA methylation level in CBMCs, buccal epithelium, and placenta show tissue-specific differences at CpG_A7. Grey shaded box = "cg05575921" CpG site of interest.

Association between AHRR intron 3 methylation and AHRR expression

The enrichment of active histone marks and the tissue-specific methylation pattern within the smoking associated differentially methylated region, suggest that it is functionally important. Using expression primers previously published by Shenker et al.,[12] we analyzed *AHRR* expression in CBMCs and placentas from smoking and non-smoking mothers. As expected, *AHRR* expression was several folds higher in CBMCs compared with placenta (**Fig. 3A**), and there was a non-significant trend toward higher expression in CBMCs exposed to smoke (**Fig. 3B**).

Maternal smoking-induced hypomethylation of AHRR is maintained at 18 mo

We next analyzed DNA methylation in matched 18-mo peripheral blood mononuclear cells (PBMCs), to determine if the DNA methylation changes within the *AHRR* gene body persist after birth. Due to the small number of longitudinally collected bloods from twins of mothers that "smoked throughout," only 3 unrelated individuals with matched birth and 18 mo mononuclear cells were available for analysis in this group, along with 8 "never smoked" control individuals (**Fig. S6**). **Figure 4** shows the mean methylation for birth and 18 mo individuals in "smoked" and "never" groups. Interestingly, both groups showed an increase in mean methylation from birth to 18 mo; however, the difference in mean methylation between the "smoked throughout" and "never smoked" remained significantly different (**Fig. 4**; **Table 3**), albeit at a slightly reduced level on average. (**Fig. 4**; **Fig. S7**). Interestingly, none from the "smoked throughout" pregnancy group analyzed longitudinally reported "household smoking" after birth, this suggests that the persistent differences in methylation were due to smoking exposure during pregnancy, and were not due to continued second hand exposure to smoking after birth. The absence of plasma cotinine in infants at 18 mo supports this conclusion (**Table S2**).

Within-pair analysis of MZ and DZ twins suggests a role for genetic factors in regulating DNA methylation at the AHRR gene body

We have previously utilized the twin model to examine the genetic and environmental influence on DNA methylation at specific genes and on a genome-scale level,[18-20] and found clear evidence for both genetic and cumulative environmental/stochastic factors in contributing to the neonatal epigenetic profile. By

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comparing within-pair DNA methylation similarity at the *AHRR* gene between monozygotic (MZ) and dizygotic (DZ) twins, the contribution of underlying genetic variation to DNA methylation at a particular locus could be estimated. Linear regression analysis of methylation levels at CpG_A7, or the mean of assay A, revealed that MZ twins as a group are generally more similar (R^2 = 0.86 and 0.83) in their DNA methylation level than DZ twins (R^2 = 0.42 and 0.54) at birth (**Fig. 5**).

Discussion

Several in utero environments have been linked to changes in neonatal epigenetic profile (reviewed extensively in Hogg et al.[21]). Many of these epigenetic studies were facilitated by the establishment and maintenance of large longitudinal birth cohorts that are now beginning to establish the case for epigenetic marks as the mediators of DOHaD mechanisms.[6] It is important to note that an epigenetic response to an in utero exposure need not always be detrimental and may in fact be a protective adaptation with the potential to confer beneficial outcomes to the progeny, or even future generations. The latter has been reported in a rat model of carbon tetrachloride-induced liver damage, associated with epigenetically mediated increased hepatic healing. Of particular note, this effect was amplified transgenerationally, such that exposure in the third generation was not associated with significant liver damage.[22] However, replication of many reported findings is generally lacking.

In order to definitively show a role of epigenetics in "programming" later health risk in humans in accordance with the DOHaD hypothesis, several requirements need to be met. First, an association between a specific intrauterine environment and a specific epigenetic change must be established, which can be difficult given the plethora of confounding factors of genetic, environmental, stochastic, and temporal origin.[23] Next, these results need to be reproducible across cohorts of similar ethnicity. In most instances, it is likely that an epigenetic change detected at birth needs to be stable over time, and must be reproducibly associated with the later onset of a specific complex disease. Finally, direct evidence for a functional role of the associated epigenetic change needs to be obtained. Given the long latency of many complex disease phenotypes implicated in DOHaD, these prerequisites are unlikely to be firmly established for many human disorders for some time due to a lack of suitable mature longitudinal cohorts with multiple biospecimen collections.

