

# Prenatal nicotine exposure leads to decreased histone H3 lysine 9 (H3K9) methylation and increased *p66shc* expression in the neonatal pancreas

## Brief Report

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## Abstract

Prenatal exposure to nicotine, tobacco's major addictive constituent, has been shown to reduce birth weight and increases apoptosis, oxidative stress, and mitochondrial dysfunction in the postnatal pancreas. Given that upregulated levels of the pro-oxidative adaptor protein *p66shc* is observed in growth-restricted offspring and is linked to beta-cell apoptosis, the goal of this study was to investigate whether alterations in *p66shc* expression underlie the pancreatic deficits in nicotine-exposed offspring. Maternal administration of nicotine in rats increased *p66shc* expression in the neonatal pancreas. Similarly, nicotine treatment augmented *p66shc* expression in INS-1E pancreatic beta cells. Increased *p66shc* expression was also associated with decreased histone H3 lysine 9 methylation. Finally, nicotine increased the expression of *Kdm4c*, a key histone lysine demethylase, and decreased *Suv39h1*, a critical histone lysine methyltransferase. Collectively, these results suggest that upregulation of *p66shc* through posttranslational histone modifications may underlie the reported adverse outcomes of nicotine exposure on pancreatic function.

## Introduction

The global prevalence of type 2 diabetes (T2D) has been increasing rapidly and is now one of the major public health challenges worldwide. While it has been thought that the development of T2D is a result of aging, genetics, and lifestyle factors, it is increasingly accepted that T2D susceptibility is established *in utero*.<sup>1</sup> Moreover, early life exposures to chemical insults may also play a key role in the development of T2D in postnatal life.<sup>2</sup> There is evidence to suggest that exposure to nicotine during pregnancy increases the risk of metabolic disorders, including T2D, in the offspring.<sup>3</sup>

T2D is characterized by peripheral insulin resistance and beta-cell dysfunction. Furthermore, in rodents, early life exposure to nicotine leads to decreased beta-cell mass, increased beta-cell apoptosis, and impaired glucose homeostasis similar to what is observed in T2D.<sup>3</sup> Interestingly, several studies have implicated *p66shc*, a member of the ShcA (Src homologous-collagen homolog) adaptor protein family, in mitochondrial dysfunction, oxidative stress, and apoptosis.<sup>4</sup> Specifically, activation of *p66shc* has been shown to lead to reactive oxygen species (ROS) production and mitochondrial-induced apoptosis,<sup>4</sup> comparable to what has been reported in the pancreas following perinatal exposure to nicotine.<sup>5</sup>

There are several lines of evidence to suggest that the pancreatic deficits reported in nicotine-exposed offspring may be mediated via *p66shc*. First, Oke *et al*<sup>6</sup> have reported increased *p66shc* expression in animals with growth restriction followed by postnatal catch-up growth; nicotine-exposed animals exhibit a similar growth trajectory.<sup>7</sup> Second, there is evidence that *p66shc* can directly influence beta-cell apoptosis; overexpression of *p66shc* in INS-1E cells enhanced glucose and palmitate-stimulated apoptosis, an effect that was partially blocked by transfection with *p66shc* siRNA.<sup>8</sup> In addition, activation of p66Shc in INS-1E cells increased mitochondrial ROS production, a key player in beta-cell apoptosis.<sup>9</sup> Finally, studies have shown that exposure to nicotine alone *in vivo*<sup>10</sup> or as a component of cigarette smoke *in vitro*<sup>11</sup> can increase the expression of *p66shc* in the kidney and in airway epithelial cells, respectively. The goal of this study was to examine the effects of nicotine *in vivo* and *in vitro* on pancreatic *p66shc* expression. Furthermore, as *p66shc* expression has been demonstrated to be influenced by posttranslational histone modifications<sup>12</sup> and nicotine has been shown to increase the transcription of *p66shc* via epigenetic mechanisms,<sup>10</sup> we also examined the effect of nicotine on epigenetic histone modifications including markers of both histone demethylation (lysine demethylase 4A (*Kdm4a*))

and 4C (*Kdm4c*) and histone methylation (suppressor of variegation 3–9 homolog 1 (*Suv39h1*) and 2 (*Suv39h2*)) and ASH1-like histone lysine methyltransferase (*Ash1l*) in the neonatal pancreas.

