# New Molecules: microRNAs and Endometriosis

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# Outline of Presentation

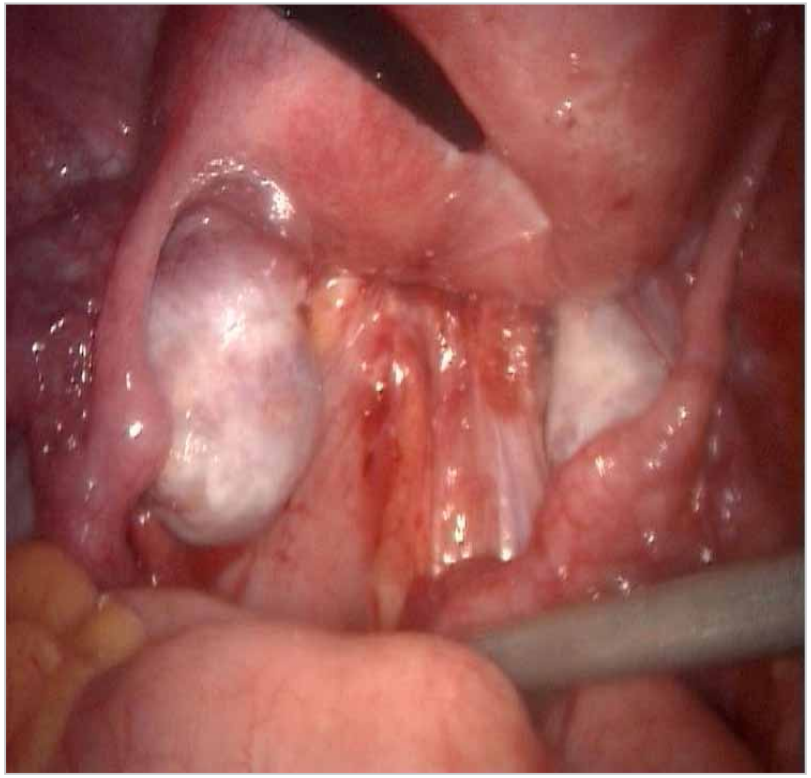
1. Background introduction

2. Eutopic vs ectopic microRNA analyses

3. Potential applications of microRNA technology
    - -Understanding the pathophysiology
    - -Diagnostic tests
    - -Therapeutics

# Endometriosis

Causes period pain and subfertility in ~10% reproductive aged women



There are significant costs to health care systems and society through loss of productivity

*Gao et al 2006*

Research into endometriosis has been an increasing priority

*Rogers et al 2009*

# Traditional Human Endometriosis Study

1. Is a small descriptive study
2. It hypothesises that a factor is upregulated in endometriotic lesions
3. The factor is identified in endometriotic tissues
4. It is concluded that the factor is likely to cause endometriosis
5. It is postulated that suppression of the factor may inhibit endometriosis

# mRNA microarray analyses

1. Have enabled us to develop a global picture of endometriosis

2. Have provided new insights into potential diagnostic and therapeutic targets


REPROMINE

*(Lam and Print unpublished data)*

Although the genelists from different studies do not correlate well there is a strong concordance in functional analysis findings between studies.

# Evidence of Post-transcriptional regulation

1. Human paired ectopic and eutopic array studies

   *(Hull et al 2008, Eyster et al 2007)*

   Anticipated transcripts were not differentially expressed such as aromatase, NFKB, TGFB

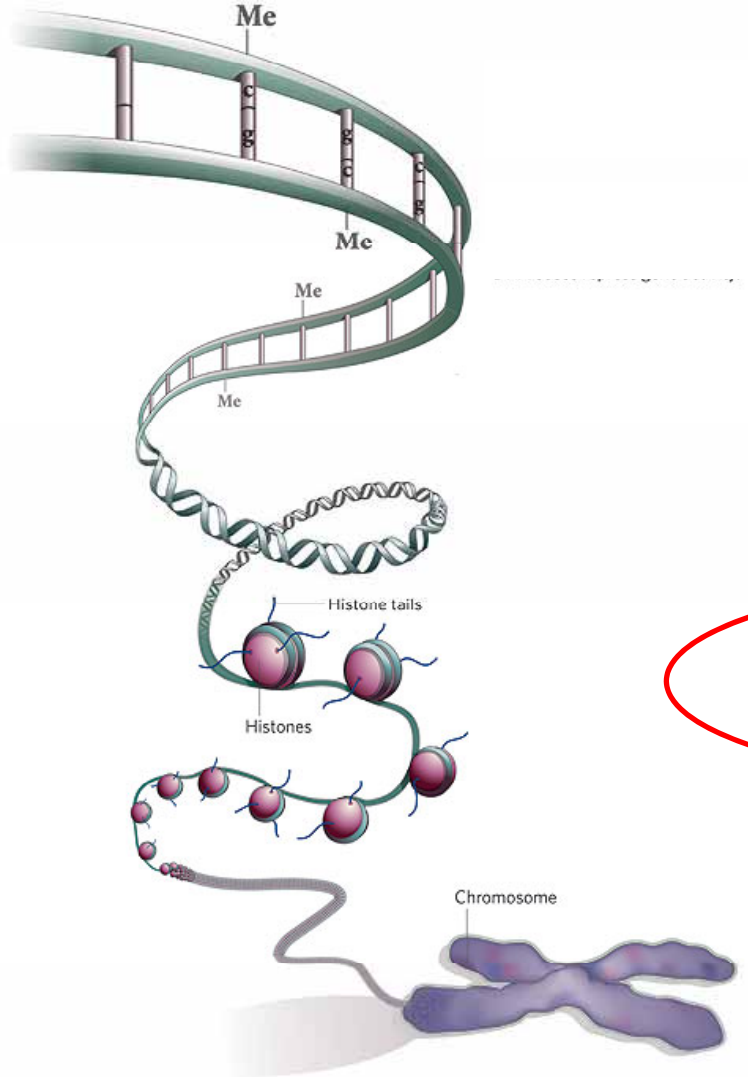
2. *In silico* IRIDESCENT analysis *(Wren et al 2007)*

   Genes and proteins associated with endometriosis in literature were not present in microarray databases

3. Proteomic studies *(Stephens et al 2010)*

   Lack of correlation between protein abundance and published mRNA gene array data

# Epigenetic regulation of gene expression




1. Methylation
2. Histone modification
3. microRNA regulation

*(reviewed in Guo et al 2009)*

# microRNAs

1. Are naturally occurring, short, non-coding RNAs

2. miRbase registry April 2010 *(http://www .mirbase.org)*
   14,197 miRNAs, 940 in humans

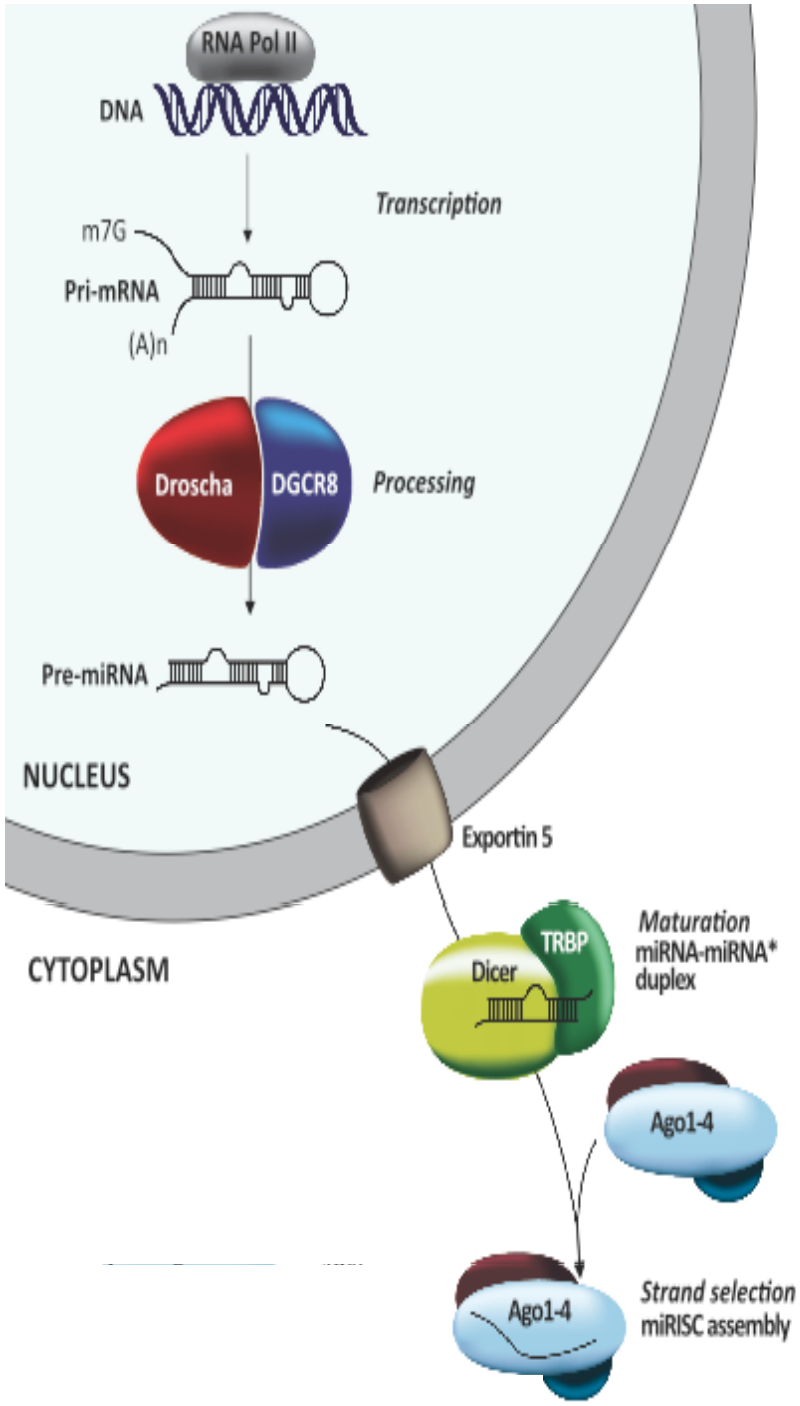
3. These microRNAs regulate ~ 8000 genes (~ 30% of genome)

4. One microRNA can regulate many mRNAs

5. Many microRNAs can regulate one microRNA

6. 5 publications on microRNAs in endometriosis (2 in Epub)

