Immune-like Mechanisms Associated with Ovulation:

- Inflammatory reactions
- Matrix remodeling
- Cytokine production and actions
- Innate immune genes

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Ovulation is an inflammatory-like reaction because levels of prostaglandins are high. Espey 1980
LH induction of prostaglandin synthase 2 (Ptgs2/COX2) is essential for ovulation; KO mice are infertile

Cumulus cell-oocyte complex expansion is a highly specialized inflammatory-related process that is obligatory for successful ovulation.

Release of a fertilizable oocyte within the cumulus oocyte complex (COC) requires:
1) The production and stabilization of an extracellular matrix
2) Genetic reprogramming cumulus cells
3) Meiotic maturation of the oocyte
Hyaluronic acid formation

4h post hCG

8h post hCG
HA binding proteins associated with inflammatory responses stabilize the matrix.

COC isolated from the oviduct

HA, IαI and Dapi
(PTX3, TNFAIP6, versican)
Mutant mouse models confirm that these genes are critical for COC expansion and fertility.
How does LH induce COC expansion?

Because cumulus cells have a distinct cell fate, do cumulus cells express a unique set of genes?
How does LH induce COC expansion?

Conti et al; Shimada et al
Mol Endocrinol, 2006
AREG, like PGE and FSH, can induce COC expansion in culture.
Do cumulus cells express a unique set of genes?

Microarray Analyses of COCs

- 343 genes
  - 9 genes
    - B
  - 170 genes
    - C
  - 136 genes
    - D
  - 423 genes
    - E

0h PMSG PO Follicle
8h hCG Periovulatory Follicle
16h hCG Ovulated Oviduct

Matrix Inflammation Innate Immunity
Oocyte Cell Signal Proliferations

Hernandez et al, Mol Endocrinol 2006
There are cell specific, as well as sequential and progressive, responses to LH.

**Rapid:** EGF-like factors: AREG, EREG, BTC
- PTGS2 and PTGER2/4
- C/EBP beta
- IL6

**Intermediate:** Toll receptor pathway
- TLR2/4, MYD88
- CD14
- IL6
- SNAP25
- RUNX1/2

**Late:** Innate immune
- PTX3
- CD34
- CD52
- RUNX1/2
- PDCD1
- CD36
**AREG** induces genes in COCs in an EGF receptor dependent manner

**Matrix genes**

- **Ptgs2**
- **Tnf aip6**

**Innate immune-related genes**

- **Pdcd1**
- **Cd52**

C, AREG, AREG + AG
The AREG-PTGS2/PGE regulatory loop is essential for the induction of matrix related genes in granulosa cells and cumulus cells.
LH/hCG activation of RAS and ERK1/2 in granulosa cells *in vivo* is rapid, transient and tightly coordinated.

*Fan et al, Development*, 2008
If LH activates both the PKA and RAS/ERK1/2 pathways, what genes that control ovulation, COC expansion and luteinization are downstream targets of each pathway?
Erk1 knockout mice are viable and fertile (Pagès et al, Science 1999).
Erk2 null mice die at E6.5
Therefore, Erk2$^{fl/fl}$ mice have been generated (Fischer et al, Immunity 2005).

To generate a double KO mouse, Erk2 was disrupted in granulosa cells of the Erk1 null strain by mating Erk1$^{-/-}$ mice with Erk2$^{fl/fl};$Cyp19-Cre mutant mice.
ERK1/2 were successfully deleted in granulosa cells
Ovulation, oocyte maturation, cumulus expansion and luteinization are blocked in Erk1/2\(^{gc/-}\) mice

![Graph showing egg and pup production with WT and Erk1/2 gc/- mice](image)

Fan et al, Science 2009
ERK1/2 globally regulate the expression of LH-target genes
LH/hCG-induced synthesis of *Il6* and *Pgr* mRNA is abolished in ERK1/2-depleted follicles.
ERK1/2 induce the expression of selected LH-target genes by activating C/EBPβ
ERK1/2 control the molecular switch by which LH reprograms granulosa cells and cumulus cells in preovulatory follicles.

- LH
  - cAMP/PKA
  - EGF-like factors
  - RAS
  - ERK1/2
  - C/EBPβ, C/EBPα and others
    - Estradiol biosynthesis
    - GC proliferation
    - Follicle growth
    - Oocyte maturation
    - COC expansion
    - Ovulation
    - Luteinization

Fan et al, Science, 2009
What other genes and signaling pathways are downstream of the ERK1/2 molecular switch?

Genes controlling:

- Inflammatory responses
- Innate immune processes
- Terminal differentiation
- Cell cycle arrest
Is the COC matrix a protective shield? Does the matrix exert specific functions? Do cumulus cells control functions beyond the matrix?
The immune system is a **surveillance** system that recognizes “self” from “non-self” or “altered-self” via pathogen recognition receptors (PRRs): CD14, Toll-like receptors (TLRs), C1q leading to transcription of *Il6*, *Tnfα* and *Ptgs2*.

