Epigenetic Programming by Maternal Behavior in the Human Infant

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abstract

OBJECTIVES: We sought to determine if variations in maternal care alter DNA methylation in term, healthy, 5-month-old infants. This work was based on landmark studies in animal models demonstrating that nurturing care by dams would alter their newborns' stress responses through epigenetic mechanisms. We used breastfeeding as a proxy for animal maternal behavior. We hypothesized alterations in DNA methylation of the glucocorticoid receptor gene and less hypothalamic stress response in infants of mothers who breastfed their infants versus infants of mothers who did not breastfeed.

METHODS: A cohort study of term, healthy infants and their mothers who did (n = 21) or did not (n = 21) breastfeed for the first 5 months was used in this analysis. Cortisol stress reactivity was measured in infant saliva by using a mother-infant interaction procedure and DNA methylation of an important regulatory region of the glucocorticoid receptor gene. Changes in DNA methylation of this gene in humans were compared to homologous regions of the rat gene. DNA samples were prepared from cheek swabs and subjected to quantitative analysis of the extent of methylation by using sensitive sequencing techniques.

RESULTS: Breastfeeding was associated with decreased DNA methylation of the glucocorticoid receptor promoter and decreased cortisol reactivity in 5-month-old infants. Decreased DNA methylation occurred in the promoter region involved in regulation of the hypothalamic-pituitary-adrenal and immune system responses.

CONCLUSIONS: Maternal care in humans may impact the hypothalamic-pituitary-adrenal stress response through behavioral programming and manifest as offspring epigenetic change. These results explain, in part, some of the positive effects observed in children who are breastfed.

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WHAT'S KNOWN ON THIS SUBJECT: Variations in early maternal care in rodents, including nursing, affect epigenetic changes (DNA methylation) in the glucocorticoid receptor gene of offspring. We report similar findings in human mothers and their infants.

WHAT THIS STUDY ADDS: This partial replication of rodent work reveals that maternal care alters the human infant epigenome through behavioral programming and hypothalamic-pituitary-adrenal stress reactivity. In addition, we show that behavioral programming could be used to partly explain the protective effects of breastfeeding.

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Epigenetics is the study of how environmental influences affect gene expression. Now, classic animal studies reveal that variations in maternal care can have profound effects on early development, stress regulation, and behavior.[1–5] Dams with high nurturing activity (licking, grooming, and arched-back nursing) have pups that display less anxiety, less fear conditioning, and less hypothalamic stress responsivity than pups that are reared by dams that are less nurturing.[2] Critically, if the subsequent litters born to dams that display these different behaviors are switched at birth, the altered stress responsivity and behavioral conditioning has been shown to be dependent on maternal behavior. Even more extraordinary is the demonstration that these effects are preserved across subsequent generations. The mechanism for these remarkable environmental effects on behavior and stress responsivity is mediated by epigenetics. Alterations in the DNA methylation of the glucocorticoid receptor promoter modify the hypothalamic-pituitary expression of this important gene and stress responsivity. Furthermore, the mechanistic exploration of these effects on DNA methylation has been shown to be mediated by alterations in the binding site for important transcription factors that regulate the expression of this gene. These effects have also been shown to be reversible after a pharmacologic modulation of chromatin (chromosome materials) remodeling. Thus, the role of maternal behavior in behavioral conditioning during early life in newborn animals is widely accepted and demonstrated. In humans, children who are exposed to physical maltreatment,[6] infants of mothers with depression,[7,8] adolescents whose mothers were exposed to intimate partner violence during pregnancy,[9] and adults with a history of child abuse[10] have increased DNA methylation of the human glucocorticoid receptor, which is the homolog of the rat glucocorticoid receptor.[11] Although the researchers in these studies did not examine hypothalamic-pituitary-adrenal (HPA) stress reactivity or parenting behavior, they did suggest that similar molecular mechanisms in rodents and humans could modulate the risk for psychopathology. We wondered whether any of these fundamental mechanisms, mediated through epigenetic effects, were demonstrable in human infants.

