miRNA biomarkers for prenatal alcohol exposure and effect in pregnant women.

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Conflict of Interest Statement:

I do **not** have an affiliation (financial or otherwise) with a pharmaceutical, medical device or communications organization.

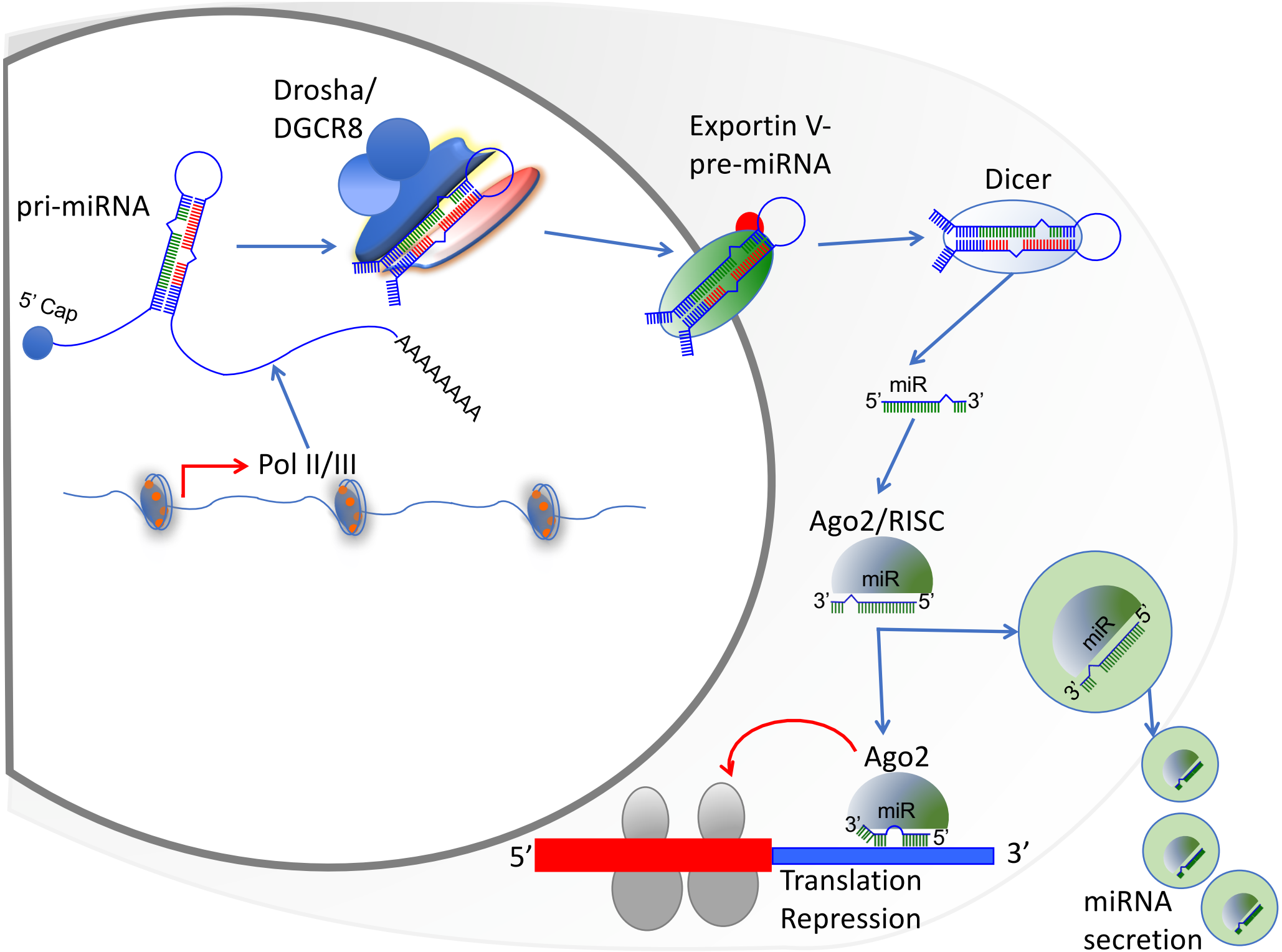
Biomarker development for FASD

- Biomarkers for exposure*
  - γ-glutamyltransferase(GGT)
  - Mean corpuscular volume (MCV)
  - Carbohydrate deficient transferrin (CDT)
  - Alcohol metabolites in newborn
    - Meconium
    - Placenta
    - Dried blood spots (Guthrie cards)
- Biomarkers that predict FASD outcomes
  - ?
- The Problem:
  - Not all prenatally exposed infants (even heavily exposed), exhibit craniofacial dysmorphia or growth deficits.
  - Nevertheless, these infants may be at risk for intellectual and secondary disabilities if undiagnosed

* Bakhireva LN, Savage DD. Focus on: biomarkers of fetal alcohol exposure and fetal alcohol effects. Alcohol Res Health. 2011;34(1):56-63. Review. PubMed PMID: 23580042; PubMed Central PMCID: PMC3860558.

Can microRNAs (miRNAs) be bio-markers for FASD outcomes?

