A wake-up call for resting follicles

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St. Marianna University School of Medicine

Kazuhiro Kawamura, M.D., Ph.D.

Contents

1. Background of POI
1. Basic and translational studies for in vitro activation of dormant follicles (IVA)
1. Clinical application of IVA
1. Future studies for IVA

Contents

Primary ovarian insufficiency (POI)

Diagnosis

1. Amenorrhea before 40 years of age
2. Hypergonadotropic hypogonadism

Symptoms

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

Primary ovarian insufficiency (POI)

Etiology

POI affects approx. 1% of women

1. Genetic—Turner syndrome, FMR1, etc.
2. Immunological—auto-immune disease
3. Iatorogenic—extensive ovarian cystectomy, partial oophorectomy, chemo-/radiation-therapies
4. Others (unknown)

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…**

Is it possible to activate residual dormant follicles in POI patients?

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

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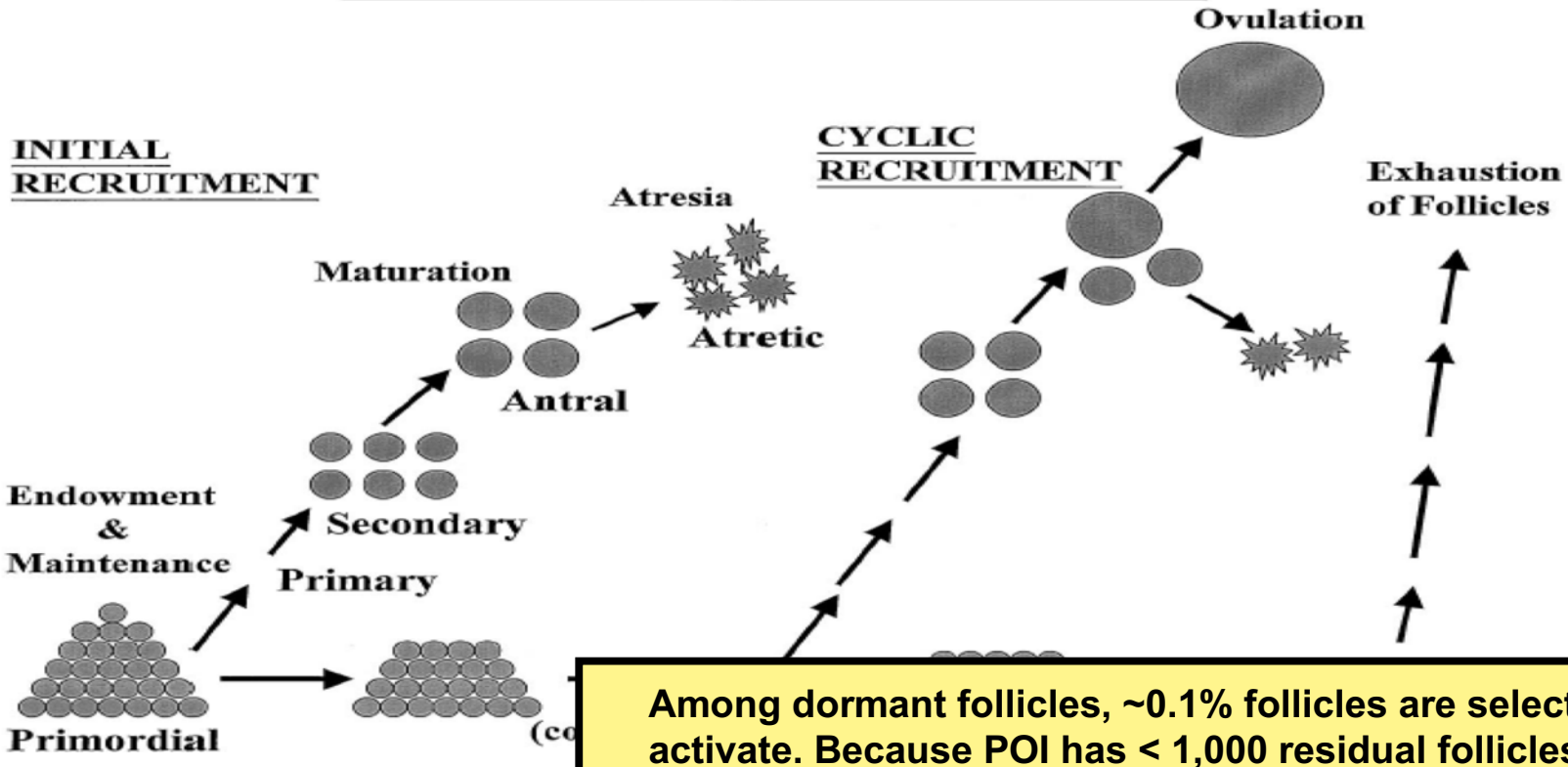
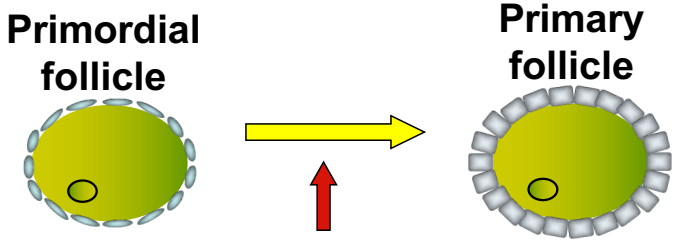
Life History of Ovarian Follicles

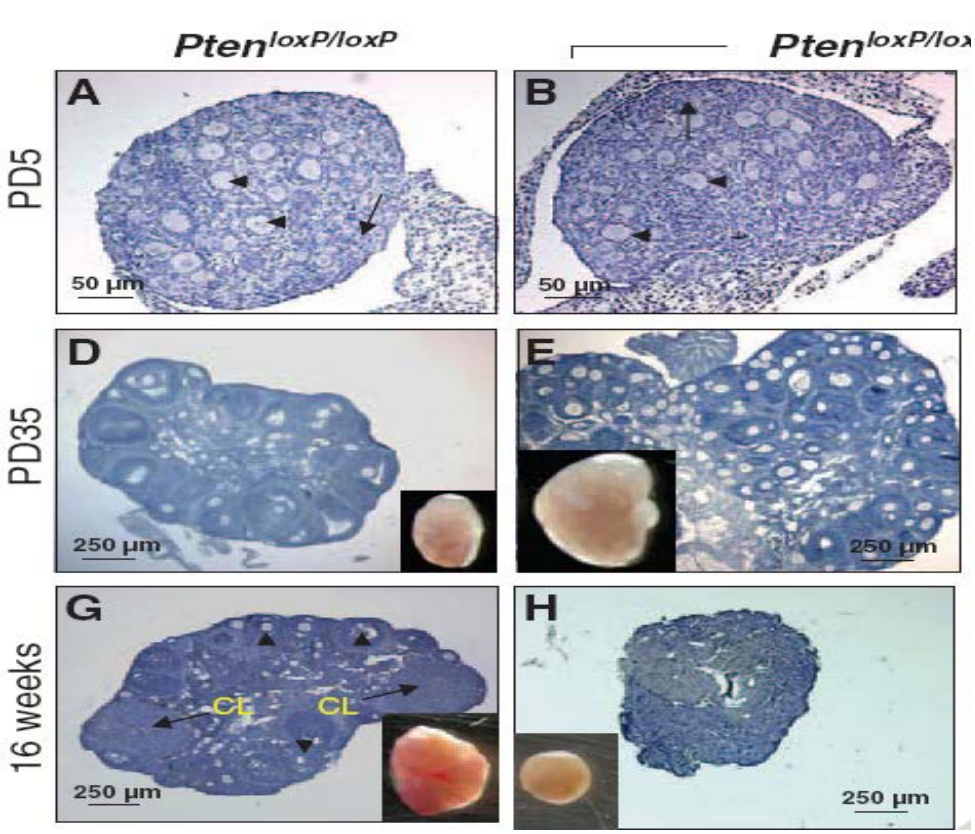




Among dormant follicles, ~0.1% follicles are selected to activate. Because POI has < 1,000 residual follicles, these growth factors can activate only 1 follicle.

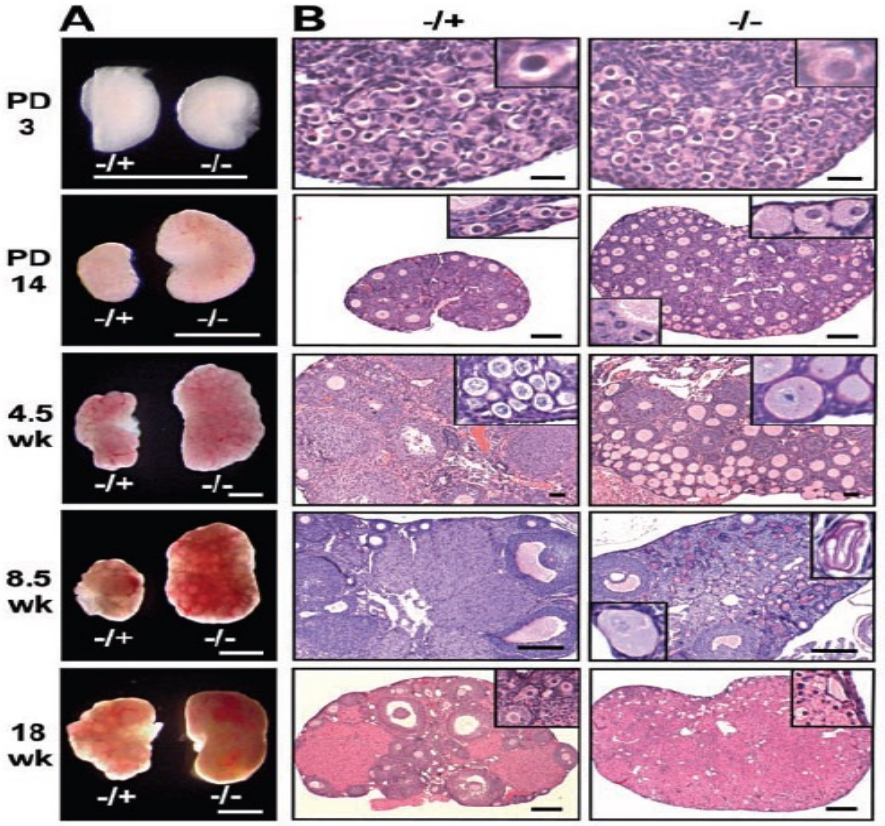
We focused on intracellular signaling system Involving in the activation.

Growth factors (kit ligand, neurotrophins, BMPs, VEGF, LIF, etc.)