TABLE 1 Patient Demographic Characteristics

	Low or No Breastfeeding Group (n = 21)	Breastfeeding Group (n = 21)	P
Age, mean			
Maternal, y	29.05	31.52	ns
Gestational, wk	39.25	39.38	ns
Infant, wk	18.76	19.14	ns
Pregnancy complications, mean	0	0	ns
Birth wt, g, mean	3389.75	3564.48	ns
Infant sex is female, %	47.6	42.9	ns
Household income, $, %			ns
0–24 999	14.3	9.5	
25 000–49 999	40	23.9	
>50 000	45.7	66.6	
Married or living with a partner, %	60	81	ns
Ethnicity, %			ns
European American	47.6	76.2	
African American	23.8	4.8	
Hispanic	14.3	9.5	
Asian American	4.8	0	
Other	9.5	9.5	

ns, not significant.

METHODS

We used breastfeeding as a proxy for high nurturing activity in the rodents to determine if human maternal care in the form of breastfeeding would elicit similar epigenetic programming in the rodents. Therefore, we hypothesized that infants of mothers who provide high levels of breastfeeding would have less DNA methylation of the glucocorticoid receptor promoter region than infants of mothers who provide low levels of breastfeeding. We further hypothesized that less DNA methylation of the glucocorticoid receptor in this region would be associated with a decreased HPA response to stress, which was measured by cortisol reactivity, in these infants.

Informed consent was obtained from all the mothers. Mothers and their infants who were born at the Women and Infants Hospital in Rhode Island were enrolled as part of the larger Rhode Island Child Health Study[12] and then recruited for this follow-up examination during the period of June 2013 through July 2014. The inclusion criteria included having an uncomplicated delivery of a singleton, viable, term infant (>37 weeks). The exclusion criteria included maternal age <18 or >40 years, the mother or child having life-threatening complications, and the infant having a congenital or chromosomal abnormality. Mothers were administered a self-report questionnaire consisting of 3 questions used to measure the extent of breastfeeding since the birth of their infants. Mothers were asked (1) if they ever breastfed their infants (yes or no), (2) the proportion of feedings that are currently breast versus bottle (mothers indicated the percentage of feedings from the breast versus percent of bottle), and (3) the age of the infants when they stopped breastfeeding. Mothers who

FIGURE 1
Mother-infant interaction procedure. A, Infant when playing as normal. B, Mother when playing as normal. C, Infant during the still-face period. D, Mother during the still-face period.

breastfed their infants continuously over the first 5 months were in the high breastfeeding group (n = 21). Mothers who did not breastfeed their infants at all or who stopped breastfeeding by 3 weeks were in the low breastfeeding group (n = 21).

Cortisol stress reactivity was elicited from the infants by using a mother-infant interaction procedure that includes a "still-face" episode, during which the mother is nonresponsive to her infant.[13] The still face is a stressor and has been reliably shown to elicit cortisol reactivity.[14] Specifically, the procedure begins with the mother and infant sitting across from each other and playing face-to-face as they normally would at home for 2 minutes (Fig 1 A and B). This is followed by a still-face episode, during which time the mother is instructed to maintain a blank "poker" face and not to smile and interact with her infant for 2 minutes. Here, the infant shows a negative response to the mother's still face (Fig 1 C and D). After the 2-minute still-face period, the mother and infant again play together as they normally would for 2 minutes, just as they might during the first play episode.

Infant saliva samples were collected before (prestress) and 30 minutes after (poststress) the still-face procedure by using a small sponge that was swabbed in each infant's mouth until it became saturated with saliva. The swab was then placed into a storage vial and frozen until analyzed. If infants ate or drank 30 minutes before the collection of the saliva sample, their mouths were first swabbed with a wet paper towel. The saliva samples were sent to Salimetrics LLC (Carlsbad, CA), and cortisol was measured by using enzyme immunoassay in units of micrograms per deciliter. The cortisol values underwent log transformation. Cortisol reactivity was computed as a difference score (poststress micrograms per deciliter minus prestress micrograms per deciliter).