## Methods

### Animal maintenance and treatment

All procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care. Nulliparous 200–250 g female Wistar rats (Envigo, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Two weeks prior to mating, the dams were randomly assigned to receive either saline (vehicle) or nicotine. Dams ( $N = 10$  per group) were injected with saline or 1.0 mg/kg/day nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) subcutaneously for 14 d prior to mating, and during pregnancy. Dams were allowed to deliver normally, and pups were euthanized at postnatal day 1 (PND1) for pancreas tissue collection. The dose of nicotine used in this animal model results in maternal serum cotinine concentrations of 136 ng/ml,<sup>13</sup> which is within the range of cotinine levels reported in women who are considered “moderate smokers” (80–163 ng/ml),<sup>14</sup> and serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth,<sup>13</sup> which is also within the range (5–30 ng/ml) observed in infants nursed by smoking mothers.<sup>15</sup> Pancreas tissue was collected from two randomly selected pups per litter, one for RNA isolation and the other for histone extraction and quantification. Tissues were excised, flash frozen, and stored at  $-80^{\circ}\text{C}$ . Total RNA was then isolated and extracted using TRIzol reagent (Invitrogen, Carlsbad, CA).

### Cell culture maintenance and treatment

INS-1E rat insulinoma pancreatic beta cells (generously provided by Dr. Claes Wollheim, University of Geneva, Geneva, Switzerland) were cultured in RPMI-1640 media containing 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2 mM L-glutamine (RPMI; Corning, Manassas, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 50  $\mu\text{M}$  2-mercaptoethanol (EMD Millipore, Etobicoke, ON), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (Gibco) at  $37^{\circ}\text{C}$  in a humidified atmosphere of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

Cells were treated for 48 h with 0 (vehicle control) and 1  $\mu\text{M}$  nicotine bitartrate (Sigma-Aldrich) diluted in supplemented RPMI as described above. This concentration was selected based on prior work showing impaired insulin secretion and mitochondrial dysfunction in INS-1E cells at this dose.<sup>16,17</sup> After 48 h, cells were washed with phosphate-buffered saline and total RNA was then isolated and extracted ( $N = 7$ –8 independent experiments) using TRIzol reagent (Invitrogen). Additional cells were collected and frozen at  $-80^{\circ}\text{C}$  for histone extraction and quantification ( $N = 5$ –7 independent experiments).

### RNA isolation and real-time quantitative polymerase chain reaction (qPCR)

Total RNA concentration from PND1 pancreas tissue and INS-1E cells was determined using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized from 2  $\mu\text{g}$  of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as