Reddy et al. Science, 2008

PTEN null mice

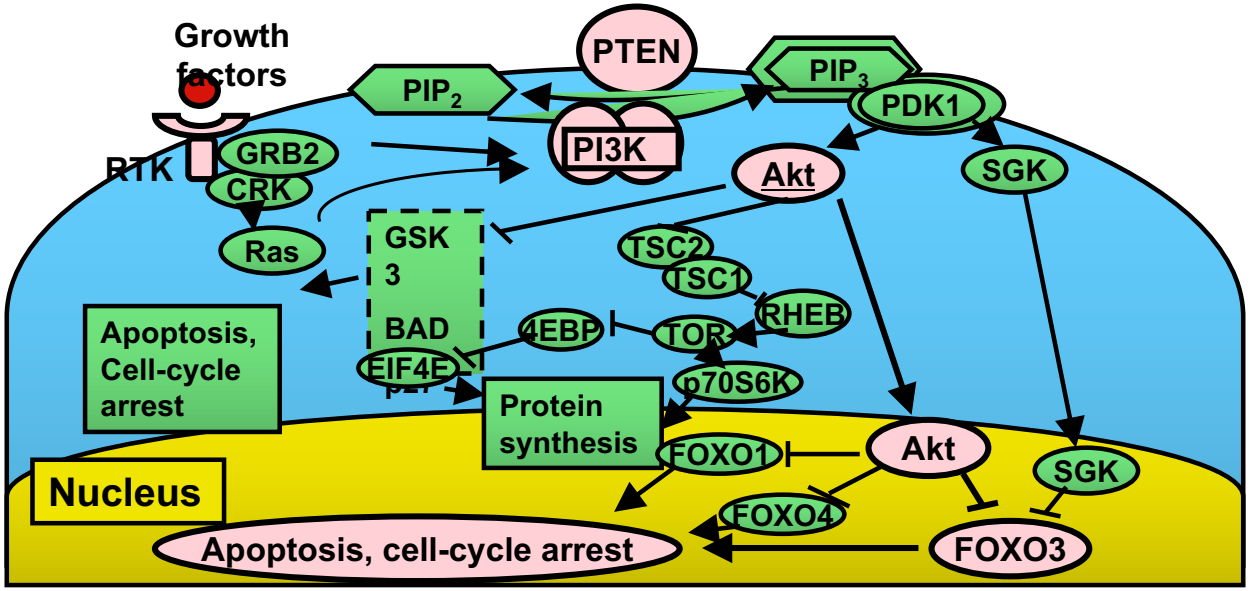


Castrillon et al. Science, 2003

Foxo3 null mice

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.

Phosphatidylinositol 3-kinase (PI3K) signaling pathway



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.

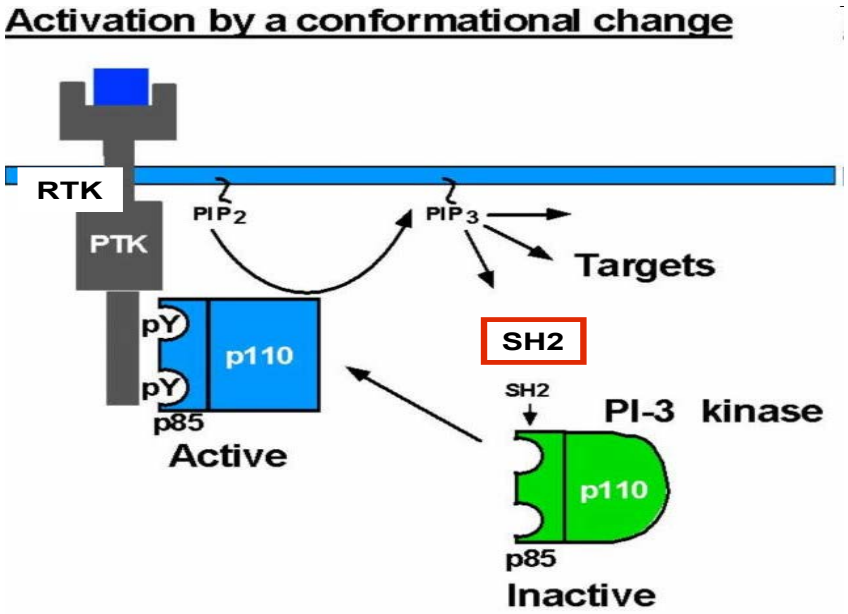
Is it possible to activate residual dormant follicles in POI patients artificially by transient PTEN suppression and/or PI3K activation using drugs?

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.



Derossi et al. BBRC, 1998

Methods--mouse

D3 mice

Ovaries

PTEN Inhibitor

&PI3K activator

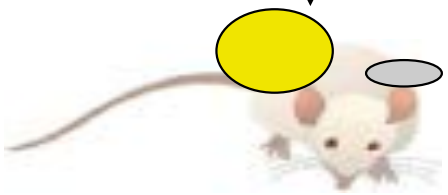
Control

Culture 2 days

transplanted into kidney capsule

Adult ovariectomized

FSH treatment



hCG treatment
Oocyte retrieval

Histological analyses

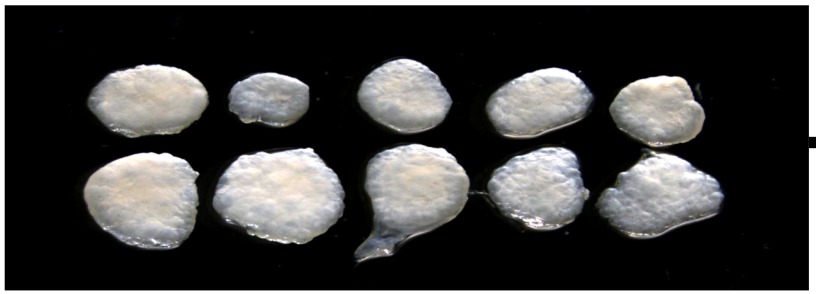
Mature oocytes

Epigenetic analyses

IVF-ET

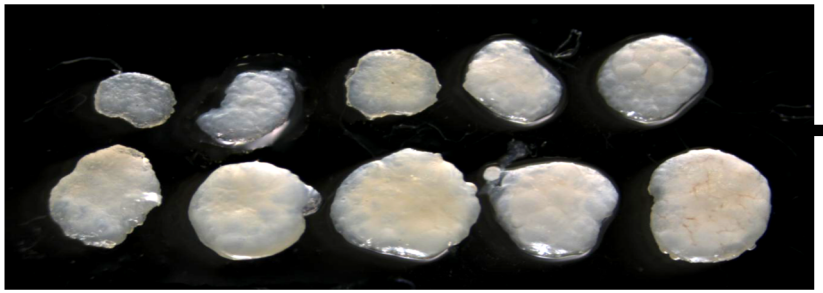
Pups

In vitro activation (IVA) - in vivo transplantation



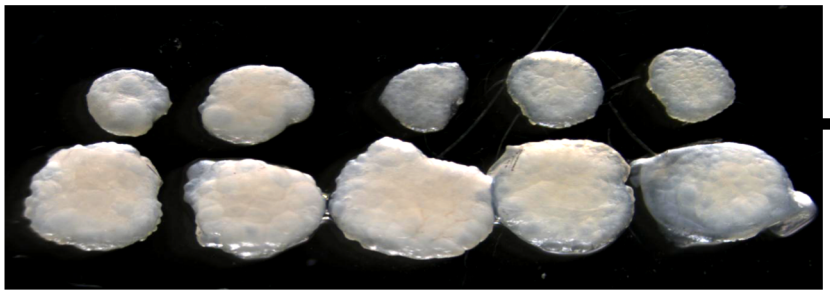
control

PTEN inhibitor



control

PI3K activator



control

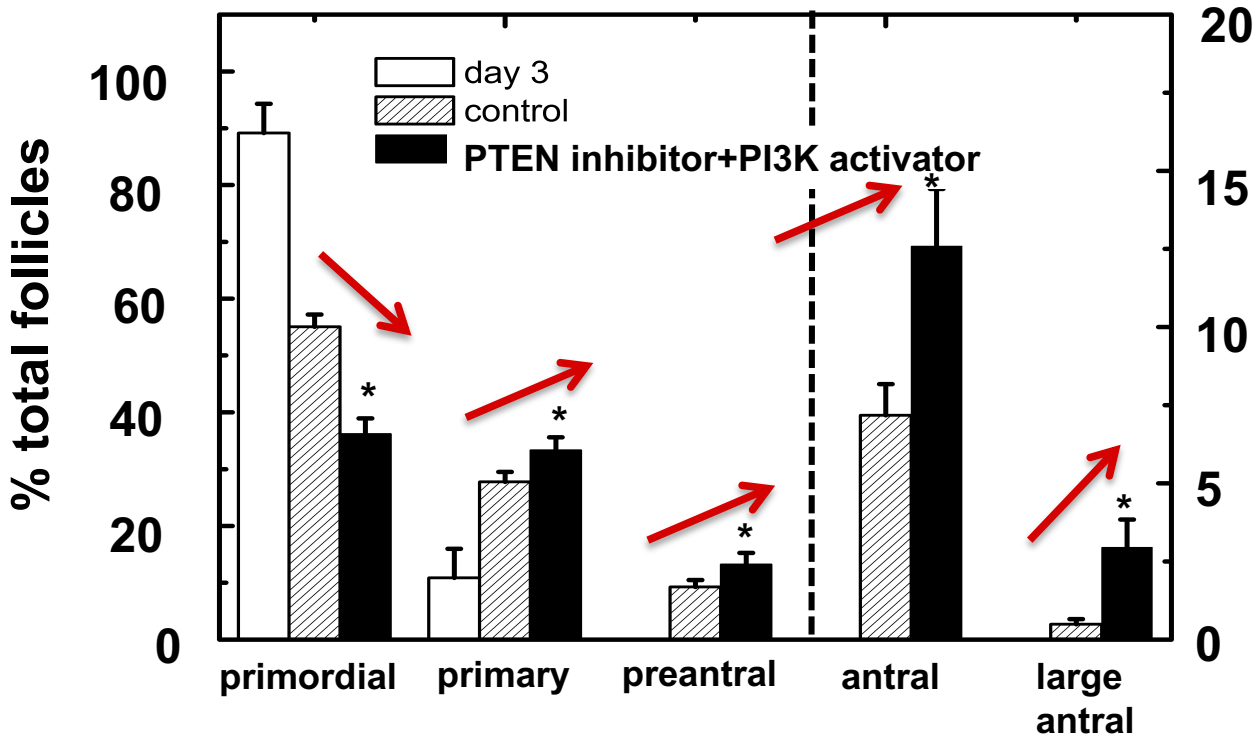
PTEN inhibitor

+

PI3K activator

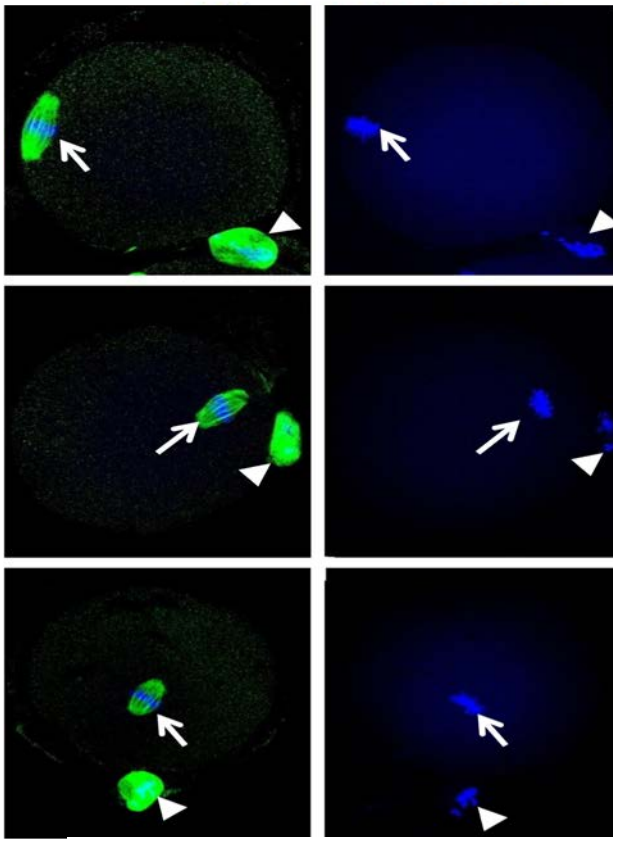
Changes in ovarian size at day 14 after transplantaion of D3 ovaries treated with PTEN inhibitor and/or PI3K activator beneath kidney capsule of host mice.

