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The Signature of Maternal Social Rank in Placenta Deoxyribonucleic Acid Methylation Profiles in Rhesus Monkeys

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Abstract

The effects of social status on human health can be modeled in captive cohorts of nonhuman primates. This study shows that maternal social rank is associated with broad changes in DNA methylation in placentae of rhesus monkeys ($N = 10$). Differentially methylated genes between social ranks are enriched in signaling pathways playing major roles in placenta physiology. Moreover, the authors found significant overlaps with genes whose expression was previously associated with social rank in adult rhesus monkeys (Tung et al., 2012) and whose methylation was associated with perinatal stress in newborn humans and rhesus monkeys (Nieratschker et al., 2014). These results are consistent with the hypothesis that system-wide epigenetic changes in multiple tissues are involved in long-term adaptations to the social environment.

Numerous studies have shown that parental socioeconomic status (SES) is strongly associated with birth outcomes (Kim & Saada, 2013) and with developmental risk (Woolfenden et al., 2014). Notably, low SES is a risk factor for pregnancy disorder, preterm delivery, and low birth weight, which in turn increases infant morbidity and mortality (de Graaf, Steegers, & Bonse, 2013; Kim & Saada, 2013). Childhood SES also has long-term consequences for adult health, as it has been associated with asthma (Barker et al., 2013; Power, Hertzman, Matthews, & Manor, 1997), mental health problems (Reiss, 2013), cardiovascular diseases, and overall mortality (Galobardes, Lynch, & Davey Smith, 2004). The underlying mechanisms potentially involve both physical (housing quality, school

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website:

Table S1. Determination of Dominance Rank

Table S2. Numbers of Differentially Methylated Probes With False Discovery Rate < 20% or Bonferroni Adjusted $ps < .05$ Detected Between Placentae of Mothers of Different Social Status

Table S3. Number of Overlapping Differentially Methylated Probes Detected Between Placentae of Mothers of Different Social Status

Table S4. List of Genes Differentially Methylated in Placenta ($p[\text{promoter}] < .05$) Between Monkeys of Low and High Status That Have a Degree of at Least 10

condition) and psychosocial (parenting practices, levels of conflict, education quality) environmental effects (Cohen, Janicki-Deverts, Chen, & Matthews, 2010). Furthermore, several studies have provided evidence that hardship during prenatal development can also lead to long-lasting effects on physical health (e.g., heart disease and diabetes; McEniry, 2013), cognitive development, and emotional health (Najman et al., 2004; Reiss, 2013).

Although it stands to reason that physical and chemical exposures such as poor nutrition can alter cellular differentiation and organogenesis and impact neurodevelopment during critical developmental windows (McEniry, 2013), mechanisms linking social adversity such as low social position of the parent and health of the child remain unclear. Of particular interest is to what degree do the effects of social status on the child emerge after birth as the child is socially exposed or whether at least some of these effects emerge prior to birth? As it is difficult to dissociate physical exposures such as suboptimal nutrition and psychosocial exposures such as stress that are characteristic of low SES in humans, nonhuman primate models were developed to study the psychosocial aspects of the surrounding environment (Sapolsky, 2004). Importantly, social status is one of the main determinants of health in nonhuman primate cohorts, such as groups of rhesus macaques, baboons, or chimpanzees (Sapolsky, 2005).

Social organization in nonhuman primates is established by dominance hierarchies, which defines how individuals submit to others in conflicts. It is noteworthy that dominance-related behaviors have also been considered a factor in structuring groups of humans as young as 2 years old (Frankel & Arbel, 1980; Savin-Williams, 1979, 1980; Strayer & Trudel, 1984). In rhesus monkeys, dominance rank is inherited from the mother, and high-ranking individuals reinforce their social position through diverse forms of aggressive behaviors and psychological threats (Linburg, 1971). There is a remarkable similarity between the effects of social positioning in humans and nonhuman primates (Sapolsky, 2005). The greater health problems of lower SES are associated with less access to resources such as nutritional diet and social support (McEniry, 2013). Moreover, women seem to be more susceptible to the deleterious effect of lower social support when facing hardship, resulting in higher rates of mental health problems (Choi & Ha, 2011). In monkeys, lower social rank is comparable to low SES in that low-ranking monkeys have restricted access to social partners, receive more aggression from other monkeys, and have less access to food resources. This combined lack of resources could affect maternal physiology and behavior, and pass to the offspring (Sapolsky, 2005). As a result, low-ranking monkeys tend to be highly stressed and further may suffer poor social support and difficulties in accessing food (Deutsch & Lee, 1991) and other resources. All these factors contribute to a high allostatic load and impaired health (Maestripieri & Hoffman, 2011). Under laboratory housing conditions food sources are not limited; however, the access to food for low-ranking monkeys might be socially restricted by the dominant monkeys.

Low social status in rhesus monkeys is associated with physiological alterations, such as perturbations of hypothalamic–pituitary–adrenal (HPA) axis activity (Michopoulos, Reding, Wilson, & Toufexis, 2012) and the immune system (Coe & Laudenslager, 2007; Paiardini et al., 2009; Tung et al., 2012). The consequences of chronic stress in low-ranking rhesus monkeys mirrors well the cardiovascular and metabolic outcomes associated with

socioeconomic disparities in humans (Sapolsky, 2004). For example, coronary artery atherosclerosis has been associated with social status in female monkeys (Kaplan, Chen, & Manuck, 2009) similarly to human cohorts (Galobardes et al., 2004), and higher consumption of caloric foods and beverages was observed in subordinate female monkeys (Arce, Michopoulos, Shepard, Ha, & Wilson, 2010) and in lower income households in the United States (Piernas, Ng, & Popkin, 2013). However, it is still uncertain when in development such status-related dissimilarities in physiology and behavior occur, and whether they require social interactions during infancy and childhood or whether they are at least partly programmed prenatally in offspring of low-ranking individuals.

The reproductive and lactating periods of rhesus macaques are particularly stressful for subordinate female monkeys (Hoffman, Ayala, Mas-Rivera, & Maestripieri, 2010). The observed hyperactivation of the HPA axis and suppression of immune functions in these prenatal and early postnatal periods could well model maternal stress and anxiety during pregnancy. Prenatal stress impacts the maternal–placental–fetal neuroendocrine axis, and this in turn affects progeny behavior as well as increases the likelihood of birth complications and health problems in offspring later in life, ranging from obesity to psychiatric disorders (O'Donnell, O'Connor, & Glover, 2009; Wadhwa, 2005). The placenta plays a critical role in embryonic development because it controls fetal exposure to the maternal environment (Jansson & Powell, 2007). In addition, perturbations in the maternal environment have been associated with alterations in placenta expression of immune genes (Bronson & Bale, 2014) and growth factors (Pankevich, Mueller, Brockel, & Bale, 2009), placenta metabolic pathways, and nutrient transport (Jansson & Powell, 2007), which all likely contribute to the fetal programming of processes involved in adult biology and pathophysiology.