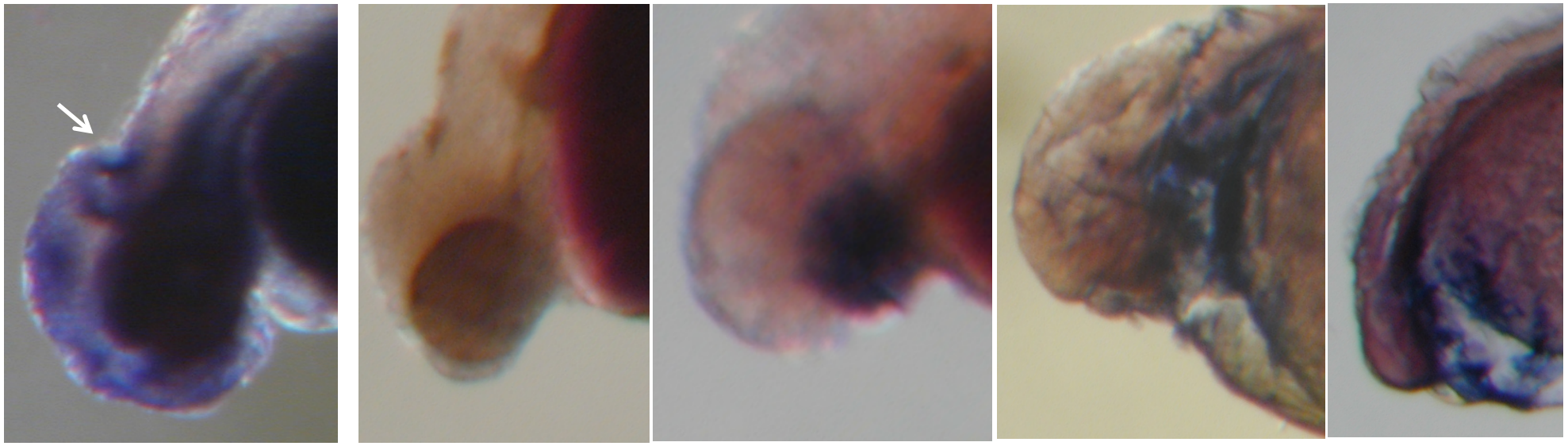
- But what are miRNAs?

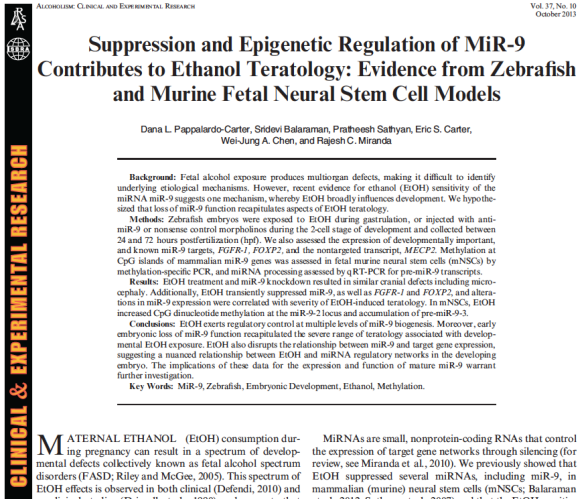

Drosha/
DGCR8
pri-miRNA
5' Cap
AAAAAAA
Pol II/III
Exportin V-
pre-miRNA
Dicer
5' miR 3'
Ago2/RISC
miR
3' 5'
miR
5'
3'
Ago2
miR
3' 5'
5'
3'
Translation
Repression
miRNA
secretion

Ethanol decreases miR-9 Expression in Zebrafish

Control

Ethanol-Exposed





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Suppression and Epigenetic Regulation of MiR-9 Contributes to Ethanol Teratology: Evidence from Zebrafish and Murine Fetal Neural Stem Cell Models

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Background: Fetal alcohol exposure produces multiorgan defects, making it difficult to identify underlying etiological mechanisms. However, recent evidence for ethanol (EtOH) sensitivity of the miRNA miR-9 suggests one mechanism, whereby EtOH broadly influences development. We hypothesized that loss of miR-9 function recapitulates aspects of EtOH teratology.

Methods: Zebrafish embryos were exposed to EtOH during gastrulation, or injected with anti-miR-9 or nonsense control morpholinos during the 2-cell stage of development and collected between 24 and 72 hours postfertilization (hpf). We also assessed the expression of developmentally important, and known miR-9 targets, *FGFR-1*, *FOXP2*, and the nontargeted transcript, *MECP2*. Methylation at CpG islands of mammalian miR-9 genes was assessed in fetal murine neural stem cells (mNSCs) by methylation-specific PCR, and miRNA processing assessed by qRT-PCR for pre-miR-9 transcripts.

Results: EtOH treatment and miR-9 knockdown resulted in similar cranial defects including microcephaly. Additionally, EtOH transiently suppressed miR-9, as well as *FGFR-1* and *FOXP2*, and alterations in miR-9 expression were correlated with severity of EtOH-induced teratology. In mNSCs, EtOH increased CpG dinucleotide methylation at the miR-9-2 locus and accumulation of pre-miR-9-3.