Replication of results has been a major issue for the field, recently highlighted for six studies that looked at the relationship between methylation and birth weight, each of which reported completely non-overlapping sets of genes.[24] The disparity may be due to differences in study design (e.g., twin or singleton), differences in birth weight range and discordance between individuals, cells analyzed, or genetic background of each cohort. It is therefore of utmost importance to attempt to replicate results in a timely manner, so as to guide future functional studies, and to eliminate false positives. To date, the association between tobacco smoking exposure and DNA methylation within the *AHRR* gene remains the most convincing example of the relationship between a specific environment and DNA methylation differences in humans.[10-14]

Table 1. Summary of the study population

Characteristic (range)	Never smoked	Smoked early	Smoked throughout
Number of twin pregnancies	23	11	12
Mother age at birth	37 (27–44)	35 (21–44)	33 (19–48)
Mother BMI	25 (20–33)	24 (18–31)	25 (18–45)
Gestational age	36 (32–38)	37 (36–38)	37.2 (34–38)
Birth weight (average of 2 twins) kg	2.60 (1.8–3.2)	2.71 (2.2–3.4)	2.61 (2.1–3.7)
Birth weight discordance (%)	9.74 (0.4–27.3)	11.22 (1.0–27.8)	9.88 (0.8 – 20.2)
Zygosity	8 MZ, 15 DZ	4 MZ, 7 DZ	4 MZ, 8 DZ
Sex	21 M, 25 F	8 M 14 F	13 M, 11 F
IVF	6	1	4
Maternal serum folate at 28 wk (nmol/L)	33.7 (14.0–64.2)	32.3 (12.5–46.2)	29.0 (8.4–42.5)
Maternal drinking early pregnancy (drinks / week)	1.6 (0–10)	4.5 (0–15)	1.6 (0–12)
No. cigarettes / day Pre-conception	0	7.0 (1–20)	13.6 (2–30)
No. cigarettes /day Knew about pregnancy	0	6.2 (1–15)	13.1 (2–30)
No. cigarettes / day at 24 wk	0	0	6.5 (1–15)
No. cigarettes / day at 36 wk	0	0	6.3 (1–15)
CBMCs	23	11	12
Buccal epithelium	9	/	8
Placenta	8	8	8

AHRR is a negative regulator of the *AHR* (aryl hydrocarbon receptor) gene, which codes for a protein that binds to a wide range of xenobiotics including nicotine and caffeine.[25] AHR induces the expression of *CYP1A1* and other genes involved in the removal of deleterious chemicals, while also playing a role in a range of other cellular pathways, including cell cycle control.[26] AHRR exerts its repressive action on AHR by binding to ARNT, a partner of AHR, and therefore higher *AHRR* expression associated with gene hypomethylation might be anticipated to attenuate the cellular responses to smoking. However, it has not been conclusively shown that higher *AHRR* expression is directly associated with lower *CYP1A1* expression (discussed in Harper et al.[27]). Interestingly, Joubert et al. identified opposite DNA methylation changes at *AHRR* and *CYP1A1* in response to smoking, suggesting that both an increase in *AHRR* and a decrease in *CYP1A1* expression may be involved in the response.[10]