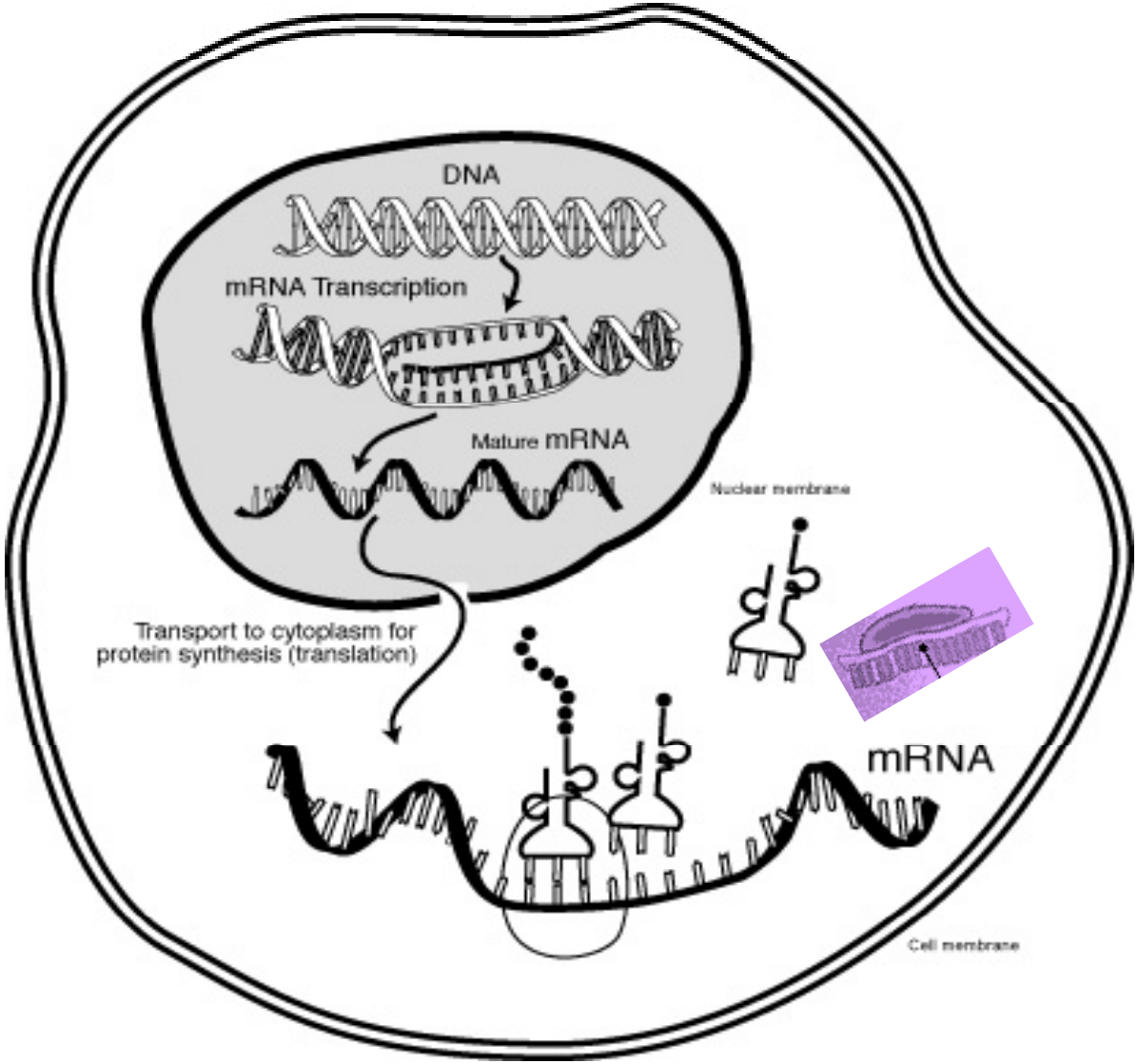
# microRNA Production




1. RNA Pol II trancribes  primary miRNAs
2. Drosha cleaves into 60nt  pre-miRNAs
3. Exportin 5 transports into cytoplasm
4. Dicer cleaves hairpin of pre-miRNA
5. Helicase unwinds sense-antisense strands
6. Mature miRNA strand selected
7. miRNA is Incorporated into RISC complex

*(Reviewed in Ohlsson Teague et al, 2010)*

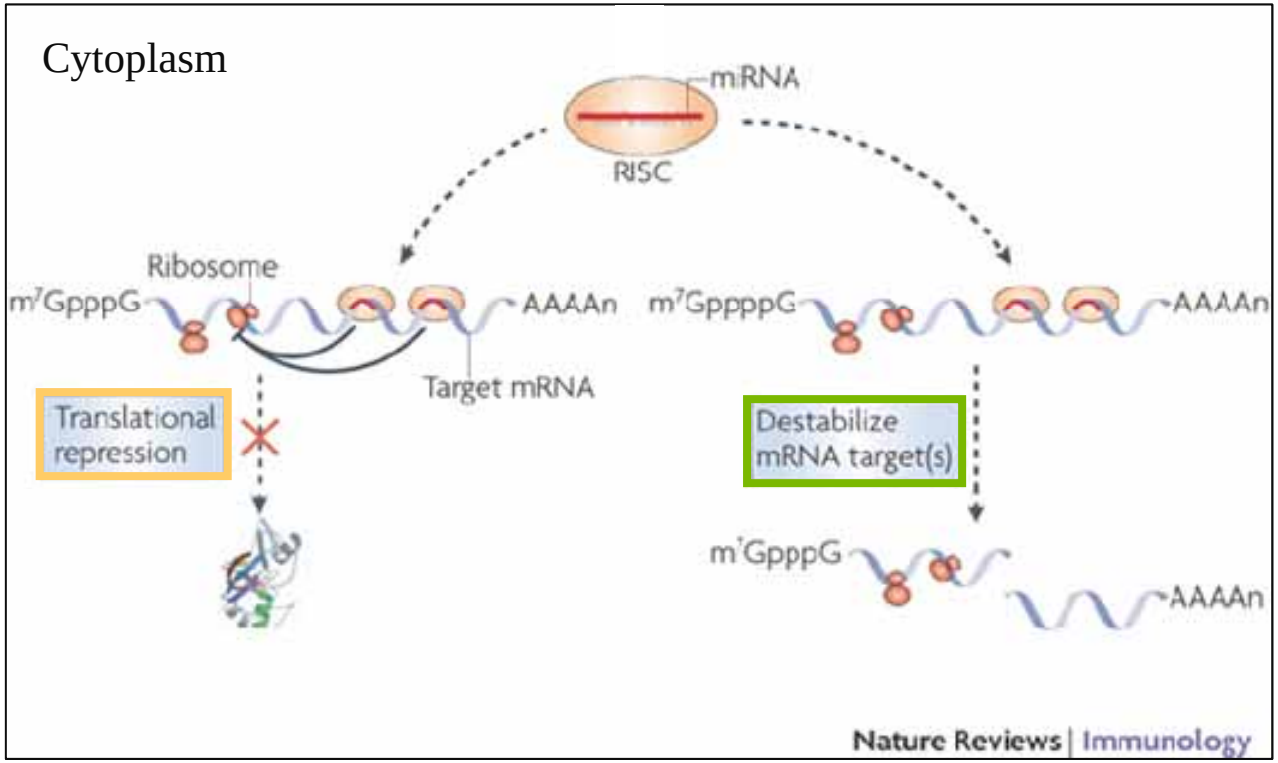
# Principles of microRNA action




Micro RNA in RISC complex

# Regulation of mRNA translation

**Incomplete homology**




**Homology (rare) Degradation**

# Hypotheses

(Ohlsson-Teague et al , Mol Endo 2009)

1. microRNAs were differentially expressed in endometriotic lesions

2. microRNA regulated mRNAs were associated with endometriotic disease

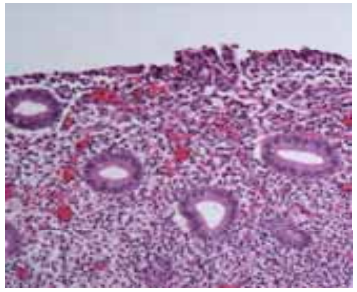
# Methods

1. Collected paired samples from 7 patients with endometriosis

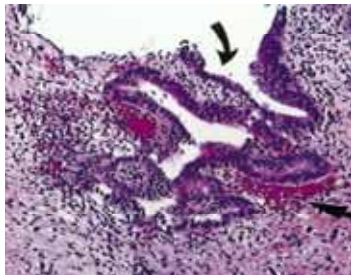
2. Hybridised to microRNA arrays (377 miRNA probes *miRvana*)