The mechanisms that mediate long-term changes in genomic programming in response to early-life social exposures are unknown. Epigenetic processes including DNA methylation, chromatin structure, and noncoding RNAs are candidates because they are known to stably modulate gene expression patterns. Furthermore, there is evidence that maternal behavior can stably alter epigenetic patterns in a variety of animal models and in humans (McGowan et al., 2009; Weaver et al., 2004). Differences in maternal care in rats led to differences in both stress responsivity and DNA methylation of the hippocampal glucocorticoid receptor gene locus in the offspring (Weaver et al., 2004). Epigenetic pharmacological manipulation reversed the effects of maternal care on DNA methylation as well as behavior supporting the idea that epigenetic differences mediate the effect of maternal care on the behavior of the offspring (Weaver et al., 2004). Several studies have since correlated perinatal stress with alterations in DNA methylation in the offspring in rodents, nonhuman primates, and humans (Cao-Lei et al., 2014; Murgatroyd et al., 2009; Nieratschker et al., 2014; Provencal et al., 2012; Wu, Patchev, Daniel, Almeida, & Spengler, 2014).

These previous observations lead to the hypothesis tested here that epigenetic processes such as DNA methylation are involved in mediating the effects of parental social rank on the offspring. First, data supporting the hypothesis that epigenetic differences in DNA methylation are associated with differences in social rank came from DNA methylation analysis of 40 individuals from high and low socioeconomic positions included in the British

Birth cohort of 1958; the data suggested a DNA methylation profile of early-life socioeconomic positioning in blood DNA methylation and a stronger signature of early-life socioeconomic positioning than socioeconomic positioning emerging later in life (Borghol et al., 2012). Second, global DNA hypomethylation of peripheral blood leukocytes of adult humans was observed in the most socioeconomically deprived group compared with the least deprived group (McGuinness et al., 2012). In rhesus macaques, differences in social rank were associated with changes in gene expression in adult lymphocytes, some with corresponding changes in promoter DNA methylation (Tung et al., 2012). However, those studies examined DNA methylation in adults, so it was unclear whether the changes in DNA methylation were downstream consequences of other effects of social rank that emerge later in life or whether they preceded the phenotypic consequences of rank. The question therefore remains whether DNA methylation differences emerge in response to parental social rank very early in life even before the fetus is engaged in social interactions. If social rank affects biological processes prenatally, it will be consistent with cross-generational transmission of biological consequences of social rank from mothers to offspring. A first study showed that maternal education, a proxy for prenatal socioeconomic adversity, is correlated with methylation levels of corticosteroid 11-beta-dehydrogenase *HSD11B2* promoter in human placenta and suggested that environmental cues transmitted from the mother during gestation may indeed participate in the programming of the developing fetus (Appleton et al., 2013). We therefore tested here whether genome-wide DNA methylation profiles in placentae immediately at birth have been altered by the status of the mother in utero. Our data suggest broad and physiologically pertinent changes in DNA methylation in placenta that correlate with maternal rank.

Method

Placentae Collection

Ten rhesus macaque (*Macaca mulatta*) mothers were studied (Table S1). All monkeys were born and reared at the Laboratory of Comparative Ethology at the NIH Animal Center in Poolesville, MD. Monkeys lived in social groups consisting of 8–10 adult females and 1 adult male and were fed twice daily, were given water ad libitum, and had daily access to foraging materials (Dettmer, Novak, Meyer, & Suomi, 2014). Within hours after birth or immediately upon C-section, placentas were collected and the sections were separately frozen at -80°C for later DNA and RNA extraction. Qiagen RNA/DNA kits were used to extract placenta DNA, regardless of whether the sample was from the maternal or fetal side. Placentas were collected from April 2010 to July 2011.

We computed gestational age at birth from ultrasound estimations of due dates, using a known gestational length in rhesus monkeys of 165 days (Table S1). One mother aborted more than 2 months early, but the placenta was salvageable; another mother required a C-section when she was determined by ultrasound to be 14 days overdue to reduce risk of harm to the fetus. Using nonparametric test (Mann–Whitney *U* test), because of the very small sample size, we found that high-ranking mothers had significantly longer gestations than low-ranking mothers ($p = .016$). Of the nine healthy, live births, six were male and three were female. Using Fisher's exact test, we found no overrepresentation ($p = 1$) of male or

female offspring in the low-rank group compared with the mid-high/high-rank group. We also reported in Table S1 the age of the mothers. We did not find any significant difference in maternal age between the low- and mid-high/high-rank groups (Mann–Whitney U test, $p = .0823$). There was no effect of rearing condition on rank in this sample ($U = 4.0$, $p = .114$).

Determination of Dominance Rank

Dominance rank was determined by animal care (AC) staff, who observed the animals at least twice daily during feeding times and for visual health inspections. Based on interactions between the subjects during repeated observations over the course of many months to years prior to the onset of this study, AC staff determined whether females were high, mid-high, mid-low, or low ranking. These ranks were based on observed interactions between individual monkeys, with high-ranking monkeys always or almost always winning competitions for food, displacing other monkeys, and receiving low rates of aggressive behaviors (e.g., threats, chases, attacks). Mid-high-ranking monkeys behaved similarly, except that they on occasion lost food competitions or received aggressive behaviors from a few higher ranking individuals. Mid-low-ranking monkeys frequently lost food competitions and often received aggressive behaviors from high-ranking individuals, and low-ranking monkeys never won food competitions and never initiated aggressive behaviors to other individuals. Female monkeys in this study were high ($n = 3$), mid-high ($n = 3$), or low ($n = 4$) ranking within their social group (Table S1). No mid-low-ranking monkeys were identified for this study (i.e., no placentas were available from mid-low-ranking monkeys).

T-Cell Isolation

Separation of CD³⁺ T cells from monkey peripheral blood was performed as described previously (Provencal et al., 2012). In brief, peripheral blood was drawn into EDTA-coated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated through centrifugation with Ficoll-Paque (GE Healthcare, Burnaby, BC, Canada), and T cells were isolated from the PBMCs using CD³⁺ Dynabeads (Life Technologies, Burlington, ON, Canada).

Rearing Conditions

Newborn rhesus monkeys were randomly divided into two groups: “mother reared” (MR, $n = 9$) and “nursery reared” (NR, $n = 8$). Briefly, MR animals were reared by their biological mothers within a social group, and NR monkeys were reared by human caregivers from the first day of life through postnatal day 30, at which time they were placed in social groups with peers (and continued receiving care from human caregivers). They lived in this setting until weaning at approximately 8 months of age (Dettmer, Novak, Suomi, & Meyer, 2012). All environmental conditions, procedures, and handling of animals were in strict compliance with the requirements of the Institutional Animal Care and Use Committee, and all experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*. Blood samples were obtained from monkeys aged 14–30 days old (born between 2010 and 2011).