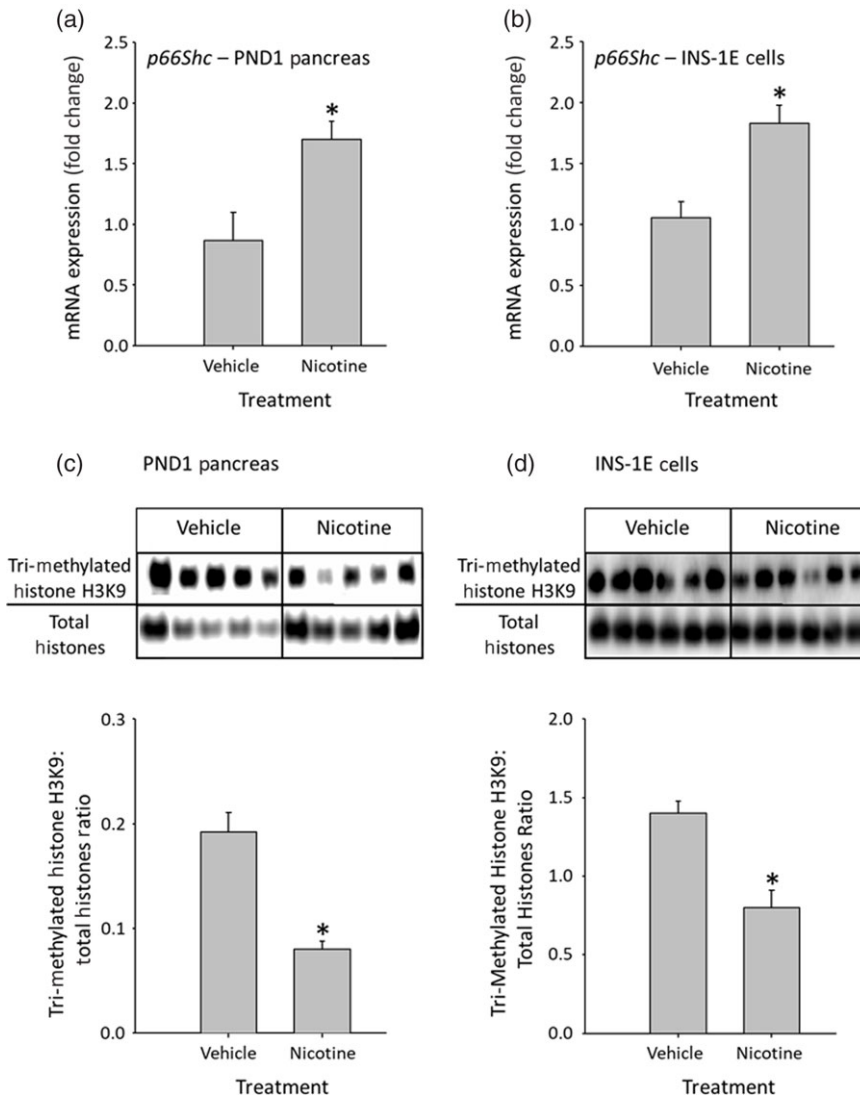
per the manufacturer’s instructions. The resulting cDNA was used as a template for qPCR, which was performed using PerfeCTa<sup>®</sup> SYBR<sup>®</sup> Green FastMix<sup>®</sup> (Quanta Biosciences, Gaithersburg, MD) on the LightCycler<sup>®</sup> 480 (Roche, Mississauga, ON). To explore expression of *p66shc* and methylation drivers in histone H3 lysine 9 (H3K9) methylation, we quantified *p66shc* and markers of both histone demethylation and histone methylation. For genes involved in H3K9 demethylation, we selected lysine demethylase 4A (*Kdm4a*) and 4C (*Kdm4c*).<sup>18,19</sup> For genes involved in H3K9 methylation, we selected suppressor of variegation 3–9 homolog 1 (*Suv39h1*)<sup>20</sup> and 2 (*Suv39h2*)<sup>21</sup> and ASH1-like histone lysine methyltransferase (*Ash1l*).<sup>22</sup> The primers were (listed as gene, forward and reverse sequence): *p66shc*, 5'-GTCCGACTACCCTGTGTTCTT-3' and 5'-CAGCAGGATTGGCCAGCTT-3'; *Kdm4a*, 5'-CTTTAGGCTCCAGGTTGTTCTT-3' and 5'-TCATCATAGGAGTTTCGAGGC-3'; *Kdm4c*, 5'-GGTGGAGAGTCCCCTAAATCC-3' and 5'-GAACTCCCGAAA-CTCCTCCAT-3'; *Suv39h1*, 5'-TTTCTGTGTACCCCTCTCAGC-3' and 5'-GAGCAACCCAGTTAACCTCT-3'; *Suv39h2*, 5'-AACCTTGATACTCGGCTTCCC-3' and 5'-TGGCAGGGCTGTAGTCAATAG-3'; and *Ash1l*, 5'-ACAGACCCAAAGAGCCGATT-3' and 5'-CAACGTAGACATCAACATGAATGG-3'. The cycling conditions included polymerase activation ( $95^{\circ}\text{C}$  for 10 min), followed by 40 cycles of denaturing ( $95^{\circ}\text{C}$  for 15 s) and annealing/elongation ( $60^{\circ}\text{C}$  for 1 min). Levels of gene expression were calculated using the  $\Delta\Delta\text{Ct}$  method<sup>23</sup> and normalized using the geometric means of two reference genes: hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) (5'-GCAGTACAGCC-CCAAAATGG-3' and 5'-GGTCCCTTTTCACCAGCAAGCT-3') and peptidylprolyl isomerase A (*Ppia*) (5'-CCGCTGTCTCTTTTCGCC-3' and 5'-GCTGTCTTTGGAACCTTTGCTCTGC-3').

