REVIEW

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Environmental exposures *in utero* and microRNA

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Purpose of review
Understanding the effects of in-utero exposures to environmental agents is of great importance as the resulting deregulation of biological processes can affect both fetal development and health outcomes that manifest later in life. Due to their established role in developmental processes and inherent stability *ex vivo*, microRNAs (miRNAs) have emerged as attractive candidates to explore the impact of such exposures during this critical window of susceptibility. In this review, we summarize the findings of studies assessing miRNAs as markers of in-utero environmental exposures and as candidates for the molecular basis through which these exposures exert their influence on children's health.

Recent findings
To date, miRNA expression profiles due to various in-utero environmental exposures, including xenochemicals, endogenous factors, and nutritional status, have been reported.

Summary
While the validity of the identified exposure-specific miRNA profiles remains to be established, the findings thus far do raise interesting questions worth addressing in future studies. Gaps that remain to be addressed include linking specific in-utero exposures to subsequent health outcomes based on established miRNA expression profiles and experimentally validating putative downstream targets of the deregulated miRNAs.

Keywords
endogenous exposures, in-utero exposures, miRNA, nutrition, xenochemicals

INTRODUCTION

The impact of environmental exposures on human health ranges from acute effects, such as skin irritation, to chronic long-term consequences, including cognitive and reproductive dysfunction. The life stage during which the exposure occurs has been deemed a critical determinant on the health consequences realized. The physiologic and metabolic characteristics of fetal development, including the immaturity of the blood–brain barrier and the reduced detoxification and elimination capacity of the internal organs such as liver and kidneys, mark the intrauterine experience as a period of heightened susceptibility [1]. In addition to impacting fetal development, environmental exposures can also modify the genome to program health outcomes experienced later in life. The molecular mechanism underlying this fetal programming, also referred to as the 'developmental origins of health and disease (DOHAD)' [2], has not been clearly delineated. However, mounting evidence suggests that epigenetics plays a vital role.

The epigenome refers to heritable, quasi-stable yet dynamic and environmentally responsive elements that regulate gene expression without changes in the DNA sequence itself [3]. Various epigenetic marks have been identified, including transcriptional regulation through DNA methylation and histone modification, as well as post-transcriptional regulation through microRNAs (miRNAs).

miRNAs are small noncoding RNAs, typically 21–23 nucleotides in length, formed from transcripts that take on a characteristic hairpin structure [4]. The process to generate miRNAs is initiated through the transcription of primary miRNA (pri-miRNA) by RNA polymerase II [5]. Processing

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KEY POINTS

- MicroRNA expression profiles reflecting in-utero environmental exposures are continually emerging.
- Published expression profiles will need to be validated to establish exposure-specific signatures.
- Additional gaps that remain to be addressed in future studies include experimentally validating putative downstream gene targets and linking miRNAs deregulated due to in-utero exposures to later health outcomes.

by DROSHA, an RNAse III endonuclease, generates a 60–70-nt stem loop intermediate with a 3' overhang [6,7]. This precursor miRNA (premRNA) is actively transported from the nucleus to the cytoplasm via Ran-GTP and Exportin-5, where it is further processed by an additional RNAse III endonuclease, DICER, into a double-stranded miRNA:miRNA* complex [8,9,10]. The mature single-stranded miRNA is loaded into the RNA-induced silencing complex (RISC), where it serves as a guide for targeting the RISC to mRNAs based on complementarity [11,12]. The degree of complementarity between a miRNA and its targets determines the mechanism of posttranscriptional regulation. Near perfect pairing results in cleavage of the target mRNA, whereas partial pairing, a phenomenon more commonly observed in animals, results in either mRNA decay or translational repression [13].