Analysis of Genome-Wide Promoter DNA Methylation

Placenta genomic DNA was extracted using the AllPrep DNA/RNA Qiagen kit (Hilden, Germany), and CD³⁺ T-cell DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). DNA samples were quantified using the Qubit system (Life Technologies). The placenta study was performed on individual samples and the CD³⁺ T cells study involved DNA pools from male and female MR and NR monkeys sampled twice, that is, at postnatal days 14 and 30. Genome-wide DNA methylation analysis was performed using a methylated DNA immunoprecipitation (MeDIP) protocol previously described (Nieratschker et al., 2014). Briefly, 2 µg of genomic DNA per sample were sonicated using a Bioruptor (Diagenode, Denville, NJ, USA), and methylated DNA was immunoprecipitated using an anti-5-methyl-cytosine antibody (Eurogentec, Fremont, CA). The DNA–antibody complex was precipitated with protein G, and the methylated DNA was resuspended in digestion buffer (50 mM Tris-HCl pH 8; 10 mM EDTA; 0.5% SDS) and treated with proteinase K overnight at 55°C. The input and bound fraction were purified, amplified using the Whole Genome Amplification kit (Sigma-Aldrich, St. Louis, MO), and labeled for microarray hybridization with Cy3-dUTP and Cy5-dUTP, respectively, using the CGH Enzymatic Labeling kit (Agilent Technologies, Mississauga, ON, Canada) in accordance with the manufacturer's instructions. Custom-designed tiling arrays were used (Agilent Technologies). All steps of the hybridization and washing were performed in accordance with the Agilent Technologies protocol for chip-on-chip analysis.

MeDIP Microarray Design, Scanning, and Analysis

For MeDIP enrichment, custom 400K promoter tiling arrays were designed for this study (Agilent Technologies). Microarray probe sequences were selected to tile at 100 bp, spacing all gene promoter regions defined as the genomic interval from –1800 bp upstream to 400 bp downstream of each transcription start site as defined for the *M. mulatta* genome by the Ensembl database (version 64.10; <http://www.ensembl.org>). All the steps of hybridization, washing, scanning, and feature extraction were performed following the Agilent protocols for chip-on-chip analysis (MeDIP). After microarray scanning, probe intensities were extracted from scan images using Agilent's Feature Extraction 10.5 Image Analysis Software (Agilent Technologies, Santa Clara, CA, USA). The extracted intensities were then analyzed using the R software environment (www.r-project.org) for statistical computing. Log ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray and then microarrays were normalized to one another using quantile normalization under the assumption that all samples have identical overall methylation levels. Linear models implemented in the “limma” package of Bioconductor were used to compute a modified *t* statistic from the normalized intensities of the probes across all samples between the two groups. For each gene promoter, we calculated the significance of enrichment for high- or low-probe *t* statistics of all probes within the promoter. For the analysis of the placenta samples, a probe was called differentially methylated if the significance of its *t* statistic was at most $p < .05$ (uncorrected for multiple tests), the associated difference of means between the groups was at least .5 and the enrichment *p* value of the promoter containing the probe was at most .001 (uncorrected for multiple tests). The false discovery rate (FDR) of these thresholds was calculated using permutation tests. In each permutation, two low and two mid-high or high ranking were

randomly selected to form one group and the remainder a second group. The number of differentially methylated probes was then calculated using the thresholds given above. Twenty of the 90 possible permutations were tested and each on average identified 5,260 differentially methylated probes. Analysis of the original groups, low compared to mid-high and high, identified 25,947 differentially methylated probes. Making the conservative that all 5,260 differences in the permutation tests are false positives, we therefore expect that at most 5,260 or ~20% of the 25,947 differentially methylated are false positives. In other words, the FDR among the 25,947 differences is at most 20%.

We compared these methylation differences in placentae to methylation differences in CD³⁺ cells of male and female monkeys. To analyze methylation differences in CD³⁺ cells of female monkeys (3 MR vs. 3 NR), we could not apply the permutation method. We therefore calculated the FDR as previously published for the male (6 MR vs. 5 NR) CD³⁺ cells (Nieratschker et al., 2014). An individual probe was called differentially methylated if the significance of its *t* statistic was at most .01 (uncorrected for multiple testing), and the associated difference of means between the groups was at least .5. Correlation between technical replicates was modeled as a random effect using the “block” variable. Significance was determined using the Wilcoxon rank-sum test comparing *t* statistics of these probes against those of all the probes on the microarray. The resulting *p* values for each gene were then corrected for multiple testing by calculating their FDR.

For biological function analyses, selected genes were overlaid on the global molecular network developed from information contained in the Ingenuity Pathway knowledge base (www.ingenuity.com). The significance of the association between the data sets and biological functions or canonical pathway is scored using a *p*-value calculation (right-tailed Fisher’s exact test).

Validation

Gene-specific real-time PCR validations of MeDIP microarrays were performed on the amplified input and bound fractions. Relative enrichment was determined after normalizing from the input fraction in each sample. SYBR green quantitative PCR (qPCR) was performed using the LightCycler[®] 480 system (Software 3.5; Roche Molecular Biochemicals, Pleasanton, CA, USA). To determine the relative DNA enrichment, the 2^{−Ct} method was used. The Student’s unpaired *t* test was used (one-tailed) and the alpha level was set at .05. When variances were significantly different between groups, the Welch’s correction was applied. Graph-Pad 5 software (La Jolla, CA) was used to perform statistical analysis.

Identification of Interaction Pathways (Cytoscape Environment)

Lists of differentially methylated genes were visualized in biological pathways using Cytoscape, an open source software. Cytoscape plugin NetworkAnalyzer (Assenov, Ramirez, Schelhorn, Lengauer, & Albrecht, 2008) was used to calculate the number of interactions (degree) of each protein corresponding to a differentially methylated gene. High degree often indicates a central role in a key cellular function such as signal amplification (kinases), signal turnover (small GTPases), or gene expression (transcription factors). Of the

8,294 genes differentially methylated at p (promoter level) $< .05$, 4,599 were successfully mapped onto the human BioGrid protein–protein interaction network (Breitkreutz, Stark, & Tyers, 2003). As expected, given the scale-free nature of interaction networks, the majority of differentially methylated genes (53%) had degree at most 2 while a much smaller number (985 or 21%) had degree at least 10 (Barabasi & Oltvai, 2004). Genes with degrees of at least 10 were selected for enrichment analyses.

