Increased DNA Methyltransferase 3b (Dnmt3b)–mediated CpG Island Methylation Stimulated by Oxidative Stress Inhibits Expression of a Gene Required for Neural Tube and Neural Crest Development in Diabetic Pregnancy

Running Title: Embryo DNA methylation in diabetic embryopathy

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ABSTRACT

Previous studies have shown that diabetic embryopathy results from impaired expression of genes that are required for formation of embryonic structures. We have focused on *Pax3*, a gene that is expressed in embryonic neuroepithelium and is required for neural tube closure. *Pax3* expression is inhibited in embryos of diabetic mice due to hyperglycemia-induced oxidative stress. DNA methylation silences developmentally expressed genes prior to differentiation. We hypothesized that hypomethylation of *Pax3* upon neuroepithelial differentiation may be inhibited by hyperglycemia-induced oxidative stress. We tested this using embryos of pregnant hyperglycemic mice and mouse embryonic stem cells (ESC). Methylation of a *Pax3* CpG island decreased upon neurulation of embryos and formation of neuronal precursors from ESC. In ESC, this was inhibited by oxidative stress. Use of shRNA in ESC demonstrated that DNA methyltransferase 3b (Dnmt3b) was responsible for methylation and silencing of *Pax3* prior to differentiation and by oxidative stress. While expression of *Dnmt3b* was not affected by oxidative stress, DNA methyltransferase activity was increased. These results indicate that hyperglycemia-induced oxidative stress stimulates Dnmt3b activity, thereby inhibiting chromatin modifications necessary for induction of *Pax3* expression during neurulation, and thus, providing a molecular mechanism for defects caused by Pax3 insufficiency in diabetic pregnancy.
[Introduction]

Maternal pregestational diabetes significantly increases risk for congenital malformations (1-6). While many organ systems can be affected, neural tube defects (NTD) and cardiac outflow tract defects (COTD) are among the most common that occur (2; 7). The malformations arise early during embryonic development, mostly within the first eight gestational weeks, when organ systems are first starting to form (8). Results of human and animal studies indicate that hyperglycemic excursions during organogenesis are responsible for malformations induced by diabetic pregnancy (9).

Work from our laboratory has demonstrated that maternal hyperglycemia inhibits expression of Pax3, a gene that is expressed in embryonic neuroepithelium and neural crest and which is required for neural tube and cardiac outflow tract formation (10-12). That homozygous mutant Pax3 mouse embryos develop NTD and COTD with 100% penetrance (13; 14) supports the notion that inhibition of Pax3 below a critical threshold is sufficient to cause NTD or COTD in embryos of diabetic mothers. Several studies have indicated that oxidative stress produced in the embryo in response to increased glucose metabolism is responsible for diabetic pregnancy-induced malformations (15-20). We have shown that oxidative stress inhibits expression of Pax3 (21; 22). The precise mechanisms by which oxidative stress inhibits Pax3 are not known.

During mammalian embryogenesis, methylation of DNA at cytosines is a dynamic process that serves several purposes, including gene silencing, chromosomal stability, and setting up parental gene imprinting patterns (23). In the inner cell mass (ICM) of the early embryo or in
undifferentiated embryonic stem cells (ESC), genes that will be expressed in a lineage-dependent fashion upon differentiation are silenced by methylation at CpG dinucleotides (24-28). Upon tissue differentiation, induced expression of these genes requires epigenetic modifications including hypomethylation of CpG dinucleotides (24-28). Dense clusters of CpG sequences, called CpG islands, are often located at mammalian gene promoters. While CpG islands differ from most chromosomal DNA by infrequent cytosine methylation, many CpG islands located around genes that are expressed in a tissue-specific fashion and that are essential regulators of embryonic development (including members of the Pax gene family) display tissue-specific methylation (29).

There are three known enzymes that regulate DNA methylation, Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 maintains DNA methylation of daughter strands during replication, and Dnmt3a and Dnmt3b regulate de novo DNA methylation, for example, during differentiation (26; 30).

Here we tested the hypothesis that Pax3 expression is silenced prior to its onset of expression during neurulation by methylation of a CpG island within its transcriptional regulatory element, and that oxidative stress, consequent to maternal hyperglycemia, preserves the hypermethylated state of this CpG island. Further, we tested the hypothesis that expression or activity of a DNA methyltransferase would be responsible for preservation of the hypermethylated state of the Pax3-associated CpG island.
RESEARCH DESIGN AND METHODS

Animal Procedures

All procedures using animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Joslin Diabetes Center. Nondiabetic female ICR mice were housed with nondiabetic ICR males and were checked daily for copulation plugs. Noon on the day that a copulation plug was found was determined to be embryonic day 0.5 (E 0.5). Transient hyperglycemia was induced in pregnant mice on E 7.5 by injecting 2 ml of 12.5% glucose dissolved in PBS at approximately hourly intervals to maintain maternal blood glucose ≥ 16.65 mmol/l as previously described (12). Oxidative stress was induced on E 7.5 using 3 mg/kg antimycin A (Sigma, St. Louis, MO), a dose which replicates the effects of maternal hyperglycemia to induce oxidative stress and inhibit Pax3 expression, as previously described (12; 21; 22). Preimplantation embryos were flushed from uteri to recover blastocysts on E 3.5, and postimplantation embryos were dissected from uteri on E 8.5.