Macrophages remove “non-self” (bacteria;LPS) or “altered self” (apoptotic cells) via scavenger receptors (**CD36**, **SCARBI/II**) that are induced by cytokines, such as **IL6**.

If cumulus cells have immune-related functions, do they express genes related to surveillance functions?
Model of matrix and immune cell related factors

Functions in vivo: Matrix molecules HA, surveillance (sperm), apoptosis (cumulus cells)
Components of the TLR receptor pathway are induced by FSH and AREG in cultured mouse COCs.
TLRs present in COCs are functional.
LPS induces expression of *Il6*, *Tnfa*, and *Ptgs2* mRNAs.
Matrix genes are mis-regulated in COC isolated from \textit{Cd14} null mice

Lui \textit{et al}, unpublished
Cumulus cells can release potent cytokines in response to pathogens.

**Pathogen Activation of TLR2/4**

- **Pam3Cys** activates TLR2
- **LPS** activates TLR4

**Cumulus Cell**

- **Ptgs2**, **Il-6**, **Tnfa**, **Ptx3**
- **PGE2**, **IL6**, **TNFα**

**Other cytokines/chemokines**

**Cumulus cells**

**Ovarian surface epithelial cells**

**Oviductal cells**

**Sperm**
Cumulus cells can release potent cytokines in response to physiological stimuli such as HA fragments.
Other cytokines and chemokines are induced in COCs (and granulosa cells) in response to pathogens and HA fragments.

*Ccl2*/MCP1: monocyte chemotactic protein 1
*Ccl4*/MIP1β: macrophage inflammatory protein 1β
*Ccl5*/ RANTES: regulated upon activation, T-cell expressed and secreted

Shimada *et al*, Development, 2008
Sperm activate the TLR2/4 pathways in COCs and induce expression of *Il-6*.
Cytokines and chemokines are rapidly released and produced by cumulus cells during IVF procedures in a TLR2/4-dependent mechanism.
Human COCs express *Tlr2* and *Tlr4* mRNAs and levels of chemokines released in IVF protocols are related to fertility success in women.
These cytokines are released from cumulus cells and granulosa cells by a progesterone receptor-dependent mechanism that involves the induction of the secretory vesicle protein SNAP25.

<table>
<thead>
<tr>
<th></th>
<th>FSH+AREG free</th>
<th>FSH+AREG RU486</th>
<th>Forskolin+PMA free</th>
<th>Forskolin+PMA RU486</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>2.31+/−1.15</td>
<td>55.67+/−11.34*</td>
<td>22.15+/−3.22#</td>
<td>168.13+/−15.41**</td>
</tr>
<tr>
<td>IL-9</td>
<td>0.98+/−0.20</td>
<td>5.35+/−1.69*</td>
<td>2.05+/−0.82#</td>
<td>4.83+/−0.25**</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.54+/−0.06</td>
<td>5.28+/−0.95*</td>
<td>4.92+/−0.55</td>
<td>11.23+/−1.84**</td>
</tr>
<tr>
<td>KC</td>
<td>33.15+/−4.64</td>
<td>51.60+/−14.46</td>
<td>35.22+/−3.51</td>
<td>81.85+/−12.28**</td>
</tr>
<tr>
<td>RANTES/CCL5</td>
<td>1.25+/−0.68</td>
<td>1.16+/−0.13</td>
<td>1.61+/−0.55</td>
<td>2.11+/−0.83</td>
</tr>
</tbody>
</table>

Shimada et al, Mol Endo, 2007
Potential roles of the TLR pathway in the female reproductive tract before and after ovulation: pathogens and matrix factors

1: Pathogen activation of TLR2/4

- Pam3Cys
- LPS

TLR2

- Ptgs2/Il-6
- Tnfa, PtX3, Pdcd1

TLR4

- PGE2/IL6
- TNFα
- other cytokines
- chemokines

CD14

Cumulus cells

Ovarian surface epithelial cells

Oviductal cells

Sperm

Ovulation, Transport, Fertilization?

HA Fragments

Polymeric HA

Hyaluronidase 1/2

2: Potential activation of TLR2/4 by hyaluronan in cumulus cells.

Richards et al, JARG, 2008
What are the functions of IL6 and other cytokines?

IL6 and OSM induce expansion of mouse COCs and oocyte maturation in culture

Liu et al, Endocrinology, 2009
IL6 induces COC expansion and matrix-associated genes but not *Il6* or *Areg* in cultured COCs.

IL6 also enhances IVF success and embryo viability.
What are some other potential roles of these cytokines and chemokines?

Sperm have receptors for cytokines and chemokines that impact sperm motility to enhance fertilization.

Sperm also express functional receptors for TLR2/4 that appear to impact sperm motility, viability and fertilization capacity.

Many infertile men and male domestic animals have infections within the genital tract that reduces fertility and impairs sperm function (causes apoptosis) in IVF protocols and in long-term sperm storage.

Antibiotics that block the action of LPS vastly improve sperm functions and viability in these infertile IVF protocols.

(Shimada et al Development, 2008 and personal communication)
Activation of TLR4/2 has also been linked recently to obesity, insulin resistance and diabetes via sensing and responding to FFA.