Results

Association Between Mother's Dominance Ranks and Placenta DNA Methylation Patterns

The association between the genome-wide DNA methylation profiles of rhesus monkey placenta at birth and the dominance rank of the mothers was examined using DNA immunoprecipitation for 5-methylcytosine followed by hybridization on microarrays tiling rhesus gene promoters. Using the extremely conservative Bonferroni adjustment for multiple testing, three probes were found differentially methylated between the placentae of low and those of mid-high and high social rank ($p < .05$). Due to our small sample size, such a conservative adjustment is likely to produce many false negatives so we also considered a combination of less conservative nominal p -value thresholds: one for a promoter-level test ($p < .001$) and another for a probe-level test ($p < .05$; see Method). With these thresholds, 25,947 probes were identified as differentially methylated (Figure 1A and Table S2). Permutation tests show that the resulting FDR is at most 20% (see Method). Our initial comparison combined mid-high and high social rank monkeys together because we hypothesized that low-ranking monkeys would be the most different from the two high-ranking groups as low-ranking monkeys never win in interactions with higher ranking monkeys. Consistent with this hypothesis, we identified fewer methylation differences between the highest ranking monkeys and the combined group of low- and mid-high-ranking monkeys (5,574 differences). Further comparisons between group pairs provided additional confirmation. Although we found 13,879 and 23,444 differences between low- and high-ranking and between low- and mid-high-ranking monkeys, respectively, only 3,421 differences were found between mid-high and high-ranking monkeys. Table S3 shows the differentially methylated probes identified in each comparison and the fraction of overlapping probes that changed methylation in the same direction between the different comparisons.

These results suggest that methylation differences are particularly pronounced between low social status and mid-high and high social status. Therefore, further analysis is restricted to these differences. These probes were associated with 4,539 genes with equal distribution of hyper- and hypomethylated probes: 12,005 had higher and 13,942 had lower methylation levels in placentae of low-status mothers. Specifically in the groups of monkeys analyzed in this study, high-ranking mothers had significantly longer gestations than low-ranking mothers ($p = .016$). We therefore performed an additional sensitivity analysis by including gestational length as a covariate in our linear model. Adjusting for gestational length reduced the number of probes associated with rank: 2,332 and 1,581 probes had higher and lower methylation levels in low-ranking monkeys, respectively. This sensitivity analysis with gestational length included provides a “lower bound” on the set of associations and the

analysis without gestational length provides an “upper bound” on the set of associations because some may be due only to gestational length. Because gestational length and social rank are confounded and because it is impossible at this stage to conclude that differences in gestational length are not directly due to differences in rank, we decided to pursue the analysis with the set of differences identified without adjusting for gestational length. We confirmed by qPCR the methylation differences in both a random selection of probes and a selection in genes known to be affected by stress or the social environment such as *MORC1* (Nieratschker et al., 2014), *MAN2C1* (Uddin et al., 2011), and *SLC6A4* (Murphy et al., 2008; Figure 1B). Overall, 60% of these differences were confirmed by qPCR analysis. We observed that *TIMP3* is less methylated in placentae of mothers of low social status. Interestingly, *TIMP3* hypomethylation in human placenta has been suggested as a biomarker of preeclampsia (Yuen, Penaherrera, von Dadelszen, McFadden, & Robinson, 2010), which is more prevalent in populations of low social status or affected by high psychosocial stress (Schneider et al., 2011). This result strengthens the hypothesis that our analysis might provide information on human placenta biology associated with social status.

The vast difference in DNA methylation between the social rank groups suggested that large gene networks involving multiple physiological systems are epigenetically reprogrammed. To gain insight into their biological functions, we first performed a gene set enrichment analysis using the ingenuity pathway analysis (IPA) software. The analysis revealed strong enrichments in genes related to a broad range of biological functions such as cell morphology ($p = 9.53E-34$), cellular growth and proliferation ($p = 1.27E-24$), cell death ($p = 5.15E-17$), and transport of molecules ($p = 8.17E-16$). In addition, fundamental signaling molecules such as adenylate cyclases, c-AMP responsive elements, protein kinases, phosphodiesterases, and phospholipases were particularly represented in the canonical pathways that most significantly associated with the differentially methylated genes between social ranks (Table 1). Other notable molecules in these signaling pathways include *IGF-1*, *IGF-R*, *PDGFA*, *EGFR*, *VEGFC*, and *NF- κ B*, which play major roles in placenta physiology such as modulation of growth, invasion, angiogenesis, as well as uptake and utilization of nutrients (Fowden & Forhead, 2009).

DNA methylation at gene promoters is believed to silence transcription (Tate & Bird, 1993). To predict whether certain pathways were activated or silenced in different social ranks, we first made the assumption that hypomethylated promoters reflected gene-activating events and hypermethylation reflected gene-silencing events. IPA analysis based on these assumptions predicted overall activation or suppression of only a few canonical pathways in placentae of mothers of low status compared to placentae of higher ranking mothers (Table 1). Most canonical pathways are enriched in genes that are both hypo- and hypermethylated. For instance, the “Hepatic Fibrosis and Hepatic Stellate Cell Activation” pathway has 27 and 31 gene promoters hypo- and hypermethylated, respectively, in placentae of mothers of low status compared to higher status, whereas the “Synaptic Long-Term Depression” pathway, which has the strongest z score predicting hyperactivation, has 11 and 25 hyper- and hypomethylated genes, respectively. This suggests complex regulation of canonical pathways by DNA methylation in placentae of mothers of different social status.

It is possible that differences in methylation modify the response of genes to specific transcription factors or that differences in methylation reflect previous perturbation of master regulators leading to differences in methylation levels. IPA identified transcription factors and hormones such as beta-estradiol, dexamethasone, growth, and nuclear factors (e.g., *TGFB1*, *TP53*, *TNF*, *HNF4A*, and *PPARG*), with surprisingly large numbers of differentially methylated targets (Table 2). For instance, *TGFB1* potentially targeted 196 hypermethylated and 153 hypomethylated genes in placenta of mothers of low rank and beta-estradiol potentially regulated 186 hypermethylated and 147 hypomethylated genes. However, only a few of the upstream regulators identified in our analysis had their activities predicted as inhibited or activated in placenta of group status relative to one another (Table 2). This suggests that differences in DNA methylation result in complex modulations of regulatory activities and not in “simple” up- or downregulation. Nevertheless, because these upstream regulators have been previously linked to stress response, sexual maturation, placenta development, invasion, angiogenesis, and metabolism (Fowden & Forhead, 2009; Hauguel-de Mouzon & Guerre-Millo, 2006; Pepe & Albrecht, 1995), we might expect a strong impact of DNA methylation differences related to dominance rank on placenta physiology.

In order to further interrogate the biological significance of the landscape of differential methylation, we mapped differentially methylated genes onto protein–protein interaction networks and calculated the degree, or number of network interactions, of each. As expected, differentially methylated genes with high degree (at least 10), also called “hubs,” are not only enriched in many of the same pathways as the full set of differentially methylated genes (Tables 1 and 2), but they also play central roles in placenta physiology such as regulation of implantation, invasion, metabolism, and angiogenesis (Fowden & Forhead, 2009; Hauguel-de Mouzon & Guerre-Millo, 2006; Pepe & Albrecht, 1995; Staun-Ram & Shalev, 2005). Some of these hubs include well-known signaling molecules such as NF- κ B, beta-estradiol, the glucocorticoid receptor, TP53, and TNF. There were also strong enrichments in genes encoding proteins involved in translation such as initiation factors and ribosomal proteins. All together, these results indicate that social rank is associated with differential methylation levels at genes playing both central and modulatory roles in cellular pathways required for placenta physiology.