Culture of Murine ESC
Murine embryonic stem cells (ESC) of the D3 line were cultured and induced to differentiate into neuronal precursors as previously described (31). Briefly, ESC were grown as undifferentiated monolayer cultures in Dulbecco’s Modified Eagle’s Media (DMEM, Life Technologies, Grand Island, NY) containing leukocyte inhibitory factor (LIF, Millipore, Billerica, MA) for 4 days, then differentiation was induced by forming embryoid bodies in nonadherent culture dishes in media without LIF, but containing 0.5 μmol/l retinoic acid (Sigma) for 4 days. Embryoid bodies were placed into adherent culture dishes with the same media as used when forming embryoid bodies for 1 day, then the media were replaced with DMEM/F-12 (Life Technologies) containing fibronectin (Becton Dickenson), insulin, transferrin, and selenium (all from Sigma) for 4 additional days to select for differentiating neuronal precursors. Oxidative stress was induced by adding 10 μmoles/l antimycin A to the media used during selection of neuronal precursors as described (31). This concentration of antimycin A has been shown to significantly increase markers of oxidative stress and to inhibit Pax3 expression by D3 ESC (31; 32). 10 μmoles/l of the DNA methyltransferase inhibitor, 5-azacytidine (Sigma), was added to the media while culturing undifferentiated ESC or while selection for neuronal precursors.

**RT-PCR Assays**

E 3.5 blastocysts were recovered from a total of 18 pregnant mice, and 3-4 blastocysts from 6 litters were pooled for 3 separate RT-PCR assays. E 8.5 embryos were recovered from 3 separate litters per treatment group and embryos from each litter were pooled for RT-PCR assay. Four 60 mm culture dishes of undifferentiated ESC or ESC-derived neuronal precursors for each treatment group were used for separate RT-PCR assays. Total RNA was extracted from embryos
or cells using Ultraspec reagent (Biotecx Laboratories, Friendswood, TX). Two hundred ng RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Foster City, CA). Real-time PCR was performed using TaqMan PCR Master Mix (Life Technologies) and primers and VIC-labeled probe to detect rRNA (Life Technologies #43189E) as the normalization control as described (21). Primers and FAM-labeled probe for Pax3 cDNA were as previously published (21). Primers and FAM-labeled probes for p53 (Mm01731290_g1), Pax6 (Mm00443081_m1), Pax7 (Mm01354484_m1), Dnmt1 (Mm01151063_m1), Dnmt3a (Mm00432881_m1), and Dnmt3b (Mm01240113_m1) cDNA were obtained from Life Technologies.

5-methylcytosine Immunoprecipitation (mDIP) Assays

E 3.5 blastocysts were recovered from a total of 18 pregnant mice, and 6-9 blastocysts from 6 litters were pooled for 3 separate methyl-DNA Immunoprecipitation (mDIP) assays. E 8.5 embryos were recovered from 3 separate litters per treatment group and embryos from each litter were pooled for mDIP assay. Three 60 mm culture dishes of undifferentiated ESC or ESC-derived neuronal precursors for each treatment group were pooled for mDIP assays. Genomic DNA was extracted and mDIP assays were performed as described (33). Briefly, genomic DNA was sonicated using 4 cycles of 70% duty, 20% output, 10 pulses/cycle on ice to generate fragments of approximately 300-1000 bp in length. Four µg of sonicated DNA were immunoprecipitated using 10 µl of 5-methylcytosine antibody (Active Motif, Carlsbad, CA). Following Proteinase K (Life Technologies) treatment, phenol chloroform extraction, and ethanol precipitation, the immunoprecipitated DNA was resuspended in 30 µl of Tris-EDTA buffer. One µl of immunoprecipitated DNA was amplified by PCR using SYBR green detection
(Life Technologies), in quadruplicate, in a 10 µl final volume. Twenty ng of unimmunoprecipitated DNA (input) were amplified in parallel as the normalization control. The primers used for amplification of the promoter-proximal Pax3 and p53 CpG islands and PCR conditions are shown in Table S1. Pax3 and p53 CpG islands were chosen using Genome Browser on the University of California Santa Cruz Bioinformatics site (http://genome.ucsc.edu). PCR primers were designed using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastDescAd).