3. Intensity dependent normalisation of array data

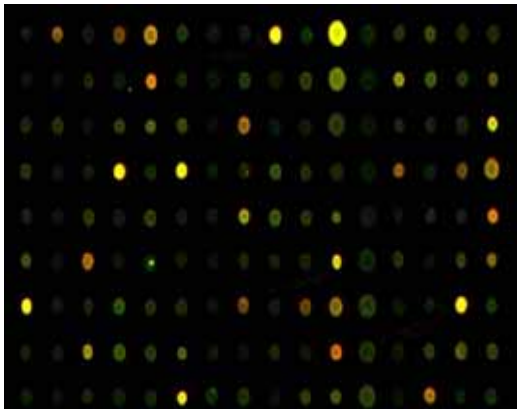
4. Bioinformatics
   ANOVA, LIMMA, ICA



7 eutopic

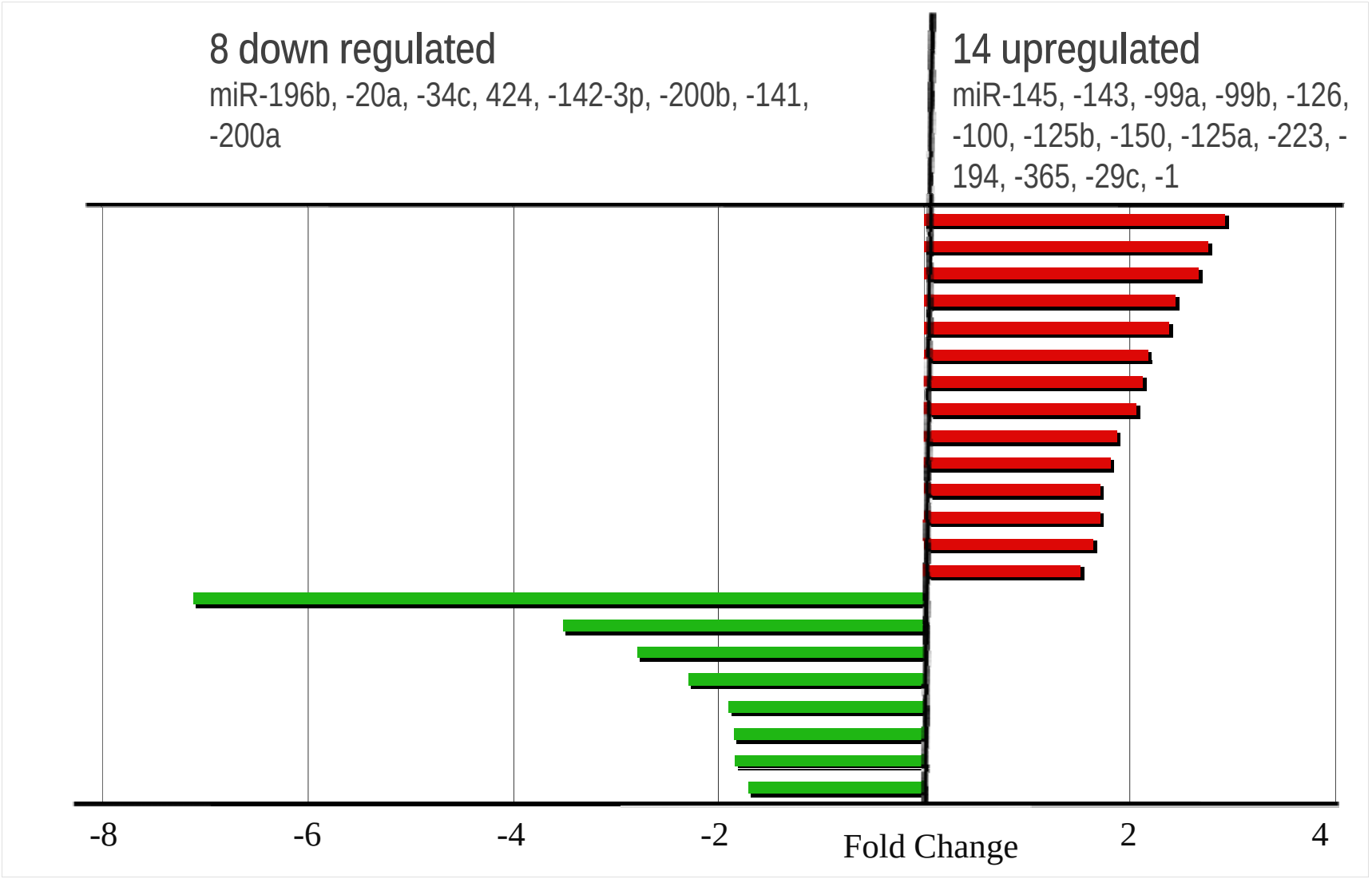


7 ectopic

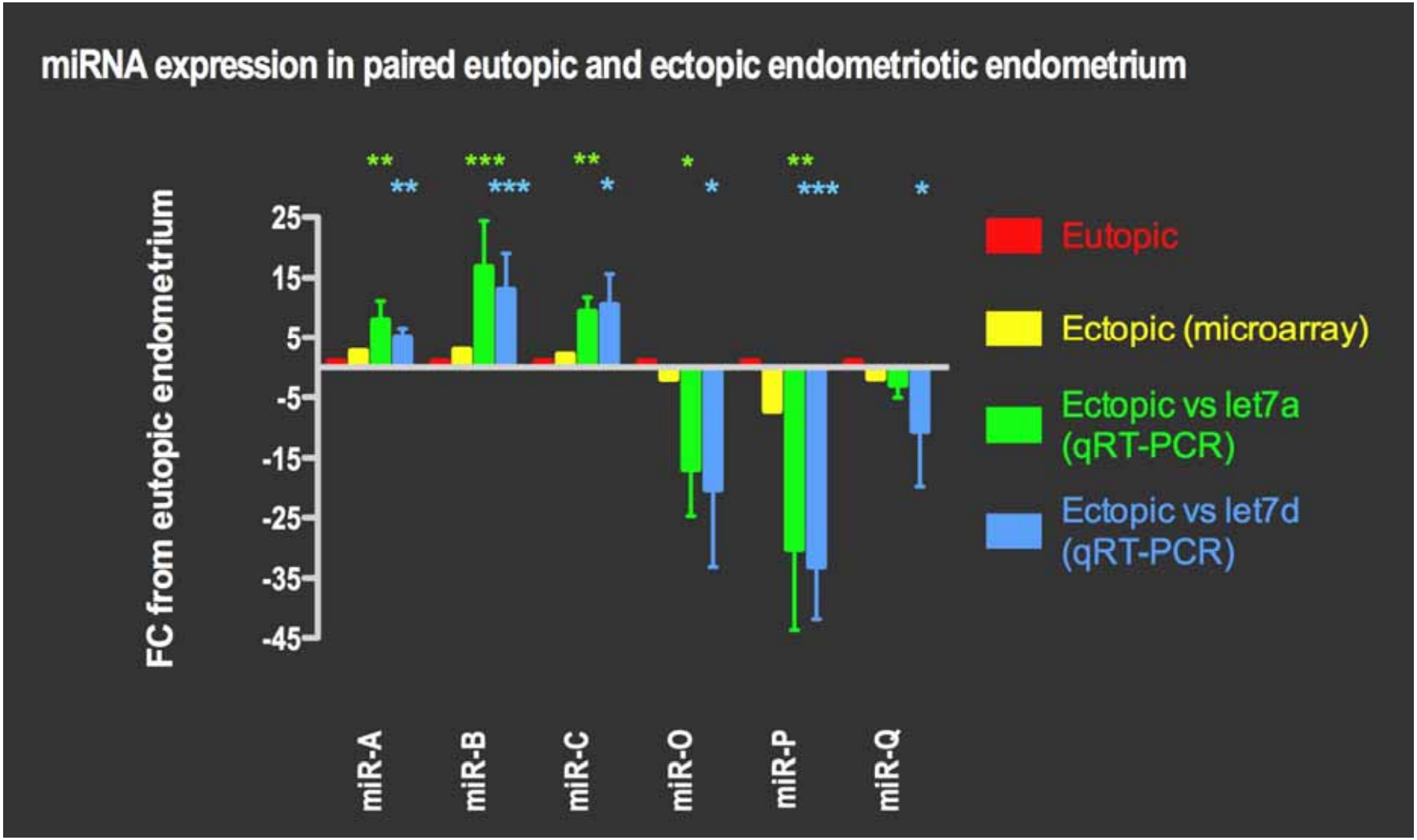


# Results: 22 microRNAs were dysregulated

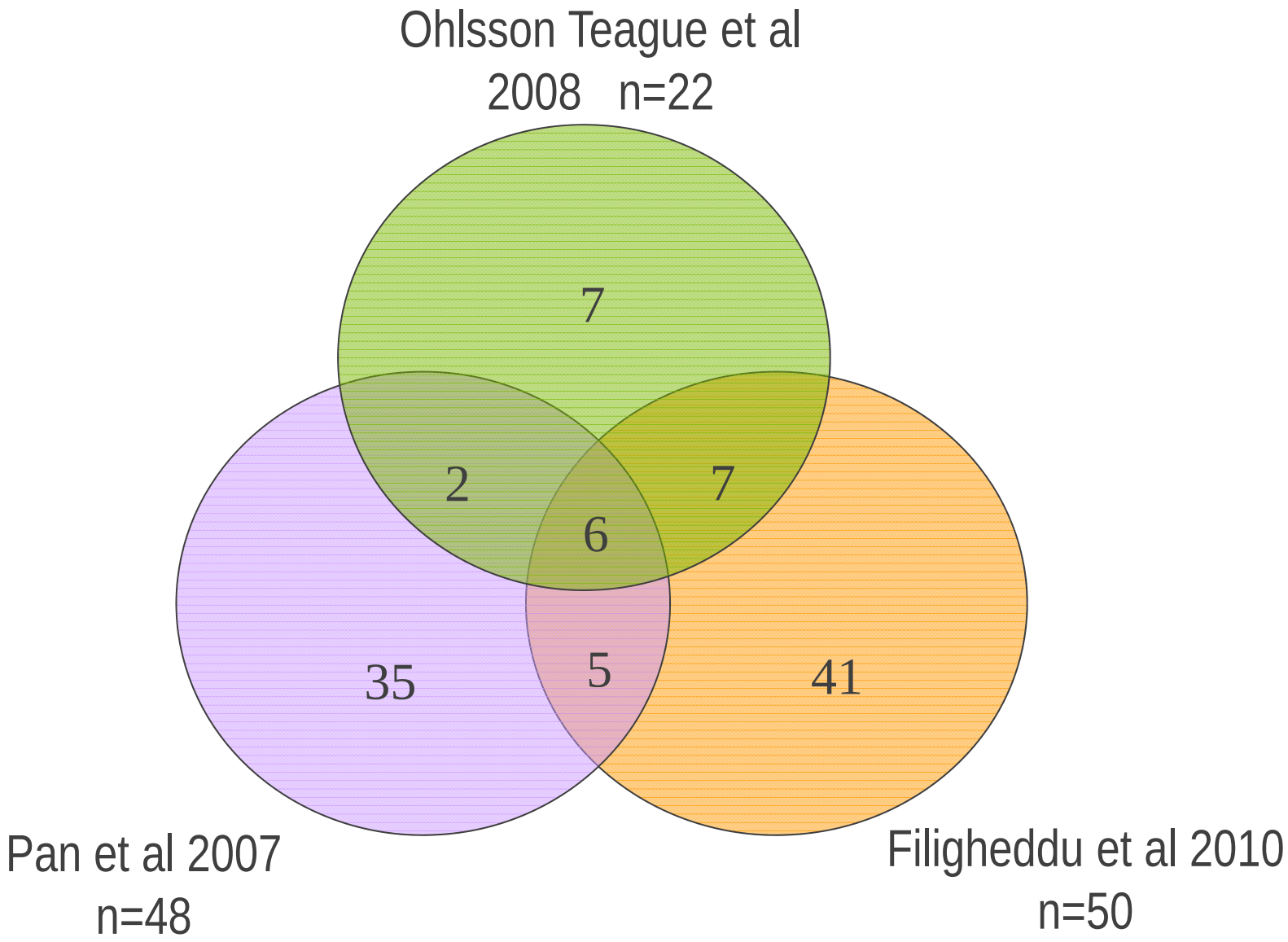
*(Ohlsson Teague et al, Mol Endocrinol 2009)*



# qRT-PCR


miRNA expression in paired eutopic and ectopic endometriotic endometrium
FC from eutopic endometrium
25
15
5
-5
-15
-25
-35
-45
miR-A
miR-B
miR-C
miR-O
miR-P
miR-Q
Eutopic
Ectopic (microarray)
Ectopic vs let7a (qRT-PCR)
Ectopic vs let7d (qRT-PCR)

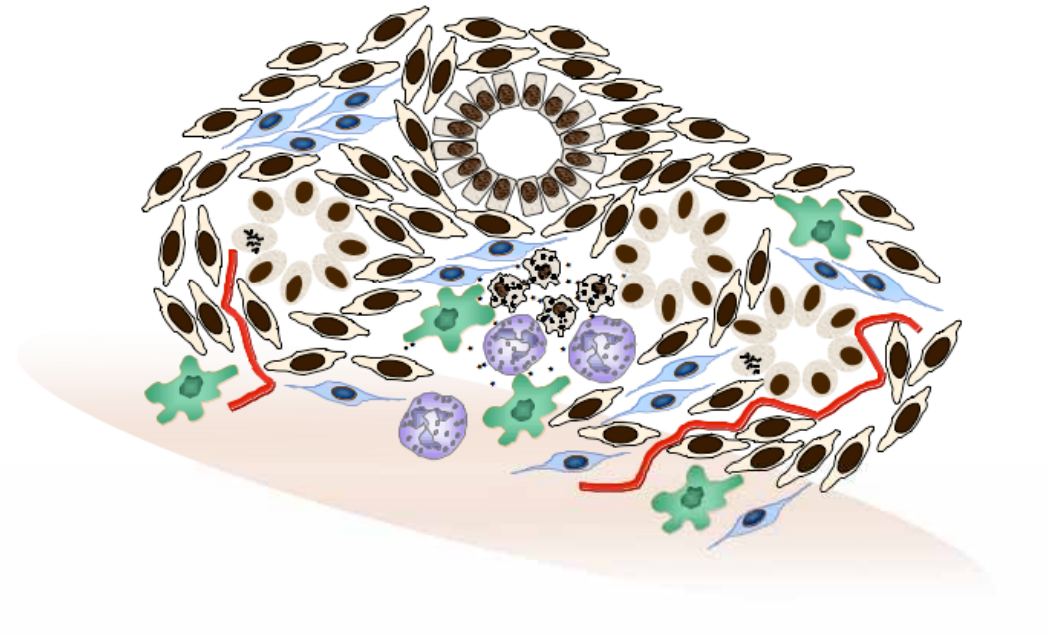
# Comparison with other studies



# Functional analyses

1. Identification of biological functions (Gene Ontology)

2. Identification of molecular signalling networks (Ingenuity Pathway Analysis)



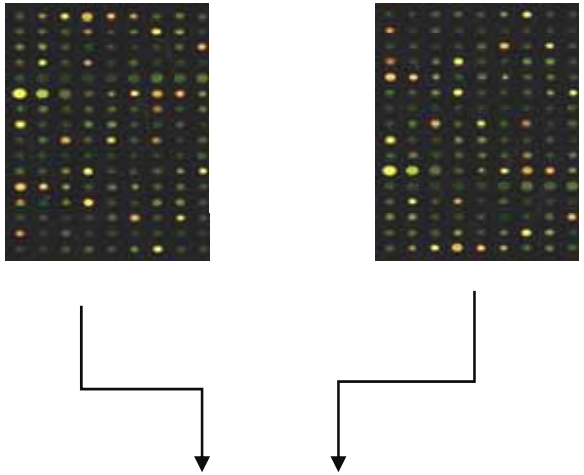
# miRNA target identification

1. *In Silico* identification of predicted miRNA Targets (PicTar, TargetScan)