Overlap Between Genes Differentially Methylated in Placentae and Genes Differentially Expressed in Mononuclear Cells in Relation to Dominance Ranks

Previously, Tung et al. (2012) reported that dominance rank is correlated with transcriptional and DNA methylation changes in PBMC of adult female macaques. Of the 1986 genes identified by Tung et al. as differentially methylated by dominance rank ($FDR < .2$), we found that 489 (at $p < .01$; $p = 5.1E-3$, hypergeometric test) and 617 (at $p < .05$; $p = 2.18E-4$, hypergeometric test) are also differentially methylated in our study. The 617 overlapping genes are enriched in pathways and upstream regulators similar to those for the set of all differentially methylated placenta genes, including regulators HNF4A, TP53, TGFB1, beta-estradiol, as well as the EGF, PDGF, glucocorticoid receptor (NR3C1), and the NF- κ B signaling (Tables 1 and 2). Some of these upstream regulators are differentially expressed in PBMC (e.g., *TP53* and *TGFB1*; Tung et al., 2012). These observations are consistent with

the hypotheses that long-term adaptations to social rank involve system-wide epigenetic changes of overlapping genes in multiple tissues at different ages and that common cellular pathways are sensitive to parental social rank at birth and the de facto social rank during adulthood.

Overlap Between Genes Differentially Methylated in Relation to Dominance Ranks and to Early-Life Stress

Our observation of enrichment for *NR3C1* targets among all differentially methylated genes in placenta (Table 2) aligns well with the observation by Tung et al. (2012) that the rank–gene expression relation was mostly explained by dexamethasone suppression. Indeed, low-ranking compared to high-ranking female monkeys exhibit diminished glucocorticoid negative feedback following administration of dexamethasone (Michopoulos et al., 2012; Tung et al., 2012) and heightened reactivity to stress during lactation (Hoffman et al., 2010). As the dysregulated HPA axis activity in low-ranking monkeys is likely due to chronic stress exposure, we hypothesized that part of DNA methylation differences in placenta of mothers of different rankings might also be related to stress. We therefore compared our list of genes associated with social status in placenta to genes associated with early-life stress in humans and rhesus monkeys (Nieratschker et al., 2014). More specifically, methylation differences were observed in human CD³⁴⁺ hematopoietic stem cells collected from the umbilical cord of newborns from mothers subjected to various levels of stress during the third trimester of pregnancy (Nieratschker et al., 2014). In addition, methylation differences were characterized in T cells derived from venous blood of male (Nieratschker et al., 2014) or female newborn (14–30 days old) monkeys, either mother- or nursery reared. We discovered a significant overlap (671 of 3405 genes, $p = 7.25\text{E-}15$, hypergeometric test) with genes associated with prenatal stress in humans (Nieratschker et al., 2014). IPA analysis of the 671 common genes revealed significant enrichments in canonical pathways (e.g., “G-protein receptor signaling,” $p = 9.3\text{E-}6$; “cAMP-mediated signaling,” $p = 7.37\text{E-}5$), upstream regulators (e.g., TGFB1, $p = 5.36\text{E-}7$; dexamethasone, $p = 8.1\text{E-}7$), and cellular functions (e.g., “cell morphology,” $p = 1.45\text{E-}11$; “molecular transport,” $p = 6.23\text{E-}7$), gene sets also enriched in our set of genes associated with social rank. Similarly, we found significant overlaps with genes associated with maternal deprivation in T cells of male monkeys (608 genes of the 1,751 significant in T cells at FDR < .01; $p = 5.04\text{E-}99$, hypergeometric test) and in T cells of female monkeys (1,023 genes of the 3,065 significant in T cells at FDR < .01; $p = 3.94\text{E-}160$, hypergeometric test). Furthermore, these overlaps included 437 genes in common. IPA analysis on these 437 genes revealed enrichments in canonical pathways (e.g., “cAMP-mediated signaling,” $p = 6.43\text{E-}3$), upstream regulators (e.g., TGFB1, $p = 6.59\text{E-}3$), and cellular functions (e.g., “cell morphology,” $p = 2.67\text{E-}4$), gene sets also enriched in our set of genes associated with social rank. These surprisingly large overlaps suggest that part of the epigenetic reprogramming related to social status might also be related to an epigenetic response to social stress.

“Prediction” of the Mother’s Status by Fetal DNA Methylation

Finally, we evaluated the possibility of predicting maternal dominance by applying a likelihood cross-validated penalized linear regression method using a LASSO penalty (Tibshirani, 1996) implemented in the R package “penalized” (Goeman, 2010). Penalized

regression was applied to average methylation levels across all promoters with at least one differentially methylated probe with the promoter p -value threshold relaxed to $p < .05$. Maternal social status was divided into three levels: low, intermediate, and high. The resulting predictor needed only two genes, *KAAGI* (kidney-associated antigen 1) and *MUSK* (muscle, skeletal, receptor tyrosine kinase) to predict maternal social status with a goodness of fit of .975 (Figure 2). Interestingly, the level of methylation of these two genes correlated across the monkeys in the different groups ($R^2 = .88$).

Discussion

The relation between social status and physical and mental health status has been recognized for some time, but most of the evidence has been epidemiological. The mechanism through which a social rank is programmed in the genome has remained a mystery. The discovery of both the overall stability but developmental plasticity of “epigenetic” mechanisms is consistent with a role for DNA methylation. A few studies documented DNA methylation patterns that differed between humans at high and low socioeconomic positions in blood cells and differences in gene expression between female nonhuman primate rhesus macaques at different social ranks (Tung et al., 2012). However, because these differences were shown in adults there are two critical questions. First, are these changes in DNA methylation indirect consequences of social rank that emerge late in life in response to differences in lifestyle and life history between the social ranks? Second, is there a biological mechanism for prenatal transmission of social rank that precedes social engagement of the individual offspring? Examining DNA methylation states early in life is essential for addressing these critical questions.

In this study, we reveal a dramatic DNA methylation difference in placentae of rhesus monkeys associated with the social ranking of the mothers. The changes in methylation are highly organized and are enriched in several networks that are critical in development and organogenesis such as cell morphology, cell differentiation, and cell death as well as cell signaling. A measure of the functional significance of the genes that are differentially methylated is the fact that many of the differentially methylated genes have high degrees of protein–protein interactions indicating central positions in gene network hubs. The broad scope of these differences covering several critical gene networks as well as the fact that it affects a tissue that is vital for the nurturing and development of the fetus illustrates the profound biological effect that maternal social rank might have on the next generation. It also provides a potential mechanistic link between the social rank of a mother and the health of the offspring.