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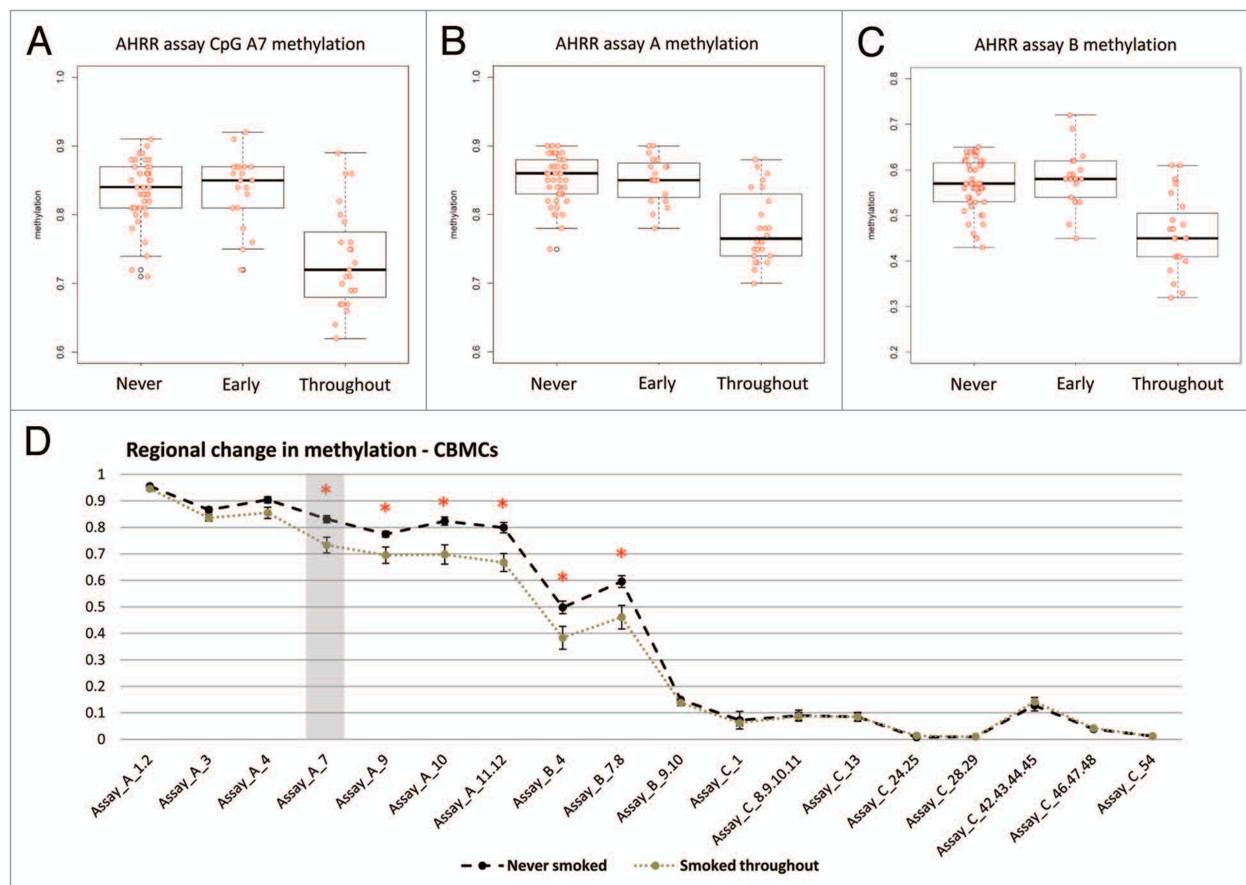

Figure 2. Association between maternal smoking and *AHRR* methylation in CBMCs. Maternal smoking throughout pregnancy is associated with lower methylation at (**A**) CpG_A7, (**B**) across assay A and (**C**) across assay B. Maternal smoking early in pregnancy was not associated with lower *AHRR* methylation. (**D**) Eight CpG sites, contained within 6 CpG units, show lower methylation (dB > 0.05, *P* < 0.05) in response to maternal smoking. The difference in methylation is limited to the CpG Island shore, whereas the CGI is hypomethylated in all CBMC samples. *Y*-axis, DNA methylation level; grey shaded box, "cg05575921" CpG site of interest; error bars, 95% CI.

AHRR may also serve as a tumor suppressor gene, with evidence for decreased expression in several human tumor types, with a concomitant increase in promoter methylation.[28] The role of gene body methylation in the expression of this gene remains unclear, yet the presence of specific chromatin "signatures" at this region supports a functional role in gene regulation. This is supported by concomitant loss of methylation and higher expression of *AHRR* observed in blood and lungs from adult smokers[11,12] It is unclear if *AHR* and *CYP1A1* expression are lowered in lungs of smokers.

