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Holocaust Exposure Induced Intergenerational Effects on *FKBP5* Methylation

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ABSTRACT

BACKGROUND: The involvement of epigenetic mechanisms in intergenerational transmission of stress effects has been demonstrated in animals but not in humans.

METHODS: Cytosine methylation within the gene encoding for FK506 binding protein 5 (*FKBP5*) was measured in Holocaust survivors ($n = 32$), their adult offspring ($n = 22$), and demographically comparable parent ($n = 8$) and offspring ($n = 9$) control subjects, respectively. Cytosine-phosphate-guanine sites for analysis were chosen based on their spatial proximity to the intron 7 glucocorticoid response elements.

RESULTS: Holocaust exposure had an effect on *FKBP5* methylation that was observed in exposed parents as well in their offspring. These effects were observed at bin 3/site 6. Interestingly, in Holocaust survivors, methylation at this site was higher in comparison with control subjects, whereas in Holocaust offspring, methylation was lower. Methylation levels for exposed parents and their offspring were significantly correlated. In contrast to the findings at bin 3/site 6, offspring methylation at bin 2/sites 3 to 5 was associated with childhood physical and sexual abuse in interaction with an *FKBP5* risk allele previously associated with vulnerability to psychological consequences of childhood adversity. The findings suggest the possibility of site specificity to environmental influences, as sites in bins 3 and 2 were differentially associated with parental trauma and the offspring's own childhood trauma, respectively. *FKBP5* methylation averaged across the three bins examined was associated with wake-up cortisol levels, indicating functional relevance of the methylation measures.

CONCLUSIONS: This is the first demonstration of an association of preconception parental trauma with epigenetic alterations that is evident in both exposed parent and offspring, providing potential insight into how severe psychophysiological trauma can have intergenerational effects.

Keywords: Cortisol, Epigenetics, *FKBP5*, Intergenerational, PTSD, Stress

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Parental trauma exposure is associated with greater risk for posttraumatic stress disorder (PTSD) and mood and anxiety disorders in offspring (1,2). Biological alterations associated with PTSD and/or other stress-related disorders have also been observed in offspring of trauma survivors who do not themselves report trauma exposure or psychiatric disorder (3–5). Animal models have demonstrated that stress exposure can result in epigenetic alterations in the next generation, and such mechanisms have been hypothesized to underpin vulnerability to symptoms in offspring of trauma survivors (6–9).

Enduring behavioral responses to stress and epigenetic alterations in adult offspring have been demonstrated to be mediated by changes in gametes (10–13), in utero effects (14), variations in early postnatal care (15,16), and/or other early life experiences (17) that are influenced by parental exposure (18). Converging data indicate that some findings in offspring may represent a biological accommodation to either the parental exposure or its biobehavioral consequences. For example, lower 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) activity was observed in Holocaust survivors compared with control subjects, presumably reflecting an enduring adaptive response to extreme nutritional deprivation (19). However, higher 11β-HSD-2 activity was noted in Holocaust offspring (20). It was suggested that if 11β-HSD-2 activity was lower during pregnancy in survivor mothers, this would expose the fetus to higher maternal glucocorticoids, resulting in the adaptation of higher 11β-HSD-2 activity in offspring (20) and in greater susceptibility to adult hypertension and metabolic syndrome (21), as has been previously observed (22).

Another model for epigenetic changes in offspring is that parental exposures produce changes that are not necessarily observed in the parent (23). Differential methylation of the glucocorticoid receptor (GR) gene associated with maternal behavior has been observed in F1 in rodents at *Nr3c1* exon 1_7 promoter (16) and in human offspring with parental PTSD at the ortholog *NR3C1* exon 1_F promoter (24). In rodents, these changes are a function of differences in postnatal maternal care, and in humans, these changes may reflect inconsistent attachments or other experiences resulting from the caregiver's symptoms.

Since parental trauma exposure has been linked with offspring trauma, particularly childhood emotional abuse (25), it has been difficult to disentangle effects of parental exposure from those potentially conferred by the offspring's early experiences (24). A major gap in the clinical literature is that parents and their adult offspring have not been studied in tandem, making it difficult to understand the origin of changes in association with parental exposure. Furthermore, whereas some aspects of the offspring phenotype are similar to those observed in parents, offspring also show a range of responses reflecting multiple contributors (24). Thus, we investigated epigenetic changes in *FKBP5* methylation in Holocaust survivors, offspring, and demographically matched Jewish parent-offspring pairs from peripheral blood samples to determine whether Holocaust exposure and/or PTSD symptoms and offspring's own experience were associated with changes in *FKBP5* methylation in the Holocaust offspring.

We focused on FKBP5, an important regulator of GR sensitivity (26), alterations of which have been associated with both PTSD and intergenerational effects (27,28). FKBP5 effectively decreases glucocorticoid binding to GR, impeding GR translocation to the nucleus (29,30). In turn, *FKBP5* gene expression is regulated by GR binding, forming an intracellular ultrashort glucocorticoid negative-feedback loop that regulates FKBP5 and GR (26,31). *FKBP5* gene expression has been shown to be altered in PTSD and major depression (32–35). *FKBP5* single nucleotide polymorphisms have additionally been shown to interact with early trauma to predict both PTSD and major depression (36,37), possibly through involvement of allele-specific, environmentally dependent changes in methylation of specific cytosine-phosphate-guanine (CpG) sites (38).