Conclusions: EtOH exerts regulatory control at multiple levels of miR-9 biogenesis. Moreover, early embryonic loss of miR-9 function recapitulated the severe range of teratology associated with developmental EtOH exposure. EtOH also disrupts the relationship between miR-9 and target gene expression, suggesting a nuanced relationship between EtOH and miRNA regulatory networks in the developing embryo. The implications of these data for the expression and function of mature miR-9 warrant further investigation.

Key Words: MiR-9, Zebrafish, Embryonic Development, Ethanol, Methylation.

MATERNAL ETHANOL (EtOH) consumption during pregnancy can result in a spectrum of developmental defects collectively known as fetal alcohol spectrum disorders (FASD; Riley and McGee, 2005). This spectrum of EtOH effects is observed in both clinical (Defendi, 2010) and

MiRNAs are small, nonprotein-coding RNAs that control the expression of target gene networks through silencing (for review, see Miranda et al., 2010). We previously showed that EtOH suppressed several miRNAs, including miR-9, in mammalian (murine) neural stem cells (mNSCs; Balaraman

Pappalardo-Carter et al (2013) ACER

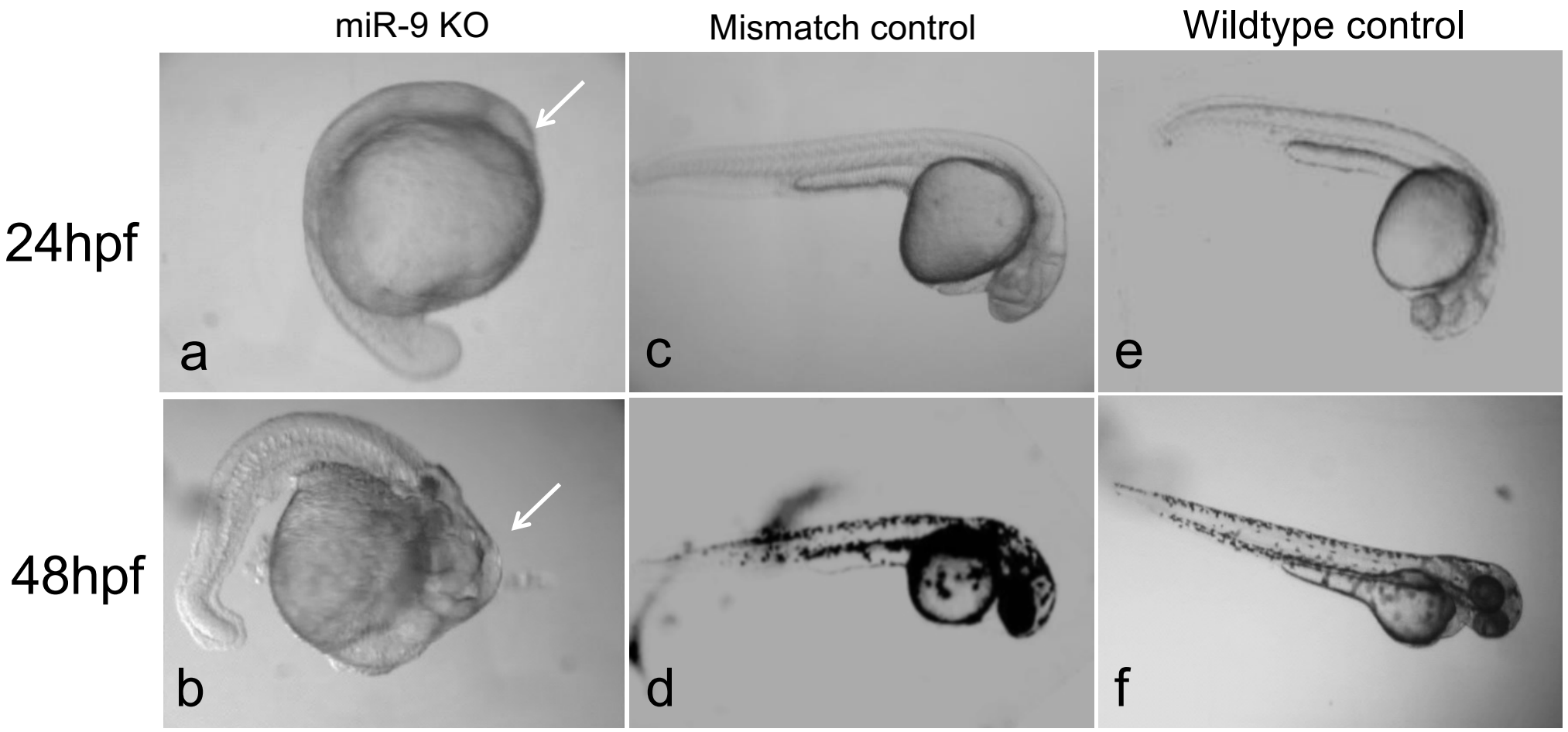
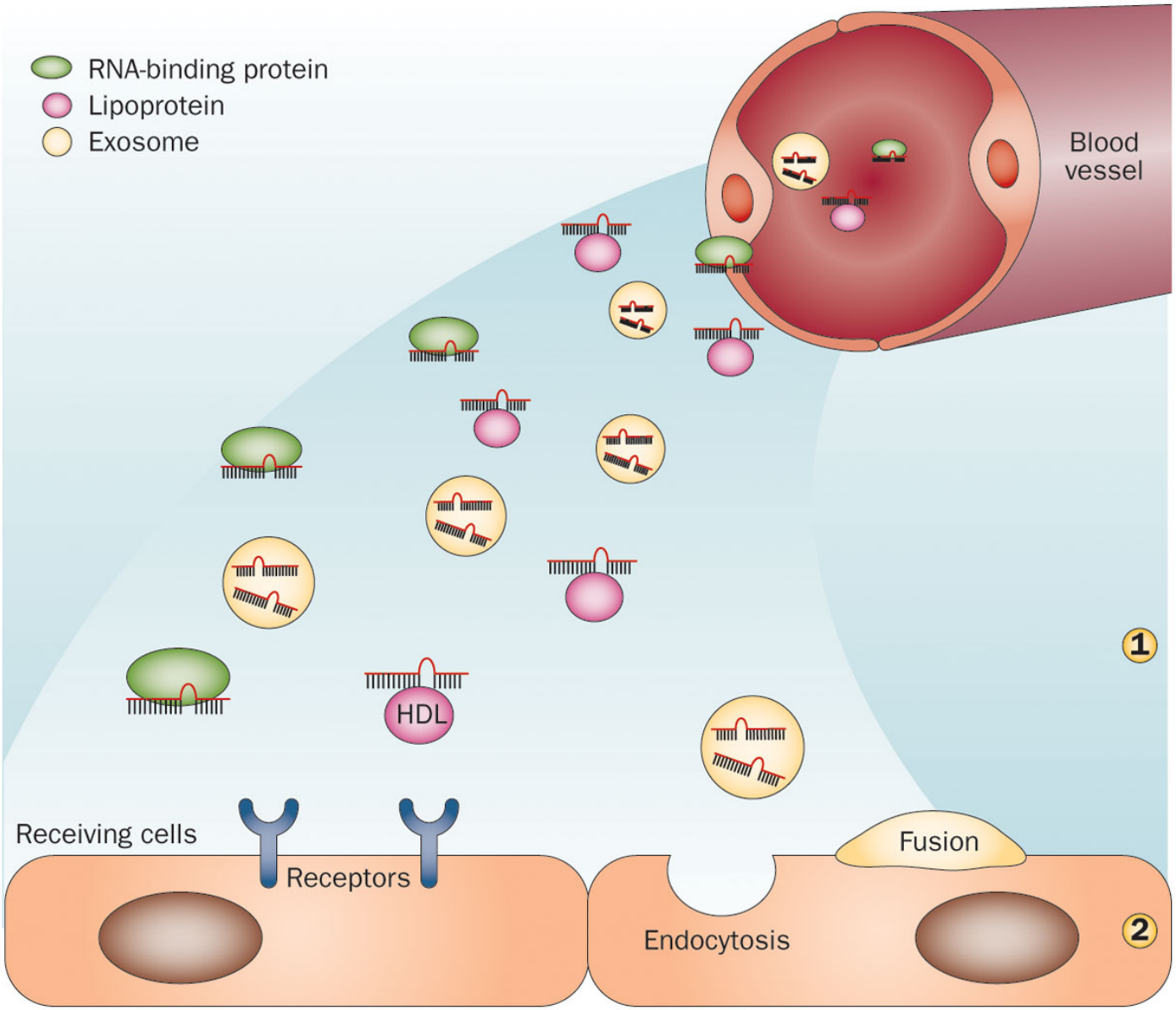