In this study we sought to replicate the DNA methylation differences in blood mononuclear cells, and to determine if other cell types are sensitive to maternal smoking in pregnancy. Our data supports previous findings, with several CpG sites within the analyzed region of *AHRR* showing lower methylation in CBMCs in association with maternal smoking (**Fig. 2**). Furthermore, we did not identify a relationship between *GFI1* and *MYO1G* methylation and smoking (**Figs. S4 and S5**). Based on the magnitude of methylation changes observed in the Joubert et al.[10] study, our study is underpowered to detect the small methylation differences observed for *MYO1G*, which may explain why we failed to find a significant association with this gene. On the other hand, our study had over 90% power to detect an association between smoking and *GFI1* methylation levels in CBMCs, based on the previously reported effect size.[10] This further highlights the need for replication of studies in different cohorts to eliminate false positive and population-specific results.

Table 2. DNA methylation level across the AHRR gene body region

CpG site	Never smoked	Smoked early	Smoked throughout	dB smoked – never (*P* value)
A1.2	0.96	0.96	0.95	−0.01 (0.007)
A3	0.87	0.85	0.84	−0.03 (<0.001)
A4	0.90	0.91	0.86	−0.05 (<0.001)
A7	0.83	0.83	0.73	−0.10 (<0.001)
A9	0.77	0.77	0.70	−0.08 (<0.001)
A10	0.82	0.82	0.70	−0.13 (<0.001)
A11.12	0.80	0.81	0.67	−0.13 (<0.001)
B4	0.50	0.51	0.38	−0.11 (<0.001)
B7.8	0.60	0.61	0.46	−0.13 (<0.001)
B9.10	0.15	0.16	0.14	−0.01 (0.05)

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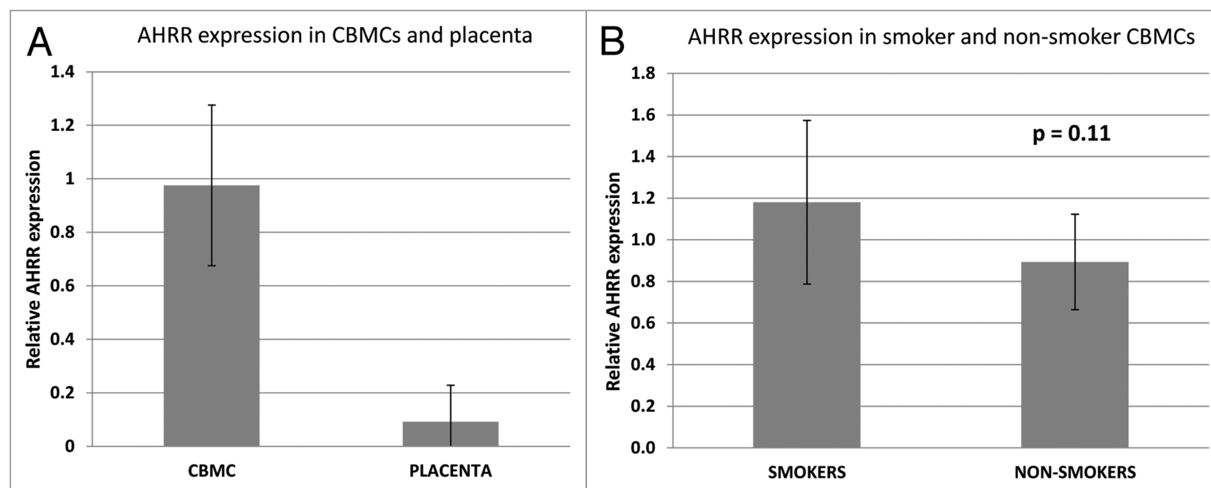

Figure 3. AHRR expression in CBMCs exposed and not exposed to smoke during pregnancy. (**A**) AHRR expression is higher in CBMCs compared with placenta tissue. (**B**) AHRR expression in CBMCs exposed to smoking during pregnancy (n = 5) is slightly higher than in non-smoking controls (n = 10), *P* = 0.11. Bars represent standard deviation.