After extensive investigation of all relevant regions in the *FKBP5* gene that had GR-binding signals including the promoter regions and introns 2, 5, and 7 (31,39), Klengel *et al.* (38) demonstrated that only intron 7 CpG sites were differentially methylated in response to early abuse in the presence of *FKBP5* rs1360780 risk allele. Intron 7 sites are located within, or in proximity to, functional consensus glucocorticoid response elements (GREs) and are demethylated by glucocorticoids, particularly during developmentally sensitive periods (38).

The transcriptional relevance of intron 7 sites was demonstrated in a series of steps. First, an experiment using chromatin conformation capture revealed that intron 7 is in direct physical contact with the *FKBP5* transcriptional start site (38). Second, reporter gene assays established that methylation of the sites affected *FKBP5* gene expression (38). Third, using a multipotent human hippocampal progenitor cell line, four of these sites (3 to 6) were shown to be demethylated by glucocorticoid administration (38). Early abuse induced demethylation in the presence of the *FKBP5* risk allele was demonstrated for three of the sites (3 to 5) in adult peripheral blood (38).

Accordingly, it was of interest to examine intron 7 methylation in the context of intergenerational trauma effects. We hypothesized differential methylation in Holocaust survivors (original parent generation [F0]) compared with nonexposed demographically matched Jewish control subjects. We further hypothesized that Holocaust offspring (first generation [F1]) would show methylation changes in the same sites as their parents as evidence of intergenerational transmission. To examine early abuse effects in offspring that might be distinguishable from transmitted effects, we evaluated childhood adversity in offspring.

METHODS AND MATERIALS

Sample

The sample represents a subset of a larger sample of Holocaust survivors, offspring, and comparison subjects for which clinical and neuroendocrine data have been previously published (4,40), based on the availability of blood samples for DNA extraction and participants' consent for DNA analysis.

The majority of Holocaust survivors was initially recruited from 1993 to 1995 and studied 10 years later as part of a longitudinal follow-up. At that time, participants were asked about the possibility of referring their offspring to evaluate potential intergenerational effects. Holocaust offspring and their parents were also recruited by advertisement for a separate project evaluating intergenerational effects of parental trauma exposure and PTSD, including future examination of molecular aspects of PTSD. All study procedures were approved by the Institutional Review Board at Icahn School of Medicine and Bronx Veterans Affairs Medical Center, and all participants provided written, informed consent.

Holocaust survivors were defined as being interned in a Nazi concentration camp, having witnessed or experienced torture, or having had to flee or hide during World War II. Demographically comparable control subjects were living outside of Europe during World War II. All F1 participants were raised by their biological parents. Following a medical and psychological evaluation, participants were excluded for serious illness or any disease requiring ongoing systemic steroid treatment, substance abuse or dependence within the previous 6 months, or lifetime history of psychosis. The full set of inclusion/exclusion criteria, including clinical evaluation procedures, is described in Yehuda *et al.* (4,40).

The present sample represents data from 40 parents and 31 offspring. Among the F0 cohort, data were available for both parents in five cases. For the F1 cohort, data were included from multiple siblings in two cases.

Procedure

Psychiatric diagnoses were determined using the Structured Clinical Interview for DSM-IV (41), and PTSD was ascertained with the Clinician-Administered PTSD Scale (42).

The Childhood Trauma Questionnaire (CTQ) (43) was completed by the F1. Holocaust F1 completed the Parental PTSD Questionnaire, which asks offspring to rate parental PTSD symptoms and severity of Holocaust exposure (44). The Beck Depression Inventory and Spielberger State-Trait Anxiety Inventory were used as measures of symptom severity (45,46).

Blood samples were obtained at 8:00 AM. F1 collected saliva into Salivette tubes (Sarstedt, Nümbrecht, Germany) at awakening and bedtime.

Biological Measures

The primary biological measure was *FKBP5* intron 7 methylation; other measures were *FKBP5* rs1360780 genotype and salivary cortisol at awakening and bedtime.

