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Maternal cigarette smoking during pregnancy is associated with downregulation of *miR-16, miR-21* and *miR-146a* in the placenta

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Maternal cigarette smoking during pregnancy is associated with poor fetal outcome and aberrant miRNA expression is associated with adverse pregnancy outcomes. In 25 human placentas, we analyzed the expression of four candidate miRNA previously implicated in growth and developmental processes: *miR-16, miR-21*, *miR-146a* and *miR-182,* and used three immortalized placental cell lines to identify if specific components of cigarette smoke were responsible for alterations to miRNA expression. *miR-16, miR-21* and *miR-146a* were significantly downregulated in cigarette smoke-exposed placentas compared to controls. TCL-1 cells exposed to both nicotine and benzo(a)pyrene exhibited significant, dose-dependent downregulation of *miR-146a*. These results suggest that *miR-146a* is particularly responsive to exposures, and that smoking may elicit some of its downstream effects through alteration of miRNA expression.

Introduction

Gene-environment interactions occur throughout the lifetime but there are few times when such interactions are more important than during intrauterine development when perturbations can affect fetal growth and development. Further, there can be lasting effects due to altered fetal programming. During in utero development, the placenta plays a critical role supporting normal growth and development, providing the fetus with nutrients, assisting in waste removal, and protecting the fetus from both maternal immune rejection and from other environmental insults. Additionally, the placenta is a metabolic and endocrine center, producing and secreting hormones which support each stage of pregnancy. The placenta also expresses metabolic compounds responsible for the reactions which ultimately protect the fetus from exposure to toxicants. A number of drugs and toxicants, including nicotine,[1] alcohol[2] and benzo(a)pyrene,[3] have been found to accumulate in placenta tissue and affect placental gene expression. Maternal cigarette smoking has been reported to be associated with increased risk for spontaneous abortion[4] and preterm delivery.[4-6] Previous studies have shown that there are placental complications linked to cigarette smoke exposure during pregnancy, including alterations to the development and function of the placenta.[7] There are more than 4,000 chemicals in a cigarette, including nicotine, benzo(a)pyrene and carbon monoxide; more than 43 of these chemicals are known carcinogens.[8] Nicotine readily crosses the placenta and can result in fetal concentrations that are 15% higher than maternal concentrations.[1] While a number of studies have shown a decrease in overall prevalence of smoking in women in the past 20 years, the prevalence of smoking in young pregnant women has increased.[9,10] Additional studies have reported that 12–15% of all women smoke during their pregnancies.[11,12] Taken collectively, these observations suggest that maternal smoking during pregnancy remains an important common exposure that can have major ramifications on not only the normal growth and development of the fetus but also on fetal programming.

The mechanisms by which exposures, such as cigarette smoke, affect the complex regulatory mechanisms of the placenta are still being characterized. One such mode of toxicity may be through the altered expression of microRNA (miRNA), small ~22 nucleotide-long noncoding RNA molecules, which are highly ubiquitous and possess conservation across many species.[13] MicroRNA posttranscriptionally regulate gene expression by base-pairing to the 3'-untranslated region of a target mRNA resulting in either translational repression or direct degradation of the mRNA, the exact mechanism of which depends largely on the degree of complementarity of the miRNA to its mRNA target. Because partial complementarity of a miRNA to an mRNA target can still lead to translational repression, a single miRNA has the capability of regulating a large number of genes.[14] By negatively regulating their mRNA targets, miRNA have been implicated in regulating cell proliferation, growth and differentiation and apoptosis.[15] Highly conserved clusters of primate-specific miRNA are expressed in

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Table 1. Demographics of the study population

	Total	Maternal smoking during pregnancy	No maternal smoking during pregnancy
Total, n (%)	25 (100)	8 (32)	17 (68)
Gender, n (%)			
Female	13 (52)	3 (37.5)	10 (59)
Male	12 (48)	5 (62.5)	7 (41)
Gestational Age in weeks, mean (SD)	38.7 (0.88)	38.4 (1.20)	38.9 (0.66)
Birthweight in g, mean (SD)	3080 (540)	3091 (682)	3071 (484)
Maternal Age in years, mean (SD)	27.3 (6.66)	24.3 (7.94)	28.8 (5.68)