To date, over 2000 unique mature human miRNAs have been annotated and catalogued in the publicly accessible database miRBase (www.miRbase.org), and up to one-third of all mRNA transcripts are believed to be regulated by miRNAs [14]. As targeting only requires partial complementarity, a single miRNA can bind multiple mRNA transcripts and each mRNA can be bound to multiple miRNAs. In this way, miRNAs are able to modulate entire gene networks, including biological processes such as development, stem cell differentiation, hematopoiesis, cardiac/muscle development, neurogenesis, insulin secretion, cholesterol metabolism, and immune response [15]. Significant modifications in the expression of miRNAs likely result in the deregulation of these processes, ultimately triggering disease. Children are a population of special interest in determining the impact of miRNA deregulation as the heightened susceptibility to early life environmental exposures may play a role in modulating miRNA expression levels. Additionally, the downstream effects owing to the resulting deregulation of miRNAs are likely more pronounced as many of the miRNA-regulated physiologic processes are most active early in the developmental process. Hence, this review focuses on miRNAs as markers of in-utero environmental exposures and as candidates for the molecular basis through which these exposures exert their influence on children's health.

microRNA AND CHILDREN'S HEALTH

A number of studies have implicated miRNAs in various pediatric health outcomes. As summarized in Table 1, these studies showcase the wide-ranging utility of miRNAs as markers of disease, in terms of diagnosis, prognosis, disease subtype classification, treatment monitoring, and agents of intervention. However, greater understanding is also required in identifying the upstream elements capable of disrupting the expression profile of miRNAs, including environmental factors, ultimately resulting in disease.

microRNA AND THE IN-UTERO ENVIRONMENT

Studies that have investigated the responsiveness of miRNAs to early life environmental exposures are summarized in Table 2. The assessed exposures fall into three general categories: xenochemicals, endogenous agents, and nutrition. The group of xenochemicals includes ethanol and tobacco smoke, established teratogens known to cross the placenta and induce fetal malformation. The remaining chemicals in this group, including the endocrine disruptor bisphenol A, have suspected but not yet verified detrimental effects on normal human fetal development. Studies revolving around endogenous factors, including parental stress and hypoxia, indicate that miRNAs are responsive not only to exogenously introduced chemical exposures, but also to the dysregulation of the internal environment. Finally, nutrition is highlighted as its own distinct category, since the importance of the nutritional state in fetal development and programming has been previously established, the best-documented of which links fetal malnutrition with later onset of metabolic diseases in studies conducted on individuals exposed to the Dutch famine [55]. The following sections highlight findings from representative exposures in each category.

Tobacco smoke

Two human observational studies have been carried out to date assessing the role of miRNAs as potential mediators of the impact of tobacco smoke on in-utero development. Maccani *et al.* [46] compared