An additional set of buccal swabs was also collected from each infant by using the Oragene Discover assisted collection system, and from those, genomic DNA was extracted by using the prepIT kit (DNA Genotek, ON, Canada). DNA was subjected to bisulfite modification by using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA), and bisulfite pyrosequencing was performed on polymerase chain reaction products that were amplified from bisulfite-modified DNA, as described previously.[15] The primers for the amplification of the human glucocorticoid receptor promoter were as follows: forward 5′-TTT TTT TTT TGA AGT TTT TTT A-3′ and reverse 5′-Biotin-CCC CCA ACT CCC CAA AAA-3′. Two primers were used to sequence the amplification product in the 1F region: sequence 1, 5′-GAG TGG GTT TGG AGT-3′; sequence 2, 5′-AGA AAA GAA TTG GAG AAA TT-3′. The percentage of DNA methylation at each cytosine guanine (CpG) site was quantified by using Pyro Q-CpG software version 1.0.11 (Qiagen, Hilden, Germany).

The mean percentage of methylation was compared between the high and low breastfeeding groups at each CpG site by using 1-way analysis of variance. Following statistically significant group differences, bivariate correlations were used to relate DNA methylation to cortisol reactivity at CpG sites 7, 10, 12, and 13. We controlled for false discovery among the 13 tests used to relate the DNA methylation of the 13 CpG sites on the glucocorticoid receptor gene and breastfeeding status by using the Benjamini and Hochberg procedure.[16] Instead of a corrected P value, a q value is obtained to determine the percentage of findings that could be false discovery. As is standard in the epigenetic literature, we chose a q value of 0.10. In the results, we present both the P and q values.

RESULTS

Participants included 42 mothers and their 5-month-old infants; 21 mothers breastfed exclusively for the first 4 months (high levels of breastfeeding), and 21 did not breastfeed for the first 4 months (low levels of breastfeeding) (Table 1). The mothers were of middle

socioeconomic status with a median income of $50 000 to $79 000. They ranged in age from 21 to 38 years (mean = 29.64 years; SD = 4.53 years), and 91% had at least a high school education. The infants were born term and healthy.

Infants of mothers in the high breastfeeding group had less methylation, with low false-discovery rates at CpG sites 7 ($P = .05$; $q = 0.03$), 10 ($P = .02$; $q = 0.008$), 12 ($P = .02$; $q = 0.02$), and 13 ($P = .04$; $q = 0.02$) in the glucocorticoid receptor gene exon 1F promoter region than infants of mothers in the low breastfeeding group (Fig 2). Lower DNA methylation at CpG sites 10 ($r = .41$; $P < .05$; Fig 3) and 12 ($r = .35$; $P < .05$; Fig 4) was associated with decreased cortisol reactivity.

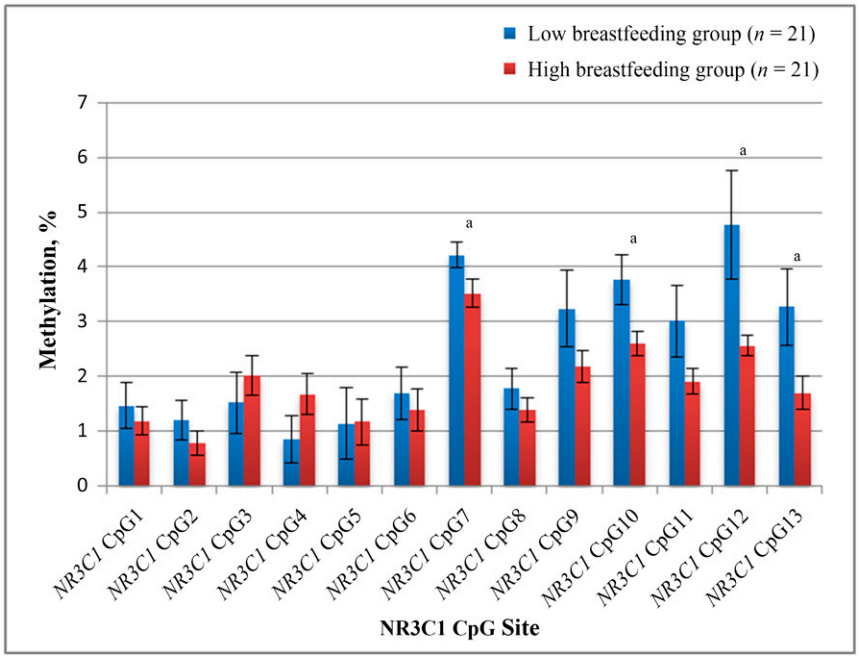

FIGURE 2

Differences in the extent of DNA methylation at CpG sites across the glucocorticoid receptor gene (*NR3C1*) in buccal cell–derived DNA from infants experiencing low (blue bar) or high (red bar) breastfeeding behaviors. Bar heights represent the mean methylation in all subjects within the group, and error bars represent the SEM. [a] False-discovery rate is $q < 0.05$.