In vitro activation (IVA) - in vivo transplantation -- ovarian histology

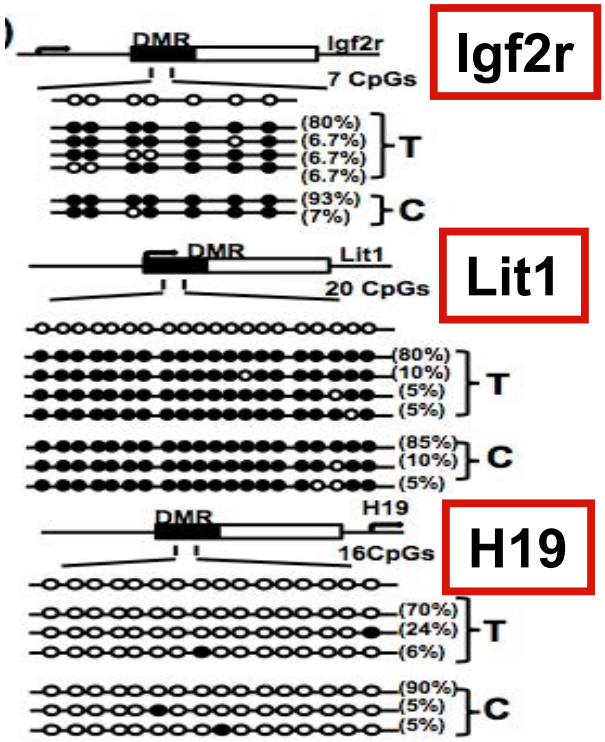


Follicular dynamics at day 14 after transplantaion of activated ovaries beneath kidney capsule of host mice.

In vitro activation (IVA) - in vivo transplantation
-- genome imprinting and meiotic spindle formation of retrieved oocyte

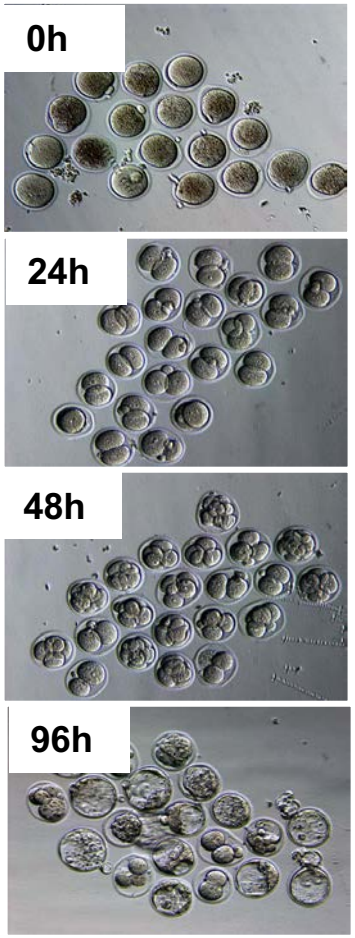


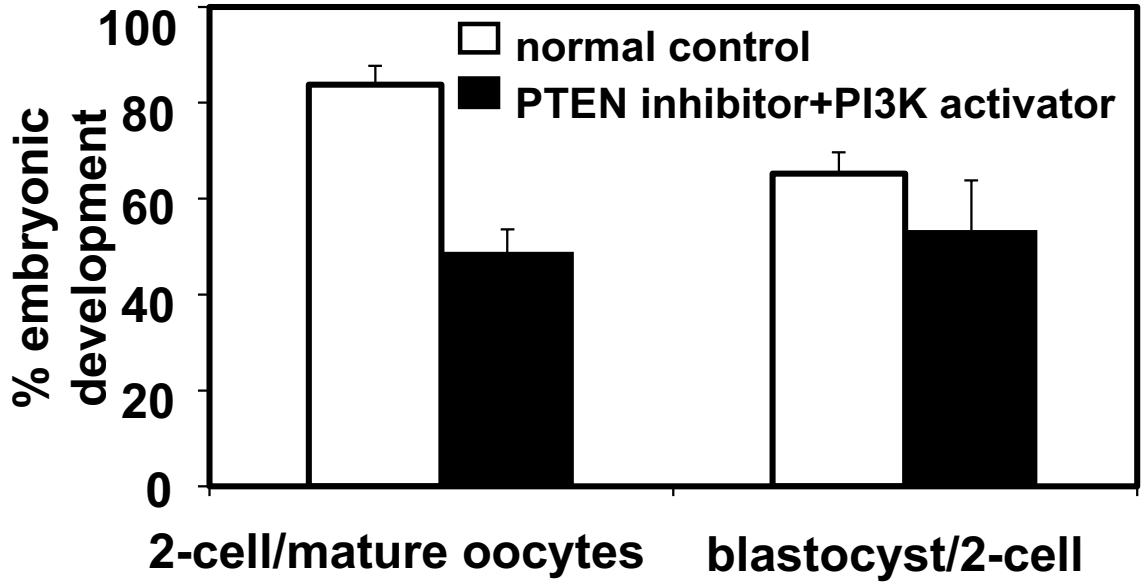
β-tubulin staining



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).

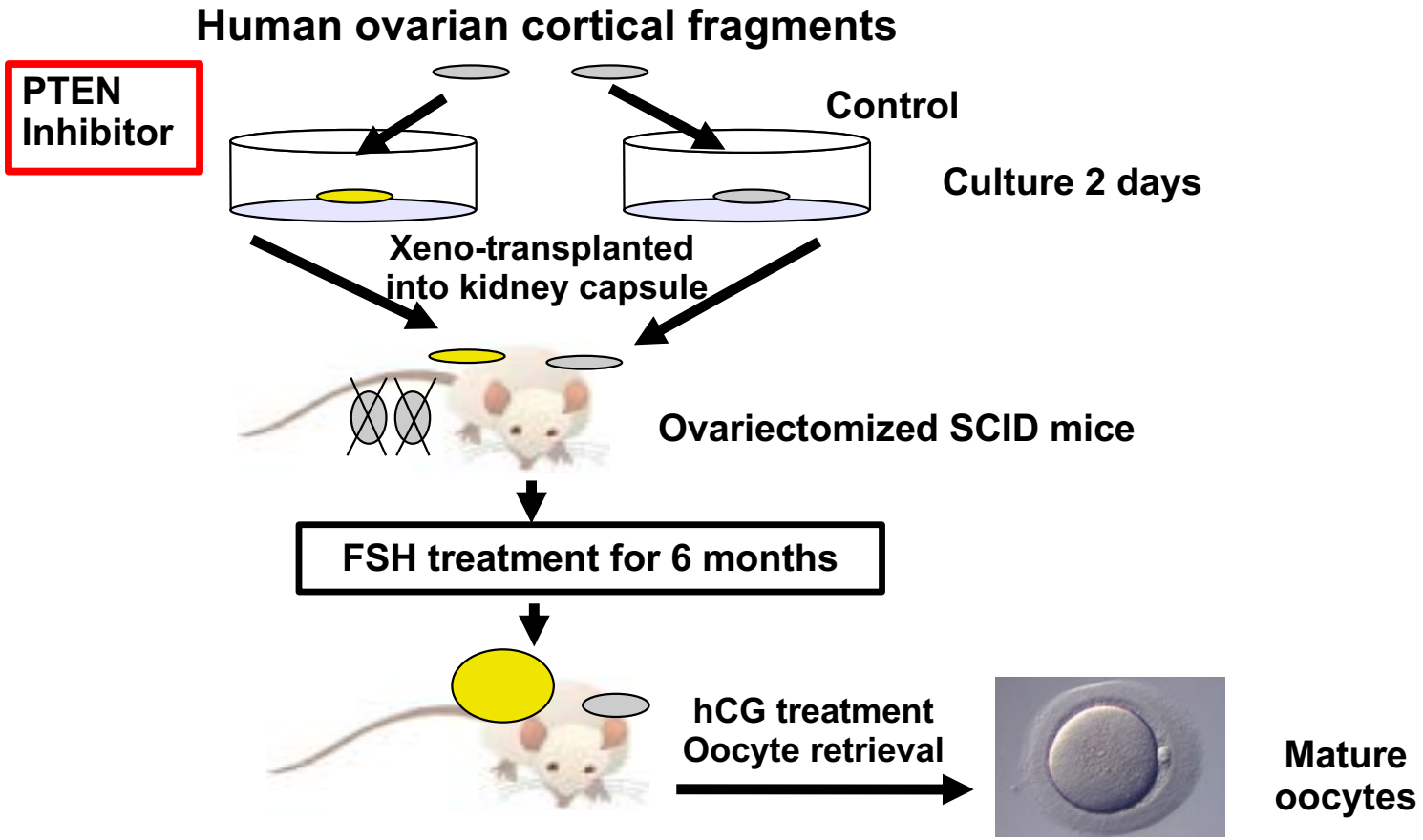
In vitro activation (IVA) - in vivo transplantation
-- early embryonic development of retrieved mature oocyte after IVF and healthy pups after embryo transfer