2. Comparison of differentially expressed mRNAs and predicted microRNA targets in endometriosis

Endometriosis associated mRNAs

*(Hever et al, 2007; Hull et al, 2008)*



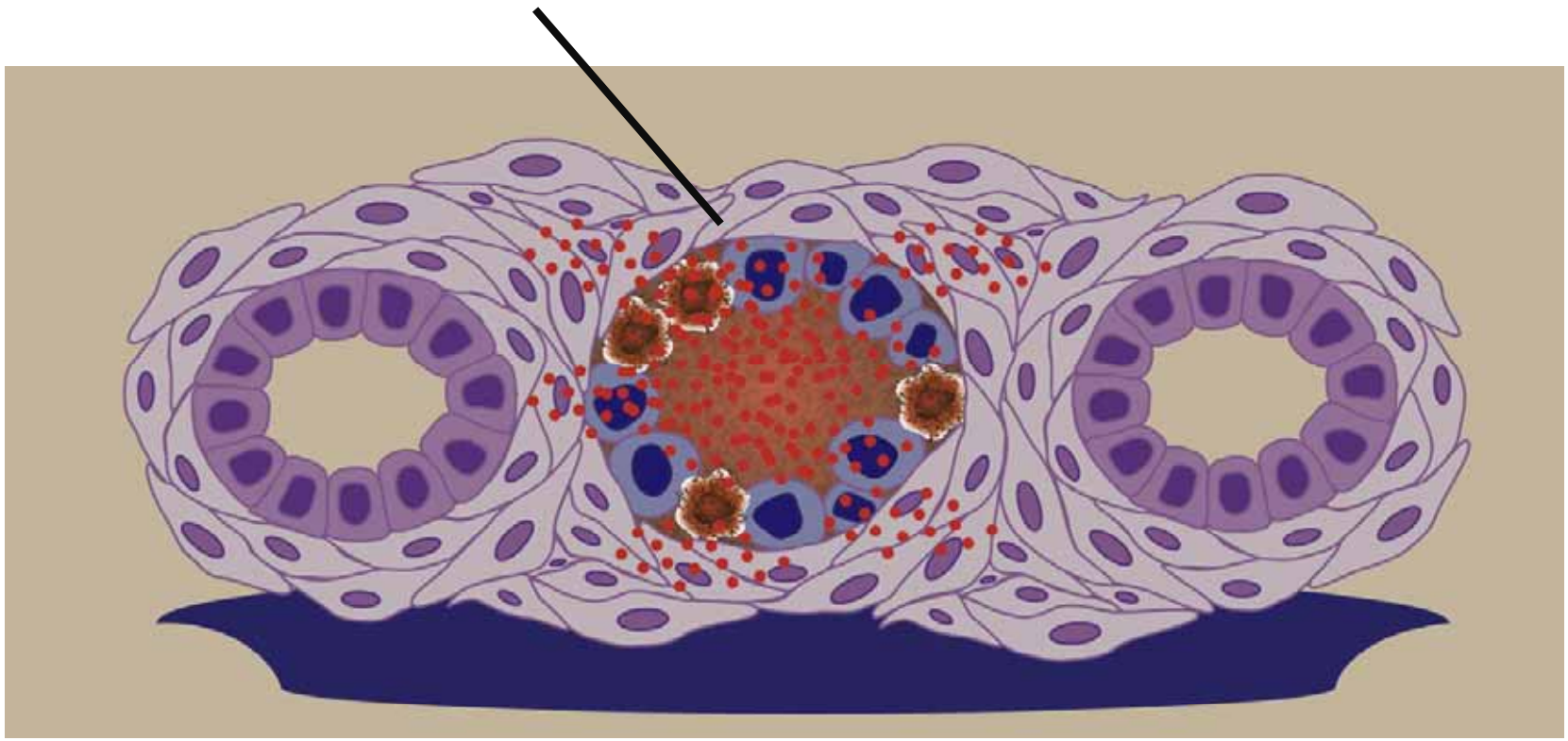
Predicted mRNA targets of endometriosis associated miRNAs

*Ohlsson Teague et al, Mol Endocrinol 2009)*

673 mRNAs

# Adhesion and Wounding

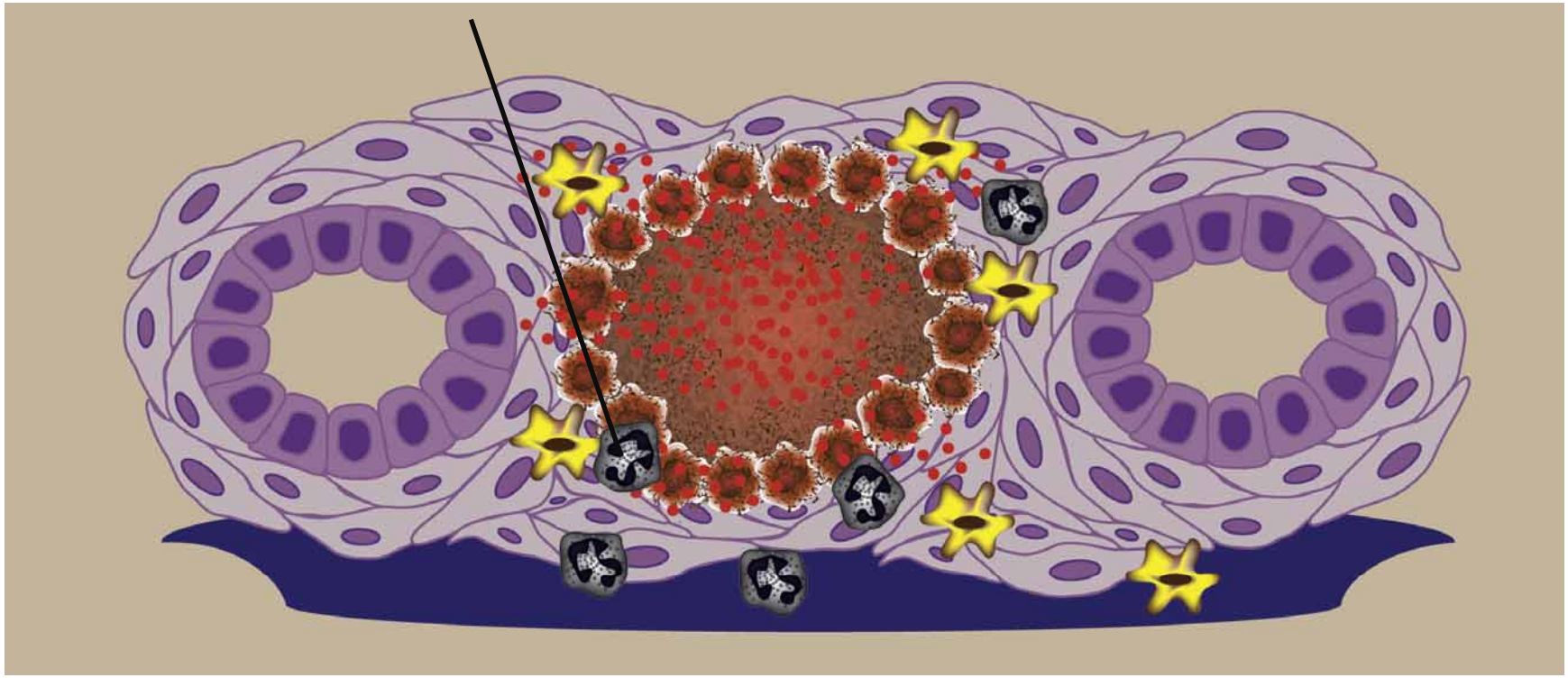
Hypoxia
miR-15b, miR-16, miR-199a
miR-20a, miR-200b



# Inflammation

Inflammation

miR-16, miR-199a



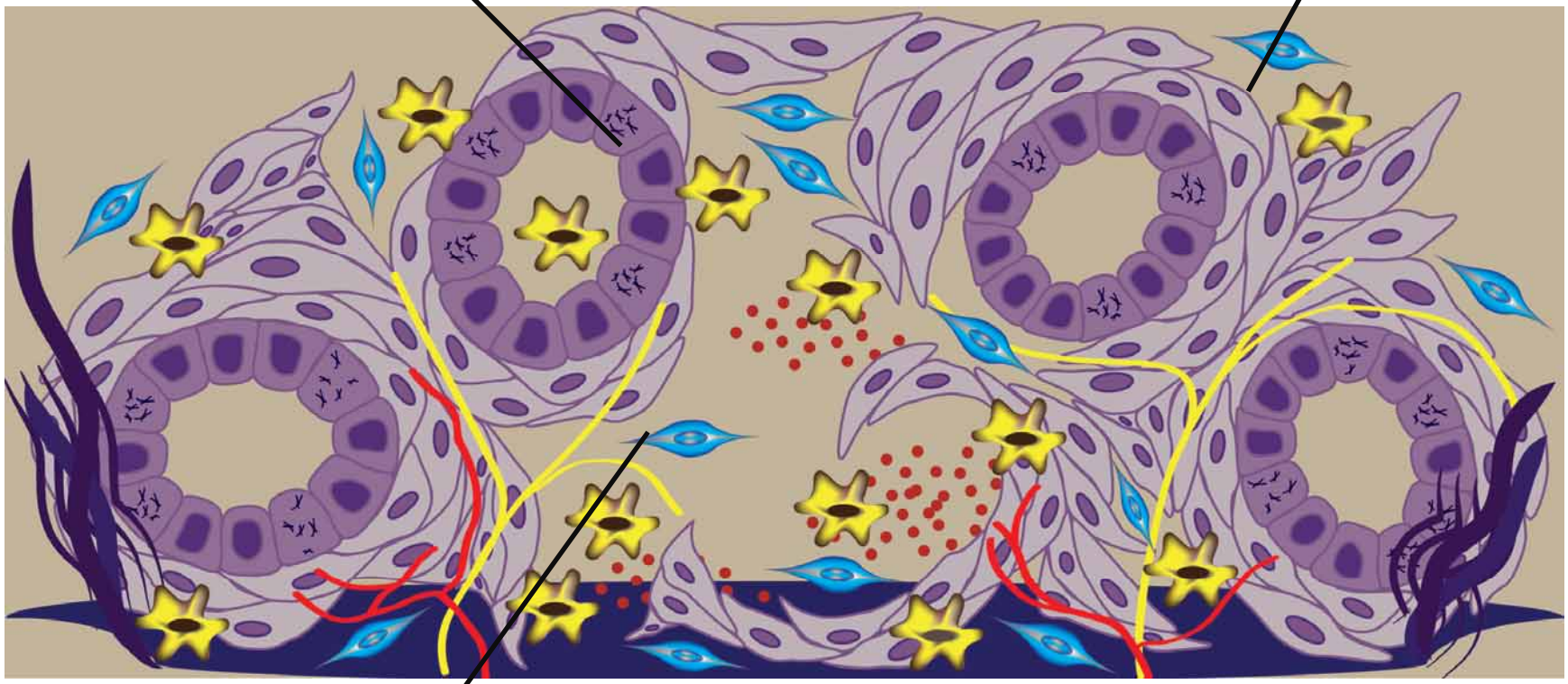
# Tissue Remodelling

Cell Proliferation

miR-125a, miR-125b, miR-143, miR-126, miR-145, miR-20a, miR-221, miR-222, miR- 26a

Extracellular Matrix Remodelling

miR-29c



Tissue Repair

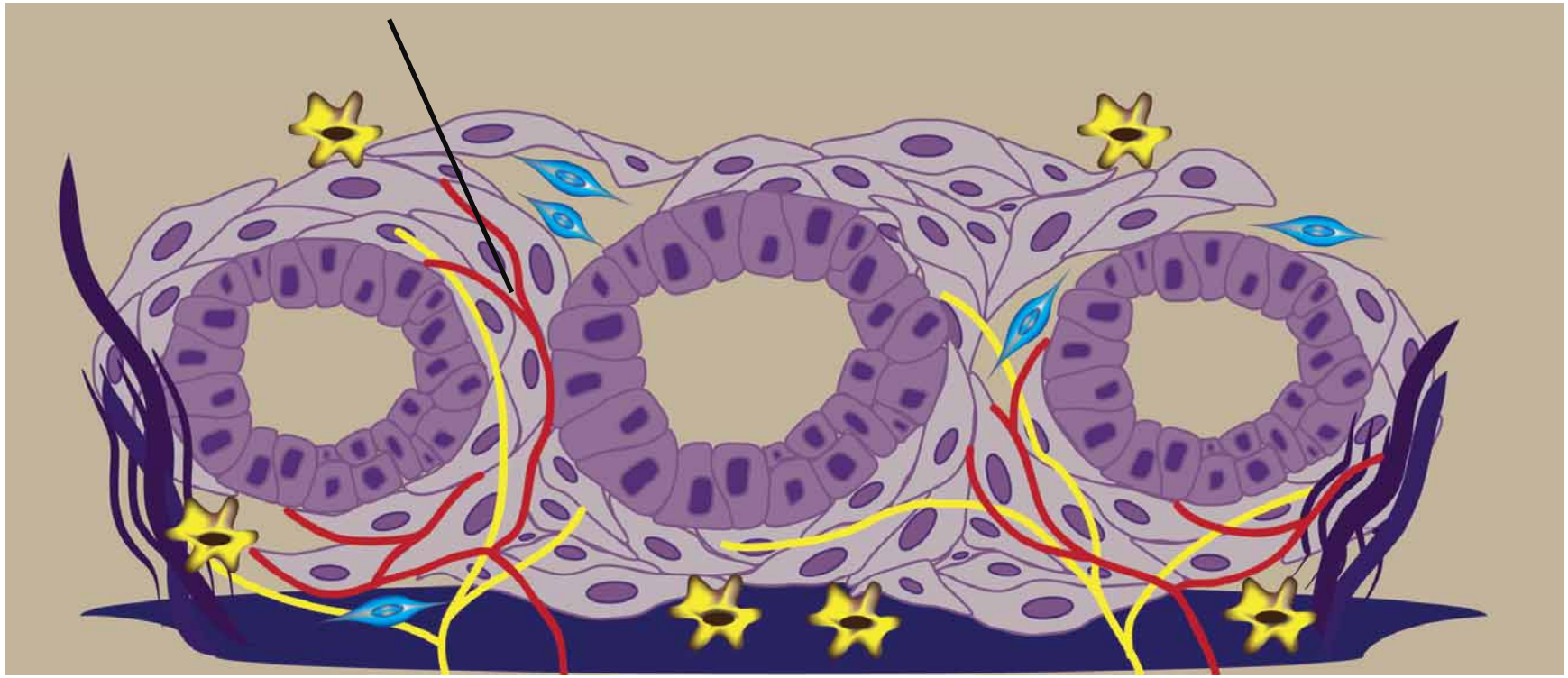
miR-200b,miR-200c, miR -141, miR-21, miR-1, miR-194