**Bisulfite DNA Modification**

Genomic DNA was prepared from cells pooled from three 60 mm culture dishes and was modified with sodium bisulfite using the BisulFlash DNA Modification Kit (Epigentek Group Inc., Brooklyn, NY) according to the manufacturer’s instructions. The bisulfite-altered DNA was amplified to generate three overlapping PCR products within the Pax3 CpG island using primers not containing CpG dinucleotides. PCR primer sequences are shown in Table S2. All PCR reactions were performed using 40 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec, and extension at 72°C for 8 sec. The PCR products were inserted into a TA cloning vector (Life Technologies) and used to transform competent DH5α E. coli (Life Technologies). DNA from 10 colonies containing each of the PCR inserts was sequenced and contiguous sequences were analyzed for retention of cytosines or conversion to thymines. CpG methylation was analyzed using Quantification Tool for Methylation Analysis (http://quma.cdb.riken.jp, Kyoto, Japan) (34).
Inhibition of DNA Methyltransferase mRNA

Short hairpin RNA (shRNA) sequences targeting \textit{Dnmt1, Dnmt3a,} or \textit{Dnmt3b} mRNA were designed using the shRNA Sequence Designer (www.clontech.com). Three shRNA sequences targeted against each of the DNA methyltransferase RNA sequences (Table S3) were inserted into the Xho1 and HindIII sites of pSingle-tTS-shRNA (Clontech, Mountain View, CA). Presence of inserts was determined by restriction digestion with MluI (Promega, Madison, WI). Transfection, selection of stably transformed cells, and induction of shRNA expression with doxycycline (Clontech) was as described (35). A scrambled sequence inserted into pSingle (35) was used as a control.

DNA Methyltransferase Activity Assay

Nuclear extracts were prepared from cells grown on 60 mm culture dishes in triplicate using an EpiQuik Nuclear Extraction Kit (Epigentek Group Inc.). DNA methyltransferase enzyme activity was assayed using a colorimetric EpiQuik DNMT Activity/Inhibition Assay Kit (Epigentek Group Inc.) according to the manufacturer’s instructions. Activity was expressed relative to nuclear extract protein that was measured using Bio-Rad Protein Dye Reagent (Bio-Rad, Hercules, CA).

Statistical Analyses

Data were analyzed by One-way ANOVA followed by Tukey’s post test or Two-way ANOVA followed by Bonferroni post test using GraphPad Prism software v. 4.0 (La Jolla, CA). Specific
tests used and comparisons made are indicated in the figure legends. P<0.05 was determined to be statistically significant.
RESULTS

Association of Pax3 CpG island methylation with Pax3 silencing in embryos and ESC

We have previously examined Pax3 expression by embryos on E 8.5, when Pax3 expression begins and the neural tube starts to form, from control pregnancies and from diabetic, transiently hyperglycemic, and oxidative stress-induced pregnancies (10; 12; 21). We hypothesized that cytosines within Pax3 regulatory elements were hypermethylated prior to the onset of Pax3 expression during embryogenesis, and that hyperglycemia-induced oxidative stress blocked differentiation-associated Pax3 hypomethylation. To test these hypotheses, we obtained embryos prior to the onset of Pax3 expression (E 3.5 blastocysts), and on E 8.5. The E 8.5 embryos were obtained from pregnant mice that had been injected with glucose to induce transient hyperglycemia, or antimycin A (AA) to induce oxidative stress, on E 7.5, or uninjected controls. We previously showed that oxidative stress induced by maternal diabetes on E 7.5 prevents normal Pax3 expression and leads to NTD (12; 21). To determine whether Pax3 is selectively regulated by hyperglycemia and oxidative stress, we assayed expression of two additional Pax genes and p53. Pax7 is a paralog of Pax3 whose spatial pattern overlaps that of Pax3 and whose expression begins slightly later than Pax3 (36). Unlike Pax3, Pax7 does not contain a promoter-proximal CpG island according to the Genome Browser on the University of California Santa Cruz Bioinformatics site. Pax6 is expressed in the ventral neural tube. Its dorsoventral expression restriction is inversely regulated to that of Pax3 by signals emanating from the notochord (36; 37). p53, like Pax3, contains a promoter-proximal CpG island, but unlike Pax3, p53 is regulated post-translationally, but not transcriptionally, by oxidative stress (38). Also, unlike Pax3, p53 mRNA does not change upon differentiation of ESC to neuroepithelial-like neuronal precursors (35). As expected, Pax3 expression significantly increased in E 8.5 embryos compared to E 3.5
blastocysts, and induction of hyperglycemia or oxidative stress on E 7.5 significantly inhibited Pax3 expression on E 8.5 (Figure 1 A). However, while expression of both Pax7 and Pax6 significantly increased between E 3.5 and E 8.5, there was no effect of hyperglycemia or oxidative stress on either Pax7 or Pax6 expression. p53 expression did not change between E 3.5 and 8.5 and was not inhibited by hyperglycemia or oxidative stress.

A 656 bp CpG island containing 49 CpG dinucleotides was identified near the Pax3 start site of transcription (-169 to +487) as described in the Research Design and Methods. This sequence overlaps an element (-1578 to +70) that is sufficient for a transgenic reporter plasmid expression in E 8.5 neuroepithelium (39). A 966 bp CpG island located upstream of and overlapping the transcriptions start site of the human PAX3 gene has 79% identity with the 656 bp mouse element, suggesting a conserved regulatory function. Two smaller CpG islands are located approximately 6.7 kb 5’ of the Pax3 coding sequence and within an intron approximately 7.3 kb 3’ of the start site of transcription, however, since neither of these elements were contained within the transgene that directed neuroepithelial expression (39), we focused on the 656 bp CpG island. A 329 bp CpG island with 29 CpG dinucleotides is located near the p53 promoter. Since p53 mRNA expression was not regulated developmentally or by hyperglycemia or metabolism, the p53 CpG island was used as a control during initial studies of the Pax3 CpG island.

