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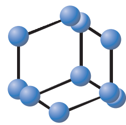

MicroRNAs as Epigenetic Targets of Cigarette Smoke During Embryonic Development

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Abstract: The adverse developmental effects of exposure to Cigarette Smoke (CS) during pregnancy are documented in this paper. These include low birth weight, congenital anomalies, preterm birth, fetal mortality and morbidity. The current biological thought now recognizes that epigenetics represents a fundamental contributing process in embryogenesis, and that the environment can have a profound effect on shaping the epigenome. It has become increasingly recognized that genes encoding microRNAs (miRNAs) might be potential loci for congenital disabilities. One means by which CS can cause developmental anomalies may be through epigenetic mechanisms involving altered miRNA expression. While several studies have focused on genes affected by CS during embryonic/ fetal development, there is a paucity of knowledge on the involvement of miRNAs in this process. This brief review summarizes the current state of knowledge in this area.

Keywords: Cigarette smoke, e-cigarettes, embryonic development, epigenome, microRNAs, placenta.

1. INTRODUCTION

Cigarette Smoke (CS) is an environmental toxicant and a major risk factor for several types of cancers [1], pulmonary and cardiovascular diseases [2-4], periodontitis [5], atherosclerosis [6], and tuberculosis [7]. CS also affects embryonic and fetal development, increasing the risk for low birth weight, underdeveloped organs, congenital anomalies, preterm birth, fetal mortality and morbidity [8-10]. It has been reported that ~10-14 percent of pregnant women in the U.S. smoke cigarettes, thereby, exposing their embryos and fetuses to nicotine and other toxicants present in CS [10, 11]. There is emerging evidence to indicate that pregnant women who smoke are also at increased risk of giving birth to children with an orofacial cleft [1, 12, 13], an association that has been supported by studies utilizing animal models [14]. These observations have led to an increased awareness of the need to understand mechanisms underlying the effects of maternal cigarette smoking on embryonic gene regulation and development. Among epigenetic factors that mediate gene-environment crosstalk are microRNAs (miRNAs) whose dysregulation has been linked to pathogenicity and craniofacial defects [15-18]. This review focuses on the current state of knowledge regarding the role CS-affected miRNAs may play in embryonic development.

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1.1. Cigarette Smoke

CS consists of mainstream smoke (smoke that is inhaled and exhaled by a smoker when puffing on a cigarette) and sidestream smoke (or secondhand smoke which emanates from the burning end of a cigarette). CS contains ~ 7000 chemicals, including nicotine, benzo(a)pyrene (BaP), formaldehyde, carbon monoxide and a number of known carcinogens [19]. Many of these chemicals contain highly oxidative radicals and redox active compounds that react with DNA [20] to create bulky adducts that can cause DNA damage [21, 22].

More recently, electronic cigarettes (e-cigs) (also called Electronic Nicotine Delivery Systems - ENDS) have been touted as a “safer alternative” to conventional tobacco smoking [23, 24], including their use during pregnancy [25-27]. This notion is based on the assumption that e-cigs produce aerosols that are devoid of many of the chemicals present in CS. Nevertheless, there is no conclusive evidence to suggest that maternal e-cig use does not affect normal embryonic and fetal development [28]. These aerosols contain chemicals, such as formaldehyde and acrolein, which can cause DNA damage and mutagenesis. Indeed, recent evidence indicates that exposure to these aerosols results in increased oxidative stress in laboratory animals and humans, cardiac defects in zebrafish larvae and craniofacial defects in frog embryos [28, 29]. Several e-cig products have also been found to be contaminated with microbial toxins [30]. Certain e-cigs have been shown to exhibit cytotoxicity attributed to the high concentrations of both nicotine and ethyl maltol, a flavor con-

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tributing chemical [31]. Prevailing evidence, thus, suggests that further studies are warranted to address the effects of e-cig exposure during pregnancy on developmental outcomes.

1.2. miRNAs

miRNAs are short ~22-nt long non-coding RNA molecules that bind to the 3' UTRs of mRNAs. They regulate a variety of biological and developmental processes, such as cell differentiation, proliferation, apoptosis, cellular responses to stress and immunity, and metabolism [32-38]. miRNAs interact with mRNAs through their short seed sequences, resulting in either mRNA degradation or suppression of transcription, the precise mechanism dependent, respectively, on whether the seed sequence matches the target sequence perfectly, or partially [39]. miRNAs are also known to function by directly activating transcription, upregulating protein expression, and targeting mitochondrial transcripts [40]. This flexibility in miRNA-mRNA interaction allows miRNAs to regulate the expression of a wide variety of target genes. A corollary to this observation is that a single mRNA can also be regulated by several miRNAs.