# Established Lesion

Angiogenesis

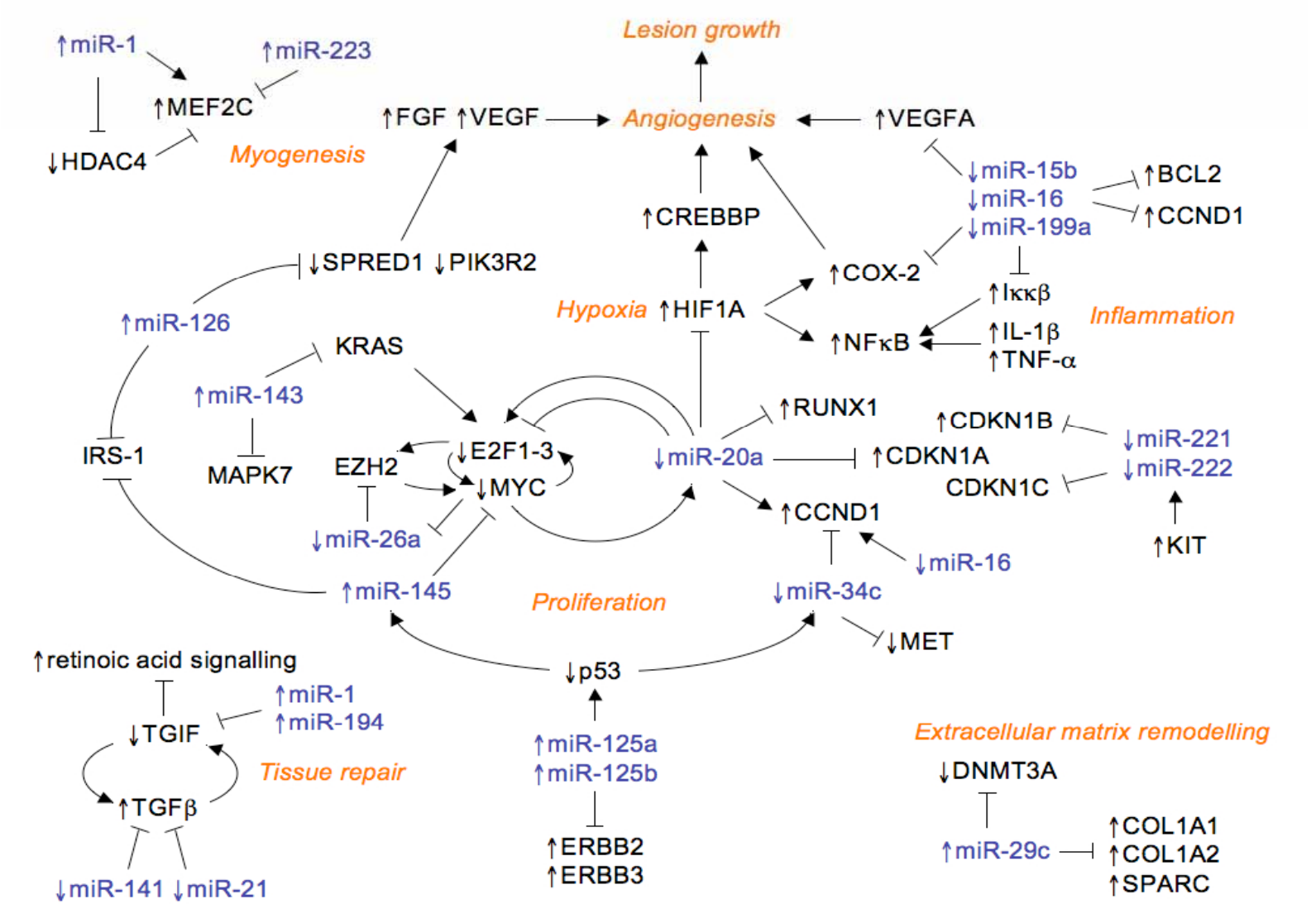
miR-145, miR-126, miR-24

miR-23a, miR-143, miR-20a



*(Toloubeydokhti et al, 2008, Estelles et al)*

# Ingenuity Analysis Pathways



# Conclusions

1. MicroRNA dysregulation is associated with endometriosis

2. mRNAs targeted by microRNAs appear to participate in the endometriotic disease process

3. Potentially microRNA manipulation could alter molecular pathways associated with endometriosis

# Application of microRNA technology to endometriotic disease

1. Understanding the pathophysiology of endometriosis
2. Developing diagnostic tests
3. Therapeutics

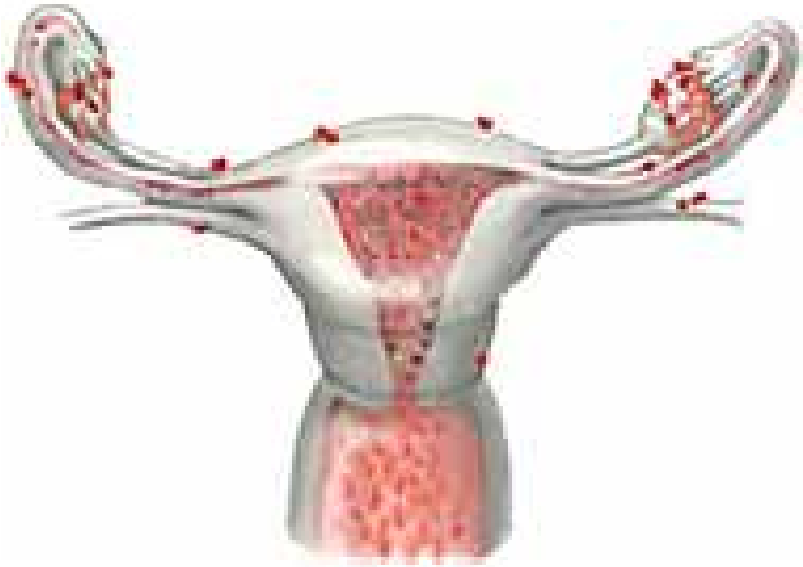
# Understanding the pathophysiology

## Sampson's theory

*Coelomic metaplasia theory*

*Induction theory*

*Embryonic rest  theory*



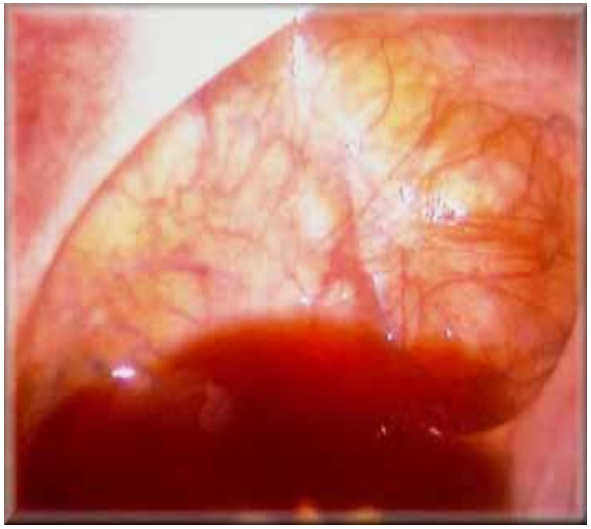
## *Other Factors*

Genetic (polygenic)

Hormonal

Environmental (dioxins)

Immune factors



# Genetic linkage studies (Endogene)

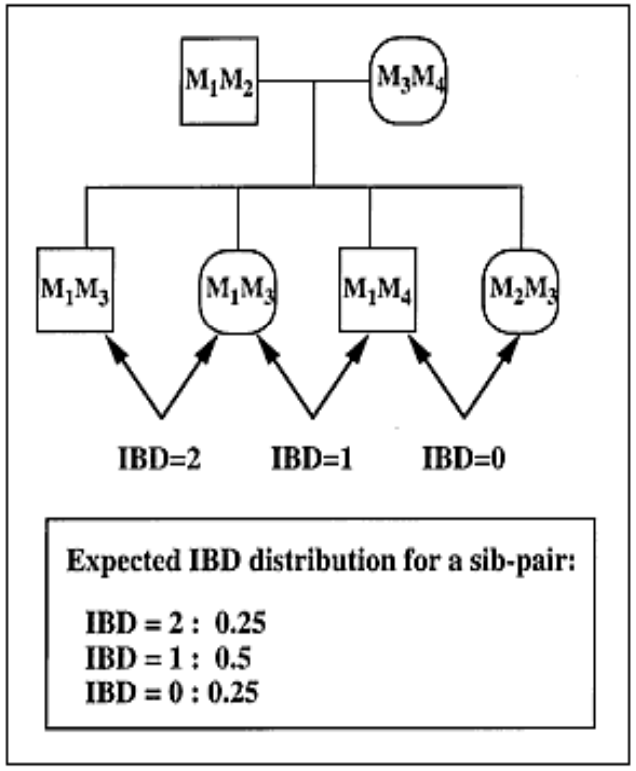
1. Quantitative genetic analysis (QTL)

2. Based on the principle that any region in the genome could encode a gene(s) of importance in endometriosis

A genome-wide approach could find these genes from among the 30 000–40 000 known human genes

# Affected sibling pair analysis

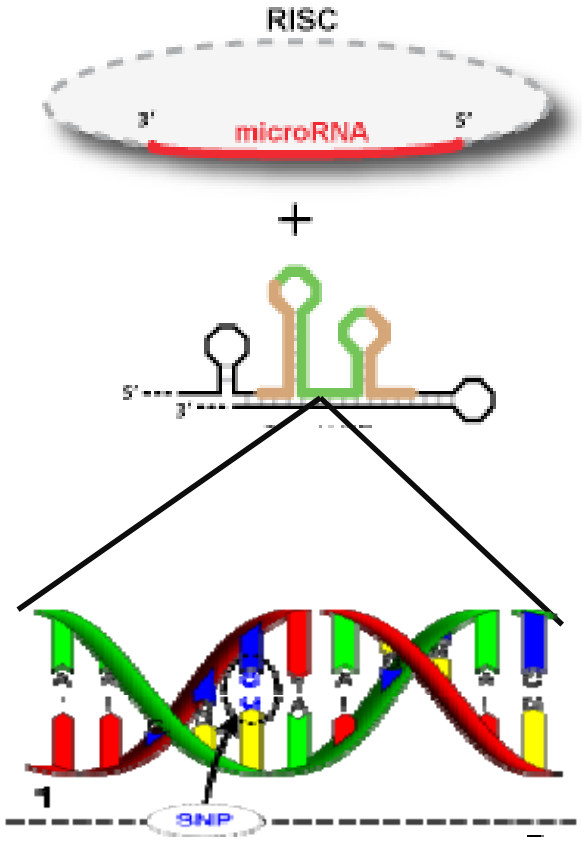
1. Based on ‘identity by descent’
2. Siblings with endometriosis will inherit identical copies of endometriosis-promoting alleles from their parents more often than by random chance.
3. Disease assignment is very important and requires laparoscopy in the probands in endometriosis studies



# Hypothesis:

*(Zhao et al, Mol Hum Reprod, Epub)*

Single Nucleotide Polymorphisms in microRNA binding sites of target genes could alter their translation and be a genetic cause of endometriosis.