5-methylcytosine genomic DNA from whole embryos was immunoprecipitated (mDIP) and then amplified by PCR with primers specific to the Pax3 or p53 CpG islands as described in Research Design and Methods. Significantly more of the Pax3 CpG island was immunoprecipitated from blastocyst DNA than from E 8.5 embryos (Figure 1 B), consistent with the hypothesis that
hypomethylation of this CpG island is involved in induction of Pax3 expression. However, there was no significant difference in Pax3 CpG island methylation in embryos from hyperglycemic or oxidative stress-induced pregnancies compared to control E 8.5 embryos. Consistent with the constant p53 mRNA expression in embryos of different developmental stages and regardless of exposure to oxidative stress, there was no difference in immunoprecipitated methylcytosine associated with the p53 CpG island from any of the embryos (Figure 1 C).

It is possible that no difference in Pax3 CpG island methylation was detected in E 8.5 embryos from hyperglycemic or oxidative stress-treated pregnancies, compared to control E 8.5 embryos, despite the significant inhibition of Pax3 expression, because Pax3 expression initiates on E 8.5, first in neuroepithelium and slightly later in somites (40), but Pax3 expression in somites does not appear to be inhibited by maternal diabetes or oxidative stress (10; 11). Thus, lack of effect of oxidative stress on methylation of the Pax3 CpG island in somites may obscure effects on the Pax3 CpG island in neuroepithelium. We then turned to murine ESC as a cell culture model that is more homogenous than the whole embryo. We previously showed that Pax3 is expressed upon induction of differentiation of neuronal precursors (resembling neuroepithelium) from undifferentiated monolayer cultures (derived from the blastocyst inner cell mass), and that Pax3 expression in ESC-derived neuronal precursors is inhibited by AA-induced oxidative stress (31; 35).

mRNA and DNA were obtained from undifferentiated (UD) or differentiating (D) ESC, or from D ESC in which oxidative stress had been induced with AA during differentiation. 5-azacytidine (AzaC), a DNA methyltransferase inhibitor, was added to UD and D cultures as a control. There
was a slight, but significant increase in Pax3 expression in UD ESC treated with AzaC (Figure 2 A), suggesting that silencing of Pax3 prior to its induction is partly due to DNA methylation. There was a significant increase in Pax3 expression in D ESC, which was further increased by AzaC. The increase in Pax3 expression in D ESC was significantly inhibited by AA. Pax3 CpG island methylation was inversely related to Pax3 expression in UD, UD + AzaC, and D ESC (Figure 2 B), suggesting that, as in embryos, induction of Pax3 expression is associated with hypomethylation of the Pax3 CpG island. There was no further decrease in methylation of the Pax3 CpG island in D ESC treated with AzaC, suggesting that the increase in Pax3 expression in D ESC treated with AzaC was due to inhibition of methylation of other genes whose expression contributes to Pax3 regulation. Notably, consistent with the hypothesis, methylation of the Pax3 CpG island was significantly increased in D ESC treated with AA compared to control D ESC. There was no significant effect of differentiation, AzaC, or AA on p53 mRNA levels or methylation of the p53 CpG island (Figure 2 C, D), suggesting that expression of p53 is not regulated by DNA methylation under these conditions.

To study localization as well as frequency of Pax3 CpG island methylation, genomic DNA from UD, D, or D ESC treated with AA was treated with sodium bisulfite. Bisulfite deaminates cytosine, converting it to uracil, but 5-methylcytosine is resistant to this reaction (41). Thus, following PCR amplification of bisulfite-modified DNA, substitutions of cytosines with thymines is indicative of unmethylated cytosines, and retention of cytosines is indicative of 5-methylcytosines. Following bisulfite modification, the Pax3 CpG island (between -194 to +510) was amplified by PCR as described in the Research Design and Methods. Ten colonies containing plasmids with CpG island fragments from each treatment group were sequenced, and
the sequences from the modified DNA were compared with the genomic sequence using the Quantification Tool for Methylation Analysis (QUMA) (34) (Figure 3 A). The percent conversion of CpG dinucleotides of the sequenced CpG island fragments was 97-100% as determined by QUMA. Notably, the mean percentage of methylated CpG dinucleotides significantly decreased between UD and D ESC (Figure 3 B). The locations of methylated CpG dinucleotides in D ESC treated with AA were similar to those in UD ESC and the mean percentage of methylated CpG dinucleotides in D ESC treated with AA was significantly greater than in D ESC (Figure 3 A, B).