Table 1. Studies assessing microRNAs in pediatric health outcomes

Outcome	miRNA-related assessment	Impact on outcome	Reference
Acute lymphocytic leukemia (ALL)	miRNA expression signature profile	Diagnostic: case–control differences	Schotte *et al.* 2009 [16]; Zhang *et al.* 2009 [17]; Oliveira *et al.* 2012 [18]
	miRNA expression signature profile	Disease subtype classification: T-ALL/MLL vs. ALL	Schotte *et al.* 2009 [16]
	miRNA expression signature profile	Disease subtype classification: T-ALL vs. B-ALL	Fulci *et al.* 2009 [19]
	miRNA expression signature profile	Treatment monitoring: response to prednisone	Zhang *et al.* 2009 [17]
	miRNA expression signature profile	Clinical prognosis	Zhang *et al.* 2009 [17]
	miRNA expression signature profile	Clinical prognosis	Kaddar *et al.* 2009 [20]
Asthma	Presence of SNPs in HLA-G impacts miRNA binding to 3′UTR region	Cause: Case–control difference	Tan *et al.* 2007 [21]
	Presence of SNPs in miRNA genetic sequence	Cause: Case–control differences	Su *et al.* 2011 [22]
	AntagomiR-targeted downregulation of miRNA	Intervention: reduces eosinic inflammation, mucus hypersecretion, TH2 cytokine production, airway hyperresponsiveness	Collison *et al.* 2011 [23]
Autism	miRNA expression signature profile	Diagnostic: Case–control differences	Abu-Elneel *et al.* 2008 [24]
	miRNA expression signature profile	Diagnostic: Case–control differences	Sarachana *et al.* 2010 [25]
	In-silico analysis	Etiology: miRNAs known to be deregulated in autism present in CNV loci	Vaishnavi *et al.* 2013 [26]
Congenital heart disease (CHD)	Presence of SNP in miRNA coding region	Etiology: Case–control differences	Xu *et al.* 2009 [27]
	In-silico analysis	Etiology: miRNAs present in CHD-related CNV loci	Xing *et al.* 2013 [28]
	miRNA expression signature profile	Diagnostic: Case–control differences	Zhu *et al.* 2013 [29]
Cystic fibrosis	miRNA expression signature profile	Diagnostic: Case–control differences	Oglesby *et al.* 2010 [30]
	In-silico/in-vitro analysis of CFTR 3′UTR binding targets	Etiology: miRNAs regulate CFTR gene expression	Megiorni *et al.* 2011 [31]
	In-silico/in-vitro analysis of miRNAs and binding targets involved in CFTR regulation	Treatment: restore function of mutated CFTR protein	Ramachandran *et al.* 2012 [32]
	miRNAs involved in CFTR regulation	Cause: miRNAs regulate CFTR gene expression	Oglesby *et al.* 2013 [33]
	In-vitro analysis: biogenesis and maturation of candidate miRNA	Cause: RNA binding proteins regulate maturation of miRNA upregulated in CF	Bhattacharyya *et al.* 2013 [34]
Diabetes, Type 1 (T1D)	Candidate miRNA expression analysis	Clinical prognosis	Sebastiani *et al.* 2011 [35]
	miRNA expression signature profile	Diagnostic: Case–control differences	Nielsen *et al.* 2012 [36]
	Candidate miRNA expression analysis	Diagnostic: Case–control differences	Salas-Pérez *et al.* 2013 [37]
Neuroblastoma	miRNA expression signature profile	Disease subtype classification	Chen and Stallings, 2007 [38]
	miRNA expression signature profile	Clinical prognosis	Bray *et al.* 2009 [39]
Obesity	miRNA expression signature profile	Diagnostic: miRNA expression pattern associated with obesity markers	Prats-Puig *et al.* 2013 [40]

3′ UTR, 3′ untranslated region; ALL, acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHD, chronic heart disease; CNV, copy number variation; HLA-G, human leukocyte antigen G; miRNA, microRNA; MLL, mixed lineage leukemia; SNP, single nucleotide polymorphism; T-ALL, T-cell acute lymphoblastic leukemia; TH2, Type 2 helper cells.

the expression level of candidate miRNAs in 25 placentas obtained at the time of delivery from women with and without a history of smoking during pregnancy. Out of the four selected targets, a significant downregulation of miR-16, miR-21, and miR-146a was observed due to maternal

Table 2. Impact of in-utero environmental exposures on microRNA expression levels

Environmental agent	miRNA	Target gene/ pathway	Function	Species	Biospecimen/ cell type	Reference
Xenochemical						
BPA	miR-146a		TLR/cytokine signaling	Human	Placenta cell line	Avissar-Whiting *et al.* 2010 [41]
Ethanol	miR-21	*Jag-1*	Notch ligand/proliferation of neuroepithelial cells	Mouse	Cerebral cortex-derived neurospheres	Sathyan *et al.* 2007 [42]
	miR-335	*Ela Vl2*	Promotes neuronal maturation			
	miR-9					
	miR-153					
	miR-10a	*HOXa1*		Mouse	Brain	Wang *et al.* 2009 [43]
	miR-10b					
	miR-9					
	miR-145					
	miR-153a	*Pou4f1*	Regulation of transcription	Zebrafish	Embryo	Soares *et al.* 2012 [44]
	miR-30d	*Pou4f1*				
	miR-725					
	let7k					
	miR-100					
	miR-738					
	miR-732					
Gold nanoparticles	let-7a		Cell proliferation	Mouse	Liver and lung	Balansky *et al.* 2013 [45]
			k-RAS activation			
			Apoptosis			
	miR-183		Apoptosis			
			Cell adhesion			
Maternal smoking	miR-146a	*BCL2L2/EDA*	Pro-survival	Human	Placenta	Maccani *et al.* 2010 [46]
			NF-κβ pathway			
	miR-16	*PLAG1/ SATB1*	Cell cycle/cell proliferation transcription factors			
	miR-21	*TRAF6*	NF-κβ/TLR4 pathway			
	miR-223		Progenitor cell proliferation/ granulocyte differentiation	Human	Cord blood	Herberth *et al.* 2013 [47▪]
PCBs	miR-762	*Wnt1*	Cardiovascular differentiation	Mouse	P19 cells embryonal carcinoma	Zhu *et al.* 2012 [48]
	miR-29a	*Gsk3β*	Cardiovascular differentiation			
	miR-324-	*Nkx2.5*	Heart development			
	5p					
PFOS	miR-19b-c	*Cdk5 Smad1 Sox11b Pou5f1*	Brain and nervous system development	Zebrafish	Embryo	Zhang *et al.* 2011 [49]
		Bax	Apoptosis			
		Vsx1				