microRNA binding sequence in target mRNA

Single Nucleotide Polymorphism

# Methodology

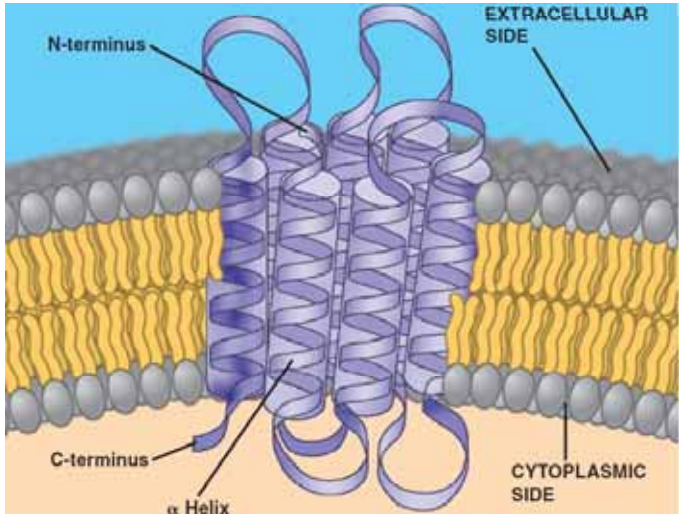
1. 958 endometriosis cases and 959 controls (Endogene dataset)
2. 2657 microRNA target sites were identified across 145 genes.
3. A total of 243 SNPs were identified within target sites.
4. A panel of 102 SNPs in predicted miRNA target sites was evaluated
5. 41 polymorphic variants in these SNPs

# Results

1. There was evidence for allelic association between endometriosis and SNPs rs35091219 and rs1736215

2. In women with advanced endometriosis and subfertility a significant association was seen with Haplotype 4 in the SLC22A23 gene microRNA binding site

3. SLC22A23 is a transmembranous transport protein



# Conclusion

Genetic alterations in microRNA binding sites in target mRNAs could contribute to the polygenic inheritance pattern of endometriosis

# microRNAs  as a diagnostic tool?

# Current diagnostic tests

1. Clinical signs and symptoms and radiological imaging are not sensitive or specific

   *(Kennedy S et al, 2005; Chamié LP et al, 2009; Bazot M et al, 2009)*

2. Diagnostic laparoscopy
   - Costly
   - Requires anaesthesia
   - Is invasive
   - Carries risks

   *(Kennedy S et al, 2005)*

3. 2/3 of women that undertake laparoscopy – do not have endometriosis

   *(Chapron C et al, 2003; Frishman GN et al, 2006)*

# A non- invasive diagnostic test:

May allow us to consider prevention

Reduction in menstrual exposure

- mirena
- implanon
- continuous COC

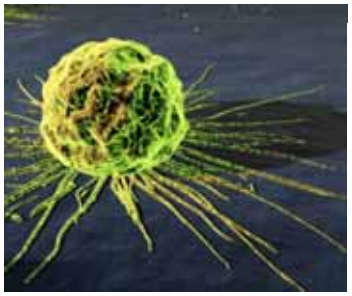
Fertility preservation

- planning of childbearing
- vitrification of eggs


But may lead to overdiagnosis and overtreatment if not used in highly selected patient populations

*(Somigliana et al, 2010)*

# microRNAs as biomarkers



1. microRNAs are stable in blood and tissues

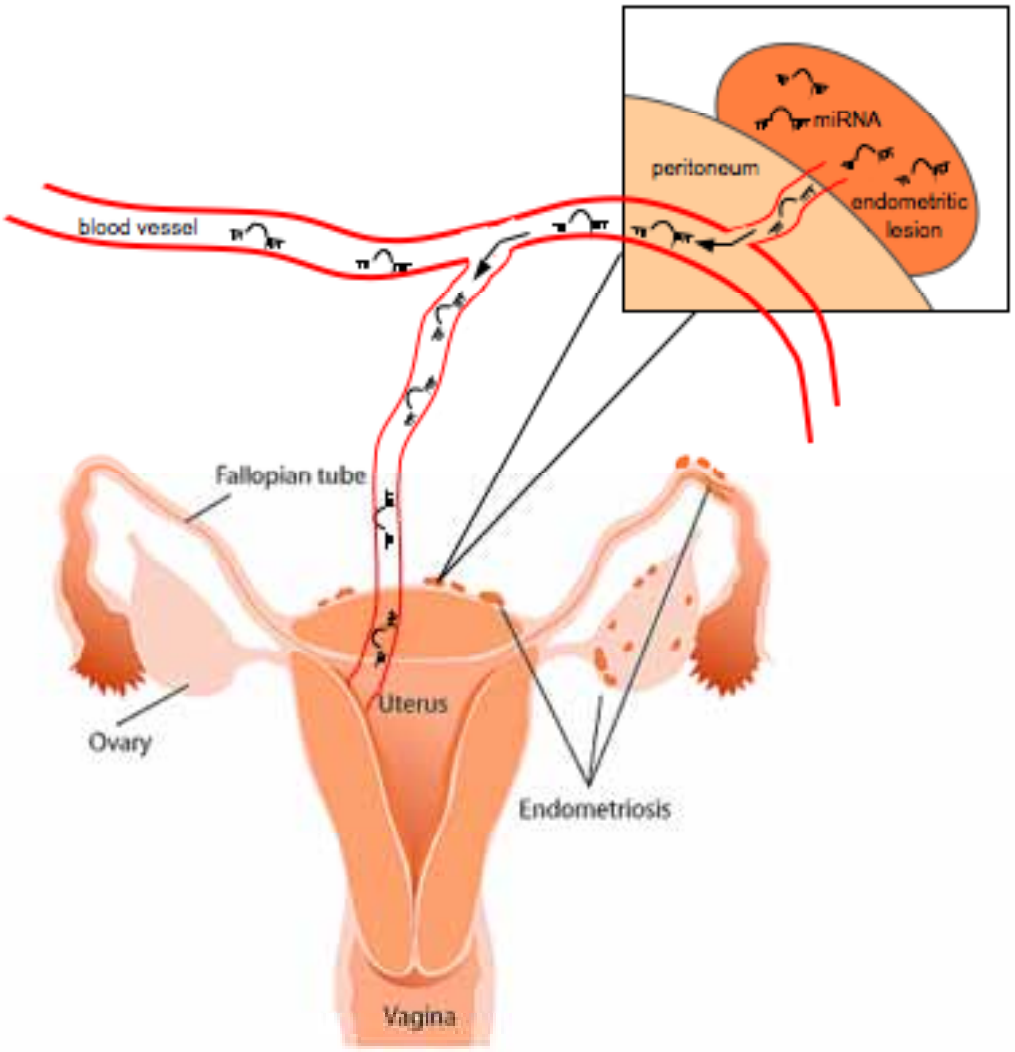
2. microRNA profiles are highly specific for the type and differentiation of ovarian cancer (*Resnick et al, 2009)*

3. microRNA profiles are different in pregnant and non-pregnant women (*Gilad et al, 2008)*

4. Human microRNAs were secreted into plasma from prostate cancer xenografts in a rodent model *(Mitchell et al, 2008)*

# microRNAs as Biomarkers in Endometriosis

Hypothesis:
Endometriosis may alter the microRNA profile of serum or eutopic endometrium



# Eutopic endometrium: endometriosis vs disease free women

(*Burney et al, Mol Hum Reprod, 2009)*

1. Retrospective study: endometriosis (n=4), controls (n=3)
2. Early secretory phase of endometrium
3. Some confounding likely from age and fibroid status

| miRNA | Microarray fold Change | |
|---|---|---|
| | Change | P-value |
| miR-34c-5p | -2.96 | 0.015 |
| miR-34b* | -2.84 | 0.019 |
| miR-34c-3p | -2.54 | 0.025 |
| miR-9 | -1.90 | 0.0032 |
| miR9* | -1.90 | 0.0152 |
| miRPlus_42780 | -1.79 | 0.038 |

# Eutopic changes in mild vs severe endometriotic disease

*(Aghajanova and Giudice, Reprod Sci, Epub)*

1. Eutopic endometrium from 19 women with mild and 44 with severe endometriosis
2. Biopsies at 3 phases of the cycle
3. Identified an up regulation in miR-21 and Dicer transcripts in severe disease

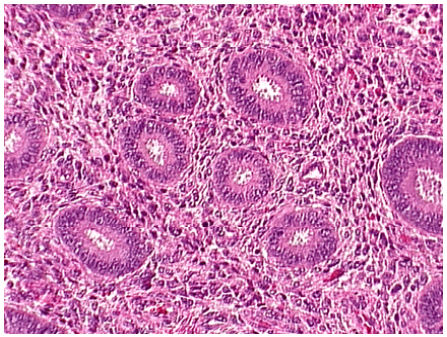
## Conclusions:

1. microRNAs may have a role in the pathogenesis of severe vs mild disease.
2. microRNA alterations may contribute to poor programming of the eutopic endometrium and implantation problems.

# Cycle phase is important in eutopic endometrium

1. In estrogen exposed mouse uteri: *(Nothnick and Healy, 2010)*
   miRs -155, -429, and -451 were upregulated
   miR -81b and -204 downregulated

2. In disease free women: *(Kuokkanen et al, 2010)*
   Cultured epithelial cells from late proliferative phase endometrium (n=4) were compared to independent cultures from the midsecretory phase (n-4)
   12 upregulated and 12 downregulated microRNAs were identified



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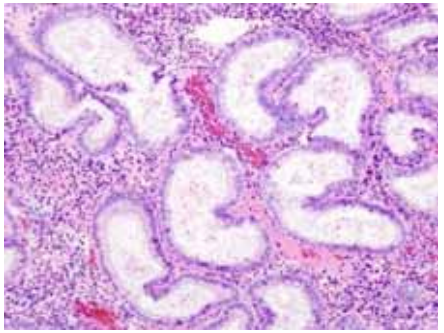
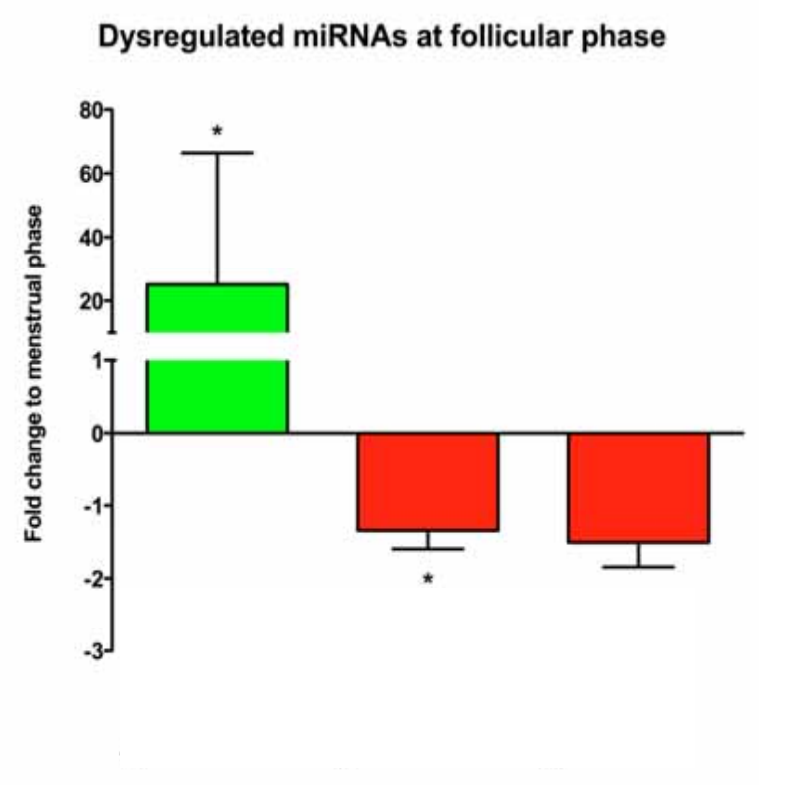