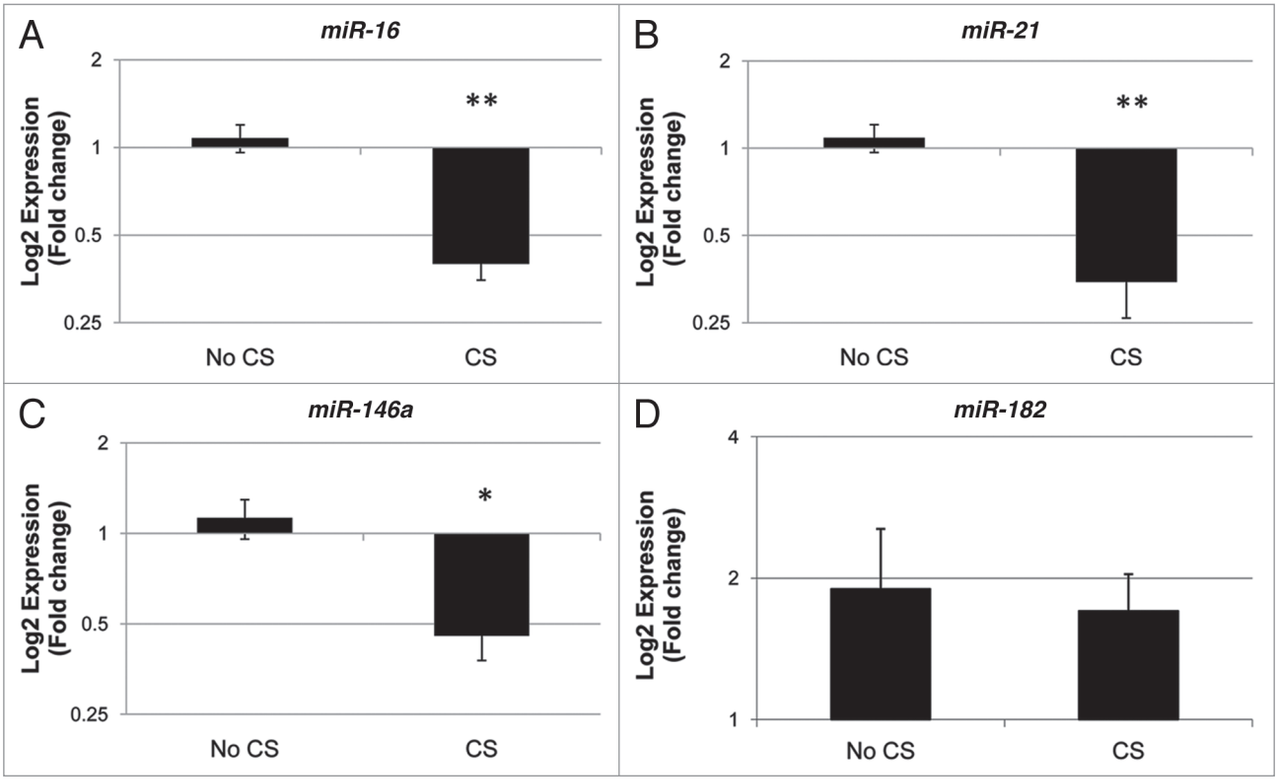

Figure 1. Maternal cigarette smoking during pregnancy is associated with downregulation of *miR-16, miR-21* and *miR-146a*. Quantitative RT-PCR analysis was used to examine the expression of the mature forms of *miR-16* (A), *miR-21* (B), *miR-146a* (C) and *miR-182* (D), in primary placenta tissue samples from infants whose mothers smoked cigarettes (CS) during pregnancy (n = 8) and infants whose mothers did not smoke during pregnancy (No CS, n = 17). *indicates $p < 0.01$ and **indicates $p < 0.0001$ as determined by t-test.

the placenta and other tissues.[16-19] Additionally, multiple groups have further characterized a number of placental miRNA whose aberrant expression is associated with derangements in the intrauterine environment or maternal condition.[20-22] These discoveries have generated much interest in the involvement of miRNA in placental gene regulation and the possible utility of discovering placental miRNA which can serve as clinical biomarkers of exposure or disease.