Analysis of buccal epithelium and placenta did not identify a smoking-associated methylation difference at *AHRR* in these tissues (**Fig. S2**). Interestingly, this region of differential methylation also showed a tissue-specific pattern, with CBMCs showing high methylation across the region (approximately 80%), while buccal epithelium and placenta showed significantly lower methylation across several CpG sites (40–70%; **Fig. 1**). This further suggests that the intron 3 of *AHRR* harbors a regulatory region, but this will require direct testing. Previous data suggest that *AHRR* is expressed at low levels in the placenta, while cord blood mononuclear cells show high interindividual variation, with no/low expression to very high expression.[29] Unfortunately, previous analysis did not explore the relationship between maternal smoking during pregnancy and *AHRR* expression in CBMCs. However, higher *AHRR* expression has previously been detected in lungs of smokers and lung cancer patients with a history of smoking.[12,30] Our expression analysis identified a trend toward higher expression in CBMCs exposed to smoking during pregnancy (*P* = 0.11; **Fig. 3**). A weakness of this analysis was the low number of individuals for which CBMC RNA was available (n = 5). Of these, only one sample showed a large drop in methylation at the *AHRR* intron 3 region. Therefore, our results suggest that smoking may not alter *AHRR* expression in CBMCs to the extent it does in lungs of patients, although this will require further testing in a larger sample set. It would be much more informative, however, to examine expression in CBMCs from individuals with a larger loss of methylation at intron 3 or to directly test for this association using appropriate in vitro reporter assays.

Another aspect of the relationship between the maternal environment and fetal epigenetics we wanted to explore was the critical time for an environment to affect DNA methylation during pregnancy. In our cohort, we analyzed a subset of women that smoked pre/peri-conception (i.e., "smoked early"), but not after. There was no difference in DNA methylation between the "never smoked" and the "smoked early" groups (**Table 2**). This suggests that early fetal exposure to maternal smoking is not sufficient to induce a DNA methylation change that is measurable at birth, at least in the tissues we examined. Furthermore, none of the mothers in the cohort smoked in the third trimester, without also smoking earlier in pregnancy, so it is difficult to pinpoint the critical period for when smoking induces DNA methylation differences. Our data support a mechanism whereby prolonged exposure is necessary to induce a detectable long lasting change in methylation. Alternatively, it is possible that short-medium term exposure later in pregnancy, possibly at a particularly sensitive point in fetal blood development, can induce a change in methylation at the *AHRR* locus. Furthermore, we did not identify a sex effect on DNA methylation level or response to maternal smoking (**Fig. 3**), which has previously been reported for some maternal environments in humans and mice.[31,32]

In accordance with previous reports,[10] we found a strong correlation between self-reported questionnaire data on smoking behavior and plasma cotinine levels, both before and after pregnancy. Furthermore, our questionnaire data and plasma cotinine levels suggest that none of the twins was exposed to household smoking after birth, suggesting that the lower methylation observed at 18 mo is an epigenetic "legacy" of intrauterine exposure. Our data are in accordance with a recent adult study that showed that *AHRR* DNA methylation in adult blood of smokers that quit approaches the levels of never smokers within the first few years of quitting, but never completely reaches normal levels, remaining on average 3–4% lower.[14] Despite the small numbers of samples available, we found evidence of a consistent effect that showed statistical significance (**Table 3**; **Fig. 4**). It will also be interesting to study the effects of paternal only postnatal smoking exposure, on *AHRR* methylation in children.

In conclusion, we have confirmed the association of maternal smoking throughout pregnancy and decreasing *AHRR* methylation in blood at birth. We have expanded the region of interest, and show that the smoking-associated DNA methylation changes