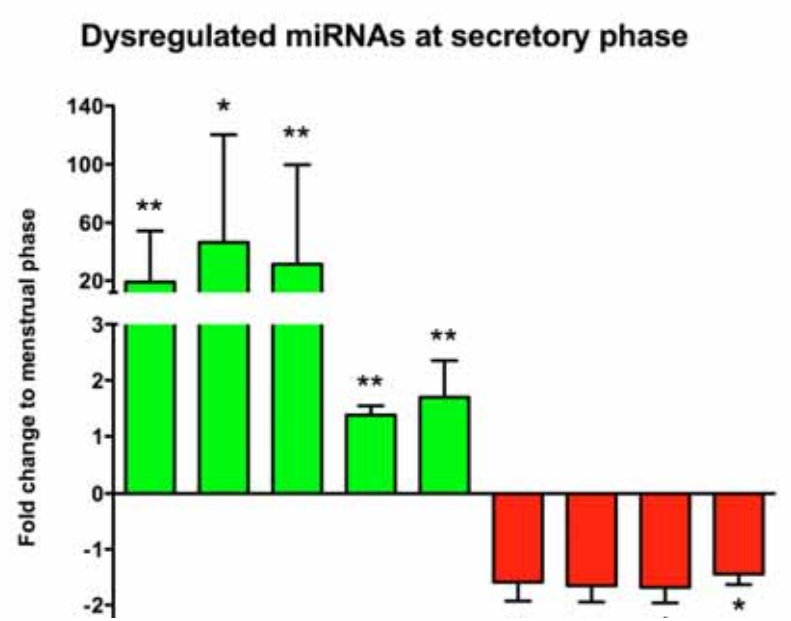
# Menstrual cycle variability in serum microRNAs

*unpublished data*

10 healthy volunteers  had serum sampling in the menstrual (control) vs follicular and secretory phase of cycle

MicroRNA multiplex-PCR (n= 677)



# Ongoing work:

Comparison of serum microRNA profiles in women with and without endometriosis

# microRNA Therapeutics in Endometriosis?

# MicroRNA characteristics

1. Are stable in blood and tissues
2. Can be transported in serum
3. One  microRNA can target many mRNAs altering several aspects of a disease process
4. Fine tune disease processes (dynamic)
5. AntagomiRs and microRNA mimics have been manufactured

But there is a risk of off target effects

# Therapeutic studies

Prostate Cancer is suppressed by miR-16

Synthetic miR-16

1. Delivered to prostate tumors
2. Downregulated miR-16 targeted genes
3. Supressed cancer growth in a xenograft model of prostate cancer *(Takeshita et al, 2010)*

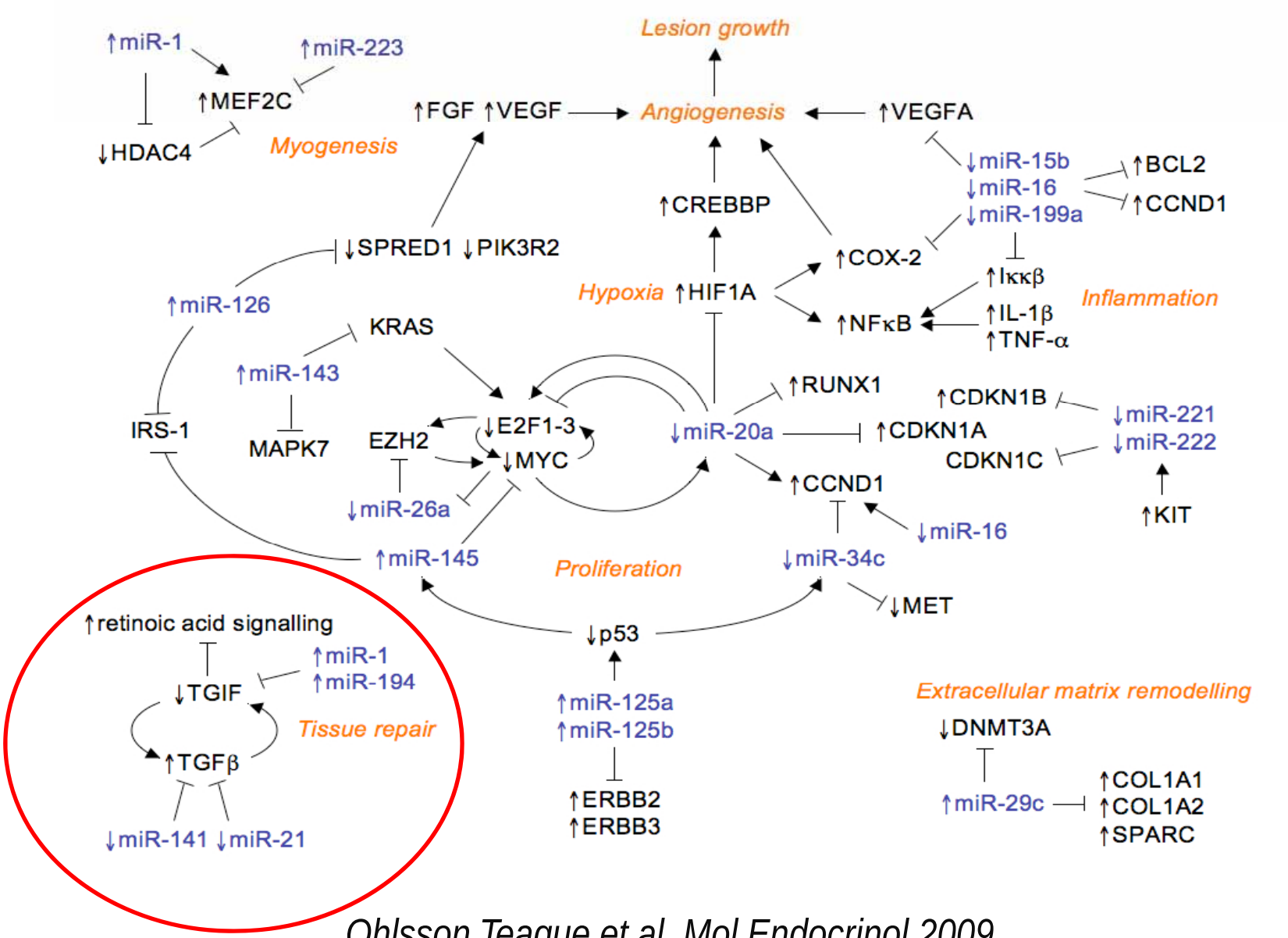

Cholesterol metabolism is supressed by miR-122

miR-122 antagomiR

1. Hybridised to miR-122
2. Upregulated 11 (miR-122 regulated) genes involved in cholesterol metabolism
3. Dose dependent lowering of plasma cholesterol in mice and non-human primates

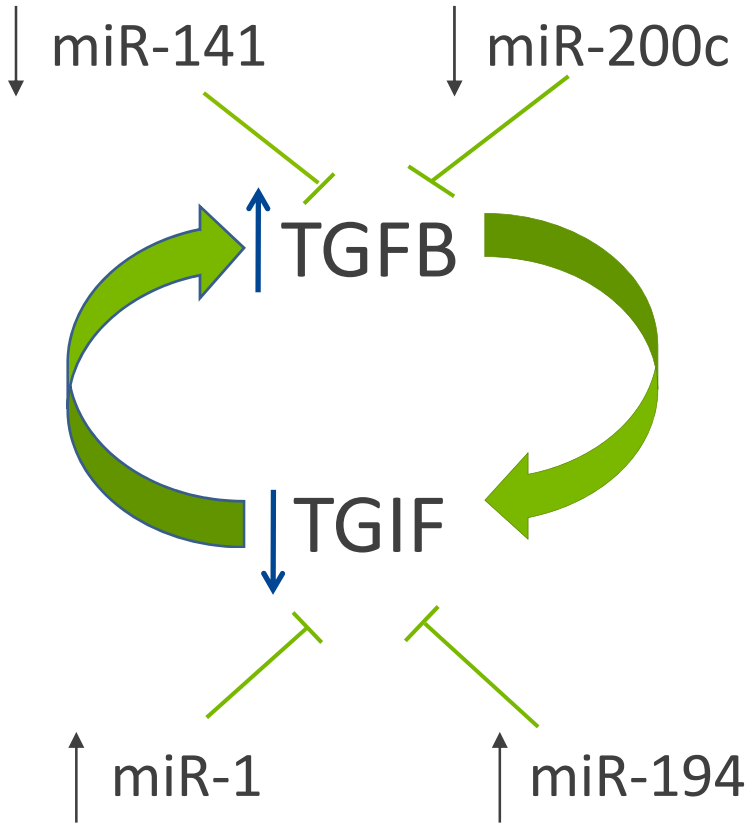
*(Krutzfeldt et al, 2005, Elmen et al, 2008, Elmen et al, 2008)*

# What should we target?




*Ohlsson Teague et al, Mol Endocrinol 2009*

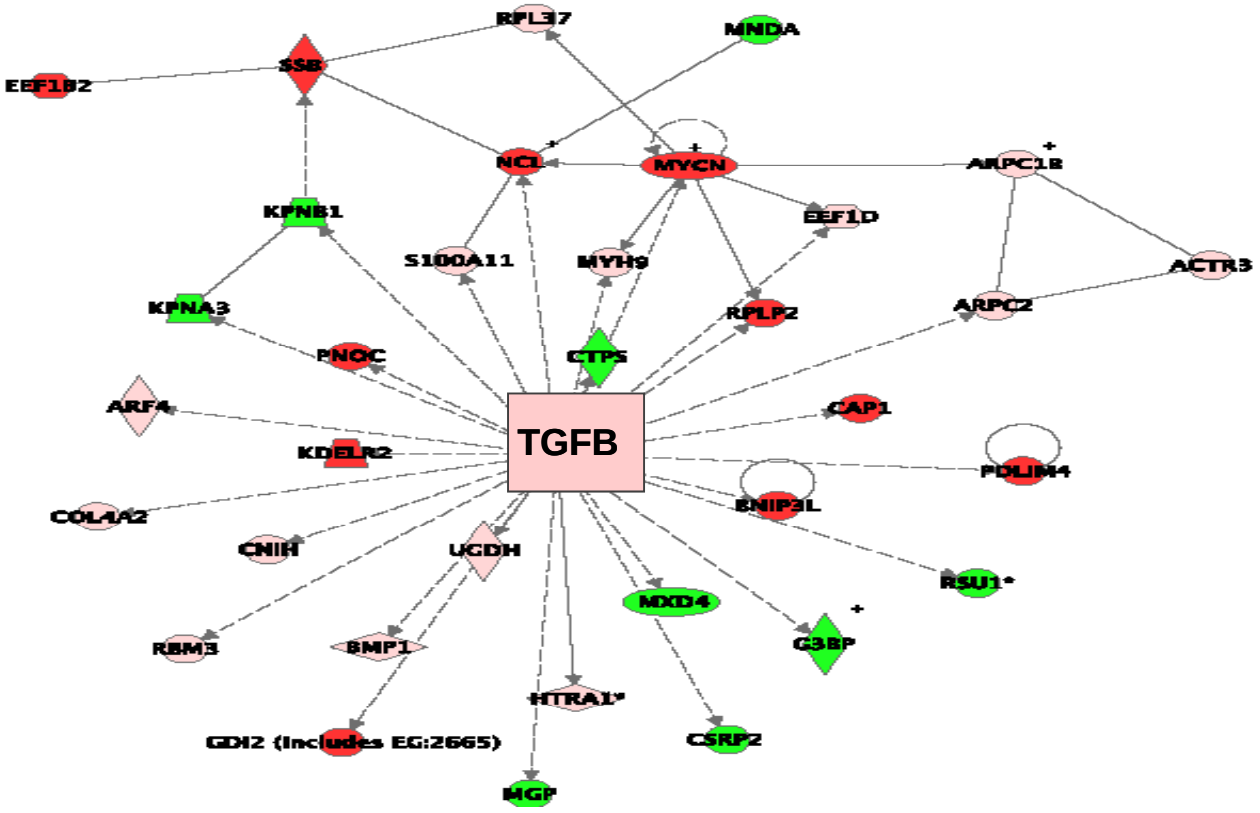
# Actions of TGFB associated microRNAs



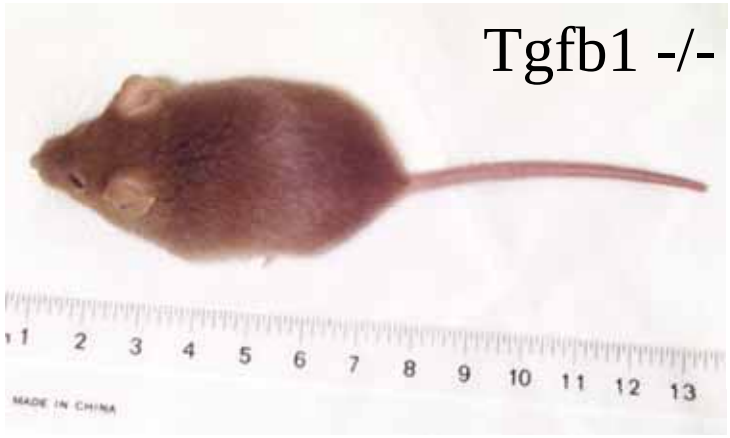

TGFB activity is enhanced by microRNAs in endometriosis

# Is TGFB important?

TGFB activity was central in peritoneal-endometrial interactions in ectopic endometrial lesion development *(Hull et al, Am J Path 2009)*