### Acid extraction of histones

Pancreas tissue and INS-1E cell pellets were homogenized with lysis buffer (10 mM HEPES pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, and protease inhibitor tablets; 1:5 ratio tissue pellet:lysis buffer) and HCl added to a final concentration of 0.2 M. Samples were placed on a rocker at  $4^{\circ}\text{C}$  for 1 h and then centrifuged at 11,000g for 10 min in an Eppendorf 5417R centrifuge at  $4^{\circ}\text{C}$ . The acid-insoluble pellet was discarded and the supernatant (approximately 500  $\mu\text{l}$ ) was dialyzed in 200 ml of dialysis buffer using the Pierce Slide-A-Lyzer<sup>®</sup> MINI dialysis units (3500 MW cut-off) in the following manner at  $4^{\circ}\text{C}$  on a rocker: twice in 0.1 M acetic acid for 1 h, once in water for 1 h, once in water for 3 h, and in water overnight. The samples were then aliquoted into separate tubes and stored at  $-20^{\circ}\text{C}$ .

### Histone quantification and immunoblotting

Protein loading samples were prepared utilizing NuPAGE lithium dodecyl sulfate Sample Buffer NuPAGE Sample Reducing Agent (Invitrogen). Loading amounts were 10–15  $\mu\text{g}$  for histone extracts. Samples were run on NuPage 4%–11.5% Bis-Tris gels (Invitrogen) in 2-(N-morpholino)ethanesulfonic acid (MES) - sodium dodecyl sulphate (SDS) running buffer at 150 V for 1–2 h. After transfer to nitrocellulose membranes, membranes were blocked for 2 h in 5% non-fat dried milk in 0.1% tris buffered saline with Tween 20 (TBST). Membranes were incubated with antibodies targeted against total nuclear histone H3 (EMD Millipore, 05-499), and trimethylated histone H3 (lysine 9, EMD Millipore, 07-422). After washing in 0.1% TBST, membranes were incubated for 2 h with



**Fig. 1.** Effect of nicotine exposure on *p66shc* mRNA in (a) PND1 pancreas and (b) INS-1E cells. Effect of nicotine exposure on H3K9 methylation (involved in chromatin silencing) in (c) PND1 pancreas and in (d) INS-1E cells. Representative histone blots are presented above bar graphs showing the quantification of blot intensity. Data are represented as mean  $\pm$  SEM,  $N=4-9$  animals per group (PND1 pancreas) or 5–8 independent experiments (INS-1E cells). Values with asterisks (\*) are significantly different to control,  $P < 0.05$ .

the appropriate secondary antibody (1:10,000 dilution). Bands were normalized to appropriate reference proteins. Beta-actin was probed with antibody (Sigma-Aldrich) for a 2-h room temperature incubation. All bands were detected with a SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) and imaged with a VersaDoc Imaging System (Bio-Rad). Densitometric analysis on blots was performed with Quantity One Software (Bio-Rad). Data were normalized to reference proteins (beta-actin for cytoplasmic proteins, or total histone H3 for nuclear proteins) by applying stripping buffer (200 mM glycine, 0.1% sodium dodecyl sulfate, and 1% Tween 20) for 10 min, and then the detection protocol was implemented as above.

