IVA: In vitro activation: A new infertility treatments for patients with primary ovarian insufficiency (POI)

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1. Basic and translational studies for in vitro activation of dormant follicles (IVA)

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Primary ovarian insufficiency (POI)

Diagnosis
1. Amenorrhea before 40 years of age
2. Hypergonadotrophic hypogonadism

Symptoms
1. Infertility
2. Estrogen deficiency-hot flashes, mood disturbances, sexual dysfunction etc.
Specific features

- Lack of follicle growth and ovulation
- Exhaustion of ovarian follicles and few residual follicles: <1,000 follicles (undetectable AMH levels)

Treatments

- Resistant to traditional gonadotropin treatments
- Egg donation is the most successful treatment option, but…
1. Background of POI

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Among dormant follicles, ~0.1% follicles are selected to activate. Because POI has < 1,000 residual follicles, these growth factors can activate only 1 follicle.

We focused on intracellular signaling system involving in the activation.

Growth factors (kit ligand, neurotrophins, BMPs, VEGF, LIF, etc.)
At early stage after birth, PTEN or FOXO3 deletion led to the activation of dormant primordial follicles and resulted in depletion of follicles within 16-18 weeks.
The PI3K signaling pathway begins PI3K activation by receptor tyrosine kinases (RTKs) after binding growth factors. PI3K activates AKT, which inhibits the activities of FOXO3, resulting in cell proliferation and survival. PTEN negatively regulates PI3K signaling.

In primordial follicles, local factors activate dormant follicles through PI3K-AKT-Foxo3 signaling pathway, whereas PTEN acts to block the signaling.
Is it possible to activate residual dormant follicles in POI patients artificially by transient PTEN suppression and/or PI3K activation using drugs?
PTEN inhibitor

A vanadyl complexed to hydroxypicolinic acid is a highly potent and specific inhibitor at nano-molar concentrations.

PI3K activator

A cell-permeable phospho-peptide (740Y-P) binds to the SH2 domain of p85 regulatory subunit of PI3K and activates enzyme activity.
Methods--mouse

D3 mice

Ovaries

PTEN Inhibitor

&PI3K activator

transplanted into kidney capsule

Control

Culture 2 days

Adult ovariectomized

FSH treatment

Histological analyses

IVF-ET

Epigenetic analyses

Pups

Mature oocytes

hCG treatment

Oocyte retrieval
In vitro activation (IVA) - in vivo transplantation

Changes in ovarian size at day 14 after transplantation of D3 ovaries treated with PTEN inhibitor and/or PI3K activator beneath kidney capsule of host mice.
In vitro activation (IVA) - in vivo transplantation -- ovarian histology

Follicular dynamics at day 14 after transplantation of activated ovaries beneath kidney capsule of host mice.
In vitro activation (IVA) - in vivo transplantation -- genome imprinting and meiotic spindle formation of retrieved oocyte

Meiotic spindle formation was evaluated by β-tubulin staining, whereas the integrity of genomic imprinting was confirmed by detecting methylation of CpG sites in Differentially methylated region (DMR) of some imprint genes (maternal: Igf2r, Lit1, paternal: H19).
In vitro activation (IVA) - in vivo transplantation
-- early embryonic development of retrieved mature oocyte after IVF
and healthy pups after embryo transfer

![Images of oocytes at different time points (0h, 24h, 48h, 96h)]

![Graph showing % embryonic development of 2-cell/mature oocytes and blastocyst/2-cell with normal control and PTEN inhibitor + PI3K activator](image-url)
Xeno-transplantation of human ovarian fragments to activate dormant follicles: IVA, in vitro activation

Ovarian cortical fragments were obtained from patients with benign ovarian tumor with informed consent from the patient and approval from local ethical human subject committee.

**Human ovarian cortical fragments**

- PTEN Inhibitor
- Control
- Culture 2 days

Xeno-transplanted into kidney capsule

Ovariectomized SCID mice

FSH treatment for 6 months

hCG treatment

Oocyte retrieval

Mature oocytes
Morphology of human ovarian fragments after 6 months of xeno-transplantation
Histology of PTEN inhibitor treated ovarian fragments

At 36 h after hCG treatment, large antral follicles in the PTEN inhibitor-treated group contained mature oocytes at metaphase II accompanied with cumulus expansion.

