New Molecules: microRNAs and Endometriosis

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University of Adelaide
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(Lam and Print unpublished data)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)
1. RNA Pol II transcribes 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
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
Results: 22 microRNAs were dysregulated

(Ohlsson Teague et al, Mol Endocrinol 2009)

8 down regulated
- miR-196b, -20a, -34c, 424, -142-3p, -200b, -141, -200a

14 upregulated
- miR-145, -143, -99a, -99b, -126, -100, -125b, -150, -125a, -223, -194, -365, -29c, -1
miRNA expression in paired eutopic and ectopic endometriotic endometrium

- **Eutopic**
- **Ectopic (microarray)**
- **Ectopic vs let7a (qRT-PCR)**
- **Ectopic vs let7d (qRT-PCR)**

FC from eutopic endometrium

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Eutopic</th>
<th>Ectopic (microarray)</th>
<th>Ectopic vs let7a (qRT-PCR)</th>
<th>Ectopic vs let7d (qRT-PCR)</th>
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<tbody>
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<td>miR-A</td>
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<td>miR-P</td>
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<td>miR-Q</td>
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**Significance Levels**
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
Comparison with other studies

Ohlsson Teague et al
2008  n=22

Pan et al 2007
n=48

Filigheddu et al 2010
n=50

35

2 7

6

5

41
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

673 mRNAs
Adhesion and Wounding

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

![Diagram](image)

Expected IBD distribution for a sib-pair:
- IBD = 2 : 0.25
- IBD = 1 : 0.5
- IBD = 0 : 0.25
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)
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

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<thead>
<tr>
<th>miRNA</th>
<th>Microarray fold Change</th>
<th>P-value</th>
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<tbody>
<tr>
<td>miR-34c-5p</td>
<td>-2.96</td>
<td>0.015</td>
</tr>
<tr>
<td>miR-34b*</td>
<td>-2.84</td>
<td>0.019</td>
</tr>
<tr>
<td>miR-34c-3p</td>
<td>-2.54</td>
<td>0.025</td>
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<tr>
<td>miR-9</td>
<td>-1.90</td>
<td>0.0032</td>
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<tr>
<td>miR9*</td>
<td>-1.90</td>
<td>0.0152</td>
</tr>
<tr>
<td>miRPlus_42780</td>
<td>-1.79</td>
<td>0.038</td>
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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
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

Cholesterol metabolism is suppressed 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
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)
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 TGFβ1 deficiency suppressed endometriosis lesion development in a xenograft knockout model

(unpublished data)
Conclusion:

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

Could microRNA manipulation be used?
Actions of TGFB associated microRNAs

(Ohsson-Teague et al Mol Endo 2009)

Synthetic microRNAs

miR-141

miR-200c

TGFB activity is enhanced by microRNAs in endometriosis

TGFB activity is suppressed 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 occurring in endometriosis?

3. Which cells are they occurring 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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