Molecular mechanisms of pathogenesis of endometriosis: Role of Rho/ROCK-mediated signaling pathway in endometriosis-associated fibrosis

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Mt. Yufu in Oita
Do you know where Japan is?
Famous hot springs in Oita, Japan
Endometriosis is characterized by endometrial glands and stroma surrounded by dense fibrous tissue. During the development of endometriotic lesions, excess fibrosis may lead to scarring and to alteration of tissue function. It has been suggested that type I collagen is a major contributor to endometriosis-associated fibrosis. One approach to understanding the pathogenesis of endometriosis is to investigate the mechanisms underlying the fibrogenesis associated with this disease.
3-D collagen gel culture

Using 3-D collagen gel culture model, we have evaluated the ECM contractility and myofibroblastic differentiation of endometriotic cyst stromal cells (ECSC). ECSC showed enhanced contractility in comparison with normal endometrial stromal cells (NESC). Activation of the Ras homology (Rho)/Rho-associated coiled-coil-forming protein kinase (ROCK)-mediated pathway and enhanced myofibroblastic differentiation is involved in this mechanism.
Research on endometriosis-associated fibrosis

1. Mechanisms of endometriosis-associated fibrosis
3. Effects of decidualization on endometriosis-associated fibrosis
Ectopic and eutopic endometrial cells

Endometriotic cyst stromal cells (ECSC)

Endometrial stromal cells with endometriosis (ESCwE)

Normal endometrial stromal cells (NESC)

Cell isolation procedure

Eutopic and ectopic endometrial tissue

- Mince

Trypsin collagenase

37°C 20 min.

150μm wire sieve

80μm wire sieve

Cell culture
Characteristics of endometriotic and endometrial stromal cells

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Status</th>
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<tbody>
<tr>
<td>Vimentin</td>
<td>positive</td>
</tr>
<tr>
<td>CD10</td>
<td>positive</td>
</tr>
<tr>
<td>Keratin</td>
<td>negative</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>negative</td>
</tr>
<tr>
<td>Leukocyte common antigen</td>
<td>negative</td>
</tr>
</tbody>
</table>

Collagen gel contraction assay

Cells were suspended in the collagen solution

Allowed to polymerize in 37°C for 30 min.

Gels were released from the dish bottom by tapping

Add culture media with 10% FBS

36-48 hr culture in floating condition

Contractility was accessed by measuring the gel surface area.

Collagen gel contraction assay


* $p < 0.0001$ vs. NESC (Bonferroni/Dunn test)
Western blot analysis

α-SMA → Marker of myofibroblastic differentiation

β-actin

Rho A

ROCK- I

ROCK- II

GAPDH

ECSC  NESC

Factors of Rho/ROCK-pathway

Relative levels of contraction-related proteins

1. Contractility of ECSC was regulated by myofibroblastic differentiation and Rho/ROCK pathway.
2. Enhanced contractility of ECSC was suggested to play roles in the formation of endometriosis-associated fibrosis.
Proposed mechanism of endometriotic stromal cell-mediated collagen gel contraction

- Stimulation by serum, ECM attachment, peptide growth factors, etc.
- PKC
- Tyrosine kinases
- Ras
- PI3-kinase
- MLCK
- Rho
- MEK
- MMPs
- Akt
- MLC phosphorylation
- ROCK
- ERK
- mTOR

ECM contraction

Research on endometriosis-associated fibrosis

1. Mechanisms of endometriosis-associated fibrosis


3. Effects of decidualization on endometriosis-associated fibrosis
Mevalonate-Rho/ROCK pathway and its inhibitors

Acetyl-CoA
↓
HMG-CoA
↓
Mevalonate
↓
Isopentenylpyrophosphate
↓
FPP
↓
GGPP
↓
Squalene
↓
Cholesterol

HMG-CoA reductase
Statins

Exoenzyme C3

RhoA
GGTase-I
GGTIs

Fasudil
Y-27632

Cell proliferation of ECSC
ECM contraction of ECSC
Apoptosis of ECSC

Heparin
Decidualization

Mevalonate-Rho/ROCK pathway and its inhibitors

Acetyl-CoA

\[ \text{Mevalonate} \]

\[ \text{HMG-CoA} \]

\[ \text{HMG-CoA reductase} \]

\[ \text{Statins} \]

\[ \text{Cholesterol synthesis pathway} \]

\[ \text{Heparin Decidualization} \]

\[ \text{Isopentenylpyrophosphate} \]

\[ \text{FPP} \rightarrow \text{GGPP} \]

\[ \text{RhoA} \]

\[ \text{GGTase-I} \rightarrow \text{GGTIs} \]

\[ \text{Activated RhoA} \]

\[ \text{ROCKs} \]

\[ \text{Fasudil Y-27632} \]

\[ \text{Cell proliferation of ECSC} \]

\[ \text{ECM contraction of ECSC} \]

\[ \text{Apoptosis of ECSC} \]