### Statistical analysis

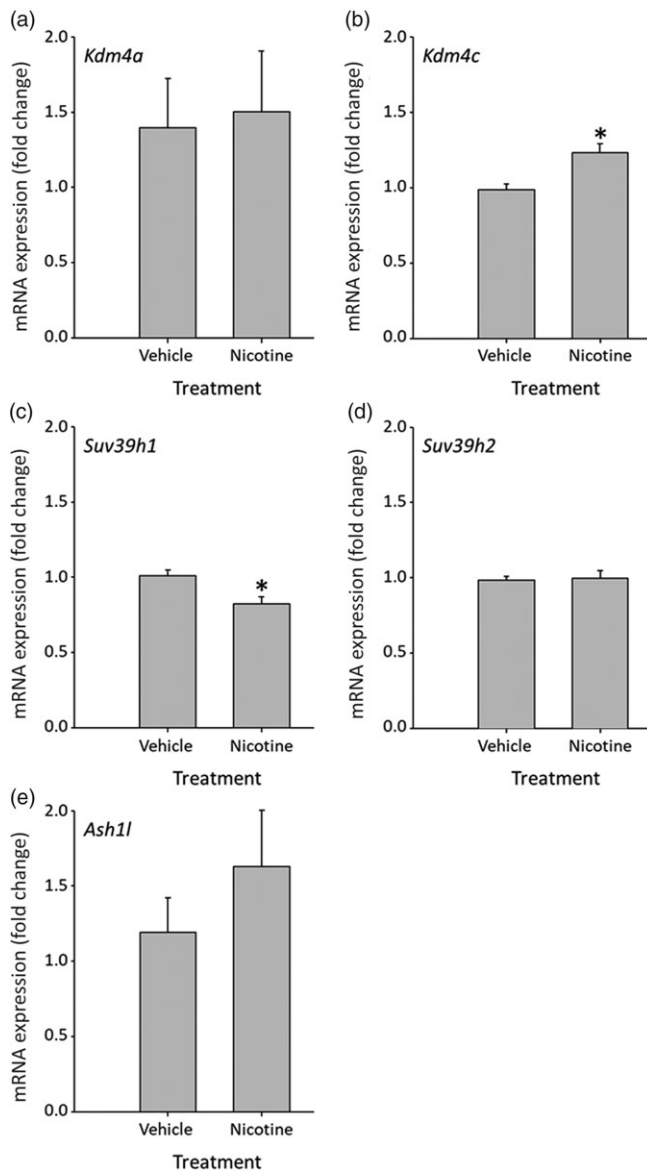
All statistical analyses were performed using SigmaPlot (v.11.2, Systat Software, San Jose, CA). Data were tested for outliers (Grubbs' test), normality, and equal variance. Means were compared between control and nicotine groups using Student's *t*-test. Data that failed normality and/or equal variance were analyzed by using the Mann–Whitney rank sum test. Values are presented as mean  $\pm$  standard error of the mean (SEM).

### Results

In PND1 nicotine-exposed rat offspring, steady-state mRNA levels of pancreatic *p66shc* were increased compared to control offspring (Fig. 1a). Similarly, nicotine exposure significantly increased *p66shc* mRNA in INS-1E cells (Fig. 1b).

Given that exposure to nicotine *in utero* has been demonstrated to lead to posttranslational histone H3 modifications in other organs including decreased H3K9 methylation in the brain,<sup>24</sup> and that nicotine has been shown to increase the transcription of *p66shc* via epigenetic mechanisms *in vitro*,<sup>10</sup> we next investigated if alterations in pancreatic *p66shc* expression were associated with the methylation of histone H3K9, which is involved in chromatin silencing. Nicotine treatment significantly decreased histone H3K9 methylation in PND1 pancreas (Fig. 1c) and INS-1E cells (Fig. 1d).

To elucidate which specific lysine methylases or lysine demethylases might mediate the decrease in histone H3K9 methylation in the PND1 pancreas, we next measured the expression of key histone methylation modifying enzymes. While the steady-state mRNA levels of lysine demethylase *Kdm4a* were unchanged in the PND1 nicotine-exposed pancreas (Fig. 2a), the mRNA expression of the lysine demethylase *Kdm4c* was significantly increased versus control



**Fig. 2.** Effect of prenatal nicotine exposure on expression of (a) *Kdm4a* mRNA (a histone lysine demethylase), (b) *Kdm4c* mRNA (a histone lysine demethylase), (c) *Suv39h1* mRNA (a histone lysine methyltransferase), (d) *Suv39h2* mRNA (a histone lysine methyltransferase), and (e) *Ash1l* mRNA (a histone lysine methyltransferase) in PND1 pancreas. Data represented as mean  $\pm$  SEM,  $N = 7-10$ . Values with asterisks (\*) are significantly different to control,  $P < 0.05$ .

animals (Fig. 2b). There was no effect of nicotine exposure on the mRNA expression of the lysine methylases *Suv39h2* and *Ash1l* (Fig. 2d and 2e), but there was a significant decrease in *Suv39h1* expression in nicotine-exposed animals (Fig. 2c).