As these miRNA play critical roles in development both of the fetus and placenta and because toxicant exposures and stress can alter the expression of miRNA,[23,24] we sought to characterize the modulation of placental miRNA by maternal cigarette smoke by analyzing the expression of four candidate miRNA for associations with maternal cigarette smoking during pregnancy.

Results

Twenty-five placentas, eight with a history of maternal cigarette smoking during pregnancy and 17 non-smoking controls, were analyzed for expression of candidate miRNA. **Table 1** illustrates the demographics of the sample population. There were no significant differences in the gender, gestational age, birth weight or maternal age between exposed and unexposed infants. Quantitative RT-PCR analysis revealed downregulation of *miR-16* ($p < 0.0001$), *miR-21* ($p < 0.0001$) and *miR-146a* ($p < 0.01$) associated with maternal cigarette smoking during pregnancy (**Fig. 1**). *miR-182* expression showed no differential expression by maternal cigarette smoking during pregnancy. To control for potential confounding effects of clinical variables in these results,

Table 2. Multivariable linear regression model of individual miRNA expression

	miR-16		*miR-21*		*miR-146a*	
	Reg. coeff.	**p**	**Reg. coeff.**	**p**	**Reg. coeff.**	**p**
Maternal Cigarette Smoking During Pregnancy						
No	Reference		Reference		Reference	
Yes	-0.058	<0.0001	-0.094	<0.0001	-0.039	0.003
Gender						
Female	Reference		Reference		Reference	
Male	-0.008	0.4	-0.003	0.9	0.001	0.9
Gestational Age, per week	0.004	0.5	0.007	0.5	0.005	0.5
Birthweight, per gram	-8.85×10^{-06}	0.4	-1.26×10^{-06}	0.9	-7.23×10^{-06}	0.5
Maternal Age, per year	-0.0003	0.7	-0.002	0.1	-0.001	0.2

Note: Each miRNA is modeled individually and the models are controlled for all co-variates in the table.