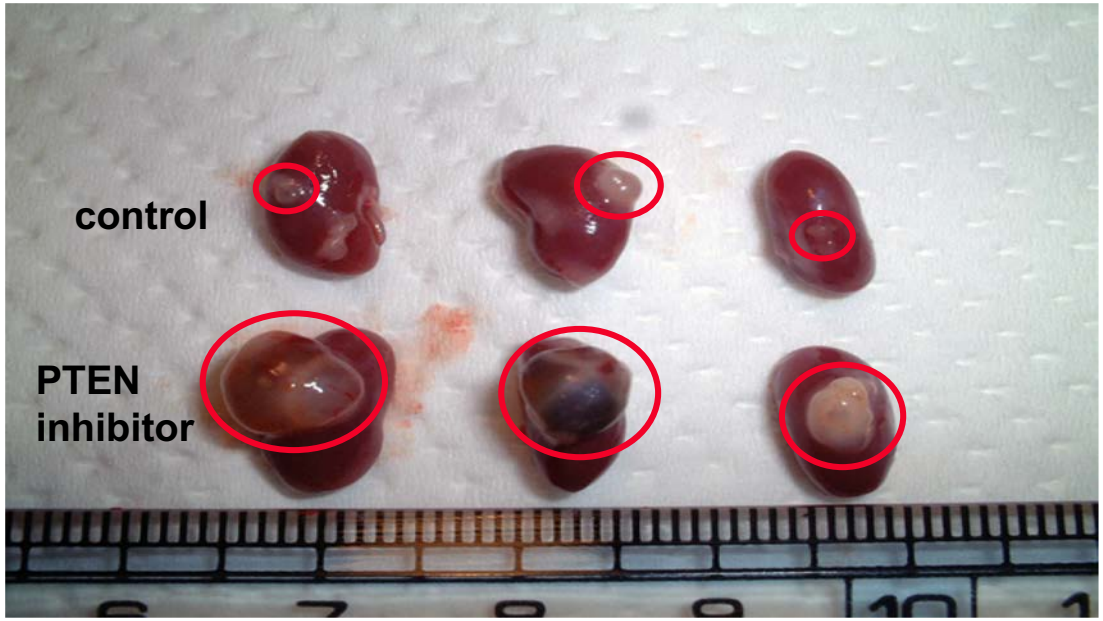


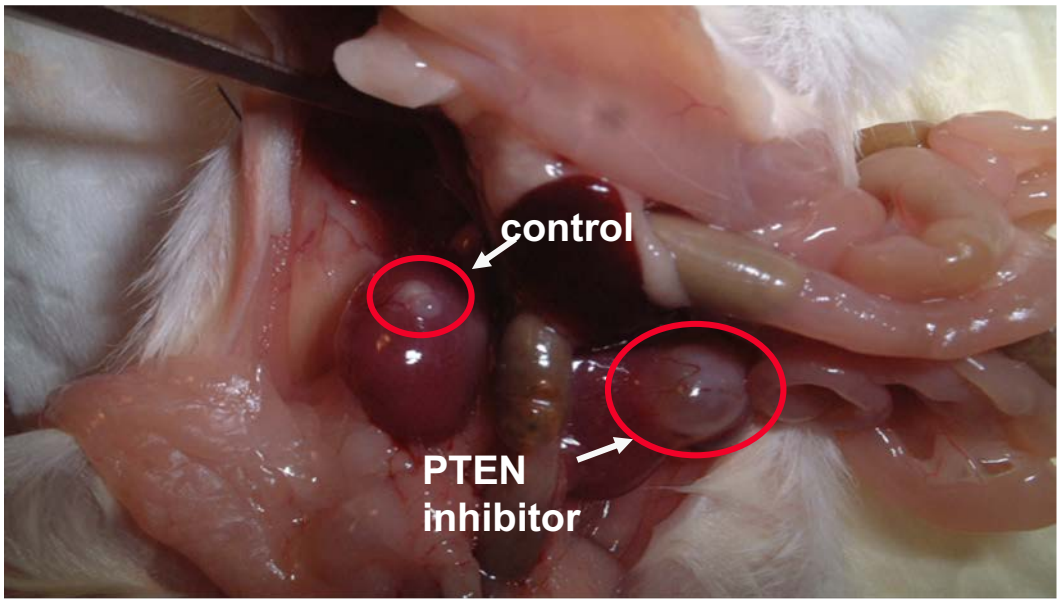
Xeno-transplantation of human ovarian fragments to activate dormant follicles: IVA, in vitro activation

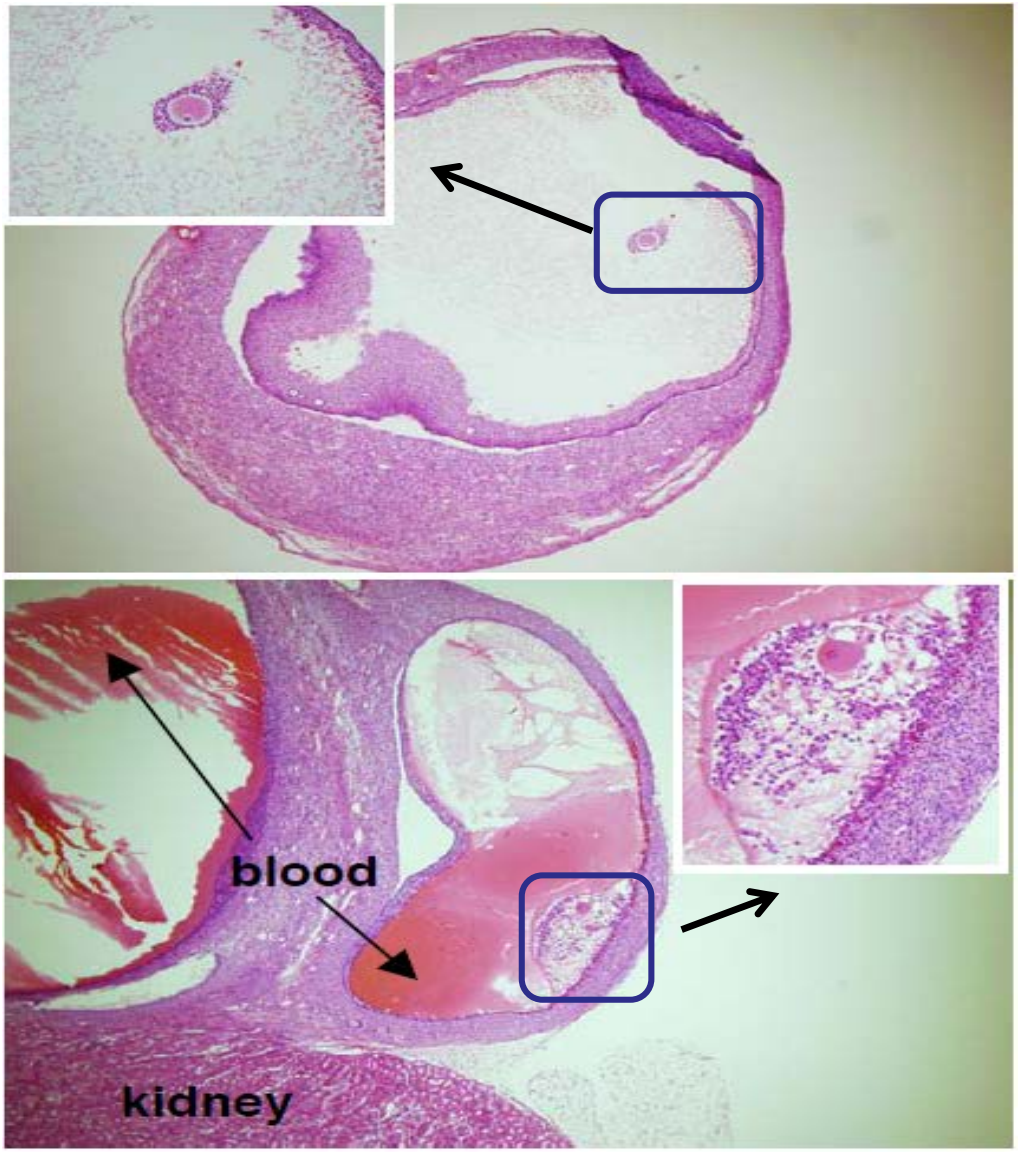
Ovarian cortical fragments were obtained from patients with benign ovarian tumor with informed consent from the patient and approval from local ethical human subject committee.



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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1. **Clinical application of IVA**

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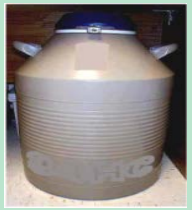
Clinical application of IVA for POI patients

Histological analyses

Cryo-preservation by vitrification



Fragmentation of ovarian strips to cubes

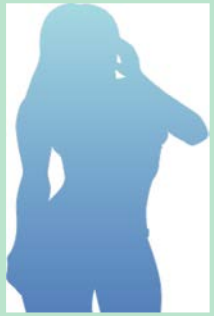
IVA: In Vitro Activation

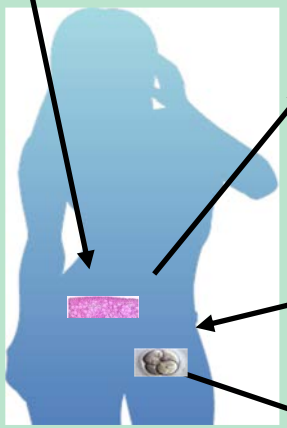
Culture of ovarian cubes with PI3K activators

Preparation of ovarian cortical strips for freezing

Ovariectomy under laparoscopic surgery

Auto-transplantation of activated ovarian cubes





Retrieve mature eggs

In vitro fertilization

Embryo cryo-preservation

Embryo transfer

Kawamura et al PNAS 2013

IRB approval:

Human Subject committee of St. Marianna University and Japan Society of Obstetrics and Gynecology

Enrolled patients

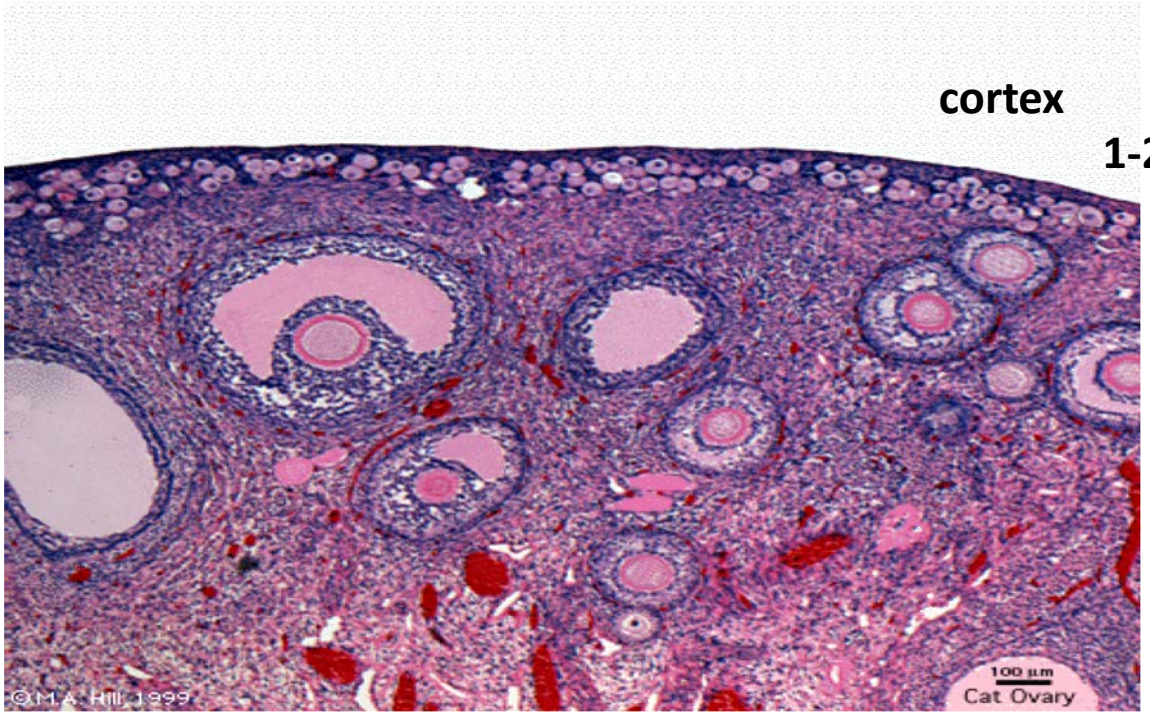
83 of POI patients (37.4 ± 4.9 years of age)

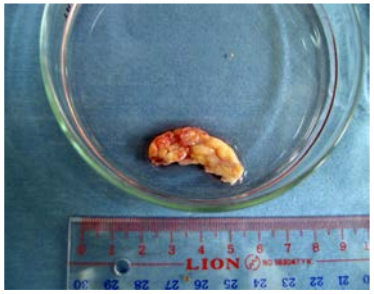
Duration of amenorrhea: 5.7 ± 3.5 years

- Ovariectomy under laparoscopic surgery
- Minimum usage of electrocautery hemostasis to avoid damage of residual follicles.