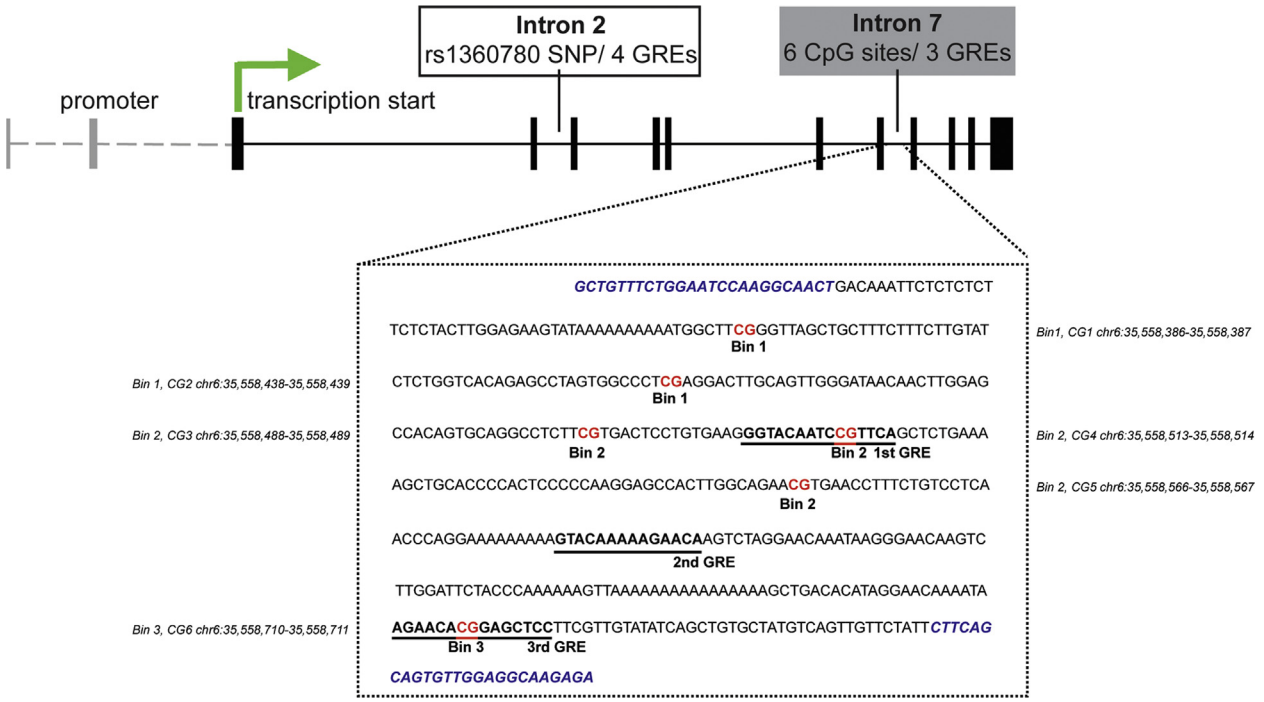

Figure 1. Schematic representation of the human *FKBP5* locus with intron 7 glucocorticoid receptor binding sequence investigated in this study. The upper panel depicts the *FKBP5* locus in 5'-3' orientation. Black bars represent the 11 exons. The transcription start site is highlighted in green. The lower panel represents the intron 7 amplicon (476 base pair) chosen for DNA methylation analysis (primer sequence dark blue/italicized). Since pyrosequencing can only reliably generate short reads, the six cytosine-phosphate-guanine (CpG) sites (red) analyzed in three bins based on the proximity to three consensus glucocorticoid response elements (GREs) are represented in bold/underlined [pyrosequencing primers are described in Klengel *et al.* (38)]. The two CpGs of bin 1 were upstream of all GREs, the three CpGs of bin 2 are surrounding the first GRE, and bin 3 represents the CpG within the third GRE. The chromosomal position (hg19) of the CpG sites is indicated on the left and the right of the lower panel. SNP, single nucleotide polymorphism.

Cytosine Methylation Sodium Bisulfite Mapping. DNA from whole blood was isolated using Gentra Puregene Kit (Qiagen, Valencia, California). Genomic DNA was bisulfite treated using the EZ-Gold Kit (Zymo Research, Irvine, California). Methylation analysis of the bisulfite-treated genomic DNA by pyrosequencing was performed by Varionostic (Ulm, Germany). Three pyrosequencing primer pairs were designed to cover three regions of the *FKBP5* intron 7 GR-binding sequence (38). Sequencing was performed on the Q24 System with PyroMark Q24 analysis software (Qiagen) and yielded percent methylation levels for the six intron 7 CpGs (sites 1 to 6) grouped into three bins (Figure 1).

Genotyping. *FKBP5* rs1360780 was genotyped using a differential hybridization protocol on LightCycler 480 (Roche, Basel, Switzerland). This method failed for 6 of 71 samples, but 5 were genotyped by pyrosequencing on a PSQ96 (Qiagen), omitting one F0 sample from analysis. Genotypes of rs1360780 were in Hardy-Weinberg equilibrium ($p > .05$). Participants were divided according to genotype data into protective genotype (GG) and risk allele carriers (AG/AA).

Hormone Measurement. Cortisol levels were determined as previously described (47). The intra-assay and interassay coefficients of variation were 2.3% and 6.1%, respectively.

Statistical Analysis

Demographic and clinical characteristics were compared, respectively, for F0 and F1 groups using analyses of variance and chi-square analyses, as appropriate. Likelihood ratio chi-square results are presented when one or more of the cells did not meet minimum sample size.

The primary analyses concerned group differences between Holocaust survivors or offspring and their respective comparison subjects in methylation for the three intron 7 bins. The multivariate analyses of covariance for F0 and F1 comparisons used age, gender, and diagnosis of current mood and anxiety disorders, as well as additional variables specified in the text, as covariates. As previously reported (38), intron 7 sites are grouped into three bins based on the pyrosequencing method that necessitates the examination of short sequencing reads (Figure 1). A main effect for bin was followed by post hoc tests investigating if the bin effect reflected a particular influence by a specific site(s). Regression analyses examined the relative contribution of parental Holocaust exposure, F0 *FKBP5* methylation, maternal and paternal PTSD, childhood adversity, and F1 *FKBP5* genotype to F1 *FKBP5* methylation.