DISCUSSION

We report, to our knowledge, the first translational study in which researchers recapitulate the effects of maternal care in rodents by demonstrating that in human infants, maternal breastfeeding impacts the infants' epigenome and is associated with altered stress responsivity. Specifically, in rodents, increased maternal nurturing behaviors led to decreased methylation of the glucocorticoid receptor promoter region in the rat hippocampus. This effect was mirrored in our population. Infants who experienced increased breastfeeding had decreased methylation in the homologous region of the human gene. Also, in parallel to observations of rodents, decreased methylation of this gene in the human infants was associated with decreased cortisol stress reactivity. In rodent pups, and presumably in human infants, this decreased methylation of the glucocorticoid receptor gene is associated with enhanced glucocorticoid receptor expression, facilitating the regulation of the HPA stress reactivity system. Our findings reveal that variations in early human maternal care alter the epigenome of the infant, which in turn alters HPA stress reactivity. These are unique results that reveal that the functional consequences of behavioral programming, because of maternal care, may be through an altered epigenetic state in the human infant.

Our findings in human infants differ only modestly from the observations of animals. The effects of breastfeeding on DNA methylation in human infants were in homologous regions of the glucocorticoid receptor gene of the rodents. This reveals that the effects of maternal behavior on the DNA methylation of the offspring and the functional consequences of DNA methylation are conserved between rodents and humans.

Mechanistic studies have been possible in animal models. In rodents, less nurturing maternal behavior is associated with increased methylation at a CpG site for a nerve growth factor binding site that is important for healthy brain development and other biological functions.[17,18] The methylation of this binding site reduces nerve growth factor occupancy, leading to reduced glucocorticoid receptor expression and subsequent disruption of the HPA system.[1] We[8] and others[7] have observed that the methylation of the human glucocorticoid receptor promoter region, including the nerve growth factor binding site, is associated with maternal mood disorders during pregnancy, infant behavior in term infants, and aberrant neurobehavior in preterm infants.[19] We found altered methylation at CpG sites 7, 10, 12, and 13. This region of the human glucocorticoid receptor gene promoter is involved in the regulation of the HPA system. CpG sites 7 and 12 are binding sites for the specificity protein transcription factor, and epigenetic variation in these binding sites can impact the binding and function