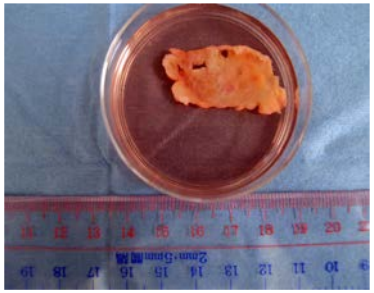
ovariectomy

Localization of early follicles in ovarian cortex

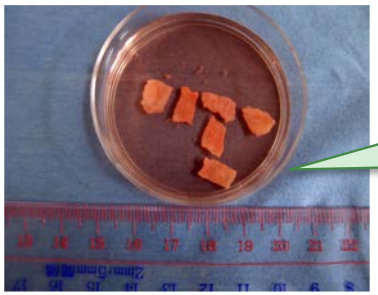




Before dissection of medulla

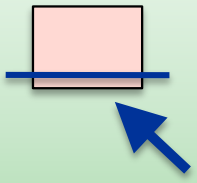


After dissection of medulla



Small ovarian stripes ready for use

- Dissect ovarian cortices containing residual follicles by removing medulla.
- Cut into small strips (1 x 1 cm^2, 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

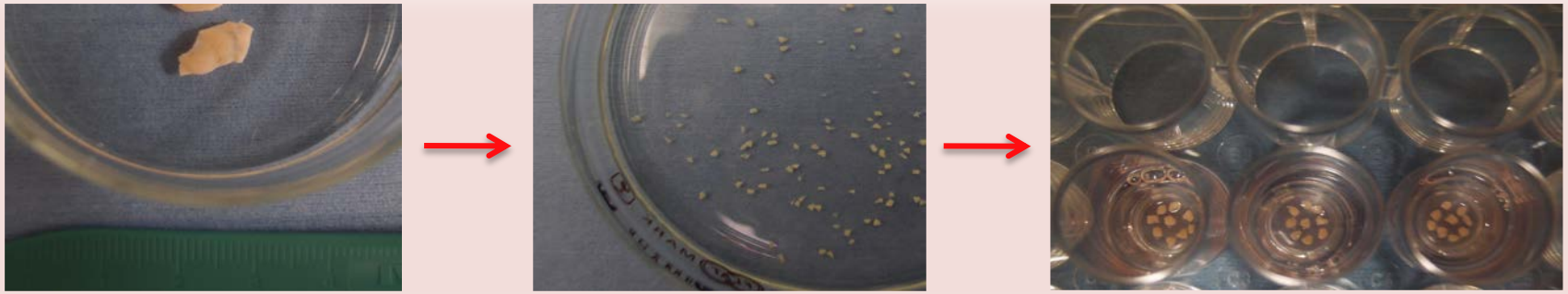
- Using 10% of volume of each ovarian stripe, detect residual follicles.

Fragmentation of ovarian strips to cubes

In Vitro Activation

Culture of ovarian cubes

- Fragment 2-3 ovarian pieces into 1-2 mm^2 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).

In Vitro Activation

Culture of ovarian cubes

Auto-transplantation of activated ovarian cubes

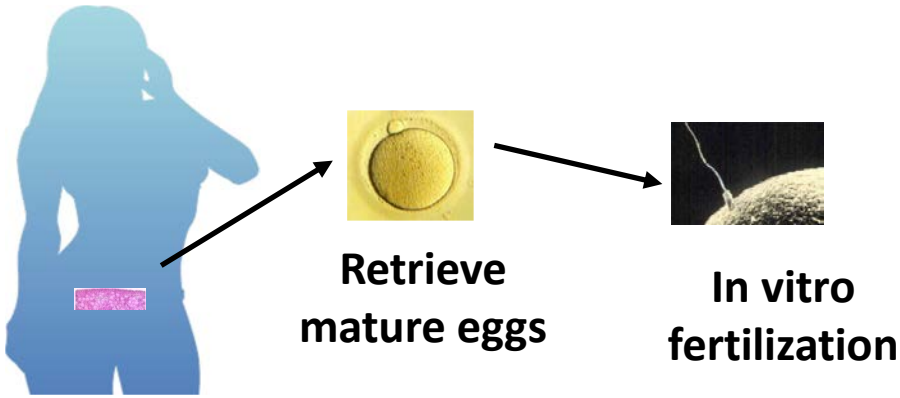
Beneath serosa of Fallopian tubes
— high vascularization,
convenience for trans-vaginal ultrasound monitoring
ease for oocyte retrieval

Patients' follow up protocols

- Monitor follicle growth weekly to biweekly: transvaginal ultrasound + serum estrogen and gonadotropin levels.

- After normalizing LH levels using EP pills and GnRHa, follicle growth was promoted by rFSH and hMG under GnRHa or GnRH AN protocols (Zhai, Kawamura, et al. JCEM 2016).

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



Tips for ovarian stimulation after IVA

In POI patients, due to absence of antral follicles before and immediately after IVA, the first sign of follicle growth is elevation of estrogen (E2) levels.

Without ovarian stimulation, follicles can grow spontaneously followed by decline in FSH and LH levels based on negative feed back of E2.
However, in most of cases, the decrease in LH levels is inadequate….

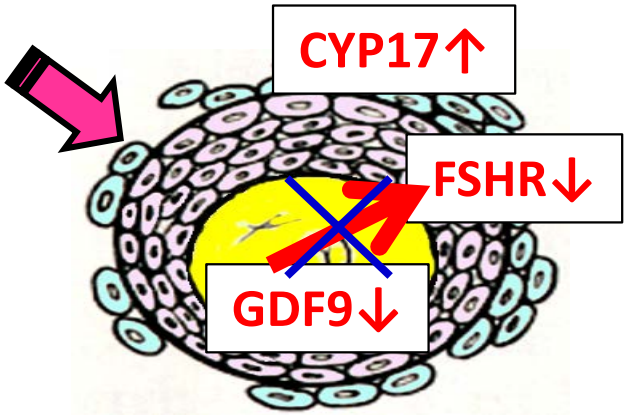
Early luteinization
Arrest of follicle growth
Oocyte degeneration

Effects of hyper-LH on oocyte-granulosa-theca cell interactions

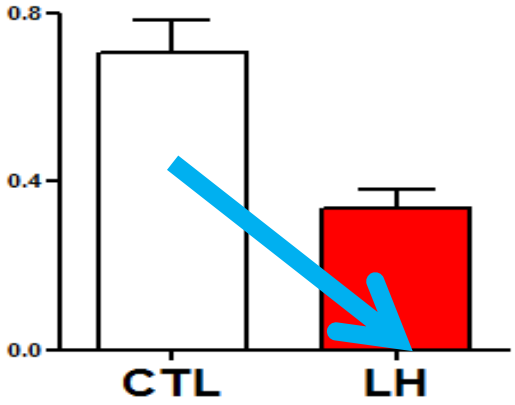
Preculture with LH

FSH stimulation

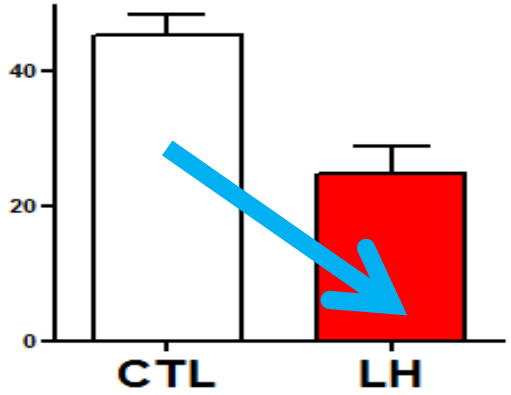
Chronic LH stimulation



cAMP production after FSH stimulation



Follicle growth after FSH stimulation



Preantral follicles exposed to high LH express low levels GDF-9 in oocyte and FSHR in granulosa cells, resulting in decreases in sensitivity of FSH stimulation and suppression of follicle growth.

Orisaka 2013 *Endocrinology*

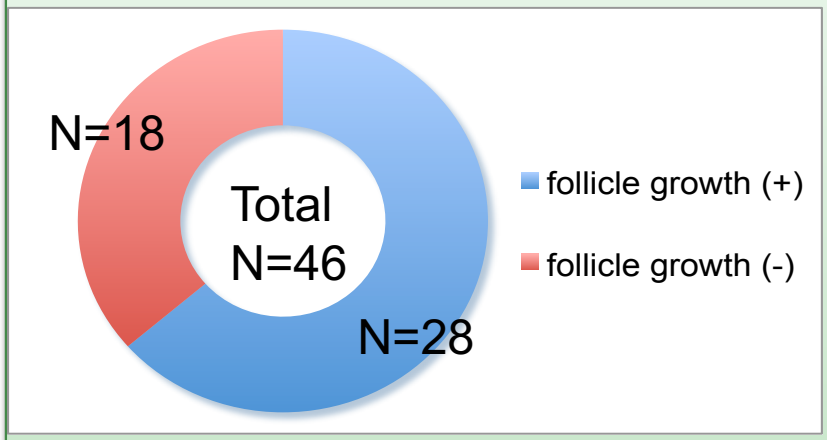
How can we stimulate ovaries after IVA?

1. Normalize LH levels by supplementation of estrogen and estrogen + progesterone with induction of withdrawal bleeding.

2. After confirmation of normal LH levels (<10 mIU/ml), maintain its low levels using GnRHa. (Daily GnRH AN injection is too expensive)

3. Similar to short protocol, treat patients with rFSH or pure HMG (low LH content) for >2 weeks.

Results



- Among 83 patients, ovary grafting was performed in 46 patients and follicle growth was found in 28 out 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

THE TIMES

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Japanese baby raises hopes for post-menopause births

The menopause occurs before the age of 40 in about 1 per cent of women Getty

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
—a female baby, 2970g

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Grafted ovaries lead to successful pregnancy

