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

Preferably, 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 before 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 short hairpin RNA in ESC demonstrated that DNA methyltransferase 3b (Dnmt3b) was responsible for methylation and silencing of Pax3 before differentiation and by oxidative stress. Although 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.

Maternal pregestational diabetes significantly increases the risk for congenital malformations (1–6). Although 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 8 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 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 (UD) 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. Although 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).

Three known enzymes 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 before 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 Joslin Diabetes Center Institutional Animal Care and Use Committee. 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 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 (AA; Sigma-Aldrich, St. Louis, MO), a dose that 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 ESC of the D3 line were cultured and induced to differentiate into neuronal precursors, as previously described (31). Briefly, ESC were grown as UD monolayer cultures in DMEM (Life Technologies, Grand Island, NY) containing leukocyte inhibitory factor (Millipore, Billerica, MA) for 4 days, then differentiation was induced by forming embryoid bodies in nonadherent culture dishes in media without leukocyte inhibitory factor but containing 0.5 μmol/L retinoic acid (Sigma-Aldrich) 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 Dickinson), insulin, transferrin, and selenium (all from Sigma-Aldrich) for 4 additional days to select for differentiating neuronal precursors.

Oxidative stress was induced by adding 10 μmol/L AA to the media used during selection of neuronal precursors, as described (31). This concentration of AA has been shown to significantly increase markers of oxidative stress and to inhibit Pax3 expression by D3 ESC (31,32). A total of 10 μmol/L of the DNA methyltransferase inhibitor, 5-azacytidine (AzaC, Sigma-Aldrich), was added to the media while culturing UD ESC or while selection for neuronal precursors.

**RT-PCR Assays**

E 3.5 blastocysts were recovered from 18 pregnant mice, and three to four blastocysts from six litters were pooled for three separate RT-PCR assays. E 8.5 embryos were recovered from three separate litters per treatment group, and embryos from each litter were pooled for RT-PCR assay. Four 60-mm culture dishes of UD 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 (Biotec Laboratories, Friendswood, TX). The High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Foster City, CA) was used to reverse transcribe 200 ng RNA. Real-time PCR was performed using TaqMan PCR Master Mix (Life Technologies) and primers, and a VIC-labeled probe was used 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 Assays
E 3.5 blastocysts were recovered from 18 pregnant mice, and six to nine blastocysts from six litters were pooled for three separate 5-methylcytosine immunoprecipitation–DNA immunoprecipitation (mDIP) assays. E 8.5 embryos were recovered from three separate litters per treatment group, and embryos from each litter were pooled for mDIP assay. Three 60-mm culture dishes of UD 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 four cycles of 70% duty, 20% output, 10 pulses/cycle on ice to generate fragments of ~300–1,000 bp in length. Sonicated DNA (4 μg) was immunoprecipitated using 10 μL 5-methylcytosine antibody (Active Motif, Carlsbad, CA). After Proteinase K (Life Technologies) treatment, phenol chloroform extraction, and ethanol precipitation, the immunoprecipitated DNA was resuspended in 30 μL Tris-EDTA buffer. Immunoprecipitated DNA (1 μL) was amplified by PCR using SYBR green detection (Life Technologies), in quadruplicate, in a 10 μL final volume. Unimmunoprecipitated DNA (20 ng; 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 listed in Supplementary Table 1. Pax3 and p53 CpG islands were chosen using the Genome Browser on the University of California Santa Cruz Bioinformatics site (http://genome.ucsc.edu). PCR primers were designed using the National Center for Biotechnology Information 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 listed in Supplementary Table 2. All PCR reactions were performed using 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 8 s. The PCR products were inserted into a TA cloning vector (Life Technologies) and used to transform competent DH5-α Escherichia 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 (QUMA) (http://quma.cdb.riken.jp, Kyoto, Japan) (34).

Inhibition of DNA Methyltransferase mRNA
Short hairpin RNA (shRNA) sequences targeting Dnmt1, Dnmt3a, or Dnmt3b mRNA were designed using the shRNA Sequence Designer (www.clontech.com). Three shRNA sequences targeted against each of the DNA methyltransferase RNA sequences (Supplementary Table 3) were inserted into the Xho1 and HindIII sites of pSingleTS-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 (Dox; 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 the Tukey post hoc test or two-way ANOVA, followed by Bonferroni post test, using GraphPad Prism v. 4.0 software (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 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 before 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 before 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 with AA to induce oxidative stress, on E 7.5, or from un.injected 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 posttranslationally, 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 with E 3.5 blastocysts, and induction of hyperglycemia or oxidative stress on E 7.5 significantly inhibited **Pax3** expression on E 8.5 (Fig. 1A). However, although expression of **Pax7** and **Pax6** significantly increased between E 3.5 and E 8.5, there was no effect of hyperglycemia or oxidative stress on **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 **RESEARCH DESIGN AND METHODS**. This sequence overlaps an element (−1,578 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 −6.7 kb 5′ of the **Pax3** coding sequence and within an intron −7.3 kb 3′ of the start site of transcription; however, because neither element was 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. Because **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.