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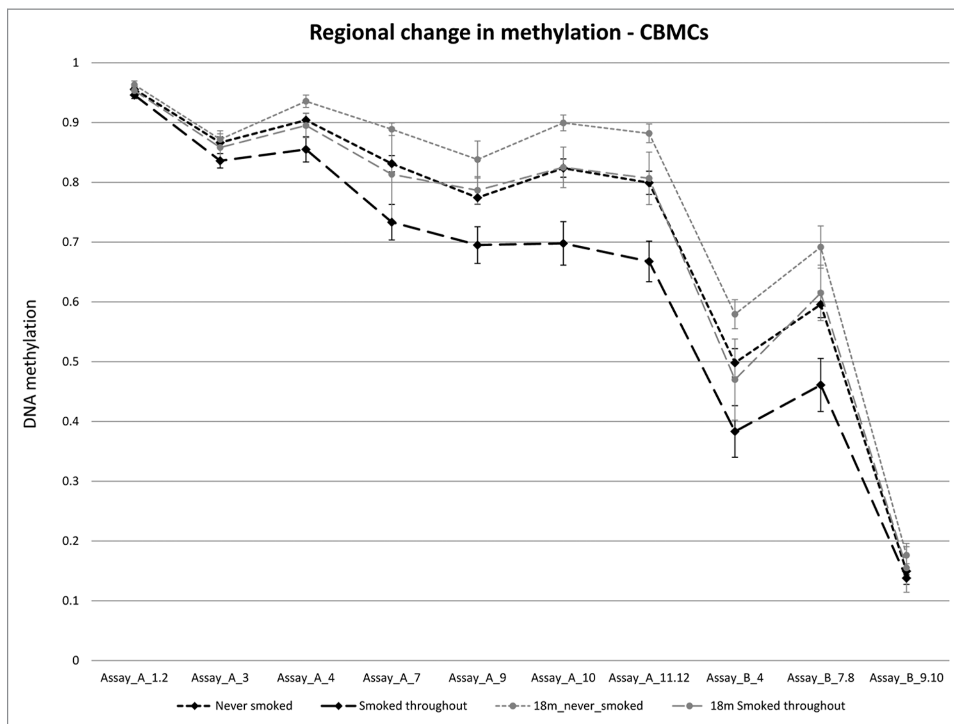

Figure 4. Maternal smoking associated DNA methylation change is maintained in peripheral blood mononuclear cells at 18 mo of age. DNA methylation levels (*y*-axis) across assay A and B at birth and 18 mo in smokers and controls. There is a general increase in methylation, at the majority smoking-associated CpG sites, from birth to 18 mo in both groups. DNA methylation differences between smoked throughout and never smoked groups remain significant at several CpG sites, however the absolute difference in methylation between the groups is lower at 18 mo.

are limited to the CpG island shore, and are tissue-specific. Finally, we show evidence that intrauterine exposure can induce persistent DNA methylation change up to 18 mo of age. Given the role of *AHRR* as a negative regulator of pathways involved in pleiotropic responses to environmental contaminants, the observed "programming" of this gene in utero following exposure to maternal smoking is likely to be of functional relevance. At present, however, it is unclear whether the observed response to smoking is an adaptive change associated with postnatal benefit, whether it is neutral, or potentially detrimental to postnatal health. This is the first step toward providing a link between environmental exposures in utero, epigenetic disruption, and DOHaD. Further analysis in a larger longitudinal birth cohort is necessary to confirm these results.

Table 3. DNA methylation at 18 mo in smokers and never smoked controls

CpG site	18 mo Never smoked	18 mo Smoked throughout	dB Smoked – never (P value)
A1.2	0.96	0.95	−0.01 (0.139)
A3	0.87	0.86	−0.01 (0.356)
A4	0.94	0.90	−0.04 (0.004)
A7	0.89	0.81	−0.08 (0.011)
A9	0.84	0.79	−0.05 (0.094)
A10	0.90	0.83	−0.07 (<0.001)
A11.12	0.88	0.81	−0.08 (0.003)
B4	0.58	0.47	−0.11 (0.005)
B7.8	0.69	0.62	−0.08 (0.055)
B9.10	0.18	0.16	−0.02 (0.321)