IMAGE COURTESY OF KAZUHIRO KAWAMURA

Current issue

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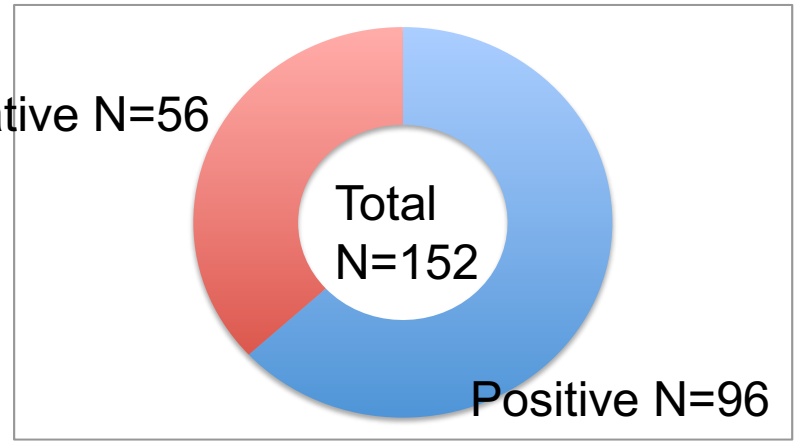
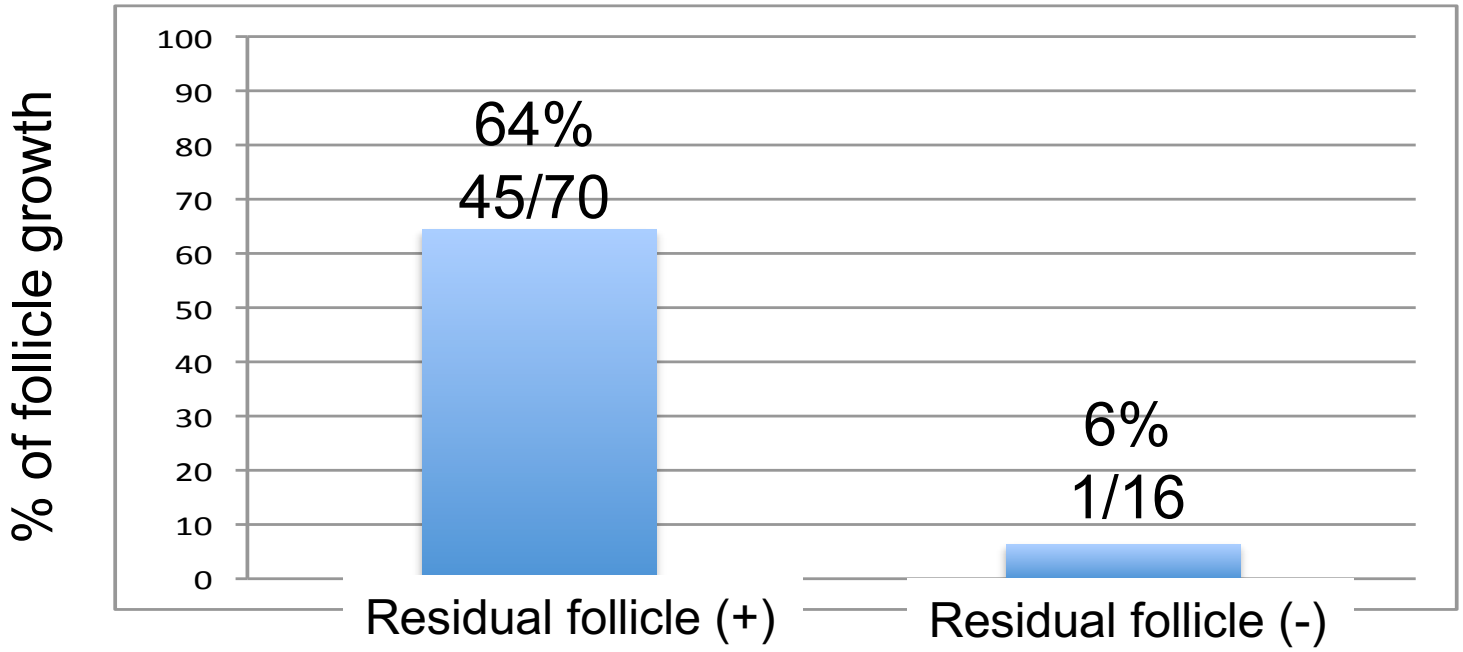
Current clinical outcome of IVA

- Ovariectomy: n=152
- Residual follicles based on histology:
  Positive: n=96
  Negative: n=56



- IVA grafting with residual follicles with follow up
  > 6 months: n=70
- IVA grafting without residual follicles with follow up
  > 6 months: n=16



Histological analyses

Our histological analyses were effective to predict IVA outcome

Indication for IVA treatment

- POI/DOR patients with residual follicles.

(Young POI/DOR patients without oocyte aging).

Results

Reproducibility of IVA was already confirmed by China, Spain, Poland groups under our guidance.

Kawamura et al Hum Reprod 2015
Zhai et al JCEM 2016

IVA pregnancy/delivery

N=2

N=3

N=2

IVA in Poland: December 2016

IVA press conference in China: May 2015

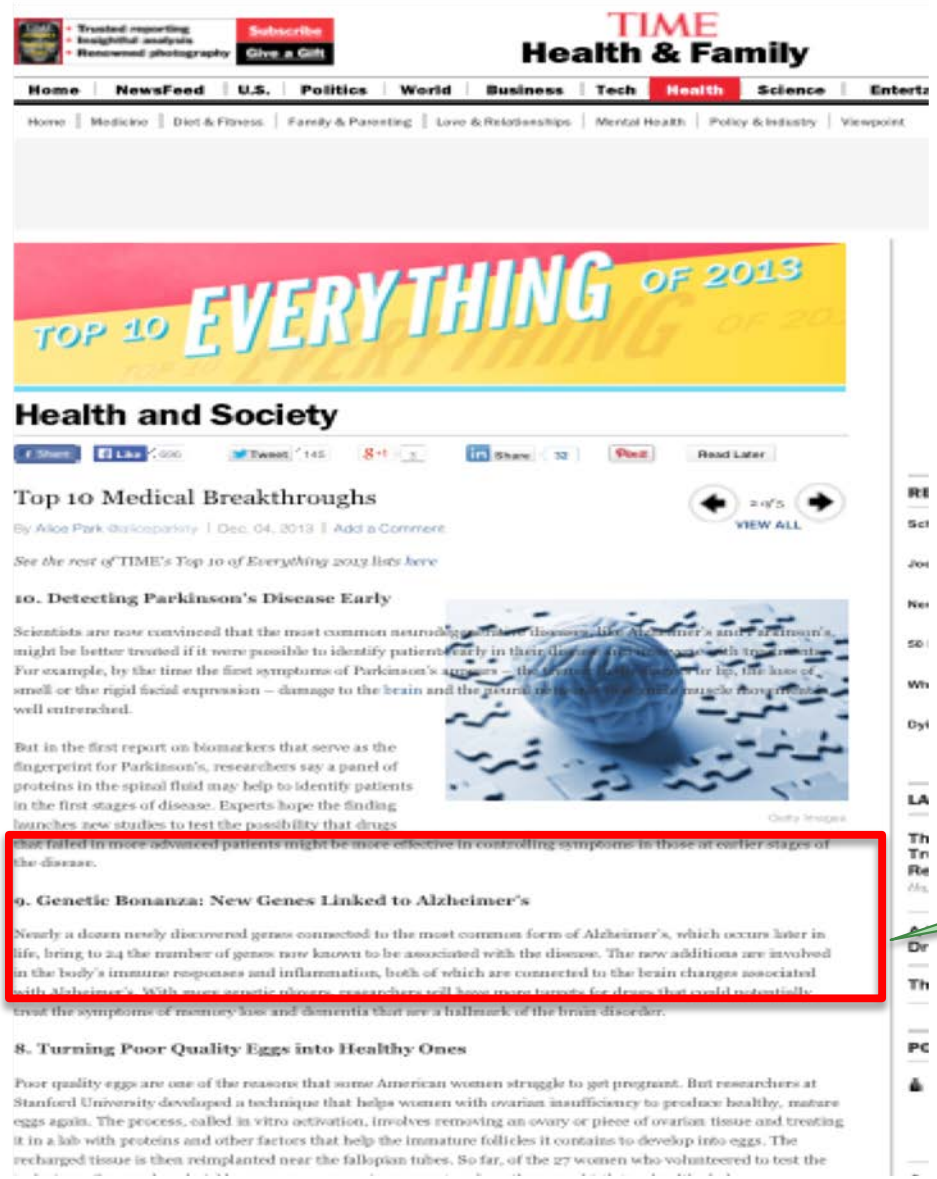
International patent :

STIMULATION OF OVARIAN FOLLICLE DEVELOPMENT AND OOCYTE MATURATION

PCT/US2013/059800 Out-licensing to Ovascience. Inc

Kawamura et al. PNAS 2013
Hsueh, Kawamura et al. Endocrine Rev 2014
Suzuki, Kawamura et al. Hum Reprod 2015
Yuan, Kawamura et al FASEB J 2015
Kawamura et al. Hum Reprod 2016
Kawamura and Hsueh Curr Opin Obstet Gynecol 2016
Zhai, Kawamura et al. JCEM, 2016
Kawamura et al. Reproduction, 2017
Haino, Kawamura et al. JAYAO 2017
Sato, Kawamura et al. J Gynecol Women's Health 2017
Kawamura et al. Syst Biol Reprod Med 2017

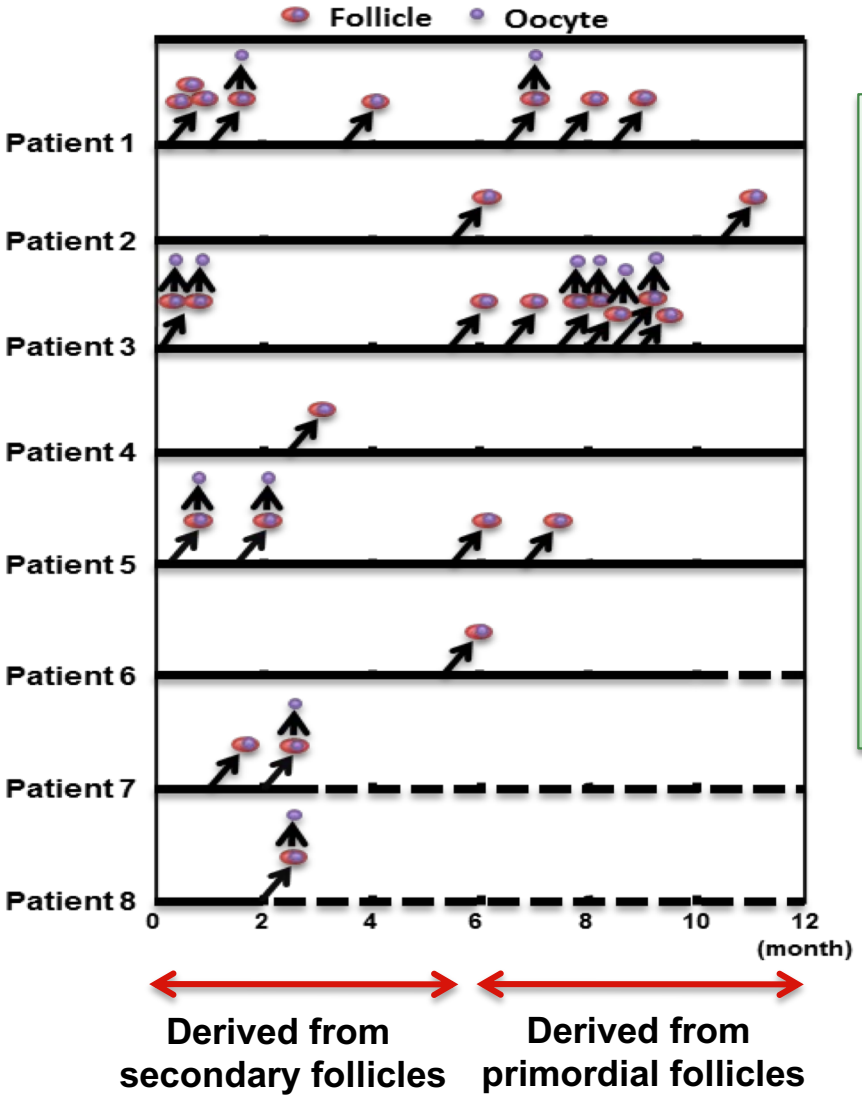
Top 10 Medical Breakthroughs | Top 10 Everything of 2013 – Health and Society | TIME.com



MARTIN SCHOELLER FOR TIME

IVA was awarded to be one of the Top 10 medical breakthrough in 2013 by TIME magazine.