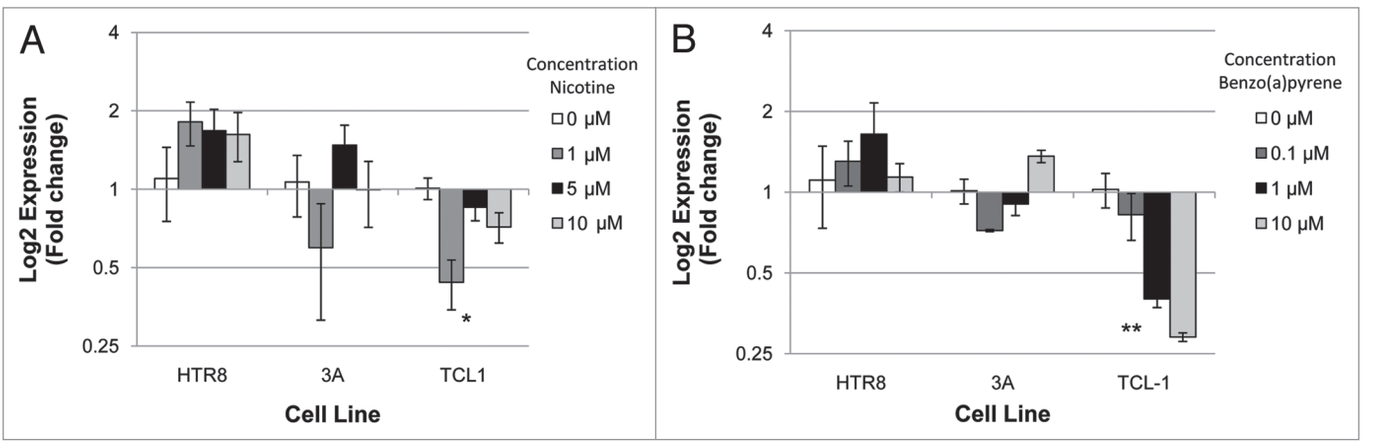

Figure 2. *miR-146a* is downregulated in TCL-1 cells exposed to nicotine and benzo(a)pyrene. Three placental cell lines (HTR8, 3A and TCL1) were exposed to increasing doses of nicotine (A) and benzo(a)pyrene (B) for 6 days, and the expression of *miR-146A* was determined through qRT-PCR. Error bars indicated standard error of the mean, *indicates a significant downregulation of *miR-146a* across doses of nicotine (ANOVA, $p < 0.03$). **indicates a significant downregulation of *miR-146a* across doses of benzo(a)pyrene (ANOVA, $p < 0.006$).

we utilized multivariable linear regression to examine the association between *miR-16, miR-21* and *miR-146a* expression with maternal smoking during pregnancy, controlled for gestational age, birth weight, gender and maternal age. The respective regression coefficients and p values are provided in **Table 2**. These models suggest that maternal smoking leads to a statistically significant 3.9% decrease in relative expression of *miR-146a*, a 5.8% decrease in the relative expression of *miR-16* and a 9.4% decrease in the relative expression of *miR-21*, each independent of infant gender, gestational age, maternal age and infant birth weight.

Three immortalized placental cell lines representing three different stages and aspects of placental development were chosen to examine the effects of nicotine and benzo(a)pyrene specifically on the expression of the three miRNA found to be significantly altered in the primary tissues in cells from these different stages and aspects of placental development. We sought to investigate the effects of nicotine and benzo(a)pyrene exposure on the expression of these three miRNA in the first trimester villous 3A cells, the first trimester extravillous HTR8 cells and the third trimester extravillous TCL-1 cells. As shown in **Figure 2A**, qRT-PCR analysis revealed that *miR-146a* was significantly altered across nicotine exposures in TCL-1 (ANOVA $p < 0.03$), with a specific significant downregulation of approximately 2.5-fold in TCL-1 exposed to 1 µM nicotine compared to mock control ($p < 0.02$). No differential expression of *miR-16* or *miR-21* occurred in TCL-1, 3A and HTR8 cells exposed to nicotine (data not shown). Similar to nicotine exposure, qRT-PCR analysis revealed that *miR-146a* was downregulated in TCL-1 cells across all benzo(a)pyrene exposure levels (**Fig. 2B** and ANOVA $p < 0.006$), with 1 µM exposures leading to a greater than 50% downregulation and 10 µM an almost 75% downregulation of the miRNA. Again, no differential expression of *miR-16* or *miR-21* occurred in TCL-1, 3A and HTR8 cells exposed to benzo(a)pyrene (data not shown).

Table 3 shows the targets as predicted by using the three-algorithm miRNA target prediction approach described and confirmed targets with respective sources[6,25-29] for the three candidate miRNA with significant differential expression in primary placenta samples exposed to cigarette smoking in utero. Predicted targets were as follows: for *miR-16*, BCL2L2 and EDA; for *miR-21*, PLAG1 and SATB1; and for *miR-146a*,

Table 3. mRNA targets predicted and/or experimentally validated for *miR-16*, *miR-21* and *miR-146a*

miRNA	Predicted target mRNA	Minimum free energy of duplex (kcal/mol)	Experimentally confirmed target mRNA	Source of target confirmation
miR-16	BCL2L2 EDA	-23.1 -25.3	Caprin-1, HGMA-1	Kaddar et al. 2009
miR-21	PLAG1 SATB1	-22.7 -23.8	PTEN PLAG1	Meng et al. 2007 Tran et al. 2007
miR-146a	TRAF6	-26.1	CCL8/MCP-2 TRAF6	Rom et al. 2010 Taganov et al. 2006; Hou et al. 2009

Note: In order to be considered a "predicted target," the target must appear in the "top 100" targets for the respective miRNA using all of the following target prediction tools: miRanda (September 2008 Release), PicTar (based on Lall, et al. 2006) and TargetScan 5.1. Minimum free energies of the miRNA-mRNA duplexes were determined using *RNA-hybrid*.