Examining the upstream regulators of differentially methylated genes provides testable hypotheses on the mechanisms that link maternal social rank with epigenetic modifications. The fact that the differentially methylated genes associated with social rank in placenta are enriched for glucocorticoid receptor target genes is consistent with a model in which variations in maternal glucocorticoids concentration or dynamics between mothers of distinctive social ranks caused differences in methylation of genes that interact with the glucocorticoid receptor. In agreement, low social rank has been previously associated with stress, differences in HPA axis reactivity of low-ranking as compared to high-ranking

monkeys have been reported (Hoffman et al., 2010; Michopoulos et al., 2012), and Tung et al. (2012) found that the rank–gene expression relation was mostly explained by dexamethasone suppression data. Moreover, binding of activated glucocorticoid receptors to target genes has been known to trigger chromatin and DNA methylation changes in its targets (Thomassin, Flavin, Espinas, & Grange, 2001). More recently, it has been proposed that increased glucocorticoid release targets demethylation of an enhancer in the *FKBP5* gene in people who suffered from early-life adversity and glucocorticoids trigger demethylation of these sites in undifferentiated neuronal precursors in culture (Klengel et al., 2013). Finally, the substantial overlaps between genes differentially methylated in placenta in association with social rank and in human cord blood CD³⁴⁺ cells and newborn rhesus monkey T cells in association with early-life stress (Nieratschker et al., 2014), as well as the common pathways and upstream regulators associated with these genes, strengthen the hypothesis that part of the epigenetic differences associated with social status is an adaptation to various levels of stress.

Stress hormones could provide an explanation of how environmental appraisal by the brain is translated into systemic DNA methylation changes. Consistent with this hypothesis, we have previously shown that exposure of guinea pig (*Cavia porcellus*) fetuses to dexamethasone in utero results in broad changes in DNA methylation in the hippocampus (Crudo et al., 2013). Particularly interesting are genes that are targets of inflammatory signaling pathways that might be activated in the mother in association with social rank. As these pathways such as NF- κ B signaling are present in several tissues, they could also direct DNA methylation modifications to their targets genes across several tissues.

To our surprise, the unbiased approach that we have taken here revealed two genes that are “predictors” of social rank that are not candidates from the “usual suspect” list. MUSK is muscle, skeletal, receptor tyrosine kinase that is involved in neuromuscular junction, whereas KAAG1 is a kidney-associated antigen with unknown function. The fact that surprisingly these genes and not known candidate genes were found to be predictors illustrated the power of unbiased analyses to discover new, unexpected candidate genes involved in social dominance and potentially new yet unknown physiological processes. Future experiments are required to dissect the biological role of these genes.

One limitation of our study is that we did not have sufficient power to compare male and female placentae for each rank. Given previous evidence from both animal and human studies on sex specificity of the relation between social status and health, we anticipate that there will be a sex-specific response in the placenta, although it is possible that these sex effects would emerge later in life and would not be detected at birth. Future experiments with a larger number of animals are required to address this question.

An important limitation of our study is that we have no information on the genome-wide genetic variance of these monkeys that would allow us to exclude gene variance effects on some of the DNA methylation changes detected here. It is possible that the DNA methylation changes detected in our study are partly driven by genetic variance. An additional limitation that needs to be considered is that processing time of the placenta tissues and cell composition differences between samples have been shown to affect

methylation levels in the human term placenta (Avila et al., 2010). It is possible that some of the DNA methylation differences might be explained by cell composition differences.

The difference in gestational age between low and high social rank may be an inherent limitation of our study because it appears that low-social rank offspring tend to have younger gestational age. The gestational age offsets (actual – expected date of birth) of our high/mid-social rank offspring are 0, 1, 13, 15, and 16, whereas for the low-social rank offspring, they are –1, –1, –4, and –4. Thus, gestational age is perfectly confounded with social rank, so there is no way to remove gestational age effects without simultaneously removing social rank effects. At least some of the effects we identify are likely gestational age effects because human studies have identified gestational age effects in cord blood and placenta DNA methylation (e.g., Simpkin et al., 2015). However, it is unlikely that all are due to gestational age differences because many are linked to genes differentially expressed by social rank in adult rhesus monkeys (Tung et al., 2012), and gestational age effects do not appear in human peripheral blood collected during childhood both before and after adolescence (e.g., Simpkin et al., 2015).

It is possible that the DNA methylation changes triggered by maternal social rank persist into adulthood across several tissues and are not limited to the placenta. We took advantage of previously published gene expression and DNA methylation profiling of social rank in adult rhesus peripheral white blood cells (Tung et al., 2012). We found a surprisingly large overlap between the genes that were differentially methylated in placenta of offspring of high- and low-social rank mothers and genes differentially methylated in adult females of high and low social rank. Interestingly, glucocorticoid as well as inflammatory signaling responsive genes were identified as common upstream regulators. This is consistent with the hypothesis presented here that differences in systemic availability of stress hormones and inflammatory mediators in response to maternal social rank trigger systemwide changes in DNA methylation and programming of their target genes in the offspring and that these alterations persist into adulthood. Alternatively, the changes in DNA methylation are maintained in the adult not as a memory of maternal social rank but in response to persistent differences in stress, inflammatory, and other mediators across social ranks. However, the significant overlap between the placenta and adult white blood cells social rank DNA methylation and expression profiles strongly supports the idea of cross-generational cross-tissue transmission of the response to social rank in the methylome. The correspondence with adult gene expression program suggests that DNA methylation profile might be involved in long-term functional consequences. Although future experiments are required for understanding the details of the molecular link between maternal social rank and DNA methylation in the offspring, our data provide important support for the hypothesis that parental social status has a profound impact on the programming of the epigenome in their offspring, providing a biological mechanism for the long-term multigenerational implications of social rank.

Our data suggest that social rank of the mother is biologically registered in the epigenome of her offspring before birth, before the child forms social bonds, and is exposed to social structures and positions. It stands to reason based on the similarity of the overall impact of social positioning on human and nonhuman primates that this effect is true for humans as

well. This has important implications to our understanding of the impact of social equality and well-being of pregnant mothers on the future generation and the need for appropriate interventions at this critical time point in life to alleviate the long-ranging consequences of social distress during pregnancy. DNA methylation markers of social distress in placenta might serve as objective markers of social distress during pregnancy and help focus interventions on the high-risk group of children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The DNA methylation landscape of maternal social rank in placenta DNA. (A) Heatmap (row distance metric: Pearson correlation, average linkage) depicting the clustering of the normalized intensities of the 26,027 microarray probes that were differentially methylated ($q < .01$) between placentae of fetuses of mothers of low and high/mid-high social status. Rows correspond to probes and columns to animals. Red indicates higher methylation in a row and blue indicates lower methylation. (B) Quantitative PCR validations of MeDIP arrays. Relative DNA methylation enrichment levels at differentially methylated probes ($M \pm SEM$) of fetuses of mothers of low social status (black bars) and high/mid-high social status (white bars). Unpaired t test were performed, # $p < 0.1$, * $p < .05$. ** $p < .01$. **** $p < .0001$. Welch's correction was applied when variances between groups were significantly different.