2. MIRNAs AND CIGARETTE SMOKE (CS)

2.1. CS and Regulation of miRNA Expression

A variety of mechanisms have been put forth to explain how CS can cause aberrant miRNA expression. Several studies indicate that the potential for CS to disrupt global expression of miRNAs resides in the fact that many miRNA encoding genes are located in vulnerable parts of the genome, such as fragile sites [20, 41]. Furthermore, these genes frequently harbor SNPs which render them susceptible to CS-induced genetic damage or altered transcript processing [20, 22]. Many components of CS can disrupt the mechanisms that regulate miRNA biogenesis. For instance, Ligorio *et al*. [42] have demonstrated *in silico* that components of CS can bind to DICER, thereby affecting function. Gross *et al*. [43] have shown that CS exposure modifies DICER post-transcriptionally *via* SUMOylation, leading to decreased production of mature miRNAs in alveolar macrophages of smokers. CS exposure can also affect DNA methylation. Breton *et al*. [44] observed that *in utero* exposure to CS leads not only to global *hypomethylation* but also to *hypermethylation* of promoter-specific CGIs (CpG islands), many of which are associated with CS metabolism. It was hypothesized that hypomethylation results from DNA damage by ROS (Reactive Oxidative Species), thus preventing the binding of maintenance methylases, whereas, CGI hypermethylation arises from incomplete erasure in methylation reprogramming during early embryonic development [44].

2.2. CS Associated miRNAs in Embryonic Development

Recent evidence indicates that miRNAs are sensitive to environmental stressors, including CS. Clinical studies [45-47] and investigations utilizing cell lines [48, 49] have revealed dysregulation of distinct panels of miRNAs associated with exposure to CS. Many additional examples of miRNA dysregulation by exposure to CS *in vivo*, or its condensate *in vitro*, have been documented [50-53]. While these observations are derived primarily from the cancer literature, far less is known regarding the epigenetic effects of exposure to CS in the developing embryo.

The placenta is an easily accessible organ, and as such, is ideally suited for studying factors that adversely affect embryonic development by disrupting normal placental function. It supports the development and growth of the embryo by providing nutrients, secreting hormones, removing waste, and acting as a protective barrier against environmental insults. The placental barrier, composed of both maternal and fetal tissue, acts as an internal barrier that can protect the embryo from xenobiotic agents, but allows more xenobiotics to pass through in comparison to the blood-brain or blood-retinal barriers [54]. CS can impinge on embryonic development through alterations in placental gene expression (Fig. **1**). Maccani *et al*. [55] observed that maternal cigarette smoking leads to the downregulation of *miR-16, miR-21* and *miR-146a* in human placental tissues, relative to unexposed controls. Extending these observations to placental cell lines, they found that the downregulation of *miR-146a* was caused by nicotine and benzo(a)pyrene (BaP), two components of CS, whereas, other CS components were presumed to target the downregulation of *miR-16* and *miR-21*. Analyses of three placental cell lines representing different stages and aspects of placental development – first trimester villous (3A) cells; first trimester extravillous (HTR8) cells; and, third trimester extravillous (TCL-1) cells – revealed a significant downregulation of *miR-146a* in TCL-1 cells when exposed to a range of doses of nicotine and BaP. A potential target of *miR-146a* is TRAF6, associated with NFKB signaling. It can thus be hypothesized that downregulation of *miR-146a* enhances NFKB signaling. Since NFKB has anti-apoptotic and pro-survival properties, increased expression of this molecule likely leads to prolonged survival of term placental cells that may cause cellular stress impinging on fetal programming.

As the development and maturation of the lungs are completed only after birth, newborn lungs are subjected to a certain amount of oxidative stress when transitioning from a state of maternal dependency to autonomic respiration [21]. Izzotti *et al*. [21] identified at least 11 pulmonary miRNAs whose expression was significantly altered when exposed to CS from birth, the post-weanling period to adult stages. These miRNAs were all found to be associated with embryological development and morphological changes, thereby implying that embryonic CS exposure might affect early lung development.