TRAF6. Minimum free energies (MFE) are listed for each respective miRNA and target mRNA duplex and were determined using *RNA-hybrid*.

Discussion

Taken as a whole, our work identifies the association of cigarette smoking during pregnancy with the aberrant expression of several miRNA involved in critical cell processes. A number of groups have previously demonstrated that nicotine[1] and benzo(a)pyrene[3] interact with the placenta and may affect placental growth and development. Previous work demonstrated the effects of toxicants and agents of cell stress on expression of *miR-146a*,[23,24,30,31] as well as on *miR-16*[32] and *miR-21*.[33,34] While our observations are limited by incomplete information regarding the duration of cigarette smoking during pregnancy, cigarette per day usage or more extensive environmental exposure information (such as alcohol usage, environmental pollutant exposure or secondhand cigarette smoke exposure), our data comprise an important first step in determining associations between maternal cigarette smoking during pregnancy and aberrant miRNA expression in the placenta.

To further investigate the effects of cigarette smoke, we utilized three placental cell lines from different stages of placental development to investigate mechanisms of aberrant miRNA expression associated with cigarette smoke exposure. Downregulation of *miR-146a* in TCL-1 cells treated with nicotine and benzo(a)pyrene suggests that *miR-146a* may be especially sensitive to agents of cellular stress. Moreover, this result suggests that two components of cigarette smoke which affect the expression of *miR-146a* in term placentas may be nicotine and benzo(a)pyrene. The lack of differential expression of *miR-16* and *miR-21* in cells treated with nicotine or benzo(a)pyrene does not necessarily rule out that their expression is not modulated by cigarette smoke. Other components of cigarette smoke may modulate the expression of these miRNA. Furthermore, the effect of the mixed exposures encountered with the myriad chemicals in cigarette smoke may be truly responsible for the modulation of expression of these miRNA. Experiments conducted with different components of cigarette smoke and complex mixtures of these components may provide more extensive information on the mechanistic effects of cigarette smoke on miRNA expression.

Previous studies on benzo(a)pyrene-exposed human choriocarcinoma cell lines BeWo and JEG-3 showed that placental cell line proliferation was largely unchanged following exposure to 10 and 50 μM doses of benzo(a)pyrene in serum-containing medium.[35] Genbacev and colleagues used nicotine-exposed placental explants to conclude that nicotine is an important molecule that can drastically placental growth and development.[36] In our cell culture experiments, we saw no detectable change in cell growth or morphology in any of our cell lines treated with any of the doses of benzo(a)pyrene or nicotine compared to respective mock treated-cells. Doses of benzo(a)pyrene and nicotine higher than the ones we used may have led to increased cell toxicity and alterations in cell growth due to increased cell stress. Future investigations using higher doses may discover miRNA whose expression and function is altered by higher levels of benzo(a)pyrene, nicotine and other agents of environmental stress.

One of the more difficult challenges is miRNA target prediction; complementarity between miRNAs and their target mRNAs is generally far from perfect and is not required for miRNA to functionally silence the expression of a protein product. As a result of this complexity, a simple search for sequence complementarity between the miRNA and its mRNA target, the basis of many target prediction algorithms, can be expected to produce many false-positive hits. Moreover, most miRNAs are thought to have potentially hundreds of targets, some of which will be targeted more strongly than others and in cell-type specific contexts. Thus, it is essential to devise a strategy which predicts targets based not solely on sequence homology, but which also incorporates additional characterizations when choosing which targets should be examined with further testing.

Our target prediction strategy suggested that PLAG1 and SATB1 were targets of *miR-21*. Both of these genes are transcription factors implicated in tissue-specific control of the cell cycle and cellular proliferation, and PLAG1 has been previously demonstrated to be a target of *miR-21*.[26] Downregulation of *miR-21* due to cigarette smoke exposure might lead to overproduction of PLAG1 and SATB1 protein, resulting in the overexpression of these key genes involved in cell cycling and proliferation.