Methods

Samples

The Peri/postnatal Epigenetics Twin Study (PETS) is a cohort of 251 young twins and their mothers, recruited mid-way through pregnancy, for which lifestyle/environment data and tissue samples were collected at birth and at 18 mo of age.[15,16] The mothers were predominantly of white European descent, and women with poor English language skills were not enrolled into the study. For the current study, we used cord blood mononuclear cells (CBMCs), buccal (inner cheek) epithelium, and placenta tissue collected at birth. Maternal smoking data from questionnaires (including number of cigarettes per week) covered pre/peri-conception (prior to knowledge of the pregnancy), from known to 12 wk, 12–24 wk, and 24–36 wk of gestation, and the presence or absence of household smoking in the first 18 mo of life. Cases were mothers who reported any smoking during these time points and controls were mothers who reported never smoking and were matched to the smoking group for gestational age, maternal BMI, birth weight, birth weight discordance, and serum folate concentration at 28 wk gestation (**Table 1**). Of a total 251 pregnant women recruited in our original study, 12 reported smoking throughout pregnancy, while 11 reported

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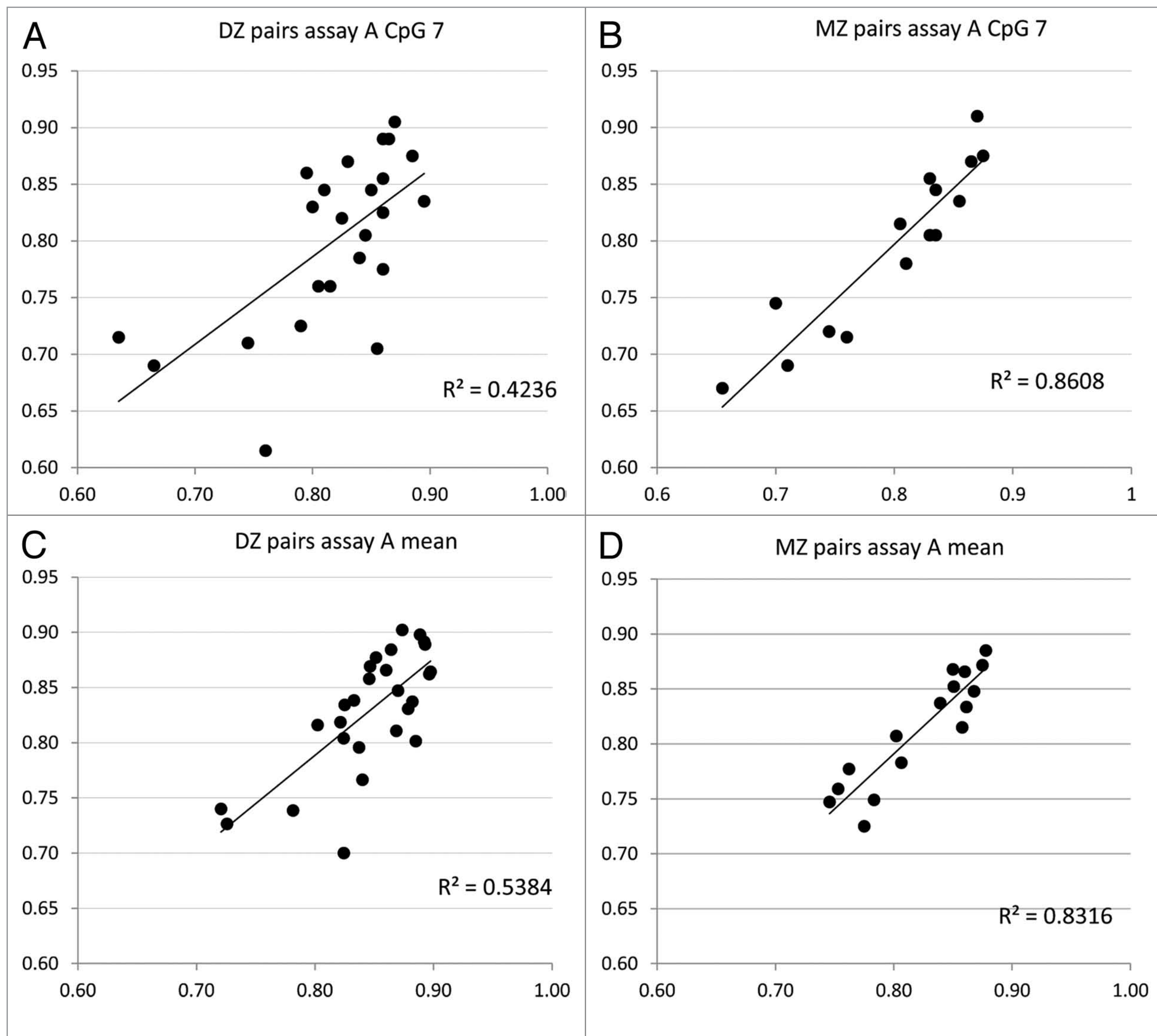