Figure 2.

Prediction of monkey social rank. (A) Social rank was predicted based on a likelihood cross-validated penalized linear regression analysis using averaged methylation levels of differentially methylated probes ($q < .2$) for each promoter. The linear regression of the prediction is shown on the left. The assigned values (1, 2, or 3) of the social status determined by animal care staff for the prediction are indicated on the x -axis. A one-way analysis of variance of the predicted values for each animal, according to social status determined by animal care staff, has been performed ($p < .0001$). Tukey's multiple comparison tests show that the predicted numbers are significantly different between groups (bar graph on the right; *** $p < .001$). (B) Relative DNA methylation enrichment levels of differentially methylated probes ($q < .2$) at KAAG1 (kidney-associated antigen 1) and

MUSK (muscle, skeletal, receptor tyrosine kinase) promoters, for each sample, according to social status determined by animal care staff. On the left, linear regression analyses of the methylation levels are shown. One-way analyses of variances of the methylation levels, according to the social status determined by animal care staff, have been performed ($p < .0001$ for both genes). Tukey's multiple comparison tests show that the methylation levels are significantly different between groups (** $p < .01$. *** $p < .001$).

Table 1

Top 40 Canonical Pathways Associated With Genes Differentially Methylated in Placenta Between Monkeys of Low and High Status ($p < .001$ or With a Degree of at Least 10) as Well as Associated With Genes for Which Methylation Levels in Placenta and mRNA Levels in Peripheral Blood Mononuclear Cell Are Associated With Social Status

$p < .001$				Degree 10		Overlapping genes		
	Pathways	p value	z score	Pathways	p value	z score	Pathways	p value
1	Hepatic fibrosis/Hepatic stellate cell activation	8.51E-09	NaN	Protein ubiquitination pathway	3.98E-24	NaN	Role of NFAT in regulation of the immune response	2.51E-05
2	Axonal guidance signaling	2.75E-07	NaN	Molecular mechanisms of cancer	2.00E-21	NaN	Glucocorticoid receptor signaling	2.75E-05
3	Epithelial adherens junction signaling	3.80E-06	NaN	Glucocorticoid receptor signaling	5.01E-20	NaN	Small cell lung cancer signaling	2.82E-05
4	Clathrin-mediated endocytosis signaling	5.62E-05	NaN	Clathrin-mediated endocytosis signaling	3.98E-19	NaN	TNFR1 signaling	4.47E-05
5	G-protein-coupled receptor signaling	9.55E-05	NaN	Huntington's disease signaling	1.58E-17	NaN	IL-8 signaling	5.25E-05
6	Tight junction signaling	1.00E-04	NaN	Small cell lung cancer signaling	5.01E-17	NaN	IL-6 signaling	7.08E-05
7	Adipogenesis pathway	1.00E-04	NaN	ErbB signaling	3.16E-15	NaN	AMPK signaling	8.71E-05
8	cAMP-mediated signaling	1.05E-04	0.289	Germ cell-sertoli cell junction signaling	6.31E-15	NaN	PTEN signaling	9.77E-05
9	Leukocyte extravasation signaling	1.26E-04	0	Pancreatic adenocarcinoma signaling	2.00E-14	-1.279	CTLA4 signaling in cytotoxic T lymphocytes	1.29E-04
10	Aggrin interactions at neuromuscular junction	1.45E-04	-0.894	Role of BRCA1 in DNA damage response	2.51E-14	-0.277	Production of nitric oxide and reactive oxygen species in macrophages	1.62E-04
11	Polyamine regulation in colon cancer	2.00E-04	NaN	B-cell receptor signaling	3.16E-14	NaN	Death receptor signaling	1.86E-04
12	Role of cytokines in mediating communication between immune cells	3.39E-04	NaN	ATM signaling	3.98E-14	1.147	Regulation of eIF4 and p70S6K signaling	2.19E-04
13	Amyotrophic lateral sclerosis signaling	3.89E-04	NaN	NF-κB signaling	3.98E-14	-1.915	iCOS-iCOSL signaling in T helper cells	2.40E-04
14	Actin cytoskeleton signaling	5.37E-04	NaN	Hereditary breast cancer signaling	7.94E-14	NaN	NF-κB signaling	2.57E-04
15	Myc-mediated apoptosis signaling	5.62E-04	NaN	Cell cycle: G2/M DNA damage checkpoint regulation	1.00E-13	1.213	GM-CSF signaling	2.75E-04
16	Protein kinase A signaling	6.46E-04	NaN	p53 signaling	1.00E-13	0.688	Acute myeloid leukemia signaling	3.55E-04
17	Sertoli Cell-Sertoli Cell junction signaling	6.61E-04	NaN	HER-2 signaling in breast cancer	1.26E-13	NaN	Ceramide signaling	4.27E-04
18	Bladder cancer signaling	7.76E-04	NaN	Chronic myeloid leukemia signaling	2.00E-13	NaN	T-cell receptor signaling	4.27E-04
19	Role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis	8.32E-04	NaN	PTEN signaling	3.98E-13	1.177	Angiopoietin signaling	4.68E-04
20	Differential regulation of cytokine production in intestinal epithelial cells by IL-17A and IL-17F	1.00E-03	NaN	IGF-1 signaling	1.00E-12	-1.279	Germ Cell-Sertoli Cell junction signaling	4.90E-04
21	Circadian rhythm signaling	1.10E-03	NaN	Glioma signaling	1.26E-12	-1.528	HIF1a signaling	5.01E-04