Temporal follicle growth in transplanted ovaries

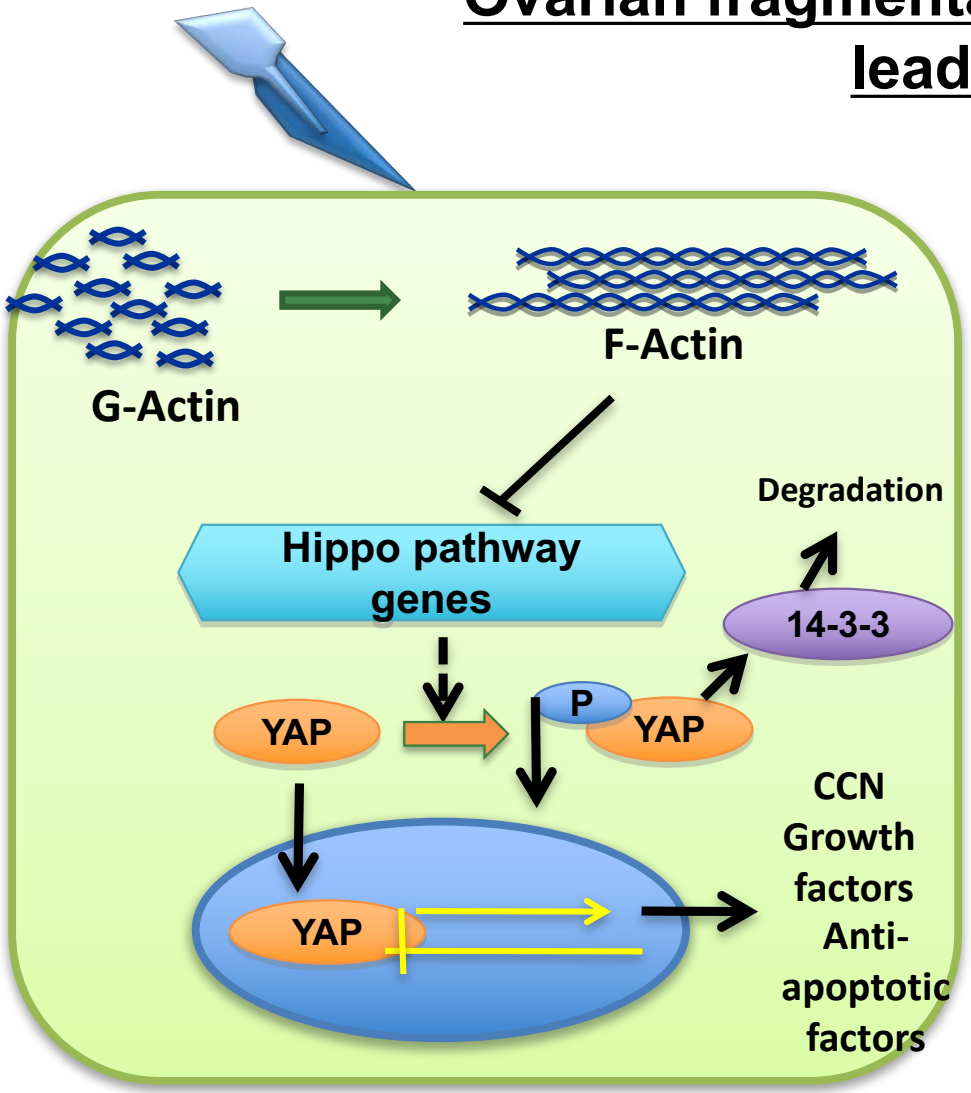


Follicle growth from primordial to preovulatory stage takes more than 4-6 months.

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 suppresses 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 in downstream CCN growth factors.

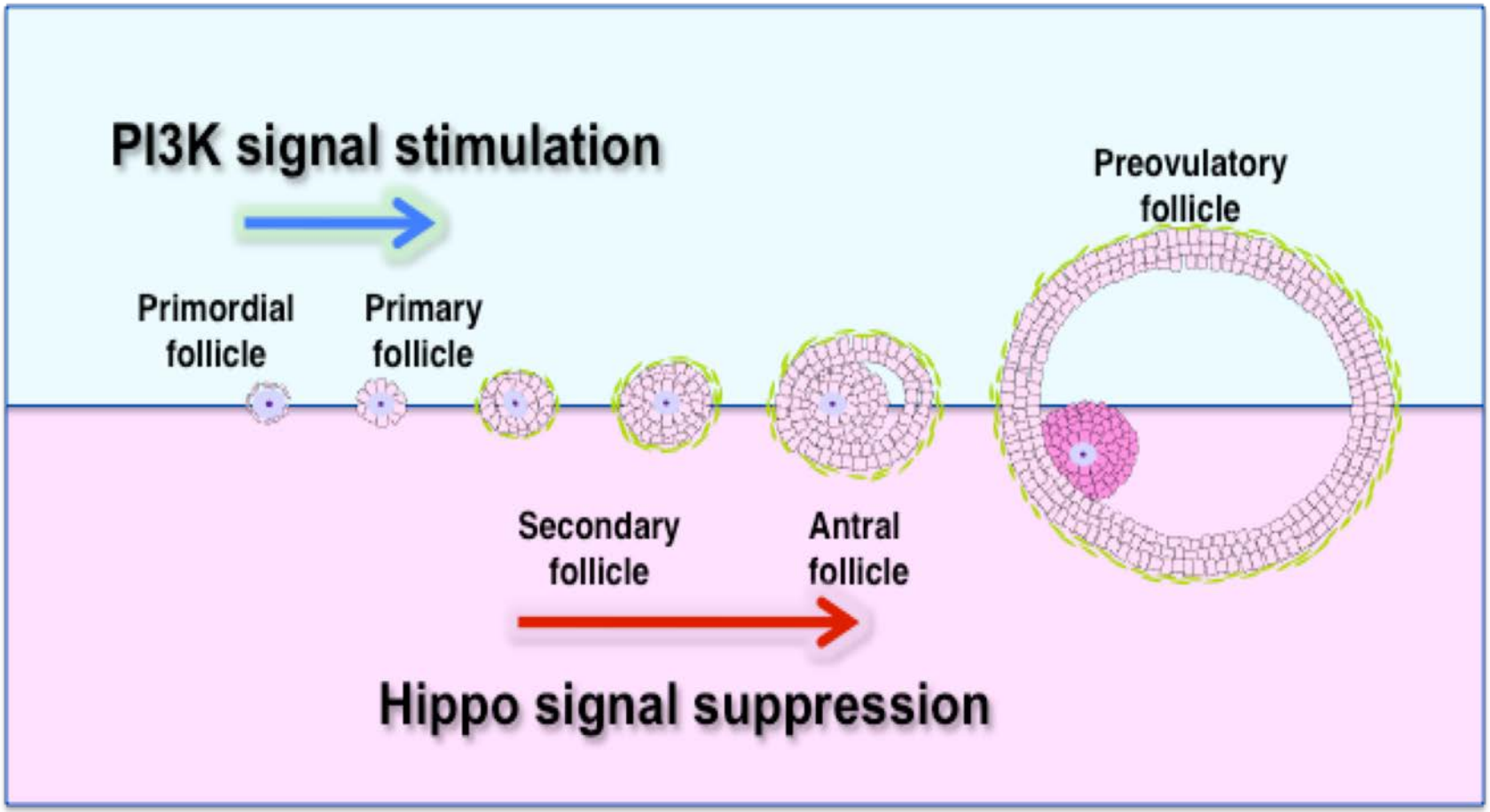
Secretion of CCN growth factors stimulated follicle growth.

Secondary follicle growth

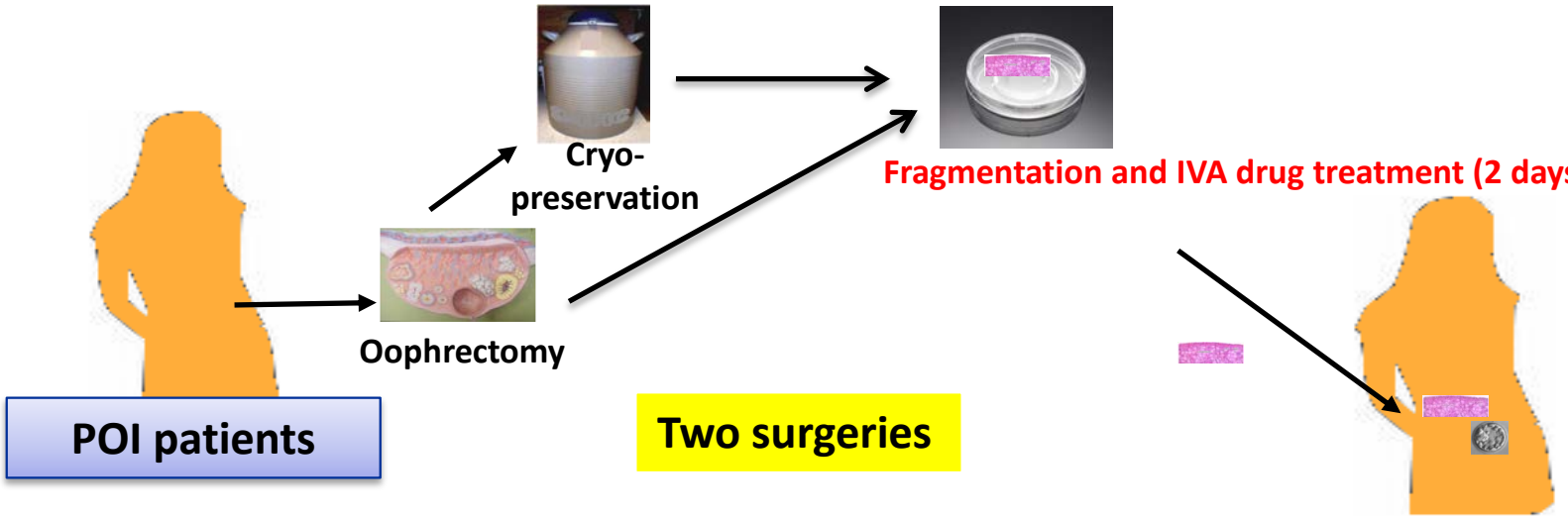
Contents

1. Background of POI
1. Basic and translational studies for in vitro activation of dormant follicles (IVA)
1. Clinical application of IVA
1. **Future studies for IVA**