Figure 5. Twin model analysis suggests a genetic component to *AHRR* methylation. The classic twin model analysis was performed to determine the genetic contribution to *AHRR* methylation at CpG_A7 and across assay A. Twin "1" methylation level is shown on the x axis, and twin "2" methylation level on the *y*-axis. The within-pair correlation (R^2) was higher in MZ twins (**B and D**) compared with DZ twins (**A and C**). This suggests that MZ twins are generally more similar in their *AHRR* DNA methylation level than DZ twins, indicating a genetic control of methylation at this locus.

smoking only in early pregnancy up to finding out they were pregnant. CBMCs were collected from all of these pregnancies (46 babies). Additionally, CBMCs from 23 matched pregnancies, with no reported smoking were also analyzed as controls (**Table 1**). In addition, we analyzed CBMCs from 23 matched pregnancies, with no reported smoking before or during pregnancy (**Table 1**). Cotinine levels were measured in maternal plasma at 28 wk and infant plasma at birth and 18 mo for 6 twin pairs for which samples and methylation data were available at birth and 18 mo. Cotinine measurements were performed with HPLC (Agilent), using a protocol adapted from Kellogg et al.[33]

DNA methylation quantification

DNA extraction from cells and tissue was performed using the phenol/chloroform method, as previously described.[20] Genomic DNA was bisulfite treated using the MethylEasy DNA conversion kit (Human Genetic Signatures). DNA methylation was quantified using the SEQUENOM MassARRAY EpiTYPER platform, as previously described.[20,34,35] Duplicate PCRs were performed, and both PCR products were analyzed using the EpiTYPER platform. To minimize technical variation, replicates that showed methylation difference greater than 0.1 (10%) were removed from analysis. Assays were designed using the EpiDesigner software (www.epidesigner.com) and cleavage patterns were determined using the "ampliconPrediction" function in R. All forward primers contain a balance tag (AGGAAGAGAG) and all reverse primers contain a T7 tag (CAGTAATACG ACTCACTATA GGGAGAAGGC T). Primers used were: Assay A F- 5′ GTTGGTAATG GTTTTGAGAT TTT 3′ R- 5′ AAAACCAACC TATCCCCTAC CTC 3′, Assay B: F- 5′ AGTGGTTTTG GTAGGGTTTT TTTT 3′

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R- 5′ AACCCCAATC TCCTCCTCTA TAAT 3′, and Assay C: F- 5′ TTTAGGTGGG ATTTTTAGGT TTAGG 3′ R- 5′ ATACAACCAA ACCCCATTAC AAAA 3′. Assay information including primer sequences is shown in and all methylation values for CBMCs are shown in **Table S1**.

Gene expression analysis

RNA from CBMCs and placenta was reverse transcribed using the Tetro cDNA synthesis kit (Bioline). AHRR expression was quantified using a previously published assay,[12] on the Roche lightcycler with the Sensifast qPCR mix (Bioline). Relative expression was calculated using the delta delta Ct method.

Data analysis

The freely available R statistical program (cran.r-project.org/) was used to produce descriptive figures, including heatmaps and box-and-whisker plots, and to determine the suitability of EpiTYPER assays to cover CpG sites of interest. Differences in methylation level between the different groups were determined using the Student *t* test. The classical twin model approach was used to determine the genetic contribution to DNA methylation level. The model is based on the fact that monozygotic (MZ) twins are genetically identical, while dizygotic (DZ) twins share about 50% of their genetic variation. DNA methylation was treated as a continuous variable, and the within-pair correlation was calculated using linear regression. Higher similarity in DNA methylation profile in MZ twins as a group relative to DZ twins as a group is supportive of a role of genetic influence on methylation levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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