Li and Kawamura et al PNAS 2010
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Clinical application of IVA for POI patients

Histological analyses

Cryo-preservation by vitrification

Preparation of ovarian cortical strips for freezing

Ovariectomy under laparoscopic surgery

Fragmentation of ovarian strips to cubes

Culture of ovarian cubes with PI3K activators

Auto-transplantation of activated ovarian cubes

Retrieve mature eggs

In vitro fertilization

Embryo cryopreservation

Embryo transfer

Kawamura et al PNAS 2013
Enrolled patients

83 of POI patients (37.4 ± 4.9 years of age)

Duration of amenorrhea: 5.7 ± 3.5 years
• Ovariectomy under laparoscopic surgery

• Minimum usage of electrocautery hemostasis to avoid damage of residual follicles.
Localization of early follicles in ovarian cortex
- Dissect ovarian cortices containing residual follicles by removing medulla.

- Cut into small strips (1 x 1 cm², 1-2 mm thickness, where residual follicles are located).

- (Option: Cryo-preserve by vitrification method.)

- 6-8 pieces of ovarian stripes could be obtained from one POI ovary.

**histological analyses**

- Use 10% of volume of each ovarian stripe to detect residual follicles.
Predictive factors for presence of residual follicles

Histological analyses

- Duration of amenorrhea
- Age for onset of amenorrhea
- Ovary size

Mean duration of amenorrhea

[Graph showing mean duration of amenorrhea with bars for residual follicle status (-) and (+)]
Fragmentation of ovarian strips to cubes

Culture of ovarian cubes

- Fragment 2-3 ovarian pieces into 1-2 mm² of cubes

- IVA drugs treatment (PTEN inhibitor and PI3K activator) for 2 days to activate dormant follicles
Before auto-transplantation, wash cultured ovarian cubes by warmed culture media alone to avoid to introduce reagents inside of body.

Transplant beneath the serosa of Fallopian tubes (20-40 cubes per site).

Beneath serosa of Fallopian tubes — high vascularization, convenience for trans-vaginal ultrasound monitoring ease for oocyte retrieval

In Vitro Activation

Culture of ovarian cubes

Auto-transplantation of activated ovarian cubes
Auto-transplantation

Cutting the serosa and making a pouch between serosa (arrows) and Fallopian tube (arrowhead).

Grafting multiple ovarian cubes (arrows) beneath the serosa of Fallopian tubes.

Wound was covered by an oxidized regeneration cellulose to avoid cube loss from the graft site.
Monitor follicle growth weekly to biweekly: transvaginal ultrasound + serum estrogen and gonadotropin levels.

Maintain LH levels to be normal range (Zhai, et al. JCEM 2016). When estrogen levels were increased, follicle growth was promoted by rFSH and hMG under GnRHa or GnRH AN protocols.

After hCG treatment, oocyte retrieval followed by IVF was performed.

Retrieve mature eggs

In vitro fertilization
Results

- Among 83 patients, ovary grafting was performed in 46 patients and follicle growth was found in 28 of 46 patients containing residual follicles based on the histological analyses.
  
  (no follicle growth was observed in patients without residual follicles)

- After IVF, embryos were cryopreserved at day 2.
Results

Thawing embryo transfer was performed in 8 patients. Others were accumulating cryopreserved embryos.

3 of 8 patients became pregnant after embryo transfer.

One miscarriage
Two successful deliveries
 —a male baby, 3254 g
 —a female baby, 2970 g
Reproducibility of IVA was already confirmed by China and Spain groups under our guidance.  

Kawamura et al Hum Reprod 2015  
Zhai et al JCEM 2016

Press conference in China: May 2015

中国第一例应用体外激活卵子（In vitro activation, IVA）及卵巢组织自体移植技术治疗卵巢早衰患者，日前在 郑州大学第一附属医院（下简称“郑大一附院”）生殖医学中心临床妊娠成功。
Follicle growth from primordial to preovulatory stage takes more than 6 months.

In contrast to our expectation, we found follicle growth before 6 months after grafting.

This result suggested that our IVA method also stimulated growth of secondary follicles in grafted ovaries.
Ovarian fragmentation suppressed Hippo signaling, leading to follicle growth.

Ovarian fragmentation led to changes in intercellular tension and facilitated the conversion of G-actin to F-actin.

Subsequent disruption of Hippo signaling decreased pYAP to total YAP ratios, leading to increased expression of downstream CCN growth factors.

Secretion of CCN growth factors stimulated follicle growth.

Kawamura et al. PNAS 2013
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Future studies

- IVA requires two times of laparoscopic surgeries.

→ Develop less invasive approach:
  intake or injection of reagents
  for disruption of Hippo signaling

- IVA can not apply for patients without residual follicles.