Table 2 (*Continued*)

Environmental agent	miRNA	Target gene/ pathway	Function	Species	Biospecimen/ cell type	Reference
	miR-19d					
	miR-181b-c					
	miR-735					
	miR-739					
	miR-297	*Ypel5*		Rat	Brain	Wang *et al.* 2012 [50]
	miR-672	*Fgfr3*				
		Syt1				
TCDD	mir-122		Metabolism	Mouse	Thymus	Singh *et al.* 2012 [51]
	miR-181a		T-cell sensitivity and selection			
	miR-23a	*Fas*	Apoptosis			
	miR-18b	*FasL*	Apoptosis			
	miR-31	*Cyp1a1*	Metabolism			
	miR-182	*AhR*	Metabolism			
Endogenous factor						
Hypoxia	mR-520c-3p			Human	Placenta	Donker *et al.* 2012 [52▪]
Paternal stress	miR-322	*β-glycan*	TGF superfamily	Mouse	Brain	Morgan *et al.* 2011 [53▪]
	miR-574-3p					
	miR-873					
Nutrition						
High-fat diet	miR-483*	*Zswim3*		Mouse	Liver	Zhang *et al.* 2009 [54]

AhR, aryl hydrocarbon receptor; BAX, BCL2-associated X protein; BCL2L2, BCL2-like 2; BPA, bisphenol A; CDK5, cyclin-dependent kinase 5; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; EDA, ectodysplasin A; ELAVL2, ELAV-like neuron-specific RNA binding protein 2; Fas, Fas cell surface death receptor; FasL, Fas ligand; GSK3B, glycogen synthase kinase 3 beta; HOXa1, homeobox A1; Jag-1, jagged 1; k-RAS, Kirsten rat sarcoma viral oncogene homolog; NFkB, nuclear factor kappa B; NKX2.5, NK2 homeobox 5; PCBs, polychlorinated biphenyls; PFOS, perfluorooctane sulfonate; PLAG1, pleiomorphic adenoma gene 1; Pou4f1, POU class 4 homeobox 1; Pou5f1, POU class 5 homeobox 1; SATB1, SATB homeobox 1; Smad1, SMAD family member 1; Sox11b, SRY-box containing gene 11b; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transcription growth factor; TLR, Toll-like receptor; TLR4, Toll-like receptor 4; TRAF6, TNF receptor-associated factor 6; Vsx1, visual system homeobox 1; Wnt1, wingless-type MMTV integration site family, member 1; ZSWIM3, zinc finger, SWIM-type containing 3.

cigarette smoking. Furthermore, miR-146a was significantly downregulated following exposure to both nicotine and benzopyrene in TCL-1 (immortalized human trophoblast cell line), a cell line derived from third trimester extravillous cells, whereas no impact due to either agent was observed on the remaining miRNAs.

The impact on miRNA expression levels due to tobacco smoke exposure during the gestational period was also addressed in a study conducted by Herberth *et al.* [47▪]. As this study was particularly concerned with the effect of tobacco smoke on immune-related responses, two candidate miRNAs, miR-155 and miR-223, previously implicated in Treg cell formation and function, were selected for analysis. In this prospective study of mother–child pairs, increasing levels of miR-223 were observed in both maternal and cord blood with increasing levels of cotinine in maternal urine.