Long chain fatty acids (palmitate) can activate TLR4 leading to production of pro-inflammatory cytokines via NFkB activation in adipocytes, hypothalamic cells and muscle cells.

Thus, inappropriate induction and activation of TLR4 by abnormal metabolic products may alter cellular homeostatic metabolic processes, leading to impaired functions of many tissues.

Loss of TLR2/4 protects mice from the effects of diet induced obesity.

(Vitseva et al, Obesity, 2008; Milanski et al, J Neuroscience, 2009; Reyna et al, Diabetes, 2008)

In the ovary, this might translate to PCOS and insulin resistance.
Macrophages remove “non-self” (bacteria; LPS) or “altered self” (apoptotic cells) via scavenger receptors (CD36, SCARBI/II) that are induced by cytokines, such as IL6.

Do cumulus cells or granulosa cells exhibit similar functions?
There are also pathological as well as physiological functions of RAS, ERK1/2 and inflammatory molecules in the ovary.

Obligatory for LH-induction of ovulation and luteinization, terminal differentiation

Fan et al, Science, 2009

Premature activation causes granulosa cell cycle arrest and abnormal follicle growth.

Fan et al, Development, 2008

Ptен/Kras mutant OSE cells develop into serous adenocarcinomas but not GCTs.

Fan et al, Cancer Res, 2009
Ovarian surface epithelial (OSE) cell tumors form in the \textit{Pten;Kras;Amhr2-Cre} double mutant ovaries.

\textbf{Tumors do not form} in the \textit{Pten;Kras;Cyp19-Cre} double mutant ovaries.

Therefore, \textbf{epithelial cells} respond to the \textit{Pten;Kras} mutations in a manner that is completely different from the response of \textbf{granulosa cells} to the same oncogenic insults. A big question is why.
Clinical Relevance

Role of EGF-like factors, RAS, ERK1/2 and IL6 in COCs, cumulus cells and oocytes as well as in growing follicles (POF) and ovarian cancer.

Role of TLR receptor pathways and HA in cumulus cells and sperm during fertilization and in infertile men.

New markers of oocyte quality and cumulus cell function/viability

Do chronic infections, such as endometriosis and autoimmune diseases contribute to PCOS and other abnormal ovarian functions, infertility and poor oocyte quality ---- cancer? Are the TLRs present on non-immune cells also involved in “sensing” and responding to abnormal cues derived from metabolic imbalance or malignant cells?
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Bacteria attach to SCARB1-positive granulosa cells after 24h in culture.
Localization of lysosomes (red) and SCARB1(green) in granulosa cells
Bacteria co-localize to lysosomes in granulosa cells

Shimada et al, 2006
What are the functions of this vast repertoire of immune-like genes?
What is the embryonic derivation of cumulus cells?
What are the physiological roles of the TLR receptor pathway in granulosa cells and cumulus cells?

Do matrix molecules regulate these receptors?

Does hyaluronan act as a ligand like LPS?

Is this pathway linked to the production and action of cytokines, such as IL6?

Is the TLR receptor pathway also functional in sperm?

Does the TLR pathway mediate infertility (sperm dysfunction) in men with bacterial infections of the reproductive tract?
IL6 regulates matrix and immune-related genes in COCs

A). COC expansion genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction</th>
</tr>
</thead>
</table>
| Ptgs2  | a  
|        | b  
|        | c  |
| Has2   | a  
|        | ab |
|        | b  |
| Tnfaip6| a  
|        | b  
|        | c  |

B). Immune related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction</th>
</tr>
</thead>
</table>
| Runx1  | a  
|        | a  
|        | b  |
| Runx2  | a  
|        | b  
|        | c  |
| Pdcd1  | a  
|        | ab |
|        | b  |

C). IL6/JAK/STAT pathway genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction</th>
</tr>
</thead>
</table>
| Il6st  | a  
|        | b  
|        | c  |
| Stat3  | a  
|        | ab |
|        | b  |
| Socs1  | a  
|        | bc |
|        | c  |
Snap mRNA and protein are induced in COCs and GC during the ovulation process.
QuickTime™ and a Video decompressor are needed to see this picture.
p38MAPK CKO mice are fertile

**AreG**

**FSH**

**control**

**p38CKO**

![Microscope images of control and p38CKO](image1)

![Graph showing superovulation and pups per litter](image2)

- **Superovulation**
  - Control: 50
  - p38CKO: 30

- **Pups per litter**
  - Control: 10
  - p38CKO: 9

**Areg**

**Ptgs2**

![Gene expression levels](image3)

- **Areg**
  - Control: 0.6
  - p38CKO: 0.6

- **Ptgs2**
  - Control: 0.06
  - p38CKO: 0.06

**Time points**

- 0h
- 1.5h
- 4h
Fertilization is compromised in the presence of TLR2/4 and CCL5 neutralizing antibodies that impair the release of cytokines/chemokines from cumulus cells that enhance sperm capacitation.

![Bar chart showing fertilization percentages](chart.png)