Dnmt3b regulation of Pax3 expression and CpG island methylation during differentiation and oxidative stress

To determine which Dnmt(s) regulated Pax3 expression and CpG island methylation, we constructed doxycycline (Dox) –inducible shRNA plasmids containing 3 different shRNA sequences that targeted each of the Dnmt transcripts. ESC were stably transformed with empty plasmid, plasmid containing a scrambled sequence, or one of the plasmids containing a Dnmt shRNA sequence. As shown in Figure 4 A-C, abundance of each of the Dnmt transcripts was knocked down both in UD and D ESC upon treatment of cells with Dox, but only in the cells transfected with specific shRNA sequences. Induction of each shRNA also decreased steady state levels of each Dnmt protein (Figure S1). Inhibition of Dnmt mRNA levels by each of the shRNA sequences was specific for the intended target Dnmt transcript and had no effect on either of the other two Dnmt transcripts (data not shown). Notably, knocking down Dnmt1 or Dnmt3a had no effect on Pax3 mRNA in either UD or D ESC (Figure 4 D, E). However, there was an increase in Pax3 mRNA in D ESC, and a trend toward increasing Pax3 mRNA in UD
ESC, upon knocking down Dnmt3b mRNA (Figure 4 F). This indicates that Dnmt3b, but not Dnmt1 or Dnmt3a, directly or indirectly suppresses Pax3 expression.

To investigate whether Dnmt3b could mediate the inhibition of Pax3 expression and increased cytosine methylation in response to oxidative stress, the effects of knocking down Dnmt3b mRNA on AA-treated differentiating ESC were examined. As shown in Figure 5 A, hypermethylation of the Pax3 CpG island in D ESC in response to AA was blocked in cells treated with Dox, but only in cells transfected with Dnmt3b shRNA plasmid. Correspondingly, AA inhibited Pax3 expression in D ESC that were untransfected, and the inhibition of Pax3 expression by AA was blocked by treatment with Dox, but only in the cell lines transfected with plasmids containing Dnmt3b shRNA (Figure 5 B). As in Figure 4, Dox treatment increased Pax3 expression by D cultures not treated with AA, but only in cells transfected with plasmids containing Dnmt3b shRNA.

**Dnmt activity regulation by oxidative stress**

Increased Dnmt3b-mediated Pax3 CpG island methylation stimulated by oxidative stress could be due to increased Dnmt3b activity or increased Dnmt3b expression or both. To investigate whether Dnmt3b activity could be stimulated by oxidative stress, total Dnmt enzyme activity was assayed using nuclear extracts prepared from UD, D, or D ESC treated with AA. As shown in Figure 6 A, total Dnmt activity decreased upon ESC differentiation, and the effect of differentiation was inhibited by oxidative stress. However, when we examined mRNA levels of each of the DNA methyltransferases, we found that only expression of Dnmt3b decreased upon
differentiation, and that there was no effect of oxidative stress on expression of any of the *Dnmt* mRNAs (Figure 6 B). Although the Dnmt activity assay could not determine which DNA methyltransferase(s) was responsible for decreased Dnmt activity in the nuclear extracts from D ESC, only expression of *Dnmt3b* decreases with differentiation. Therefore, unless there are processes that regulate activity of any of the DNA methyltransferases during differentiation, the decreased abundance of Dnmt3b is sufficient to explain the decreased Dnmt activity upon differentiation. Moreover, while our results cannot exclude the possibilities that activities of Dnmt1 and/or Dnmt3a are stimulated by oxidative stress, because *Dnmt3b* expression is unaffected by oxidative stress, this indicates that the increased Dnmt3b-mediated *Pax3* CpG island methylation during oxidative stress is because Dnmt3b enzymatic activity is stimulated by oxidative stress.
DISCUSSION

*Pax3* is a gene whose expression in embryonic neuroepithelium and neural crest is essential for neural tube closure and cardiac outflow tract formation (13; 14). And yet, its regulation during normal embryonic development is poorly understood. It is expected that induction of *Pax3* expression in temporal- and tissue-specific fashion involves multiple coordinated processes, including induction and assembly of transcription factors and co-activators, modifications of histones by acetylation and methylation, and modification of cytosine methylation within the *Pax3* CpG island, or even other regulatory elements such as enhancers. However, which of these processes might be affected by excess glucose metabolism in embryos of diabetic mothers, thereby causing abnormal gene expression and congenital malformations, has not previously been reported. The data reported here indicate that hypermethylation of a *Pax3* CpG island by Dnmt3b contributes to *Pax3* silencing prior to induction of embryonic neuroepithelium and neural crest, and that oxidative stress stimulates Dnmt3b-mediated methylation of the *Pax3* CpG island, thereby preserving the methylated state of the same cytosines as in undifferentiated embryo cells. This, then, suppresses *Pax3* expression. A schematic diagram of the regulation of the *Pax3* CpG island during embryonic development and oxidative stress is shown in Figure 7.