Parental stress

In a study by Morgan and Bale [53▪], the impact of prenatal stress on brain development was assessed. For this purpose, the offspring of male mice that were prenatally exposed to maternal stressors were analyzed. Microarray analysis of brain-derived miRNAs revealed that the profile of F2 male offspring stemming from a stress-exposed lineage was more similar to the profile of nonexposed F2 female offspring than nonexposed F2 male offspring, suggesting dysmasculinization of the brain

among the F2 stress-exposed males. Specifically, three miRNA species, miR-322, miR-574-3p, and miR-873, were observed to be downregulated among the F2 stress-exposed offspring, whereas the expression level of β-glycan, a predicted target common to all three miRNAs, was determined to be upregulated compared with F2 control offspring. Finally, following treatment with the aromatase inhibitor formestane, the brain miRNA profile of formestane-treated male mice clustered more closely to control female mice than control male mice, further suggesting that the miRNA environment is sensitive to hormonal dysregulation. The overall findings from this study, therefore, suggest that the changes incurred during in-utero development can be transmitted transgenerationally.

Nutrition

The effects of a maternal high-fat diet on the miRNA profile of the resulting offspring were assessed in a murine model by Zhang *et al.* [54]. Microarray analysis of liver-derived miRNAs from female offspring identified 10 miRNAs that were upregulated and 23 miRNAs that were downregulated among offspring exposed to a high-fat diet. Although all targets were not successfully validated, the downregulation of miR-483∗ was consistently observed as the greatest fold-change among high-fat diet-exposed offspring. The authors determined that the genetic location of miR-483∗ lies within the intron of *Igf2,* a gene known to be critical in regulating fetal growth, indicating that the transcription of both is likely regulated by the same promoter. Paradoxically, among high fat-exposed mice, the downregulation of miR-483∗ was observed alongside an upregulation of *Igf2*.

FUTURE PERSPECTIVE

Findings relaying the impact of in-utero exposures on miRNA expression levels thus far have raised several interesting questions that will need to be further addressed. Principal among these is determining whether the observed changes in miRNA expression levels reflect a causal pathway between exposure and outcome, identifying the downstream gene targets impacted by deregulated miRNAs, and delineating variability introduced because of methodological and biospecimen specifications.

Role of microRNAs in linking exposure to outcome

To address this question, it will first have to be established whether the observed changes in miRNA expression levels due to environmental exposures are the result of a direct effect or a surrogate indication of a different mechanism. This issue was highlighted in the study by Herberth *et al.*, in which the expression level of miR-223 in blood was found to vary among different blood cell-types, leading the authors to question whether the observed tobacco-related increase in miR-223 levels is indeed a direct response to the exposure or a bystander effect of smoke-induced changes in blood cell-type composition [47▪]. Although the utility as a marker of exposure is not diminished in either case, the former would more directly associate miRNAs to the causal pathway linking exposures to putative deregulated physiological processes.

Given a substantiated impact of environmental exposures on miRNA expression levels and related physiological processes, more efforts into how these changes in expression profiles translate into health outcomes will also be warranted. To date, studies relating changes in miRNAs to various health outcomes and studies relating exposures to changes in miRNAs are often conducted separately. Few studies have linked the observed changes in miRNAs due to environmental exposures to known health effects. One such example includes the finding by Herberth *et al.* [47▪] that higher levels of miRNA-223 in cord blood were also correlated with decreasing levels of Treg cells in newborns, which in turn was shown to be associated with a significantly higher risk of developing atopic dermatitis by the age of 3. Such findings pave the way to further understand the etiologic processes underlying the exposure–outcome relationship and offer possibilities for remediation and intervention.

Prediction of microRNA targets

In order to identify potential physiological processes that are affected by the exposure-incurred changes in miRNA profiles, studies often employ various existing web-based programs (e.g., miRBase, Targetscan, PicTar) to identify putative mRNA targets of the aberrantly expressed miRNAs. Using slightly varying algorithms, these programs scan a library of mRNA 3′UTR regions to identify potential binding sites for the miRNAs of interest. However, as binding of miRNAs to their targets requires only partial complementarity, prediction of targets typically yields up to 20% false positives [56]. The substantial rate of false positives and the observed discrepancy in predicted targets across the various algorithms highlight the need to experimentally validate reported putative targets.