Our target prediction strategy predicted BCL2L2 and EDA as targets for *miR-16*. BCL2L2 or B-cell CLL/lymphoma 2 like 2 protein, is a pro-survival molecule previously reported to be targeted by *miR-133b*.[37] Like other anti-apoptotic proteins in the

BCL-2 family, BCL2L2 has been shown to be regulated both pre-transcriptionally and post-transcriptionally.[38] The prediction that BCL2L2 is a target of *miR-16* supports hypotheses that by downregulating *miR-16*, cigarette smoke upregulates BCL2L2 and may enhance signaling through anti-apoptotic pathways. The EDA gene is mutated in anhidrotic ectodermal dysplasia and under normal circumstances, produces the protein ectodysplasin, a member of the TNF superfamily that is involved in activation of the NFκβ signaling pathway.[39] Again, *miR-16* downregulation by cigarette smoke exposure, could lead to an upregulation of EDA protein thereby enhancing NFκβ signaling and increased tendency toward survival.

Our in-silico analysis predicted TRAF6 as a target for *miR-146a*, suggesting that downregulation of *miR-146a* could result in an upregulation of TRAF6, a protein also important for signaling through both the NFκβ signaling pathway[40] as well as a mediator of inflammation in the toll-like receptor 4 (TLR4) pathway.[41,42] Previous reports using different target prediction strategies have hypothesized TRAF6 as a target of *miR-146a*[28] and have validated TRAF6 as a target in murine macrophages.[29] TLR4 mediates the inflammatory response, and aberrant TLR-4 signaling is associated with inflammation-induced preterm delivery.[43] Such a consequence could be the result of dysregulated TRAF6 production as a result of downregulation of *miR-146a*. TRAF6 overexpression due to downregulation of *miR-146a* could result in overactive TLR signaling which could have a number of downstream consequences for both placenta and fetus. Further investigation into such relationships would prove vital into better understanding both the role of TRAF6 in TLR4 signaling, as well as the association of aberrant expression of *miR-146a* with adverse pregnancy outcomes.

As with *miR-16*, downregulation of *miR-146a* can lead to enhanced signaling through the anti-apoptotic and pro-survival NFκβ pathway, resulting in placental cells that avoid apoptosis and prolong their survival. Components of cigarette smoke have been suggested to function through the NFκβ pathway[44] and, in light of our results with both placental tissues as well as cell culture experiments, both benzo(a)pyrene and nicotine may be acting through the NFκβ signaling pathway through downregulation of *miR-146a*. Studies by other groups have demonstrated that an NFκβ-modulated pathway upregulates *miR-146a* when *miR-146a* expression is induced with IL-1β.[45] While this may be true under normal circumstances under which conditions of cellular stress are held to a minimum, our data suggest that agents of cell stress, such as nicotine and benzo(a)pyrene, may downregulate *miR-146a* expression which, by consequently upregulating TRAF6 expression, enhances signaling through NFκβ and promotes cell survival. The complexities of this potential duality of effect, namely the notion that *miR-146a* both negatively regulates and can be regulated by the NFκβ pathway, remain to be further elucidated and future experiments involving knock-downs of various components of the pathway, including the miRNA acting as negative regulators of the pathway, may clarify the mechanisms at play. Our results suggest then that TRAF6, and therefore the NFκβ and TLR4 signaling pathways, may be important mediators of the effects of cigarette smoke exposure on the placenta.

Our results indicate a potential cascade of molecular changes that occur in the placenta upon exposure to cigarette smoke. We suggest that cigarette smoke exposure during pregnancy is associated with downregulation of *miR-16, miR-21* and *miR-146a* in the placenta. Collectively, aberrant repression of these miRNA upregulates the targets of these miRNA and may affect cell cycle regulation, growth, immunomodulation and development in the placenta. These changes in target gene expression may have further effects downstream for both placenta and fetus, ultimately resulting in altered fetal programming. Future studies are ongoing to further characterize the effects of environmental exposures on placental miRNA, on understanding the downstream phenotypes of aberrant miRNA expression and in investigating the placenta as a record of the intrauterine environment.