# TGFB1 deficient/ immune deficient mouse model of endometriosis





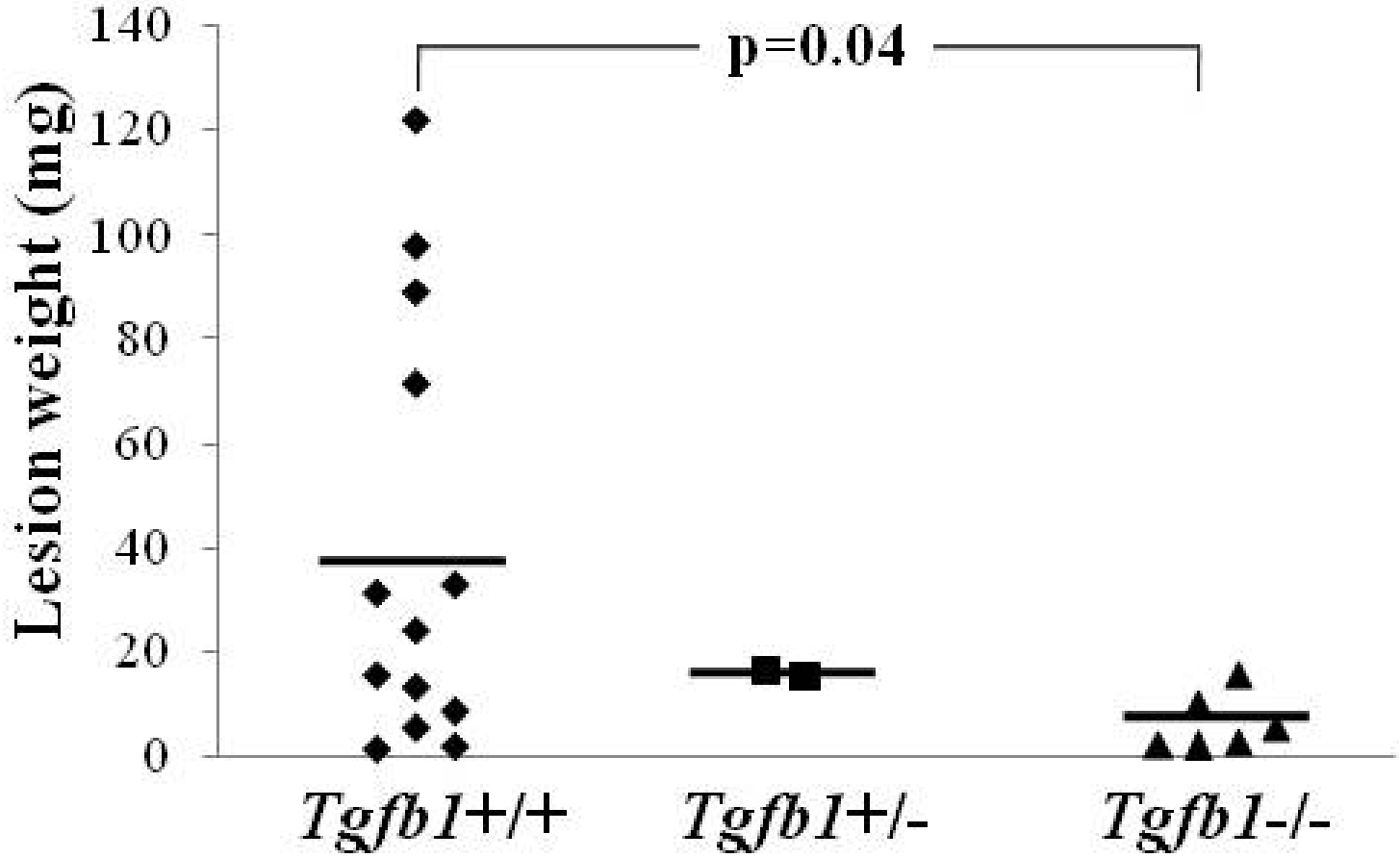
1. Tgfb1-/- mice with and intact immune system - no survivors
   50% die in utero, 50% die from an autoimmune wasting disease

2. Tgfb1-/- mice on a immunocompromised background
   20% of live offspring are Tgfb-/- which survive to 12 weeks

3. These mice have functional TGF B2, TGF B3 and macrophages

# Host TGFB1 deficiency suppressed endometriosis lesion development in a xenograft knockout model

(unpublished data)



# Conclusion:

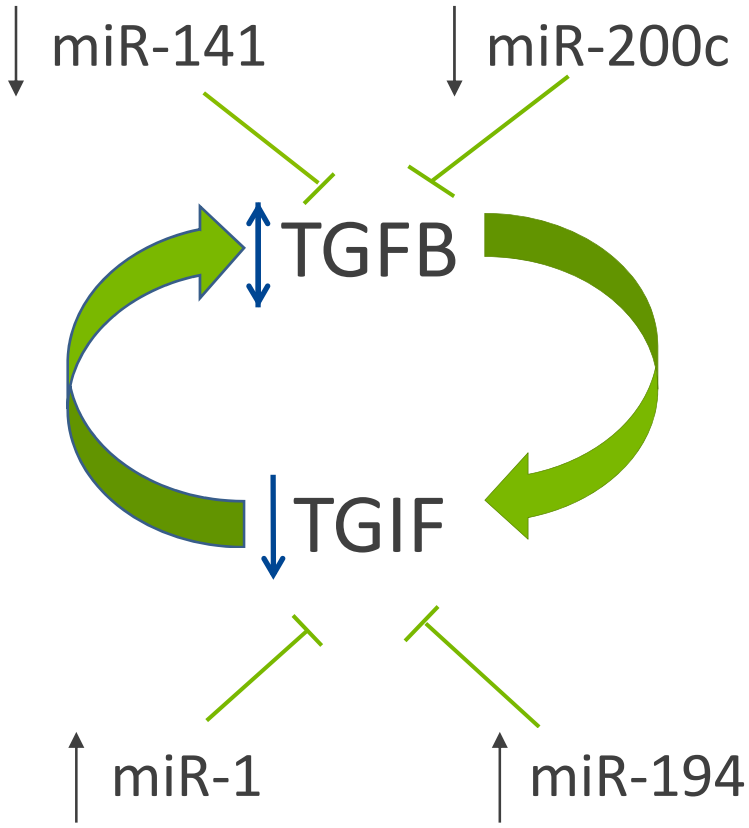
Suppressing host TGFB1 activity may be an effective strategy  for threating endometriosis.

Could microRNA manipulation be used?

# Actions of TGFB associated microRNAs

(*Ohlsson-Teague et al Mol Endo 2009)*

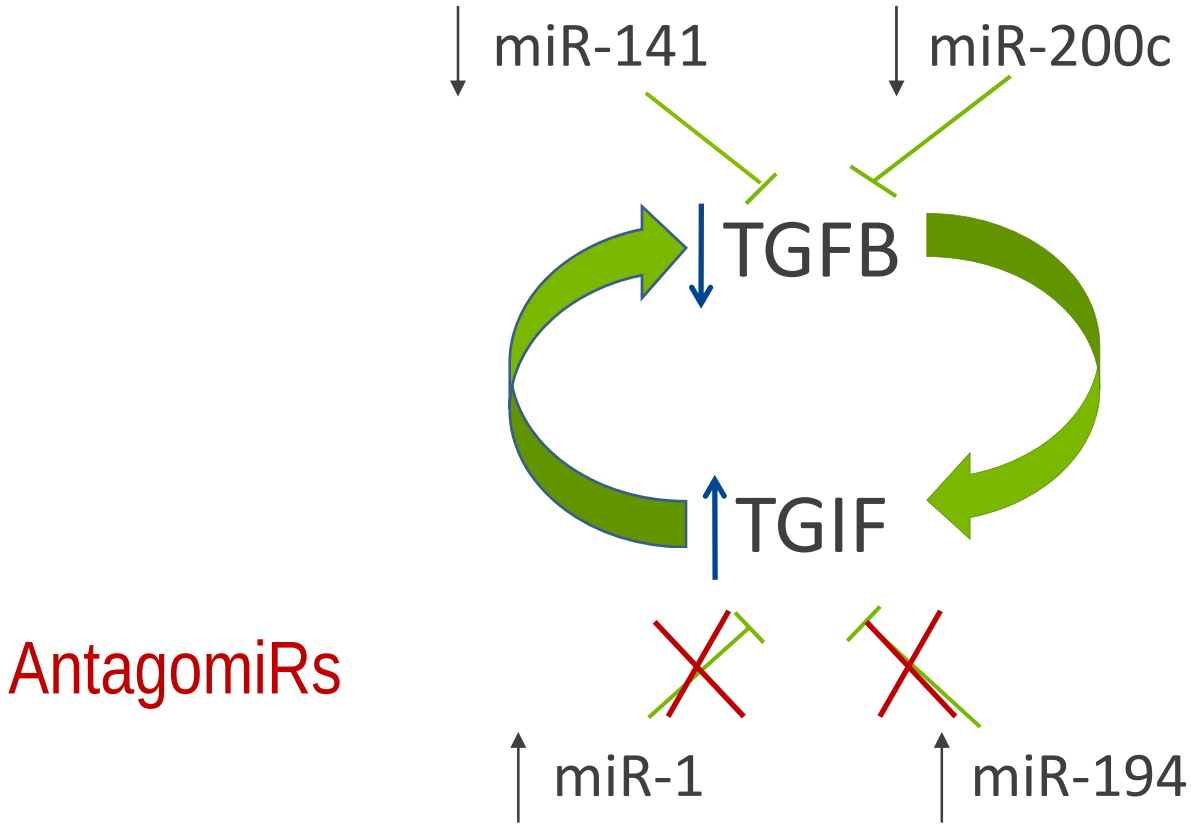
Synthetic microRNAs




TGFB activity is enhanced by microRNAs in endometriosis

TGFB activity is supressed by synthetic microRNAs in endometriosis

# Actions of TGFB associated microRNAs




TGFB activity is enhanced by microRNAs in endometriosis
TGFB activity is suppressed by AntagomiRs in endometriosis

# The role of microRNAs in endometriosis is only starting to be evaluated.

# Our knowledge of microRNAs in endometriosis?

1. Endometriotic tissues has a different microRNA profile compared to eutopic endometrium.
2. microRNAs are likely to regulate mRNAs and molecular networks that contribute to the pathophysiology of endometriosis.
3. The menstrual cycle phase is likely to alter serum and eutopic endometrial microRNA profiles.
4. Genetic differences in microRNA binding sites may contribute to the inheritance of endometriosis
5. There may be differences in the eutopic endometrial microRNA profile between women with and without endometriosis

# Further Research Questions?

1. Are there other microRNAs that are not detected in our microarrays studies (deep sequencing)
2. Are our predicted microRNA/mRNA interactions actually occuring in endometriosis?
3. Which cells are they occuring in?
4. Does eutopic endometrium or serum have a characteristic microRNA profile in endometriosis?
5. Which stage of the cycle is a distinguishing microRNA profile best tested?
6. Can we establish therapeutic models to evaluate microRNA activity in vivo?
7. Can microRNA delivery systems specifically target endometriosis?
8. Are antagomiRs and synthetic microRNAs effective?
9. Are antagomiRs and synthetic microRNAs safe?

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