Oxidative stress does not affect all gene expression regulating embryogenesis, as morphology of the neurulatung E 8.5 embryo is normal (10), and, as shown here, expression of *Pax7* and *Pax6*, which are also expressed in the neural tube beginning on E 8.5, is unaffected by oxidative stress. Rather, *Pax3* appears to be selectively regulated by oxidative stress resulting from excess glucose metabolism. Because knocking down *Dnmt3b* mRNA blocks both the hypermethylation of the *Pax3* CpG island and the inhibition of *Pax3* expression caused by oxidative stress, this
indicates that the CpG island surrounding the *Pax3* transcription start site is an oxidative stress-responsive regulatory element. This is not a characteristic of all CpG islands of embryo-expressed genes, as methylation of the *p53* CpG island (and *p53* expression) were unaffected by oxidative stress. This said, the responsiveness of the *Pax3* CpG island to oxidative stress seems to be limited to neuroepithelium and neural crest, as *Pax3* expression by somites is not inhibited by maternal diabetes (10;11), and hypermethylation of the *Pax3* CpG islands following hyperglycemia or oxidative stress was not observed in whole E 8.5 embryos, which contained a greater abundance of somitic progenitors than neuroepithelium and neural crest. The 1.6 kb element that is sufficient for *Pax3* expression in neuroepithelium and neural crest is not sufficient for *Pax3* expression in somites (39). Therefore, differential transcriptional regulation of *Pax3* in somites compared to neuroepithelium and neural crest is a likely explanation for the lack of effect of hyperglycemia and oxidative stress on *Pax3* expression in somites. Further investigation will be needed to understand the tissue-specific regulation of the *Pax3* CpG island by oxidative stress.

The mechanism by which the *Pax3* CpG island becomes hypomethylated during differentiation is not known. The CpG island could be passively demethylated, due to decreased methylation of daughter strands during DNA synthesis. This could be due to decreased expression of *Dnmt3b*, decreased activity of *Dnmt3b*, or other processes, such as histone modifications (24), that divert *Dnmt3b* from the *Pax3* CpG island. Alternatively, the CpG island could be actively demethylated, initiated by oxidation of 5-methylcytosine to 5-methylcytosine by the ten eleven translocation (TET) family of enzymes (42). The latter process could occur independent of DNA synthesis. Because embryo cells and embryonic stem cells are rapidly proliferating when they
begin to adopt a neuroepithelial cell fate, passive demethylation would seem the most likely mechanism. This is consistent with the decreased expression of Dnmt3b in differentiating ESC. If this is the case, stimulation of Dnmt3b activity by oxidative stress might increase Pax3 CpG island methylation of daughter strands. On the other hand, if demethylation is active, Dnmt3b might compete with a TET enzyme for binding to the Pax3 CpG island. Additional research is necessary in order to understand how the Pax3 CpG island becomes demethylated during differentiation, and how oxidative stress antagonizes this process.

We previously showed that Pax3 negatively regulates the p53 tumor suppressor protein by stimulating its degradation in neuronal precursors (35). This appears to be the sole Pax3 function that is required for neural tube and neural crest development (43; 44). We have speculated that Pax3 is regulated by the transition from predominantly glycolytic to increasingly aerobic metabolism that occurs as stem cells start to differentiate, so that it can titrate the abundance of p53, which promotes aerobic metabolism and terminal differentiation (9). Thus, oxidative stress resulting from excess glucose metabolism may disturb the metabolic cues that lead to Pax3 gene activation. We have also shown that increased embryo glucose metabolism, resulting from maternal hyperglycemia, causes embryo hypoxia, that embryo hypoxia induces oxidative stress, that oxidative stress stimulates activity of the enzyme, AMP-activated protein kinase (AMPK), and that resulting AMPK activity inhibits Pax3 expression (22; 31). Activation of enzymes, such as AMPK, that can translocate to the nucleus (45) and activate transcription factors and co-activators (46-48) can explain how fuel metabolism can regulate Pax3 expression. However, whether regulation of Pax3 by AMPK might be mediated by increased Dnmt3b activity still remains to be determined.
Others have shown that transient hyperglycemia causes persistent changes in histone methylation patterns that can explain “metabolic memory” despite normoglycemia (49; 50). It is intriguing to speculate that stimulation of Dnmt3b activity by transient hyperglycemia could also have long-lasting effects on cytosine methylation of cells involved in diabetic complications in general.

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**Author Contributions.** D.W. designed and performed the experiments. M.R.L. designed the experiments and wrote the manuscript. M.R.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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FIGURE LEGENDS

Figure 1. Embryo gene expression and CpG island methylation during differentiation from blastocysts to neurulating embryos and in response to maternal hyperglycemia or oxidative stress. A. RT-PCR of Pax3, Pax7, Pax6, or p53 mRNA normalized to rRNA from E 3.5 blastocysts or E 8.5 embryos. E 8.5 embryos were obtained from control pregnant mice or mice in which transient hyperglycemia had been induced on E 7.5 (Glucose) or in which oxidative stress had been induced with antimycin A (AA). mRNA was expressed relative to gene expression in control E 8.5 embryos. B. mDIP assay of the Pax3 CpG island from E 3.5 blastocysts or E.5 embryos from pregnancies treated as in A. C. mDIP assay of the p53 CpG island from E 3.5 blastocysts or E.5 embryos from pregnancies treated as in A. In B and C, immunoprecipitated DNA was normalized to total DNA before immunoprecipitation (input) and expressed relative to immunoprecipitated DNA from E 8.5 embryos. (n = 3 pools of blastocysts from 18 separate pregnancies or pooled embryos from 3 separate E 8.5 pregnancies. Data were analyzed by One-way ANOVA followed by Tukey’s post test. **, P<0.01; ***, P<0.001.)