Methodological differences: next generation sequencing vs. microarray

Several high-throughput methodologies are currently in use to determine miRNA expression levels.

Utilization of microarrays relies on nucleic-acid hybridization of labeled miRNA-derived cDNAs to complementary oligonucleotide probes immobilized on the array, with fluorescence intensity indicating the level of expression. This methodology is currently the most widely applied means of high-throughput determination of miRNA expression levels. However, innate properties of miRNAs limit the sensitivity and specificity achieved with this method. For example, miRNAs of low abundance likely fall below the limit of detection on the array. There is also considerable sequence homology among miRNAs, which probes on the array may not be sensitive enough to distinguish. Next generation sequencing is a methodology that is able to address several of these limitations. Additionally, unlike array-based methods, profiles generated using sequencing are not limited to previously identified miRNAs. However, the multiple steps involved in setting up the sequencing reaction offer various opportunities to introduce bias [57]. Furthermore, although the price is continuously dropping, the cost of sequencing at present is still higher than array-based methods.

Specimen issues: placenta, cord blood, and maternal plasma

As with any biomarker-related study, the biospecimen source of miRNAs greatly influences the interpretation of meaningful results. Placental tissue, umbilical cord blood, and maternal sera are common sources for biomarkers reflecting the in-utero experience in human observational studies. All three serve as easily obtainable, noninvasive sources of miRNAs. However, there are factors driving distinctions in the expression profile generated from these biospecimens that need to be considered. Both placenta and blood are composite tissues consisting of heterogeneous cell types. Therefore, minimizing variability in cell-type composition across samples needs to be accounted for prior to analysis to reduce the likelihood of introducing bias into the study. Cord blood does provide access to specific cell lineages that make this particular biospecimen an attractive source for studies focusing on the impact of environmental exposures on the differentiation potential of stem cell populations, such as neuronal precursors, and on the immune response of cytokines.

While the expression profile generated from placental tissue and cord blood reflects the exposure experience toward the end of pregnancy, maternal plasma offers a means to monitor dynamic changes in expression level throughout pregnancy, enabling the focus on specific gestational periods. However, the proportion of placental miRNA in circulation likely fails to account for the comprehensive expression profile of the placenta. Therefore, maternal plasma is best suited to develop screening markers, whereas cord blood and placental levels can also be analyzed to further etiologic understanding.

CONCLUSION

The implementation of miRNAs as indicators of environmental exposures relevant to children's health is still a nascent field, and the promise of their utility is just beginning to be realized. While mRNA transcript levels, the ultimate targets of miRNAs, can also be utilized for this purpose, one of the major attractions over their labile transcript counterparts is the inherent stability and robustness of miRNAs in bodily fluids under various conditions [58,59]. Furthermore, technological advances have made high-throughput assessment affordable, providing a means of feasible and sensitive detection.

The relevance of this marker in these types of studies is also established by the fact that miRNAs are known to be involved in processes with heightened activity during early development. Hence, beyond reflecting extent of exposure to an environmental agent, an observed deregulation of miRNA levels can also point to the etiologic mechanism, ultimately linking a given exposure to an outcome.

Already various exposure and outcome-related signatures have been identified. However, the lack of comparability in experimental conditions prevents deriving meaningful conclusions from the findings reported thus far. Even in studies focusing on the same exposure of interest, variability exists in the form of dosage, study population, biospecimen analyzed, detection methods utilized, and definitions for fold cut-offs. Hence, while technological advances yet to come will further facilitate the ability to assay and analyze expression profiles under various conditions, resulting in novel contributions to the literature, future studies should also focus on replicating existing expression profiles. Only by establishing reproducible expression profiles and validating putative gene targets can the ultimate goal of building a cohesive narrative tying environmental exposures to children's health outcomes be realized.

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Conflicts of interest

There are no conflicts of interest.

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- ■■ of outstanding interest

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