Many of the mechanisms whereby maternal smoke exposure may adversely affect proper embryonic growth and development are thought to be mediated by alterations in miRNA expression. For example, maternal cigarette smoking during pregnancy has been associated with dysregulated expression of miRNAs in the embryo [56], placenta [55] and cord blood [57]. The expression of *miR-140*, known to regulate zebrafish palatal development *in vivo* [58], has been shown to be reduced by environmental smoke exposure *in vitro* [21]. In a case-control study, infants with CA/AA genotypes at rs7205289 (located in the *miR-140* gene) exposed to maternal passive smoking during the first trimester exhibited

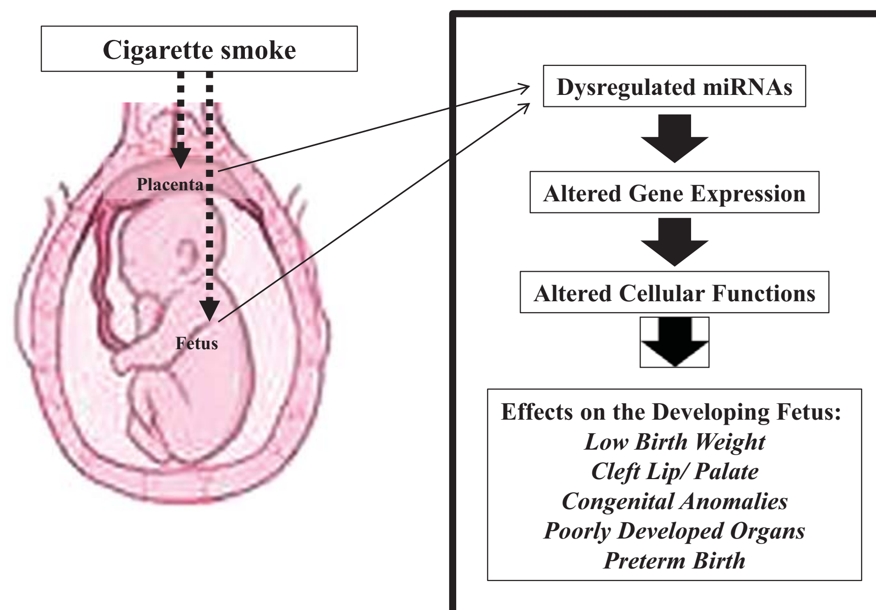

Fig. (1). Effects of Cigarette Smoke (CS) on miRNA expression during embryonic/fetal development. CS can directly affect miRNA expression during embryonic development in, at least, two ways. Components of CS can affect miRNA expression in the placenta, a crucial organ required for normal embryonic development (bold arrow, left), or, penetrate the placental barrier and affect the developing embryo/fetus directly (bold arrow, right). CS can also affect the developing embryo *indirectly* by contributing to poor quality of miRNAs in parental germ cells or through transgenerational passage of affected miRNAs. Aberrantly expressed miRNAs can alter gene expression and developmental pathways, thereby enhancing the risk for abnormal embryonic/ fetal development. These include low birth weight, cleft lip/palate, congenital anomalies, poorly developed organs, and pre-term birth, to name a few.

synergistically increased cleft palate risk [59]. As this SNP is located close to the cleavage site of Drosha, it can be speculated that the processing of pri-miRNA may be affected. Importantly, human epidemiological studies indicate that infants with the A-allele (rs7205289) when exposed to maternal passive smoking during the first-trimester exhibit inhibition of *miR-140* expression and an increased risk for non-syndromic cleft palate [59]. The cleft palate risk is attributed to increased signaling by Pdgf (a molecule necessary for proper palate development [60]) following downregulation of *miR-140*. Indeed, one of the functionally validated targets of *miR-140* in mouse palatal mesenchymal cells is PDGFRA [58, 59]. A linkage has also been revealed between exposure to CS, expression of specific miRNAs and TGFß-dependent developmental processes [61]. Moreover, significant interactions have been identified between maternal smoking, a TGFß gene variant, and isolated cleft palate [62]. Taken together, these data offer support for the notion that exposure to CS may be associated with increased cleft palate risk *via* dysregulation of miRNA levels during palatal development.