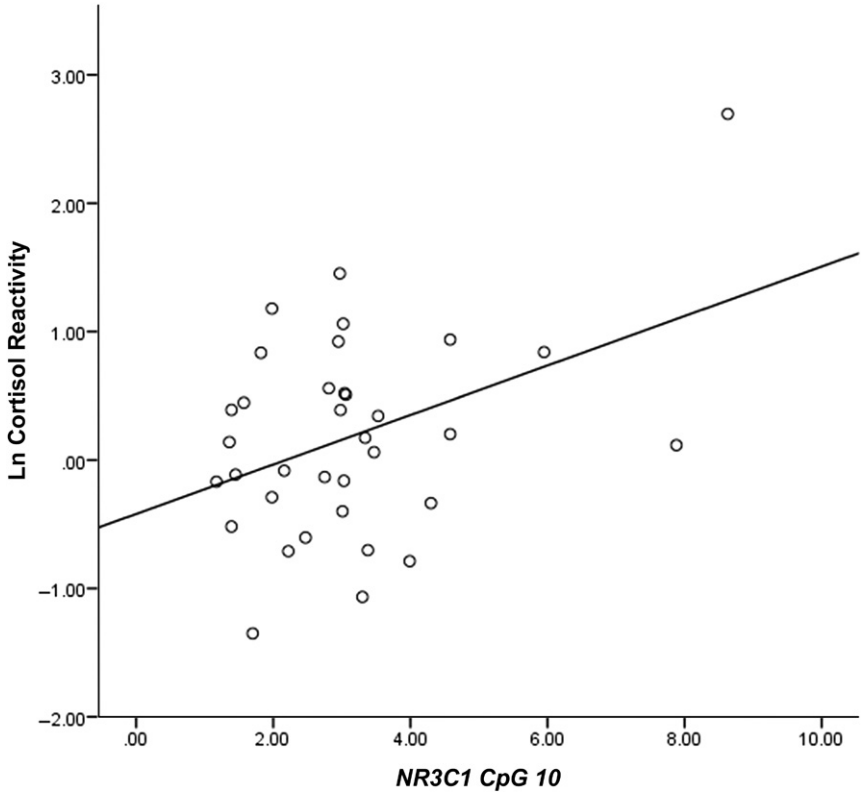

FIGURE 3

Scatterplot of the correlation between amount of DNA methylation of the glucocorticoid receptor gene (*NR3C1*) at CpG site 10 and cortisol reactivity. Cortisol was measured from saliva collected before and after a mother-infant interaction still-face stress procedure. Cortisol reactivity was computed as a difference score (poststress micrograms per deciliter minus prestress micrograms per deciliter) from the log transformation shown on the y-axis. The mean percentage of DNA methylation of the glucocorticoid receptor gene (*NR3C1*) at CpG site 10 is shown on the x-axis. The line in the figure is the linear regression that was statistically significant ($r = 0.41$; $P < .05$), and the circles are the individual subjects. R^2 linear = 0.167.

of this transcription factor.[20] The specificity protein transcription factor is activated for a number of cellular processes, including cell differentiation, cell growth, apoptosis, response to DNA damage, chromatin remodeling, and, interestingly, immune responses. With respect to the HPA axis, the specificity protein is a mediator of steroid hormone nuclear signaling. We would expect the increased DNA methylation to have effects on chromatin, leading to decreased specificity protein binding, a reduced rate of transcription, interference with cellular processes, and disruption of the HPA axis. This argument is supported by our finding that increased DNA methylation is associated with increased cortisol stress reactivity.

We used breastfeeding as a proxy for a high level of maternal nurturing behavior in the rodents to replicate the epigenetic effects of maternal care on the glucocorticoid receptor gene promoter region. That the specificity protein binding factor is involved in immune responses could suggest epigenetic associations related to the positive effects of breastfeeding. Breastfeeding and the accompanying tactile stimulation have effects that are comparable to a high degree of maternal nurturing in young animals. Higher levels of maternal care in both rodents and humans decrease DNA methylation in analogous regions of the glucocorticoid receptor promoter, which results in decreased reactivity of the HPA system response to stress. As in the rodents, behavioral programming by the mother affects the epigenome of the infant. There are undoubtedly multiple pathways for behavioral programming within species. We would expect additional pathways for behavioral programming that are conserved between rodents and humans to be identified, including pathways that alter, for example, serotonin activity.[21]

Our study had some limitations. For obvious reasons, we could not measure DNA methylation in hippocampal cells or conduct the kind of cross-fostering, intergenerational transmission or pharmacologic studies that can be conducted in animal models. Although we recognize that there may be differences in DNA methylation at specific CpG sites between hippocampal tissues and the cells that are available from a buccal swab, we note that buccal cells come from the same primordial lineage and that findings are similar to those from buccal and brain specimens that are used in studies of psychiatric traits.[22] We also highlight the comparability of our findings in human buccal cells to animal work in which researchers use brain cells, suggesting that they could be measuring similar processes. We did not study gene expression, and we used salivary cortisol, which is a peripheral glucocorticoid. Although DNA methylation is correlated with gene expression in human epigenetic studies[23,24] and salivary cortisol is a well-established measure of HPA stress reactivity[25] that is correlated with serum cortisol, our findings would be strengthened by using these measures, and they certainly should be considered in future studies in this area.