A secondary analysis investigated the impact of physical and sexual abuse on *FKBP5* methylation at intron 7, bin 2 based on prior work (38). A composite CTQ score for physical and sexual abuse was used to explore differential associations with methylation in the entire offspring sample and in subjects identified by the presence and absence of the *FKBP5* risk allele. When an effect was found, further analyses investigated if single or multiple sites were driving the effect. The significance of the difference between two correlation coefficients was evaluated using the Fisher r-to-z transformation.

F0 and F1 methylation correlations were performed only for bin 3/site 6, as this was the single site for which an effect of Holocaust exposure was observed. The functional effect of intron 7 methylation was assessed in relation to the intron 7, bin average based on the experiments of Klengel *et al.* (38) showing transcriptional effects when the three bins/six sites were experimentally methylated and tested concurrently. Finally, statistical significance for all analyses was set at two-tailed, $p < .05$.

RESULTS

Holocaust Survivor and Comparison Group Demographic Characteristics

Demographic and clinical characteristics of Holocaust survivor and comparison groups are detailed in Table 1. There were no significant differences between the groups in age, gender, years of education, current anxiety or mood disorder diagnoses, or frequency of the *FKBP5* risk allele. Holocaust survivors differed from control subjects in presence and severity of current PTSD as anticipated.

Holocaust Offspring and Comparison Group Demographic Characteristics

Demographic and clinical characteristics of Holocaust offspring and comparison groups are displayed in Table 2. There were no significant differences between the groups in age, gender, years of education, childhood trauma scores, psychiatric diagnoses, frequency of the *FKBP5* risk allele, or cortisol levels. However, there were significant differences in a composite score of depression and anxiety self-ratings.

FKBP5 Intron 7 Methylation in Holocaust Survivors and F0 Comparison Subjects

A multivariate analysis of covariance comparing Holocaust survivors and F0 control subjects on intron 7 methylation showed 10% higher levels in bin 3/site 6 in survivors (control subjects: 51.33 ± 2.10, survivors: 56.39 ± 1.03; $F_{1,31} = 4.34$, $p = .046$; Figure 2A). The finding remained significant when the presence of PTSD was entered as an additional covariate ($p = .049$). The addition of presence/absence of the *FKBP5* risk allele as a covariate did not appreciably alter the finding ($p = .053$), suggesting that bin 3/site 6 methylation changes in F0 were driven more by exposure than genetic variation in this single nucleotide polymorphism. There was no significant main effect of F0 PTSD diagnosis on bin 3/site 6 methylation ($F_{1,31} = .33$, not significant [ns]). No differences between Holocaust survivors and F0 control subjects were apparent for bins 1 and 2.

FKBP5 Intron 7 Methylation Holocaust Offspring and F1 Comparison Subjects

When a similar analysis was performed for F1, significantly lower intron 7 methylation (7.7% difference) was observed at bin 3/site 6 in Holocaust offspring than comparison subjects (control subjects: 57.58 ± 1.63, offspring: 53.13 ± 1.00; $F_{1,25} = 5.03$, $p = .034$; Figure 2B). The finding was unchanged without covariation ($F_{1,29} = 4.91$, $p = .035$) and when presence of lifetime PTSD in offspring ($p = .036$) or severity of childhood trauma ($p = .049$) were added, respectively, as additional covariates. In contrast, the effect was reduced when controlling for parental PTSD ($F_{1,23} = 2.59$, $p = .121$), suggesting an influence of parental PTSD symptoms.

A linear regression confirmed that parental Holocaust exposure was the salient predictor of offspring's bin 3/site 6 methylation ($\beta = -.368$, $p = .034$). Serial regression models were used to identify additional predictors of bin 3/site 6 methylation. Neither parental PTSD ($\beta = .134$, ns) nor the presence of the *FKBP5* risk allele ($\beta = -.044$, ns) contributed to the model. In each of these, however, parental exposure remained a significant predictor (respectively, $\beta = -.461$, $p = .054$; $\beta = -.362$, $p = .043$). Adding childhood adversity as measured by the CTQ total score did not alter the predictive significance of parental exposure ($\beta = -.359$, $p = .049$) and was not a predictor of bin 3/site 6 methylation ($\beta = -.034$, ns). Since emotional abuse is reported substantially more often by Holocaust survivor offspring (25) and was reported in this sample at a trend level of significance ($t = -1.98$, $df = 24.89$, $p = .059$), we tested emotional abuse in the regression model. However, like the CTQ total score, emotional abuse was not significantly associated with bin 3/site 6 methylation ($\beta = .018$, ns) and did not alter the significance of Holocaust exposure at this site ($\beta = -.375$, $p = .045$).