## Discussion

This study investigated the effects of maternal nicotine exposure on the expression of pro-oxidative adaptor protein *p66shc* in the pancreas of nicotine-exposed rat offspring. To reinforce that the effects *in vivo* are attributable to direct effects of nicotine in the pancreatic beta cells, we confirmed our results *in vitro* using the INS-1E beta-cell line. Prenatal exposure to nicotine increased mRNA expression of *p66shc* in the neonatal rat pancreas. Similarly, *p66shc* expression

was increased following nicotine treatment *in vitro*. The increase in *p66shc* expression *in vivo* occurred in association with diminished histone H3K9 methylation. Furthermore, this reduced methylation occurred with changes in the expression of histone-modifying enzymes; specifically, an increase in lysine demethylase and a decrease in lysine methyltransferase. As other studies have demonstrated that elevated *p66shc* mediates oxidative stress, mitochondrial dysfunction, and cell injury in general including beta-cell apoptosis,<sup>4,8,25</sup> the increased expression of *p66shc* likely underlies pancreatic deficits reported following early life exposure to nicotine.<sup>5,7</sup>

Given that fetal growth-restricted offspring exhibit higher postnatal hepatic *p66shc* protein abundance in postnatal life,<sup>6</sup> it is plausible that the increase in *p66shc* expression in the pancreas of nicotine-exposed animals is indirectly related to nicotine-induced reductions in fetal growth.<sup>7,26</sup> However, our *in vitro* studies using INS-1E cells demonstrate that nicotine can directly increase *p66shc* expression in pancreatic beta cells. Moreover, a previous study has demonstrated that *p66shc* expression is upregulated following nicotine exposure in renal proximal tubule cells.<sup>10</sup> As nicotine readily crosses the placenta and is present in breast milk,<sup>15</sup> it is likely that the increased expression of *p66shc* in the neonatal pancreas was a direct result of exposure to nicotine *in utero* and during the first day of neonatal life. Upregulation of *p66shc* leads to pancreatic beta-cell dysfunction, apoptosis, and cytotoxicity,<sup>8</sup> and higher levels of *p66shc* have been reported in T2D patients,<sup>27</sup> suggesting that this nicotine-induced increase in pancreatic *p66shc* expression early in postnatal life may be central to the metabolic deficits seen in nicotine-exposed animals in adulthood.<sup>7</sup>

The mechanisms by which early life exposure to nicotine can transcriptionally regulate pancreatic *p66shc* expression are not fully understood, but may involve epigenetic modifications. In renal proximal tubule cells, nicotine increased *p66shc* transcription via epigenetic modifications.<sup>10</sup> In the present study, it was found that there was a decrease in the ratio of trimethylated histone H3K9 to total histones following nicotine exposure in both PND1 pancreas and INS-1E cells, demonstrating that exposure to nicotine *in utero* may lead to an active chromatin environment permissive for *p66shc* transcription. Similarly, prenatal nicotine exposure and adult cigarette smoke exposure in rodents have been shown to result in decreased H3K9 methylation in the brain.<sup>24</sup> Decreased lysine 9 trimethylation has been shown to promote a euchromatin state,<sup>18</sup> which is consistent with our observed increased *p66shc* mRNA expression.

It has been suggested that the histone modifications that occur *in utero* are sustained by a diverse range of histone-modifying enzymes whose expression levels are governed by external environmental insults (i.e., drugs, hypoxia, and low nutrients) during key perinatal windows of development.<sup>18</sup> With respect to histone H3 methylation, histone-modifying enzymes exist to either methylate or demethylate the lysine 9 residue, ultimately influencing overall gene transcription.<sup>18</sup> In the nicotine-exposed PND1 pancreas, we observed an increase in the steady-state mRNA levels of the histone lysine demethylase *Kdm4c* and a decrease in the expression of the histone lysine methyltransferase *Suv39h1*. Collectively, these changes have the potential to promote a decrease in histone H3K9 methylation resulting in a euchromatin environment. It is noteworthy that, in mutant obese C57BL/6 mice, *Suv39h1* expression decreased concomitantly with an increase in *Kdm4c* expression, resulting in lower histone H3K9 methylation of the *p66shc* promoter and a corresponding increase in *p66shc* gene expression.<sup>20</sup>



Taken together, results of this study suggest that nicotine exposure during fetal development can increase the expression of *p66shc* in the neonatal pancreas via histone modifications. As *p66shc* is associated with mitochondrial oxidative stress and beta-cell apoptosis,<sup>8</sup> an increase in *p66shc* provides a potential link between *in utero* nicotine exposure and beta-cell dysfunction in postnatal life, and may be a potential therapeutic target to ameliorate the long-term metabolic deficits seen in children born to women who smoke during pregnancy.

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**Conflicts of interest.** None.

**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Canadian Council of Animal Care) and have been approved by the institutional animal care committee at McMaster University.

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