Materials and Methods

Placenta samples. Placenta samples were collected within two hours of delivery at Women and Infants' Hospital in Providence, RI, USA. An approximately 1 g biopsy of placenta was excised, free of maternal decidua, from the maternal side of the placenta 2 cm from the umbilical cord insertion site, and the sample was placed immediately in RNAlater and stored at 4°C. At least 72 h later, placenta samples were removed from the RNAlater, blotted dry, aliquoted and stored in sample tubes at -80°C until needed for examination. Medical information, including cigarette smoking during pregnancy, available on patient charts was also collected. All samples were collected in under appropriate IRB protocols for Women and Infants' Hospital and Brown University.

RNA extraction. RNA was extracted from placenta samples and cultured cells using the miRvana miRNA Isolation Kit (Ambion) following the manufacturer protocols. For tissue samples, a 200 mg piece was cut and placed in ice cold, sterile PBS and homogenized using a PowerGen 125 Tissue Homogenizer (Fisher Scientific). The homogenized sample was removed from PBS and RNA was extracted using manufacturer protocols. Extracted RNA was quantified using a Nanodrop spectrophotometer and then aliquoted into single-use aliquots and stored at -80°C.

Quantitative RT-PCR (qRT-PCR) for mature miRNA. Expression of mature miRNAs was measured using commercially available TaqMan MicroRNA Assays or TaqMan Gene Expression Assays (Applied Biosystems, Valencia, CA, USA) on an Applied Biosystems 7500 Real Time PCR system and analyzed with 7500 System Software. All reactions were run in triplicate, with RNU44 serving as the referent for miRNA expression. In addition, a no-RT control was run with each plate.

Cell culture. 3A and TCL-1 placental cell lines were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS and 1% Pen-Strep. HTR8 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 5% FBS. These three placental cell lines represent three different stages and aspects of placental development and were selected to examine the effects of components of cigarette smoke in models of different timepoints during pregnancy. 3A cells are first-trimester villous cells, HTR-8s are first trimester extravillous cells and TCL-1s are third

trimester extravillous cells. Placental cell lines were exposed to 0, 1, 5 and 10 μM doses of nicotine (Sigma-Aldrich) in medium for 6 days, with 1 μM being closest to a physiologically relevant dose and higher doses used to investigate the effects of more concentrated doses of nicotine on placental cells.[46,47] Placental cell lines were exposed to 0.1, 1 and 10 μM doses of benzo(a)pyrene (Sigma-Aldrich) or DMSO (<0.1%) alone in medium for 6 days based on previously published methods and physiologically-relevant doses.[23,48] None of the doses of nicotine or benzo(a)pyrene led to increased cell toxicity compared to controls. Cells were cultured for 6 days, with exposure medium refreshed on days 2 and 4. The 6-day period of exposure was chosen to mimic a long-term chronic exposure, but to avoid sub-culturing of the cells, and has been previously described.[23] All experimental and control conditions were performed in triplicate. Following exposure, cells were harvested and RNA was extracted for analysis by qRT-PCR as described above.

miRNA target prediction. Three target prediction algorithms were used to predict targets for miRNA of interest, and a fourth algorithm was used to evaluate predicted targets for base-pairing and minimum free energy. The three algorithms used for target prediction were *miRanda* (September 2008 release, available online at www.microrna.org/microrna/home.do), *PicTar* (as cited in ref. 49 and available online at http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi) and *TargetScan 5.1* (available online at www.targetscan.org/). In order to be considered a predicted target for further investigation, the target must have appeared in the top 100 targets in all three prediction algorithms. The *RNA-hybrid* algorithm was used to evaluate predicted targets for information on secondary structure and thermodynamic stability of the miRNA-mRNA duplex.[50] *RNA-hybrid* is available online at http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html.

Statistical analysis. Two-tailed t-tests were used to compare miRNA expression levels determined by real-time PCR in human tissue samples. In order to examine the association between exposure to cigarette smoke and miRNA expression, controlling for potential confounders, we employed multivariable linear regression modeling, in SAS 9.1 (SAS Institute, Cary, NC, USA). ANOVA was used to determine differential expression of miRNA across treatments of nicotine and benzo(a)pyrene on cells in cell culture experiments.

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