	<i>p</i> < .001	Pathways			Degree 10			Overlapping genes		
		<i>p</i> value	<i>z</i> score	Pathways	<i>p</i> value	<i>z</i> score	Pathways	<i>p</i> value	<i>z</i> score	Pathways
22	UVA-induced MAPK signaling	1.10E-03	-1.043	Paxillin signaling	1.58E-12	-2.041	Inhibition of angiogenesis by TSP1	5.75E-04		
23	IL-12 signaling and production in macrophages	1.17E-03	NaN	Estrogen receptor signaling	1.58E-12	NaN	Clathrin-mediated endocytosis signaling	6.03E-04		
24	Molecular mechanisms of cancer	1.29E-03	NaN	Non-small cell lung cancer signaling	2.51E-12	-1.604	ILK signaling	6.03E-04		
25	Atherosclerosis signaling	1.38E-03	NaN	Cell cycle: G1/S checkpoint regulation	2.51E-12	-2	RANK signaling in osteoclasts	6.61E-04		
26	Aryl hydrocarbon receptor signaling	1.51E-03	0	Myc-mediated apoptosis signaling	3.16E-12	NaN	Apoptosis signaling	6.61E-04		
27	Oxidative ethanol degradation III	1.55E-03	NaN	Integrin signaling	3.98E-12	-3.182	Pancreatic adenocarcinoma signaling	6.76E-04		
28	T helper cell differentiation	1.58E-03	NaN	FAK signaling	6.31E-12	NaN	IL-17 signaling	6.92E-04		
29	HIPPO signaling	1.82E-03	1.807	Leukocyte extravasation signaling	6.31E-12	-0.354	JAK/Stat signaling	6.92E-04		
30	Remodeling of epithelial adherens junctions	1.82E-03	0	RAR activation	1.00E-11	NaN	PI3K/AKT signaling	7.08E-04		
31	Mechanisms of viral exit from host cells	1.86E-03	NaN	Role of CHK proteins in cell cycle checkpoint control	1.26E-11	0.535	FAK signaling	7.08E-04		
32	Pancreatic adenocarcinoma signaling	2.19E-03	-0.626	T-cell receptor signaling	1.58E-11	NaN	Wnt/B-catenin signaling	7.24E-04		
33	LPS/IL-1-mediated inhibition of RXR Function	2.24E-03	0.471	Aggrin interactions at neuromuscular junction	1.58E-11	-2.357	Lymphotoxin B receptor signaling	7.41E-04		
34	Synaptic long-term depression	2.82E-03	2.333	Regulation of eIF4 and p70S6K signaling	2.00E-11	-1.387	EGF signaling	1.12E-03		
35	FAK signaling	2.95E-03	NaN	CD28 signaling in T helper cells	2.51E-11	-0.408	CXCR4 signaling	1.35E-03		
36	Gap junction signaling	3.16E-03	NaN	Adipogenesis pathway	6.31E-11	NaN	PDGF signaling	1.78E-03		
37	Germ Cell-Sertoli Cell junction signaling	3.31E-03	NaN	PI3K/AKT signaling	6.31E-11	-0.408	CD40 signaling	1.78E-03		
38	Cellular effects of sildenafil (viagra)	3.31E-03	NaN	Glioblastoma multiforme signaling	1.26E-10	-0.962	CD28 signaling in T helper cells	1.82E-03		
39	Agranulocyte adhesion and diapedesis	3.39E-03	NaN	Cyclins and cell cycle regulation	1.35E-10	1.604	Tight junction signaling	1.95E-03		
40	Paxillin signaling	3.47E-03	-1.279	Virus entry via endocytic pathways	1.41E-10	NaN	IL-17A signaling in airway cells	1.95E-03		

Table 2

Top 40 Potential Upstream Regulators of Genes Differentially Methylated in Placenta Between Monkeys of Low and High Status ($p < .001$ or With a Degree of at Least 10) as Well as Top Potential Regulators of Genes for Which Methylation Levels in Placenta and mRNA Levels in Peripheral Blood Mononuclear Cell Are Associated With Social Status

p < .001				Degree 10		Overlapping genes	
Regulators		p value	z score	Regulators		p value	p value
1	TGFB1	1.76E-18	ns	TP53		6.97E-57	HNF4A
2	TNF	2.13E-15	ns	Beta-estradiol		2.96E-38	TP53
3	Tretinoin	3.90E-15	ns	MYC		3.90E-33	ESR1
4	Beta-estradiol	3.80E-14	ns	ESR1		1.16E-32	ERG
5	TP53	6.92E-14	ns	TGFB1		1.12E-31	Camptothecin
6	Dexamethasone	6.54E-13	ns	E2F1		8.48E-29	FSH
7	INHA	3.87E-12	ns	Tretinoin		9.19E-29	MYC
8	Lipopolysaccharide	4.05E-12	ns	Camptothecin		5.71E-27	NR3C1
9	CTNNB1	7.16E-12	ns	HNF4A		3.68E-26	TCOF1
10	ESR1	1.31E-11	ns	Benzoyloxycarbonyl-Leu-Leu aldehyde		6.97E-26	IL2
11	SRF	1.46E-11	ns	Doxorubicin		7.37E-25	Dexamethasone
12	PD98059	1.48E-11	ns	APP		1.59E-24	TGFB1
13	Ethanol	1.81E-11	ns	CD3		4.97E-22	FN1
14	YY1	4.47E-11	ns	NR3C1		2.80E-21	Beta-estradiol
15	Forskolin	7.66E-11	ns	FTEN		4.18E-19	Let-7a-5p (and other miRNAs w/ seed GAGGUAG)
16	Decitabine	1.08E-10	ns	Methylselenic acid		6.62E-19	LMNA
17	IGF1	1.91E-10	ns	STAT5A		2.60E-18	ETV4
18	Calcitriol	5.02E-10	ns	TP63		2.78E-18	IL5
19	Wortmannin	5.55E-10	ns	TNF		4.13E-18	Tanespimycin
20	NR3C1	2.05E-09	ns	E2F4		5.46E-18	IL6
21	HNF4A	3.04E-09	ns	EGF		9.72E-18	NFYB
22	APP	5.99E-09	ns	Dihydrotestosterone		3.97E-17	Collagen type IV
23	IL1	6.91E-09	ns	5-Fluorouracil		5.45E-17	CXCL12
24	FGF2	7.52E-09	ns	HGF		7.85E-17	RBFOX2
25	Tetrachlorodibenzo-dioxin	1.00E-08	ns	Cisplatin		1.20E-16	KRAS

	$p < .001$			Degree 10		Overlapping genes	
	Regulators	p value	z score	Regulators	p value	Regulators	p value
26	IFNG	1.07E-08	ns	HRAS	1.37E-16	FAS	1.56E-04
27	Cisplatin	1.66E-08	ns	FAS	1.38E-16	NUPR1	1.96E-04
28	BDNF	1.78E-08	ns	Butyric acid	1.77E-16	Prednisolone	2.14E-04
29	OSM	1.79E-08	ns	RB1	4.53E-16	miR-122-5p (miRNAs w/ seed GGAGUGU)	2.32E-04
30	ACT	1.84E-08	ns	Tanespimycin	5.08E-16	JUN	2.72E-04
31	CREBBP	2.11E-08	ns	Sirolimus	5.83E-16	HGF	2.79E-04
32	LY294002	3.56E-08	ns	HSF1	6.45E-16	PDGFB	2.96E-04
33	IKZF1	3.91E-08	ns	CD 437	2.10E-15	Lonafarnib	3.16E-04
34	Morphine	5.02E-08	Inhibited	HTT	2.92E-15	Gambogic acid	3.16E-04
35	AKT	5.35E-08	ns	BRCA1	3.14E-15	<i>Salmonella enterica</i> serotype abortus equi lipopolysaccharide	3.61E-04
36	D-glucose	5.77E-08	ns	IGF1	3.40E-15	1'-acetoxychavicol a cetate	4.22E-04
37	HTT	5.99E-08	ns	CDKN1A	3.65E-15	NREP	4.22E-04
38	EP300	7.09E-08	ns	RNA polymerase II	3.67E-15	FLT1	4.25E-04
39	CREB1	8.12E-08	ns	Dexamethasone	3.89E-15	TCR	4.70E-04
40	RARA	1.14E-07	ns	Lipopolysaccharide	3.90E-15	TREM1	5.07E-04