miR-9 KO
Mismatch control
Wildtype control
24hpf
48hpf
a
b
c
d
e
f

Figure 2 Blood and other body fluids contain active miRNAs

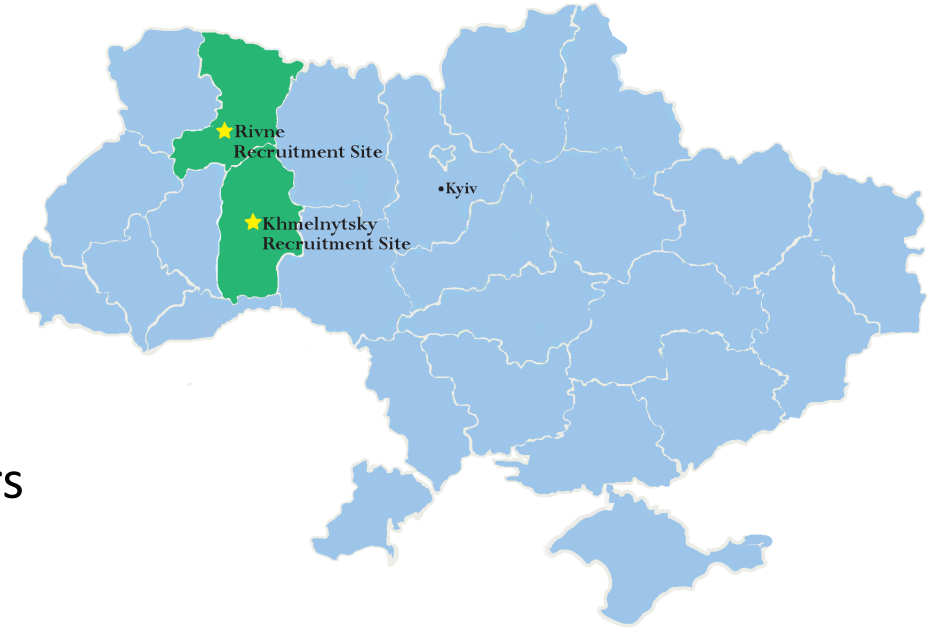


Guay, C. & Regazzi, R. (2013) Circulating microRNAs as novel biomarkers for diabetes mellitus *Nat. Rev. Endocrinol.* doi:10.1038/nrendo.2013.86

nature REVIEWS ENDOCRINOLOGY

Can maternal circulating miRNAs predict infant outcomes?