Table 1. Demographic and Clinical Characteristics for Holocaust Survivors and F0 Comparison Subjects

Parents (F0)	Holocaust Survivors ($n = 32$) Mean ± SD or *n* (%)	Jewish Comparison ($n = 8$) Mean ± SD or *n* (%)	Group Comparisons *F* (*df*) *p* or χ^2 (*df*) *p*
Age	77.9 ± 5.2	73.1 ± 8.5	$F_{1,38} = 3.98$, $p = .053$
Gender			$\chi^2_1 = .46$, ns
Male	12 (37.5%)	2 (25.0%)	
Female	20 (62.5%)	6 (75.0%)	
Years of Education	9.2 ± 4.1	12.3 ± 3.0	$F_{1,35} = 3.91$, $p = .056$
Current PTSD[a]	16 (51.6%)	0 (0%)	$\chi^2_1 = 9.86$, $p = .012$
Current Anxiety Disorder Except PTSD[b]	4 (13.8%)	0 (0%)	$\chi^2_1 = 2.08$, ns
Current Mood Disorder[b]	9 (31.0%)	0 (0%)	$\chi^2_1 = 5.13$, ns
Biological Information			
FKBP5 risk allele	18 (58.1%)	3 (37.5%)	$\chi^2_1 = 1.08$, ns

ns, not significant; PTSD, posttraumatic stress disorder.
[a]Clinician-Administered PTSD Scale (42).
[b]Structured Clinical Interview for DSM-IV (41).

Table 2. Demographic and Clinical Characteristics for Offspring of Holocaust Survivors and F1 Comparison Subjects

Offspring (F1)	Holocaust Offspring (n = 22) Mean ± SD or n (%)	Jewish Comparison (n = 9) Mean ± SD or n (%)	Group Comparisons F (df) p or χ^2 (df) p
Age	46.0 ± 8.0	47.0 ± 8.5	$F_{1,30}$ = .10, ns
Gender			χ^2_1 = 1.06, ns
Male	6 (27.3%)	1 (11.1%)	
Female	16 (72.7%)	8 (88.9%)	
Years of Education	16.4 ± 2.7	16.8 ± 1.8	$F_{1,30}$ = .14, ns
Parental PTSD[a]			
Maternal PTSD	11 (52.4%)	0 (0%)	χ^2_1 = 10.37, p = .011
Paternal PTSD	11 (52.4%)	0 (0%)	χ^2_1 = 10.37, p = .011
Any parent with PTSD	16 (76.2%)	0 (0%)	χ^2_1 = 18.40, p < .001
Childhood Trauma[b]			
Total score	36.7 ± 11.5	30.2 ± 6.9	$F_{1,30}$ = 2.46, ns
Emotional abuse	9.0 ± 5.4	12.0 ± 3.3	$F_{1,30}$ = 2.57, ns
Emotional neglect	10.3 ± 4.1	8.0 ± 4.0	$F_{1,30}$ = 1.97, ns
Physical abuse	6.1 ± 2.5	5.4 ± 1.0	$F_{1,30}$ = .55, ns
Physical neglect	5.5 ± 1.4	5.2 ± .7	$F_{1,30}$ = .32, ns
Sexual abuse	5.6 ± 3.0	5.0 ± .0	$F_{1,30}$ = .40, ns
Self-Reported Anxiety and Depression Ratings[c]	40.0 ± 24.6	22.0 ± 12.3	$F_{1,30}$ = 4.32, p = .047
Current Anxiety Disorder (except PTSD)[d]	8 (36.4%)	0 (0%)	χ^2_1 = 6.56, p = .041
Current Mood Disorder[d]	3 (13.6%)	0 (0%)	χ^2_1 = 2.19, ns
Biological Information			
FKBP5 risk allele[e]	14 (63.6%)	4 (44.4%)	χ^2_1 = .10, ns
Wake-up cortisol[f]	738.4 ± 463.9	743.3 ± 337.5	$F_{1,21}$ = .01, ns
Bedtime cortisol[f]	250.3 ± 556.3	144.6 ± 72.9	$F_{1,21}$ = .21, ns

F0, original parent generation; F1, first generation; ns, not significant; PTSD, posttraumatic stress disorder; SNP, single nucleotide polymorphism.

[a]Assessed using the Parental PTSD Questionnaire (46).

[b]Childhood Trauma Questionnaire (43).

[c]Combined scores of the Beck Depression Inventory and Spielberger State-Trait Anxiety Inventory scales.

[d]Structured Clinical Interview for DSM-IV (41).

[e]Presence of risk-allele (A) in *FKBP5* rs1360780 SNP genotype.

[f]Salivary cortisol (ng/dL).

Childhood Adversity Effects on *FKBP5* Intron 7 Methylation in Offspring

An analysis was then performed to attempt to replicate a previous report on a traumatized inner city population showing significant effects of the interaction of physical and sexual abuse with the *FKBP5* risk allele on methylation at intron 7, bin 2/sites 3 to 5.