Effect of simvastatin on the morphology and contractility of ECSC

*\( p < 0.0005, ** p < 0.0001 \) vs. controls (Bonferroni/Dunn test)

Effect of simvastatin on the cell viability of ECSC

*\( p < 0.0005 \), **\( p < 0.0001 \) vs. controls
(Bonferroni/Dunn test)
Mevalonate-Rho/ROCK pathway and its inhibitors

**Cholesterol synthesis pathway**

Acetyl-CoA → HMG-CoA → Mevalonate → Isopentenylpyrophosphate → FPP → GGPP → RhoA → ROCKs

- **Statins** inhibit HMG-CoA reductase.
- **GGTIs** activate GGTase-I.

**Exoenzyme C3**

- **Heparin** and Decidualization are involved.

**ROCKs**

- **Fasudil** (Y-27632) inhibits ROCKs.

**ECM contraction of ECSC**

**Apoptosis of ECSC**

**Cell proliferation of ECSC**

Collagen gel contraction assay treated with Y-27632

Inhibition of contractility by Y-27632

Y-27632 (μM)

* p < 0.0001 vs controls (Bonferroni/Dunn test)

Mevalonate-Rho/ROCK pathway and its inhibitors

Cholesterol synthesis pathway

Acetyl-CoA → HMG-CoA → Mevalonate → Isopentenylpyrophosphate

HMG-CoA reductase → FPP → GGPP → RhoA → GGTagase-I → GGTIs

Squalene → Cholesterol

ROCKs → Cell proliferation of ECSC

Activated RhoA → ECM contraction of ECSC

Apoptosis of ECSC

Statins

Heparin

Decidualization

Collagen gel contraction assay treated with fasudil

ECSC

Control

Fasudil (100 μM)

NESC

Control

Fasudil (100 μM)

Tsuno et al. J Clin Endocrinol Metab (2011)
Effect of fasudil on the morphology of 3-D cultured ECSC

Control

Fasudil (100 μM)

Giemsa stain (x100)

Tsuno et al. J Clin Endocrinol Metab (2011)
Effects of fasudil on the proliferation of ECSC

**BrdU incorporation assay**

*\( p < 0.001 \), **\( p < 0.005 \) vs. unstimulated controls (Bonferroni / Dunn test)

**Modified MTT assay**

*\( p < 0.001 \), **\( p < 0.005 \) vs. unstimulated controls (Bonferroni / Dunn test)

Tsuno et al. J Clin Endocrinol Metab (2011)
Effects of fasudil on apoptosis of ECSC

![Graph showing the effect of fasudil on apoptosis of ECSC](image)

* P<0.001 vs. unstimulated controls (Bonferroni/Dunn test)

Tsuno et al. J Clin Endocrinol Metab (2011)
Effects of fasudil on the cell cycle of ECSC

Tsuno et al. J Clin Endocrinol Metab (2011)
Effects of fasudil on the levels of contraction-related proteins

α-SMA

RhoA

ROCK-I

ROCK-II

Bcl-2

Bcl-xL

GAPDH

Fasudil (μM) 0 100

Tsuno et al. J Clin Endocrinol Metab (2011)
Mevalonate-Rho/ROCK pathway and its inhibitors

Acetyl-CoA → HMG-CoA → Mevalonate → Isopentenylpyrophosphate → FPP → GGPP → RhoA → Activated RhoA → ROCKs

Cholesterol synthesis pathway:

- HMG-CoA reductase
- Statins
- Squalene
- Cholesterol
- Exoenzyme C3
- Heparin
- Decidualization

GGTase-I → GGTIs

ROCKs:

- Fasudil
- Y-27632
- siRNA

Cell proliferation of ECSC

ECM contraction of ECSC

Apoptosis of ECSC

Effect of heparin on the ECSC-mediated 3-D gel contraction

Control

Heparin sodium 1 μg/mL

Heparin sodium 10 μg/mL

Heparin sodium 100 μg/mL

Inhibition of ECSC contractility by heparin

* * p<0.0001 vs untreated controls (Bonferroni/Dunn test)

Effect of heparin on the morphology of 3-D cultured ECSC

Control

Heparin sodium 100 μg/mL

LSM5 Pascal Ver. 4.2 (Carl Zeiss)

Effects of heparin on the levels of contraction-related proteins

1. Simvastatin, a Rho inhibitor, Fasudil, a ROCK inhibitor, and heparin significantly inhibited the collagen gel contractility of ECSCs.