Methods



- Prospective Ukrainian cohort
  - Recruited between 2006-2011
  - Collaboration with Omni-Net Centers
- Participants were recruited from:
  - The Rivne Regional Medical Diagnostic Center
  - The Khmelnytsky Perinatal Center
- Screening for prenatal alcohol consumption was carried out by prenatal care providers
  - Infants were evaluated at 6 and 12months of age
- This study was approved by IRBs:
  - University of California, San Diego
  - Lviv Medical University in Ukraine
  - Texas A&M University

Methods Continued

- Maternal Plasma Samples were obtained at mid and late pregnancy (136 samples from 68 pregnant women)

ID	Group	Mid Pregnancy	Late Pregnancy	Totals
HEa	Alcohol-exposed, Infant Affected	22	22	44
HEua	Alcohol-exposed, Infant Unaffected	23	23	46
UE	Unexposed Control	23	23	46
	Totals	68	68	**136**

- Exiqon qRT-PCR miRNA arrays (752 miRNAs)

ANOVA Models

miRNA	MIMAT#	Exposure Group p<(BH)	UE		HEua		HEa	
			Mid Preg	Late Preg	Mid Preg	Late Preg	Mid Preg	Late Preg
hsa-miR-222-5p	MIMAT0004569	0.006						
hsa-miR-187-5p	MIMAT0004561	0.006						
hsa-miR-299-3p	MIMAT0000687	0.038						
hsa-miR-491-3p	MIMAT0004765	0.038						
hsa-miR-885-3p	MIMAT0004948	0.038						
hsa-miR-518f-3p	MIMAT0002842	0.038						
hsa-miR-760	MIMAT0004957	0.038						
hsa-miR-671-5p	MIMAT0003880	0.038						
hsa-miR-449a	MIMAT0001541	0.038						
hsa-miR-204-5p	MIMAT0000265	0.038						
hsa-miR-519a-3p	MIMAT0002869	0.038						
hsa-miR-363-3p	MIMAT0000707	0.065						
hsa-miR-378a-5p	MIMAT0000731	0.065						
hsa-miR-539-5p	MIMAT0003163	0.074						
hsa-miR-518b	MIMAT0002844	0.074						
hsa-miR-133b	MIMAT0000770	0.074						
hsa-miR-10b-5p	MIMAT0000254	0.074						
hsa-miR-517c-3p	MIMAT0002866	0.076						
hsa-miR-518e-5p	MIMAT0005450	0.076						
hsa-miR-524-3p	MIMAT0002850	0.088						
hsa-miR-147b	MIMAT0004928	0.097						

ANOVA Model: HEa>(HEua ≅ UE)

Can We Classify the HEua group?

- Random Forest Analysis
  - Machine learning classification strategy
- 5% of miRNAs with highest variance irrespective of class membership
- Included clinical/demographic variables
- Initial classification of HEa *vs*. UE