For protective genotype carriers (n = 13), physical and sexual abuse were positively associated with bin 2 methylation (r = .698, p = .008). For risk allele carriers (n = 18), there were significant negative associations with bin 2 methylation (r = −.479, p = .044). These associations were driven by the association at site 3 with CTQ scores for physical abuse (for protective genotype carriers: r = .707, p = .007 and for risk

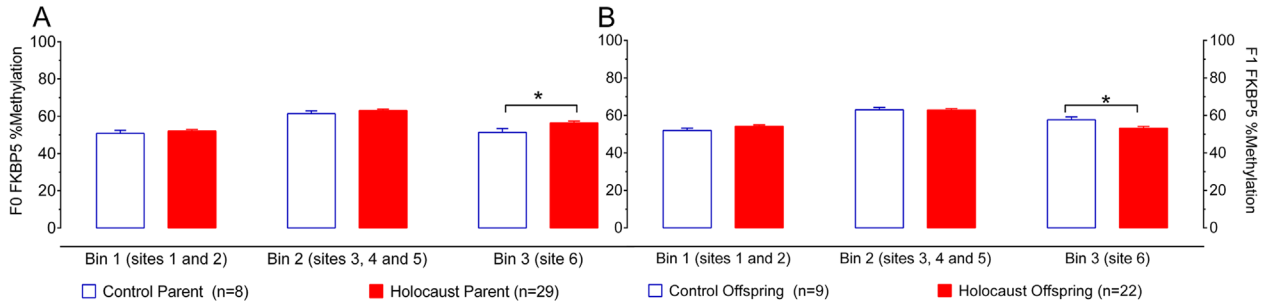

Figure 2. Methylation at *FKBP5* intron 7, bins 1, 2, and 3 for Holocaust survivors **(A)**, Holocaust survivor offspring **(B)**, and their respective comparison subjects. The percent methylation (mean ± SEM) is represented by red bars for Holocaust survivor parents (F0) and their offspring (F1) (F0: n = 32, F1: n = 22) and by white bars for F0 and F1 control subjects (F0: n = 8, F1: n = 9). Division of sites into bins is indicated. *p < .05.

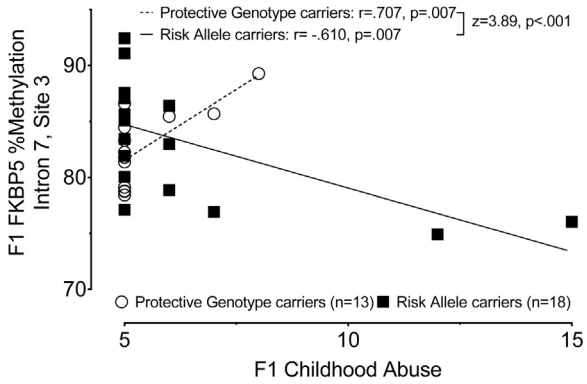

Figure 3. Relationship between *FKBP5* intron 7, site 3 percent methylation and Childhood Trauma Questionnaire physical abuse subscale score. The carriers of the *FKBP5* rs1360780 risk allele ($n = 18$) are depicted by black squares and the carriers of the protective genotype ($n = 13$) are depicted by black open circles. Significance was set at $p < .05$. F1, first generation offspring.

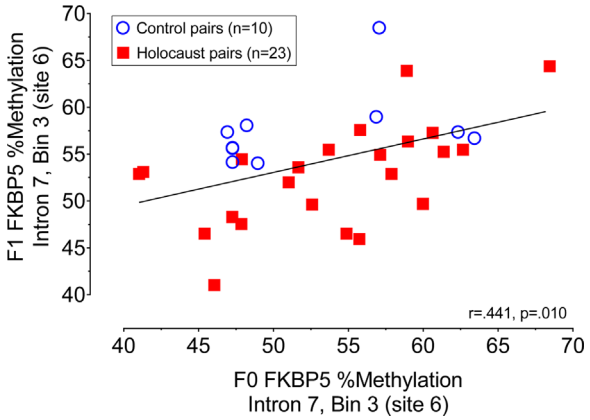

Figure 4. Relationship between original parent generation (F0) and first generation (F1) *FKBP5* intron 7 bin 3/site 6 percent methylation. Parent-offspring pairs are represented by red squares for Holocaust survivors ($n = 23$) and by blue open circles for control subjects ($n = 10$). Significance was set at $p < .05$.

allele carriers: $r = -.610$, $p = .007$; Figure 3). The correlations at site 3 with physical abuse differed significantly according to the presence of the risk allele ($z = 3.89$, $p < .001$; Figure 3).

F0 *FKBP5* Intron 7 Methylation at Bin 3 Predicts F1 *FKBP5* Intron 7 Methylation at Bin 3

F0 intron 7 bin 3/site 6 methylation was correlated with F1 methylation at the same site ($r = .441$, $n = 33$, $p = .010$; Figure 4). This association was primarily driven by the Holocaust-exposed families ($r = .569$, $n = 23$, $p = .005$ for Holocaust-exposed compared with $r = .370$, $n = 10$, ns for control subjects). Covariation for the presence of the *FKBP5* risk allele in both generations did not substantially alter the association of bin 3/site 6 methylation between survivors and offspring ($r = .438$, $df = 29$, $p = .014$) or within the Holocaust-exposed families ($r = .559$, $df = 19$, $p = .008$).