→ Regeneration oocytes:
  iPS cell?,
  oocyte precursor/stem cell?
Recent progress **in less invasive IVA**

- Regular IVA needs two times of laparoscopic surgeries.

Develop less invasive approach: intake or injection of reagents for disruption of Hippo signaling.

Although this approach can not apply for severe POI patients without secondary follicles, we can treat DOR/POI patients.
Candidate molecule: Sphingosine 1-phosphate (S1P)

S1P is a bioactive sphingolipid, acting on GPCR to suppress Hippo signaling.

S1P is a physiological substance and exists in follicular fluid in ovaries.
Effects of S1P on disruption of Hippo signaling in D10 mouse ovarian tissue culture

S1P stimulates nuclear translocation of YAP in granulosa cells followed by increase in expression of downstream CCN2 growth factor.
Effects of S1P on secondary follicle growth in D10 mouse ovarian tissue culture

S1P increased ovarian weight and stimulated early secondary follicle growth.
Effects of S1P on CCN2 expression in human ovarian tissue culture

Human ovarian cortex containing early secondary follicles were cultured with S1P for 3h.

S1P increased expression of CCN2 growth factor.
Summary

S1P disrupts Hippo signaling in early follicles leading to stimulation of secondary follicle growth.

Yuan, Kawamura et al FASEB J 2015

Because S1P is physiological substance existing in follicular fluid, intake or injection of S1P expects to stimulate follicular growth in POI/DOR patients including aging without severe adverse reactions.

Patent: PCT/US2013/059800
Future studies

- IVA requires two times of laparoscopic surgeries.

Develop less invasive approach:
intake of injection of reagents
for disruption of Hippo signaling

- IVA can not apply for patients without residual follicles.

Regeneration oocytes:
iPS cell?,
oocyte precursor/stem cell?
The method for isolation of oogonial stem cells by Dr. Tilly

- Frozen human ovarian cortex obtained from Japanese patients
- Disperse the tissue by treatment with collagenase for 1 h
- FACS isolation of oogonial stem cells using DDX4 antibody
- Culture for 1 mo

Difficult reproducibility in other labs!

Woods & Tilly, 2013

FACS

DDX4 (+)

DDX4 antibody

Day 0
Day 1
Day 2
Day 4
Day 8
Day 16
Day 30

5.55%

Development novel method for regenerate oocytes

Our method

Human ovary

Tissue dispersion

DDX4 antibody

Culture with 10%FBS

Tissue dispersion

Collagenase

medulla

Our oocyte-like cells did not complete meiosis.
Gene expression changes during human oogenesis

**PRDM1**

**PGC formation**

**SYCP1**

**SYCP3**

**DAZL**

Meiosis initiation

Follicle formation

He J et al. PLoS ONE 2013
Differentiation of PRDM1 positive cells by tissue dispersion of medulla


Our method

Tissue dispersion could differentiate PRDM1 positive cells from medulla.
PRDM1 positive cells have an ability for self-replication

Doubling time: 2.5 days

Cell number

Day 0  Day 30  Day 60

POI patient 1

POI patient 2
Induction of meiosis related genes during culture in PRDM1 positive cells

Real-time qPCR

**PRDM1**

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**DAZL**

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DAZL expression could not induce following Tilly’s method in our cells.
DAZL expression could be induced by BMP4 treatment supplemented with KSR, but retinoic acid treatment could not induce SYCP3 expression.
Prediction of important factors responsible for meiosis based on microarray analyses of murine female germ lineage cells

1) Up-regulated genes (>2-fold) were extracted from microarray data by comparison of those in 11.5dpc and 13.5dpc.

2) Identify transcriptional factors related to the up-regulated genes.

3) Predict upstream signals based on the transcriptional factors.
Transcriptional factors

Factor A

Factor B

Factor C

Factor D

Predict upstream signals based on published data base and papers
Identification of agonists and ligands to induce SYCP3 expression

Agonists/Ligands

KSR
BMP4

Day6
Day12

DAZL
SYCP3

SCYP3 expression could be induced by treatment of agonists and ligands for predicted signaling systems A-D for meiosis.
Presence of PRDM1 positive cells in medulla obtained from POI patients

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<td>SYCP3(+)</td>
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NA: under investigation

PRDM1 positive cells could obtain from medulla tissue of normal and POI ovaries in all cases including no residual follicles, and our method could induce meiosis in the cells.
Collaborators

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Thank you for your kind attention.