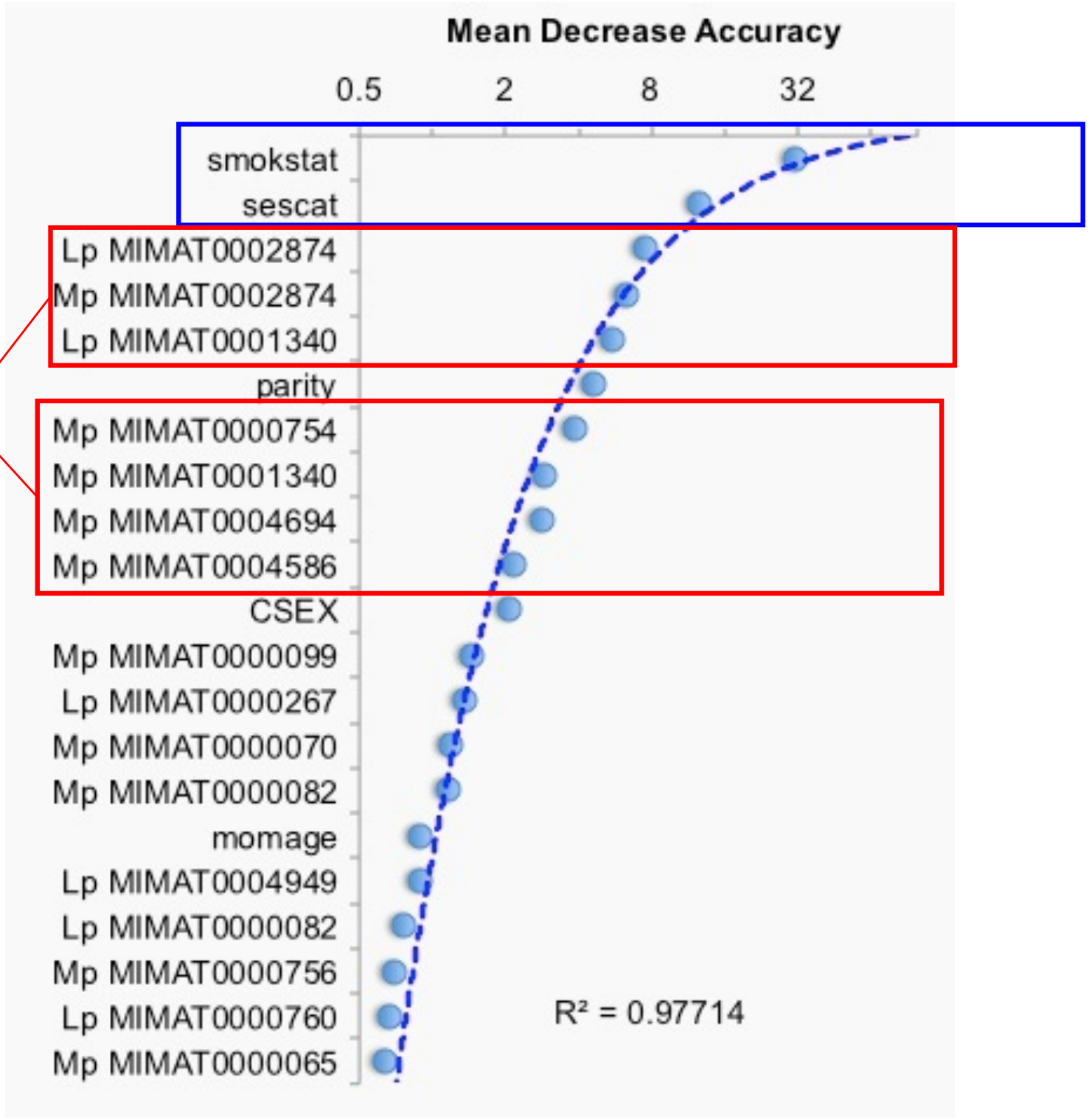
Top 5% high-variance miRNAs[*,#]

Confusion Matrix for Group HEa *vs.* UE

	Classified as HEa	Classified as UE	Classification error
True HEa	18	4	0.182
True UE	2	21	0.087

*With demographic and clinical variables. Overall mis-classification rate = 13.33