Rodents are the most frequently used species in both experimental and translational studies. The risks involved in the translation of rodent findings to human findings are well

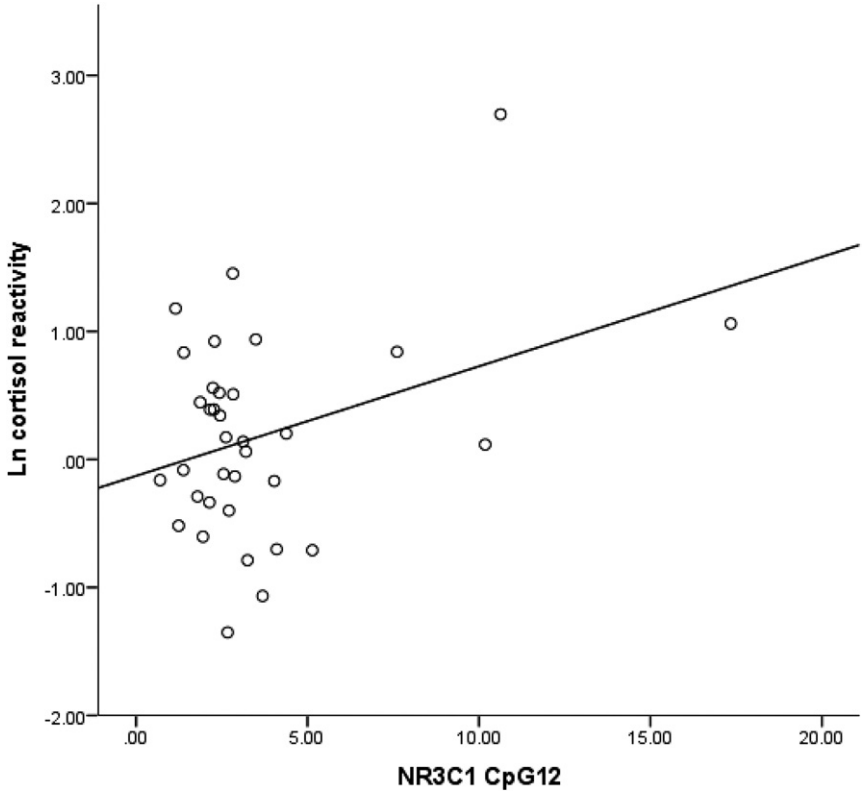

FIGURE 4

Scatterplot of the correlation between amount of DNA methylation of the glucocorticoid receptor gene (*NR3C1*) at CpG site 12 and cortisol reactivity. Cortisol was measured from saliva collected before and after a mother-infant interaction still-face stress procedure. Cortisol reactivity was computed as a difference score (poststress micrograms per deciliter minus prestress micrograms per deciliter) from the log transformation shown on the y-axis. The mean percentage of DNA methylation of the glucocorticoid receptor gene (*NR3C1*) at CpG site 12 is shown on the x-axis. The line in the figure is the linear regression that was statistically significant ($r = 0.35$; $P < .05$), and the circles are the individual subjects. R^2 linear = 0.124.

recognized. Although determining the exact equivalence in the development between these species is difficult, we can say that in terms of age, the rodent at 6 postnatal days, the age used in the rodent work,[1] roughly corresponds to the human infant at 5 months,[26] the age of the infants whom we studied. This underscores the translational importance of our study.

Despite these limitations, we have identified a conserved pathway of maternal behavioral programming in humans that is associated with the altered epigenetic state of a gene that affects HPA stress reactivity, which is an important functional consequence. Dysregulation of the HPA axis has been implicated in the etiology of a range of psychological disorders.[27–31] Interestingly, such disruptions are also related to the responsivity of immune systems.[32] Understanding how variations in maternal care alter the infant epigenome through behavioral programming and the modification of HPA stress reactivity provides insight into the molecular mechanisms that could confer risk or protection for psychopathology. It may be too early, but we can also speculate that future translational work could lead to the development of interventions that are used to increase maternal care and reprogram the HPA system.

CONCLUSIONS

We have presented translational evidence that as in developing animals, variation in maternal care in humans may impact the epigenome of the offspring through behavioral programming, which in turn could alter HPA stress reactivity and may partly provide a mechanistic link for the positive impacts of breastfeeding on infant development.

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ABBREVIATIONS

CpG: cytosine guanine
HPA: hypothalamic-pituitary-adrenal

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