Figure 2. Gene expression and CpG island methylation during differentiation of ESC to neuronal precursors and during oxidative stress. A. RT-PCR of Pax3 mRNA normalized to rRNA from undifferentiated (UD) ESC or ESC induced to differentiate into neuronal precursors (D), treated or not with 5-azacytidine (AzaC) or AA. B. mDIP assay of the Pax3 CpG island from cultures treated as in A. C. RT-PCR of p53 mRNA normalized to rRNA from the same cultures as in A. D. mDIP assay of the p53 CpG island from the same cultures as in B. (For A and C, n = 4 culture dishes. For B and D, n = 3 culture dishes pooled, assayed in quadruplicate. Data were analyzed by One-way ANOVA followed by Tukey’s post test. *, P<0.05; **, P<0.01; ***, P<0.001.)
**Figure 3.** A. Bisulfite sequencing of genomic DNA (-194 to +510, relative to the *Pax3* transcription start site) from UD ESC, or D ESC that had been treated or not with AA. Methylated CpG’s are shown as closed circles and unmethylated CpG’s are shown as open circles. B. The mean percent methylated CpG’s determined from 10 sequenced clones from each treatment group were analyzed by One-way ANOVA followed by Tukey’s post test. ***, P<0.001.**

**Figure 4.** RT-PCR of RNA from ESC stably transfected with Doxycycline (Dox)-inducible plasmids expressing shRNA targeting *Dnmt1* (A, D), *Dnmt3a* (B, E), or *Dnmt3b* (C, F). Cells were untransfected (Control), transfected with empty pSingle plasmid (Vector), pSingle containing a scrambled shRNA sequence (Scrambled), or pSingle containing one of three different shRNA sequences targeting each of the DNA methyltransferases (-sh-1, -sh-2, -sh-3). RNA was assayed from UD or D ESC that had been treated or not with Dox. *Dnmt* mRNA was normalized to *rRNA* and expressed relative to control UD ESC. *Pax3* mRNA was normalized to *rRNA* and expressed relative to control D ESC. A. *Dnmt1* expression by cells transfected with Dnmt1 shRNA plasmids or controls. B. *Dnmt3a* expression by cells transfected with Dnmt3a shRNA plasmids or controls. C. *Dnmt3b* expression by cells transfected with Dnmt3b shRNA plasmids or controls. D. *Pax3* expression by cells transfected with *Dnmt1* shRNA plasmids or controls. E. *Pax3* expression by cells transfected with shRNA plasmids to knock down *Dnmt3a* mRNA or controls. F. *Pax3* expression by cells transfected with shRNA plasmids to knock down *Dnmt3b* mRNA or controls. (n = 4 replicate culture dishes. UD = open bars; UD + Dox = right diagonal hatched bars; D = solid bars; D + Dox = left diagonal hatched bars. Data from each
transformed cell line were analyzed individually by One-way ANOVA followed by Tukey’s post test. ***, P<0.001.)

Figure 5. A. RT-PCR of Pax3 mRNA normalized to rRNA from ESC stably transfected with shRNA plasmids targeting only Dnmt3b mRNA. Differentiating cultures were treated or not with AA to induce oxidative stress and treated or not with Dox to induce shRNA expression. B. mDIP assay of ESC that were cultured along side the cultures used in A. (Numbers of culture plates used for RT-PCR and mDIP assays are the same as in Fig. 2. Data from untransfected control cultures (UD, D, D + AA) were analyzed by One-way ANOVA and Tukey’s post test. In panel A, Pax3 CpG island methylation was significantly different (P<0.001) in all treatment groups. In panel B, Pax3 expression was significantly different between UD and D, and between D and D + AA cultures (P<0.001), but was not different between UD and D + AA cultures (P>0.05). Significant differences from One-way ANOVA are not indicated in the figure.) Data from shRNA transfected cells were analyzed by Two-way ANOVA (treatment group vs. Dox administration) followed by Bonferroni post test to determine which cultures were affected by Dox treatment. Significant differences between – Dox (open bars) and + Dox (closed bars) in each treatment group are indicated. (***, P<0.001)). Diff: differentiated state (U, undifferentiated, D, differentiating); AA: antimycin A added (+) or not (-) to differentiating neuronal precursor cultures; shRNA: untransfected (-), empty vector (V), scrambled shRNA (Sc), Dnmt3b-sh-1 (1), Dnmt3b-sh-2 (2), Dnmt3b-sh-3 (3).