[#]Mid- and late-pregnancy miRNAs included in model as separate variables



hsa-miR-15b-3p

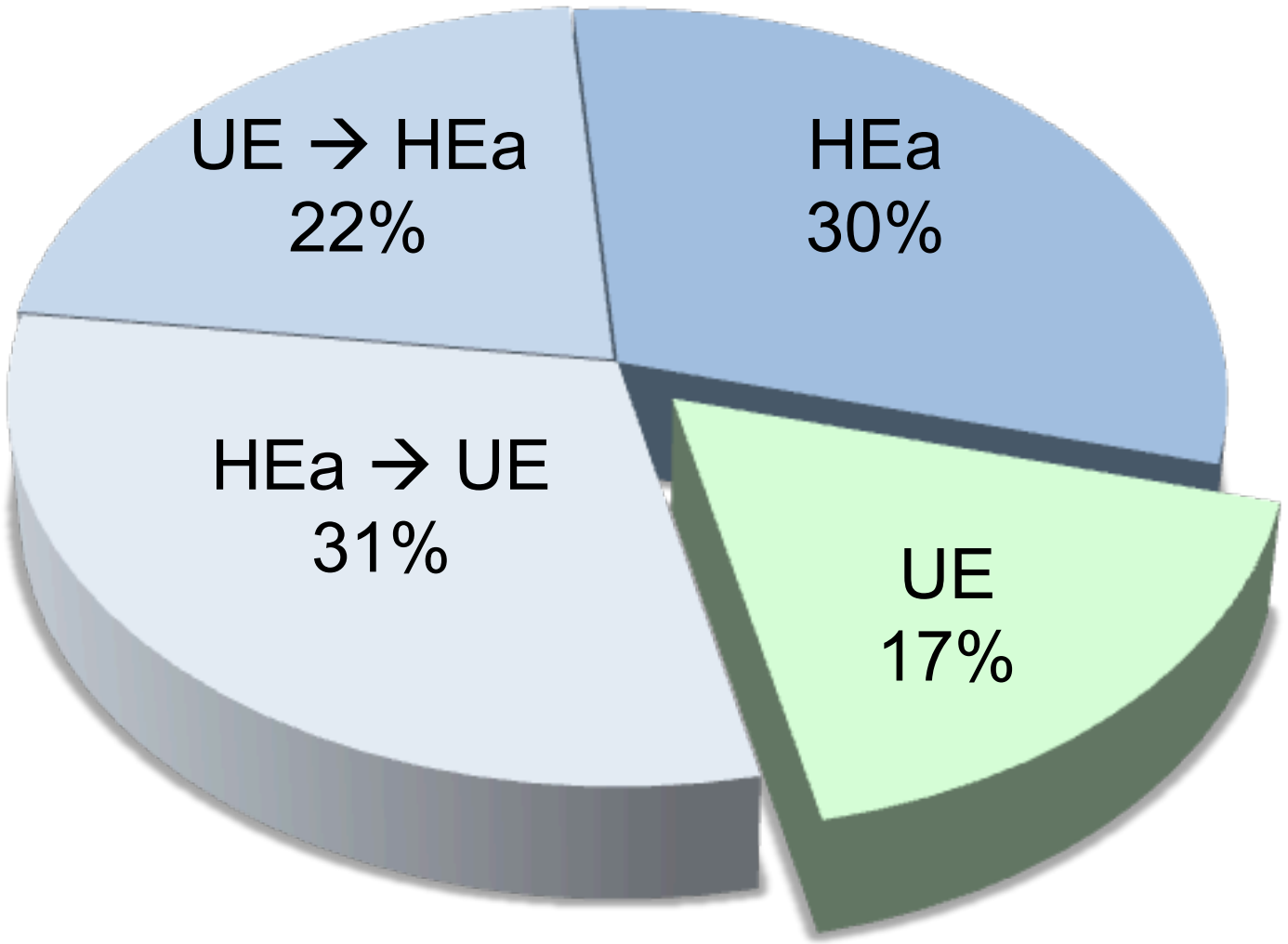
hsa-miR-337-3p

hsa-miR-342-5p

hsa-miR-423-3p

hsa-miR-503-5p

HEua Group Prediction



RFA model: (HEa ≅ HEua)≠UE

Understanding the biological underpinnings of maternal miRNA prediction models for FASD outcomes:

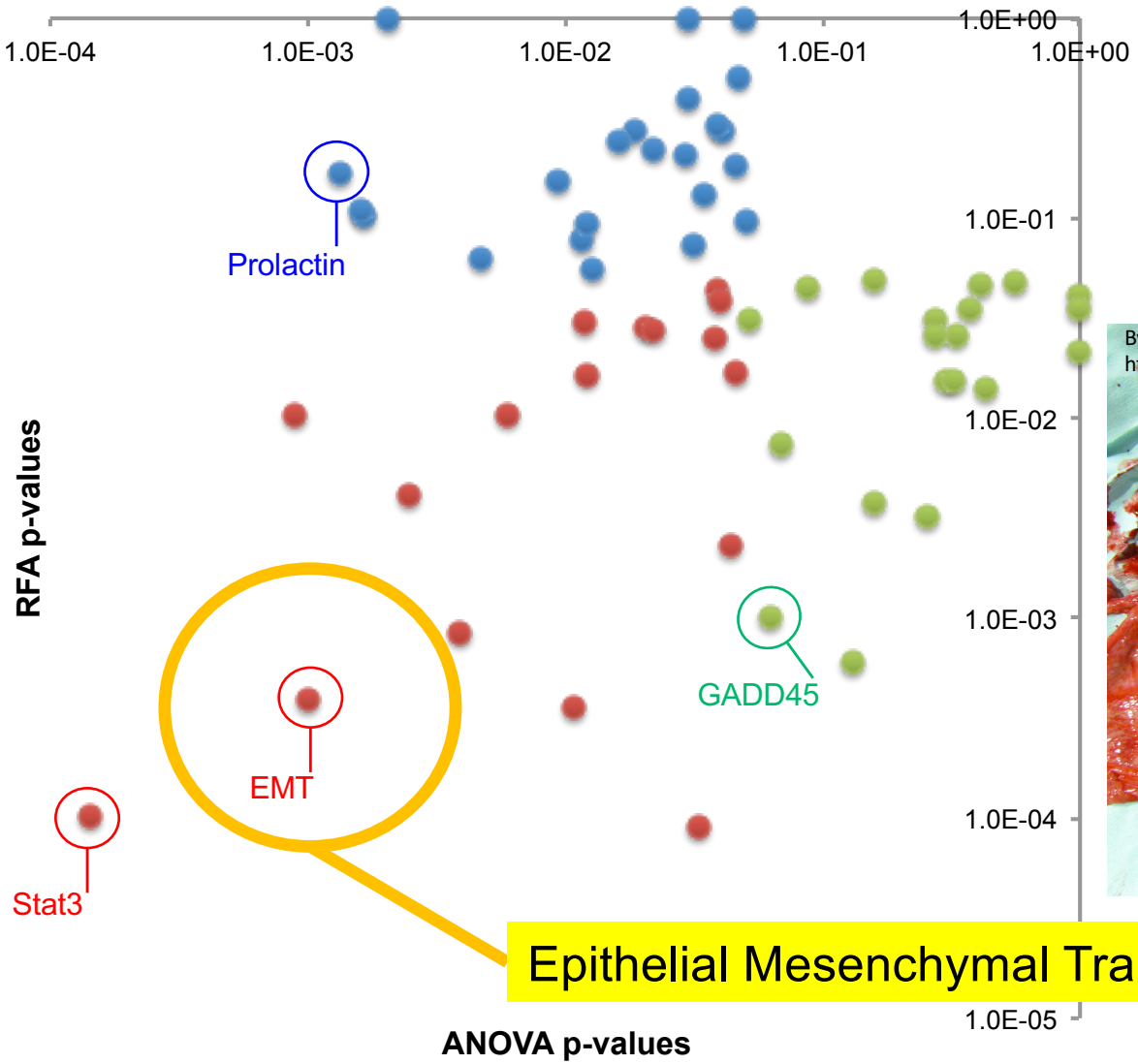
Are maternal miRNAs functional in the fetus?