Marczylo *et al*. [63] observed that CS can induce significant differential expression of miRNAs in the spermatozoa of smokers, compared to that of non-smokers. Out of a total of 130 miRNAs found to be expressed in spermatozoa, these authors identified 28 that were affected by CS. Many of the affected miRNAs were associated with sperm quality and processes involved in normal embryonic development, such as cell differentiation, cell death and cell proliferation, thereby linking paternal CS exposure to possible reproductive defects. Among altered miRNAs, there were several epi-miRNAs (miRNAs associated with epigenetic modification) that target several DNA methyltransferases (DNMTs) and Histone deacetylases (HDACs). While similar studies of the ovum are not available, it has, nevertheless, been reported that ovarian development can be severely affected by CS as well. Evaluation of ovarian tissues in mice exposed to CS, compared to control mice, revealed changes in 152 miRNAs [64]. One of the primary targets of these miRNA changes appears to be the MAPK signaling pathway, associated with cell proliferation, differentiation, apoptosis, survival and motility, all of which may contribute to ovarian dysregulation, and possibly, to altered miRNA content of the ovum.

The examples described above and summarized in Table **1** indicate the significant CS-induced alterations that miRNAs exert in a developing embryo. In most of these studies, the effects of miRNA on target gene expression should be considered speculative unless validated by functional experimental evidence (Table **1**). It is also evident that our knowledge in this area is limited warranting additional studies.

CONCLUSION

A plethora of reports, focused mainly on adults and cancer, indicate that CS-associated miRNAs alter basic developmental processes, such as cell proliferation, apoptosis, and cell differentiation, thereby underscoring the view that embryonic development may be susceptible to alterations in miRNAs caused by maternal CS exposure (Fig. **1**). Extensive lists of miRNAs affected by CS exist in the literature [20, 65] and a computational network associating tobacco com-

Table 1. Summary of microRNAs affected by CS and implicated in development.

miRNAs	Tissue	Validated*/ Predicted Gene Targets	Effects of CS Exposure	Birth Risk	Refs.
miR-: *[27b; 124a; 138; 148; 196;199a; 214; 219; 335; 337; 341; 345; 376; 411]*	Mouse lung	-	miRNAs variably expressed in newborns, post-weanling females, and adult females.	Lung development affected	[21]
miR-140	Mouse / human palate	*Pdgfra**	Down-regulation implicated in cleft palate.	Cleft palate	[59]
hsa-miR-: *16* *21* *146a*	Human placenta	*BCL2L2; EDA* *PLAG1*; SATB1* *TRAF6**	All 3 miRNAs down-regulated in CS-exposed tissues.	Altered fetal programming	[55]
hsa-miR-: *[365; 944; 1267; 340; 4513; 576-3p; 576-5p; 1246; 30c[1]; 933; 7[2]; 1285; 1270; 509-5p; 146b-3p; 4748; 519d; 550a; 550b; hsa-let-7a-2-3p]*	Human spermatozoa	*PHC2*[1]* *CBX5*[2]* *EZH2*[3]* *HDAC9*[4,5]*	28 differentially expressed miRNAs in CS: 21 up-regulated (top); 7 down-regulated (bottom).	Transmission of harmful phenotypes to progeny	[63]
[574-5p[3]; 3145-5p; 146b-5p[4]; 634; 129-3p[5]; 652; 4723-5p]					
hsa-miR-223	Human maternal and cord blood	-	High *miR-223* expression correlates with low regulatory T cell numbers.	Atopic dermatitis	[57]

*, validated targets; *[1-5], numbers correlate miRNAs (in column 1) with their respective targets (in column 3);

ponents with 'environment' and 'miRNAs' has identified at least 58 miRNAs and 7 diseases [66]. Whether the identification of these miRNAs can be extrapolated to embryonic or fetal systems in the context of CS exposure remains to be clarified. To affect the developing embryo, toxicants present in CS should be able to either penetrate the placental barrier, like nicotine [67], or influence placental gene expression (Fig. **1**). miRNAs may also be subjected to spatial or temporal regulation during embryonic/fetal development – *i.e.*, they may not all be expressed in an embryonic tissue at the time of CS exposure. CS-mediated changes in embryonic development could also be indirect. These include poor quality of miRNAs present in parental germ cells and intergenerational passage of affected miRNAs to offsprings. In summary, more studies are warranted to clearly understand how the effects of CS impinge on embryonic development *via* altered miRNA expression. Identifying CS-affected miRNAs during embryonic/fetal development will be a critical step in identifying dysregulated signaling pathways and the genes that regulate them.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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