As a result of the above analyses, we added F0 bin 3/site 6 methylation to the regression analyses testing predictors of F1 bin 3/site 6 methylation. F0 methylation did not alter the significance of parental Holocaust exposure as a predictor of bin 3/site 6 methylation in F1 ($\beta = -.418$, $p = .022$).

FKBP5 Methylation Is Associated with Wake-up Cortisol in Offspring

Intron 7 *FKBP5* bin average methylation was negatively correlated with wake-up cortisol ($r = -.630$, $df = 16$, $p = .005$, controlling for age, gender, and current mood/anxiety disorder). This association was also significant without covariation ($r = -.432$, $n = 22$, $p = .044$; Figure 5). The correlations of bin average methylation with bedtime cortisol were not significant ($r = -.216$, $df = 13$, ns, and $r = -.137$, $n = 19$, ns, respectively).

DISCUSSION

The main finding in this study is that Holocaust survivors and their offspring have methylation changes on the same site in a functional intronic region of the *FKBP5* gene, a GR-binding sequence in intron 7, but in the opposite direction. To our knowledge, these results provide the first demonstration of an association of preconception stress effects with epigenetic changes in both exposed parents and their offspring in adult humans. Bin 3/site 6 methylation was not associated with the FKBP5 risk allele and could not be attributed to the offspring's own trauma exposure, their own psychopathology, or other examined characteristics that might independently affect methylation of this gene. Yet, it could be attributed to Holocaust exposure in the F0.

It is not possible to infer mechanisms of transmission from these data. It was not possible to disentangle the influence of parental gender, including gamete or in utero effects, since 21 of 22 Holocaust parents were survivors. Epigenetic effects in maternal or paternal gametes are a potential explanation for epigenetic effects in offspring (10–13), but blood samples will not permit ascertainment of gamete-dependent transmission. What can be detected in blood samples is parental and offspring experience-dependent epigenetic modifications.

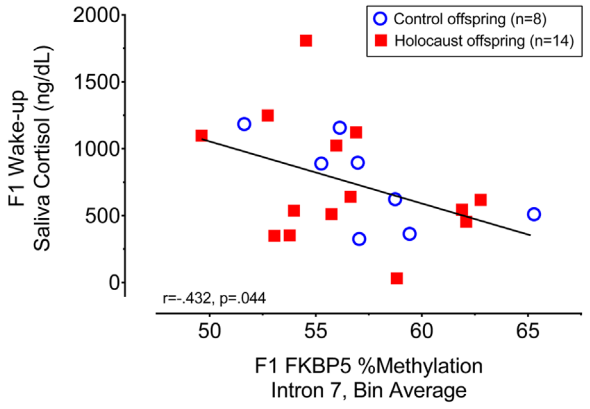

Figure 5. Relationship between *FKBP5* intron 7, bin average percent methylation, and wake-up salivary cortisol (ng/dL) in first generation (F1). Holocaust survivor offspring ($n = 14$) are depicted by red squares and control subjects ($n = 8$) are depicted by blue open circles. Significance was set at $p < .05$.

Future prospective, longitudinal studies of high-risk trauma survivors before conception, during pregnancy, and during postpartum may uncover sources of epigenetic influences. In addition, it would be of interest to replicate our findings on *FKBP5* intron 7 methylation in other populations with substantial trauma exposure. For example, peripheral blood from female survivors of the Tutsi genocide who were pregnant at the time of exposure and their adolescent offspring was analyzed for *NR3C1* and *NR3C2* promoter methylation (48). Interestingly, in that study, exposure effects were identified at specific sites in both exposed mothers and offspring, and mothers' methylation correlated with offspring methylation. It is also necessary to investigate multiple generations to differentiate among exposure effects, epigenetic inheritance, and social transmission (9). Animal models can provide further mechanistic understanding of how extreme stress effects mediate changes in offspring.

Despite the potential limitations of our cross-sectional approach, a significant effect of severe parental trauma was observed in both generations at the same site of a transcriptionally relevant region of a stress-related gene. The Holocaust effects in F0 and F1 methylation are not likely driven by respective differences in control subjects. F0 and F1 control subjects differed substantially in age (F0: ~75 years vs. F1: ~45 years), which could explain their 12% methylation difference at bin 3/site 6, since *FKBP5* site-specific demethylation occurs with age (49). The directional difference in bin 3/site 6 methylation between Holocaust survivors and their offspring was unexpected but may reflect an intergenerational biological accommodation. We previously reported a directional difference between parents and offspring in 11β-HSD-2 activity, which was interpreted as an accommodation in the offspring during a sensitive developmental window to the biological consequences of parental trauma exposure (20). Like FKBP5, 11β-HSD-2 is a moderator of glucocorticoid action, and in the aforementioned study, the increase in offspring 11β-HSD-2 activity was interpreted as a protective adaptation to mitigate exposure to elevated maternal glucocorticoids associated with reduced maternal 11β-HSD-2 activity (20,50). Although no claims regarding gender-specific effects can be made in this study, it is conceivable that *FKBP5* hypermethylation, leading to decreased *FKBP5* expression and increased GR sensitivity in the F0 mothers, would result in lower circulating glucocorticoid levels during pregnancy, promoting demethylation in the fetus to optimize or increase glucocorticoid levels. However, preconception or postnatal influences are also possible, and offspring low cortisol levels may further regulate *FKBP5* methylation levels. At present, it is not clear whether glucocorticoid programming in offspring reflects intergenerational consequences of parental exposure or offspring recalibration of glucocorticoid regulation. Although imperfect, such studies provide an opportunity to understand development-dependent adaptations to environmental influences that contribute to individual stress-reactivity set points and ultimately vulnerability to psychopathology or resilience.