Effects of ANOVA and RFA miRNAs on placental trophoblasts

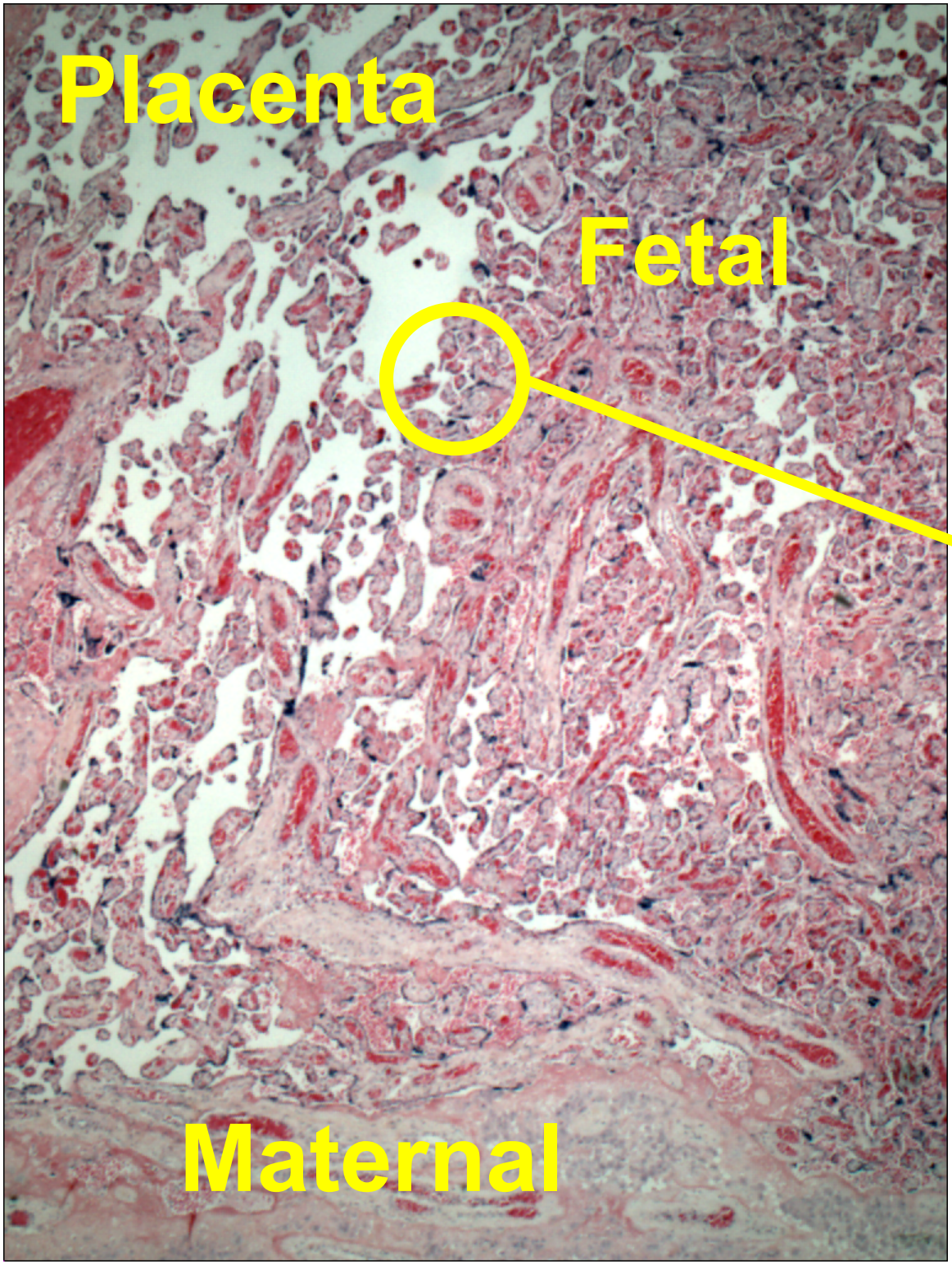
Common pathways

ANOVA-specific pathways HEa>(HEua ≅ UE)

RFA-specific pathways (HEa ≅ HEua)≠UE



Epithelial Mesenchymal Transition



Chorionic Villi

Trophoblasts

Maternal miRNAs control Placental growth

Conclusions: maternal miRNAs may be useful predictors of infant FASD outcomes.

- ANOVA models:
  - Plasma miRNAs in mid and late pregnancy separate HEa (Heavily exposed, affected) from other groups
  - miRNAs elevated in HEa group
- Random Forest Classification Models:
  - May be used to categorize HEua infants into risk subpopulations
- Identified miRNAs are functional in fetal tissues
  - Maternal miRNAs control placental invasion and cell proliferation

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