2. Inhibition of the mevalonate-Rho/ROCK-mediated signaling pathway may provide a novel therapeutic strategy for the treatment of endometriosis-associated fibrosis.
1. Mechanisms of endometriosis-associated fibrosis
3. Effects of decidualization on endometriosis-associated fibrosis
Mevalonate-Rho/ROCK pathway and its inhibitors

Acetyl-CoA → HMG-CoA → Mevalonate → Isopentenylpyrophosphate → FPP → GGPP → RhoA → ROCKs → Cell proliferation of ECSC, ECM contraction of ECSC, Apoptosis of ECSC

Cholesterol synthesis pathway:

HMG-CoA reductase → HMG-CoA → Mevalonate

Statins

GGTase-I → GGTIs

Exoenzyme C3

siRNA

Heparin

Decidualization

Fasudil, Y-27632, siRNA

Hormonal therapy has been used for endometriosis to create a hypoestrogenic acyclic hormone environment. Progestins either alone or combined with estrogens are now used as the first choice of hormonal therapy on a long-term basis. The administration of a combined OCs or progestins alone induces decidualization of both the endometriotic lesions and the eutopic endometrium.

In vitro decidualization of ECSC

DMEM + 10% chacoal- stripped FBS

+ dibutyryl-cyclic adenosine monophosphate (db-cAMP) (0.5 mM)
  + dienogest (100 nM)

  or

  + medroxyprogesterone ascetate (MPA) (100 nM)

12 days culture

Production of PRL by ECSC, ESCwE and NESC

*\( p < 0.0001 \), vs. untreated controls (Bonferroni/Dunn test)

Production of IGFBP-1 by ECSC, ESCwE and NESC

*\( p<0.0001 \), vs. untreated controls (Bonferroni/Dunn test)

# Results of 3-D gel contraction assay

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>db-cAMP + MPA</th>
<th>db-cAMP + dienogest</th>
<th>db-cAMP</th>
<th>MPA</th>
<th>Dienogest</th>
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</thead>
<tbody>
<tr>
<td><strong>ECSC</strong></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td><strong>ESCwE</strong></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td><strong>NESC</strong></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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</tbody>
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Collagen gel contraction mediated by ECSC, ESCwE and NESC

*\( p<0.0001 \), vs. untreated controls (Bonferroni/Dunn test)

Morphology of ECSC, ESCwE and NESC in 3-D culture

<table>
<thead>
<tr>
<th></th>
<th>db-cAMP + db-cAMP + db-cAMP</th>
<th>MPA</th>
<th>dienogest</th>
<th>MPA</th>
<th>Dienogest</th>
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</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ECSC</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td><strong>ESCwE</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>NESC</strong></td>
<td></td>
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</table>

 LSM5 Pascal Ver. 4.2 (Carl Zeiss)

Cell density of ECSC, ESCwE and NESC in 3-D culture

Tsunou et al. J Clin Endocrinol Metab (2009)
Expression of contractility-related molecules in 3-D cultured ECSC, ESCwE, and NESC

<table>
<thead>
<tr>
<th></th>
<th>ECSC</th>
<th>ESCwE</th>
<th>NESC</th>
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<td>db-cAMP+MPA</td>
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RhoA
ROCK-I
ROCK-II
α-SMA
GAPDH

Expression of integrins in ECSC, ESCwE and NESC

<table>
<thead>
<tr>
<th></th>
<th>ECSC</th>
<th>ESCwE</th>
<th>NESC</th>
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<td><img src="image9.png" alt="Image" /></td>
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Integrin α1
Integrin α2
Integrin β1
GAPDH

Summary

1. Decidualization inhibits the contractility of ectopic and eutopic endometrial stromal cells through downregulation of collagen I receptor expression and suppression of Rho-ROCK-mediated signaling pathways.

2. Decidualization inhibits the differentiation of ectopic and eutopic endometrial stromal cells into myofibroblastic phenotype and induces the differentiation of these cells into epithelioid decidual phenotype.
Proposed mechanisms of endometriotic stromal cell-mediated collagen gel contraction

Stimulation by serum, ECM attachment, peptide growth factors, etc.

- PKC
- Tyrosine kinases
- Ras
- PI3-kinase

- MLCK
- Rho
- MEK
- MMPs
- Akt

- MLC phosphorylation
- ROCK
- ERK
- mTOR

ECM contraction

平成21年度日本産科婦人科学会学術奨励賞を与えて頂き、理事長の吉村泰典教授をはじめ日本産科婦人科学会の皆様方に御礼申し上げます。

第62回日本産科婦人科学会学術講演会にて、受賞講演として「子宮内膜症の病態解明と新しい薬物療法の開発」の講演の機会を与えてくださった会長の稲葉憲之教授、座長の久保田俊郎教授にお深謝いたします。

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Collaborators of the present research
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Yukie Kawano  Wakana Abe
Kentaro Kai  Mamiko Okamoto
Harunobu Matsumoto
Thank you very much for your attention!

Oita University Hospital