In contrast to the findings at bin 3/site 6, which relate to parental exposure, bin 2/sites 3 to 5 methylation was associated with offspring trauma exposure (childhood physical and sexual abuse), but the direction depended on the presence of the *FKBP5* risk allele. Further analysis indicated that the effect was driven by a physical abuse induced site 3 demethylation in *FKBP5* risk allele carriers. These results partially replicate previous findings (38) in which bin 2 demethylation was associated with physical and sexual abuse, the *FKBP5* risk allele, and their interaction in two separate large cohorts. It should be noted that mean CTQ subscale and total scores for the Holocaust offspring were generally lower than reported in other population samples and only a small minority of offspring in this study would be considered traumatized according to previously reported criteria. However, this minority contributed clinically relevant variance to CTQ scores for physical and sexual abuse. Klengel *et al.* (38) and other investigators (51,52) focusing on early childhood trauma effects and the influence of the gene × early life environment interactions on methylation did not assess the contribution of parental experiences, particularly trauma exposure. Our findings suggest that it is important to assess parental exposure characteristics since they may exert profound influences. Although different sites may be involved in mediating parental versus offspring's own early trauma, it is possible that the effect of offspring abuse may be an indirect consequence of parental trauma. Indeed, parental trauma exposure has been shown to result in an increased prevalence of childhood abuse, most strongly emotional abuse, in offspring (25).

Despite different associations with methylation at bins 2 and 3, both appear to be functionally important in this sample, as previously reported (38). Furthermore, both bins within intron 7 appear to be equally sensitive to glucocorticoid-dependent *FKBP5* demethylation in a multipotent human hippocampal progenitor cell line (38). Bin 2 contains the first consensus GRE and bin 3/site 6 is located within the third GRE of *FKBP5* intron 7. These GREs come into contact with the transcriptional start site of *FKBP5* via chromatin interactions; alterations in methylation at bins 1, 2, and 3 affect GR-induced *FKBP5* gene transcription as shown by reporter-gene assay (38). Although we were unable to disentangle functional effects of methylation at distinct CpGs in intron 7, there is reason to believe that methylation at individual sites in the *FKBP5* gene may contribute to transcriptional and functional effects. Indeed, for the *NR3C1* gene, associations of individual sites with exposures to several types of adversity are increasingly recognized (52).

While the effects were relatively small, with 10% or less difference in DNA methylation, small differences of around 1% to 2% have been previously associated with differential gene expression of the closest gene in a mixed tissue with many cell subtypes, such as peripheral blood (53). Therefore, there are likely specific immune cell subtypes with more pronounced effects. In addition, even within the same cell type, observed global changes reflect diverse changes in single cells, as has been reported in a number of articles using clone-based sequencing for methylation analysis [for example (16,17,54)]. Lower methylation leading to higher *FKBP5* messenger RNA and protein levels has been linked to decreased GR sensitivity (38), as supported in this study by the negative correlation between F1 intron 7, bin average methylation and wake-up cortisol levels. These effects could be mediated by the *FKBP5*-associated changes in GR function and hypothalamic-pituitary-adrenal axis regulation or indirectly through the interaction partners of this immunophilin in other neuroendocrine regulatory circuit mechanisms (29,31,55,56).

In summary, our data support an intergenerational epigenetic priming of the physiological response to stress in offspring of highly traumatized individuals. These changes may contribute to the increased risk for psychopathology in the F1 generation. Two sites anticipated to operate similarly to regulate *FKBP5* gene expression were demonstrated to have different environmental influences. The mechanism of intergenerational transmission of epigenetic effects at bin 3/site 6 is not known but does not appear to be mediated by childhood adversity, as is the case for bin 2. From a biological perspective, accommodation to multiple environmental influences at distinct and potentially redundant sites on genes central to stress regulation would facilitate maximal stress responsivity and adaptation. Future studies should focus on assessing the effects of trauma at various developmental stages, as well as potential differences in maternal and paternal effects. Additionally, the mechanism of intergenerational transmission of trauma and functional importance of site specificity remain to be explored. Early detection of such epigenetic marks may advance the development of preventive strategies to address the intergenerational sequelae of exposure to trauma.

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RY, NPD, FH, and EBB designed the study. RY and EBB supervised the project and data collection. LMB supervised the clinical assessments, and RY and LMB supervised the biological sample collection. NPD and TK performed the biological assays. HNB was responsible for data management of the project. RY, NPD, LMB, and HNB did primary analyses and drafted the manuscript. EBB did additional analyses and edited the manuscript. All the authors discussed the results and commented on the final version of the manuscript.

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