Figure 6. A. Total Dnmt activity using nuclear extracts from UD ESC or D ESC treated or not with AA. Dnmt activity was performed using a colorimetric assay kit as described in Research
Design and Methods and is expressed as O.D./mg protein per hour. (n = 3 culture dishes. **P<0.01; ***P<0.001). B. RT-PCR of Dnmt1, Dnmt3a, or Dnmt3b, normalized to rRNA from UD ESC, or D ESC treated or not with AA. ***, P<0.001).

Figure 7. Schematic diagram of regulation of Pax3 CpG methylation and Pax3 expression. A. In embryonic cells that do not express Pax3 (blastocysts and undifferentiated ESC), the Pax3 CpG island surrounding the Pax3 transcription start site is hypermethylated, which contributes to gene silencing. B. During normal differentiation (of E 8.5 embryos and ESC induced to form neuronal precursors), decreased Dmnt3b expression contributes to decreased Pax3 CpG island methylation and increased Pax3 expression. C. During differentiation under conditions of oxidative stress, increased reactive oxygen species (ROS) stimulates Dnmt3b activity, which preserves Pax3 CpG island methylation and suppresses Pax3 expression.
Figure 1. Embryo gene expression and CpG island methylation during differentiation from blastocysts to neurulating embryos and in response to maternal hyperglycemia or oxidative stress. A. RT-PCR of Pax3, Pax7, Pax6, or p53 mRNA normalized to rRNA from E 3.5 blastocysts or E 8.5 embryos. E 8.5 embryos were obtained from control pregnant mice or mice in which transient hyperglycemia had been induced on E 7.5 (Glucose) or in which oxidative stress had been induced with antimycin A (AA). mRNA was expressed relative to gene expression in control E 8.5 embryos. B. mDIP assay of the Pax3 CpG island from E 3.5 blastocysts or E 8.5 embryos from pregnancies treated as in A. C. mDIP assay of the p53 CpG island from E 3.5 blastocysts or E 8.5 embryos from pregnancies treated as in A. In B and C, immunoprecipitated DNA was normalized to total DNA before immunoprecipitation (input) and expressed relative to immunoprecipitated DNA from E 8.5 embryos. (n = 3 pools of blastocysts from 18 separate pregnancies or pooled embryos from 3 separate E 8.5 pregnancies. Data were analyzed by One-way ANOVA followed by Tukey’s post test. **, P<0.01; ***, P<0.001.)

108x65mm (300 x 300 DPI)
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95x51mm (300 x 300 DPI)
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SUPPLEMENTAL MATERIALS FOR:

Increased DNA Methyltransferase 3b (Dnmt3b) –mediated CpG Island Methylation Stimulated by Oxidative Stress Inhibits Expression of a Gene Required for Neural Tube and Neural Crest Development in Diabetic Pregnancy

By Dan Wei and Mary R. Loeken

SUPPLEMENTAL RESEARCH DESIGN AND MATERIALS

Immunoblot of Dnmt1, Dnmt3a, and Dnmt3b

One plate per treatment group was grown in parallel with the 4 plates that were used for assay of RNA by RT-PCR (Figure 4 in the main text) and was extracted for protein. Twenty µg protein from each culture dish were resolved by SDS-polyacrylamide electrophoresis and proteins were detected by immunoblot as described (1). Antibodies against Dnmt1 (ab 13837), Dnmt3a (ab 13888), and Dnmt3b (ab 16049) were obtained from Abcam (Cambridge, MA) and were used at a 1:1000 dilution. Horse radish peroxidase-coupled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used to detect the primary antibodies bound to Dnmt1 and Dnmt3a, and horse radish peroxidase donkey anti-rabbit IgG (GE Healthcare Life Sciences, Pittsburgh, PA) was used to detect the primary antibodies bound to Dnmt3b. Both secondary antibodies were used at a 1:4000 dilution.

REFERENCES

Supplemental Figure Legend

Figure S1. Immunoblot of representative culture dishes of cells transfected with shRNA plasmids or untransfected (Control), and treated or not with Dox to induce shRNA expression, as in Figure 4 in the main text.
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103x91mm (300 x 300 DPI)
Table S1. Primer sequences and PCR conditions for Pax3 and p53 CpG Islands

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>PCR Condition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
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</tbody>
</table>
| Pax3 | Forward: 5'-AAAGCGAGCACAGTGCAGCG-3'  
Reverse: 5'-GGCCGATCTGCTAGACTCGCAC-3' | 95°C, 15 sec | 62.5°C, 30 sec | 72°C, 30 sec | 44 |
| p53  | Forward: 5'-CAGAGGTCGCGCAAGTCTCGC-3'  
Reverse: 5'-GCCTTCCCCGCTCGGAAATCGGAG-3' | 95°C, 15 sec | 62.5°C, 30 sec | 72°C, 30 sec | 44 |
Table S2. Primers used for PCR of the Pax3 CpG island after Bisulfite modification

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<td>5'-AGCTTACGCGTAAAAAGGAAGAAGTGACACCTAAGTTCAAGAGACTTAGGTGTCACTTCTCTCTCCC-3’</td>
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