![Figure 1](image-url)

**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 with glucose or in which oxidative stress had been induced with 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. 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). The error bars indicate the standard error. Data were analyzed by one-way ANOVA, followed by the Tukey post test. **P < 0.01; ***P < 0.001.
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 (Fig. 1B), consistent with the hypothesis that hypomethylation of this CpG island is involved in induction of *Pax3* expression. However, *Pax3* CpG island methylation in embryos from hyperglycemic or oxidative stress-induced pregnancies did not differ significantly compared with 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 (Fig. 1C).

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 with 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 UD monolayer cultures (derived from the blastocyst ICM), and that *Pax3* expression in ESC-derived neuronal precursors is inhibited by AA-induced oxidative stress (31,35).

mRNA and DNA were obtained from UD or differentiating (D) ESC, or from D ESC in which oxidative stress had been induced with AA during differentiation. 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 (Fig. 2A), suggesting that silencing of *Pax3* before 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. AA significantly inhibited the increase in *Pax3* expression in D ESC. *Pax3* CpG island

![Figure 2](image)

**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 UD ESC or ESC induced to differentiate into neuronal precursors (D), treated or not with 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 and assayed in quadruplicate.) The error bars indicate the standard error. Data were analyzed by one-way ANOVA, followed by the Tukey post test. *P < 0.05; **P < 0.01; ***P < 0.001.
methylation was inversely related to Pax3 expression in UD, UD + AzaC, and D ESC (Fig. 2B), 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 with control D ESC. There was no significant effect of differentiation, AzaC, or AA on p53 mRNA levels or methylation of the p53 CpG island (Fig. 2C and 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, after 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. After bisulfite modification, the Pax3 CpG island (between −194 and 510) was amplified by PCR, as described in 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 QUMA (34) (Fig. 3A). 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 (Fig. 3B). 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 (Fig. 3A and 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 Dox-inducible shRNA plasmids containing three 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 Fig. 4A–C, abundance of each of the Dnmt transcripts was knocked down both in UD and in 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 (Supplementary

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**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 CpGs, ●; unmethylated CpGs, ○). B: The mean percentage of methylated CpGs (mCpGs) determined from 10 sequenced clones from each treatment group were analyzed by one-way ANOVA, followed by the Tukey post test. The error bars indicate the standard error. ***P < 0.001.
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 UD or D ESC (Fig. 4D and 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 (Fig. 4F). 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 Fig. 5A,
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 the 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 (Fig. 5B). As in Fig. 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 Fig. 6A, 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 oxidative stress had no effect on expression of any of the Dnmt mRNAs (Fig. 6B). 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, although our results cannot exclude the possibilities that activities of Dnmt1 and/or Dnmt5a 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 coactivators, 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 before 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 UD 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 Fig. 7.

Oxidative stress does not affect all gene expression regulating embryogenesis, because morphology of the neurulating 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 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, because methylation of the p53 CpG island and p53 expression were unaffected by oxidative stress. This said, the responsiveness of the Pax3 CpG island to

**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 expressed as optical density/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. The error bars indicate the standard error. ***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 UD 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 Dnmt3b 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.
oxidative stress seems to be limited to neuroepithelium and neural crest, because Pax3 expression by somites is not inhibited by maternal diabetes (10,11), and hypermethylation of the Pax3 Cpg islands after hyperglycemia or oxidative stress was not observed in whole E 8.5 embryos, which contained a greater abundance of somatic 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 with 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 caused by 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-hydroxymethylcytosine by the ten-eleven translocation family of enzymes (42). The latter process could occur independent of DNA synthesis. Because embryo cells and ESC 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. However, if demethylation is active, Dnmt3b might compete with a ten-eleven translocation enzyme for binding to the Pax3 Cpg island. Additional research is necessary 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 AMPK, and that resulting AMPK activity inhibits Pax3 expression (22,31). Activation of enzymes, such as AMPK, which can translocate to the nucleus (45) and activate transcription factors and coactivators (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 diabetes 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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