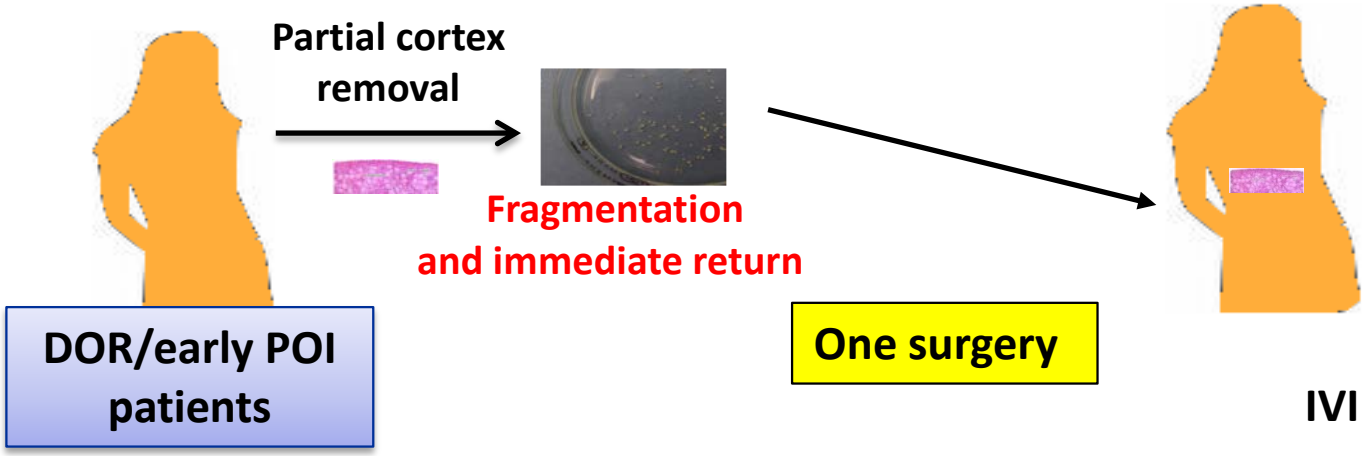
Two-step follicle stimulation in IVA



1. Original IVA (PI3K stimulation and Hippo disruption)



2. Drug-free IVA (Hippo disruption only)



IVI workshop, Bilbao, 2017

Drug-free IVA and orthologous grafting

Limitation:

Only applicable to DOR/early POI patients who likely have secondary follicles

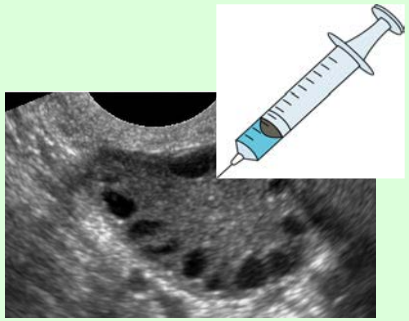
Advantages:

- Minimal damages to ovarian blood supply
- No need to use Akt-stimulating drugs
- Avoid potential follicle loss during culture
- One laparoscopic surgery
- Spontaneous pregnancy possible

IVI group, Spain (Bilbao workshop 2017)
3 spontaneous pregnancies/14 patients

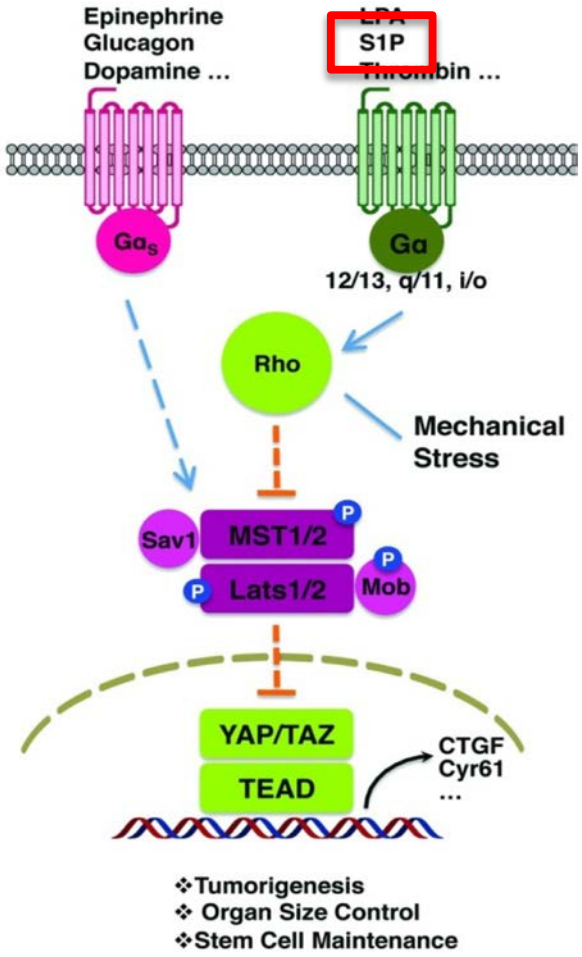
Surgery-free Hippo signal disruption

Develop less invasive approach:
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.

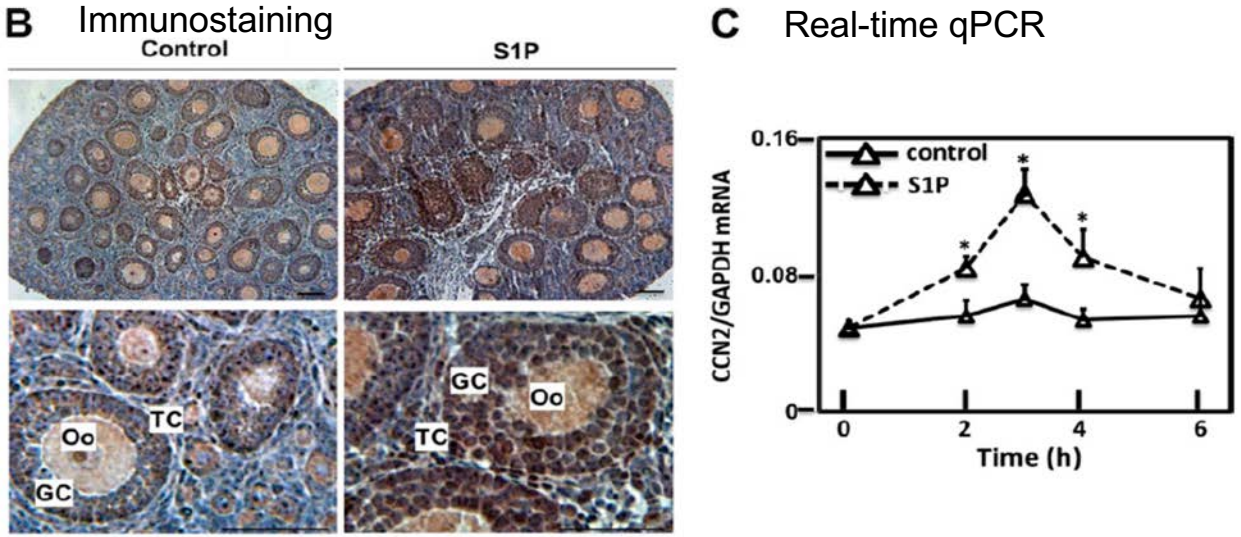
Candidate molecule: Sphingosine 1-phosphate (S1P)



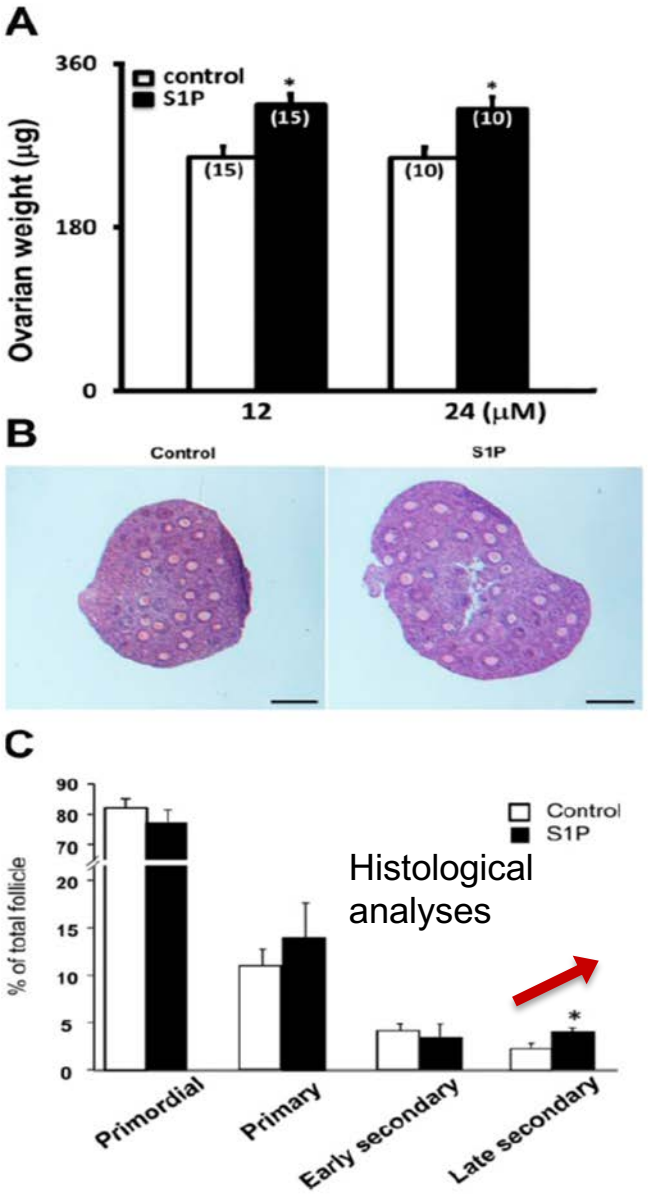
S1P is a bioactive sphingolipid, acting on GPCR (G12/13-coupled receptors) to suppress Hippo signaling.

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



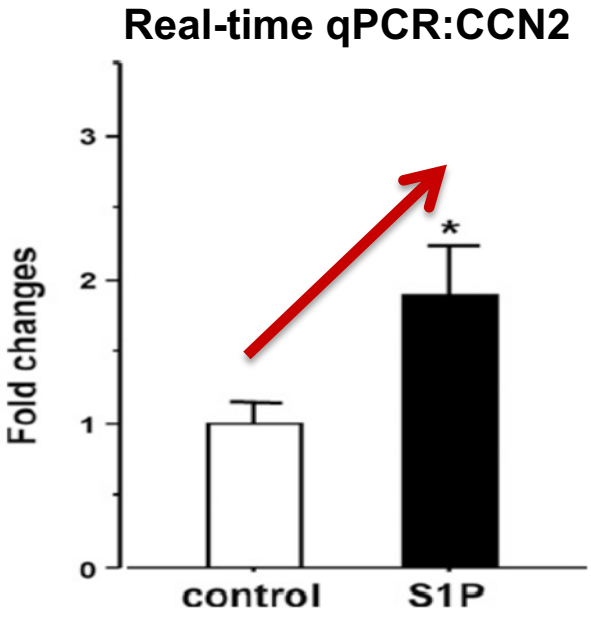
S1P stimulates nuclear translocation of YAP in granulosa cells followed by increase in expression of downstream CCN2 growth factor.



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.

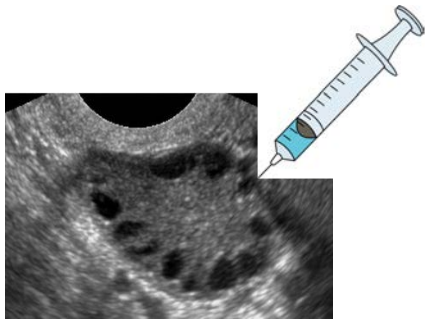
Human ovarian cortex was obtained from patients with ovarian tumor with IRB approval and informed consent.

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

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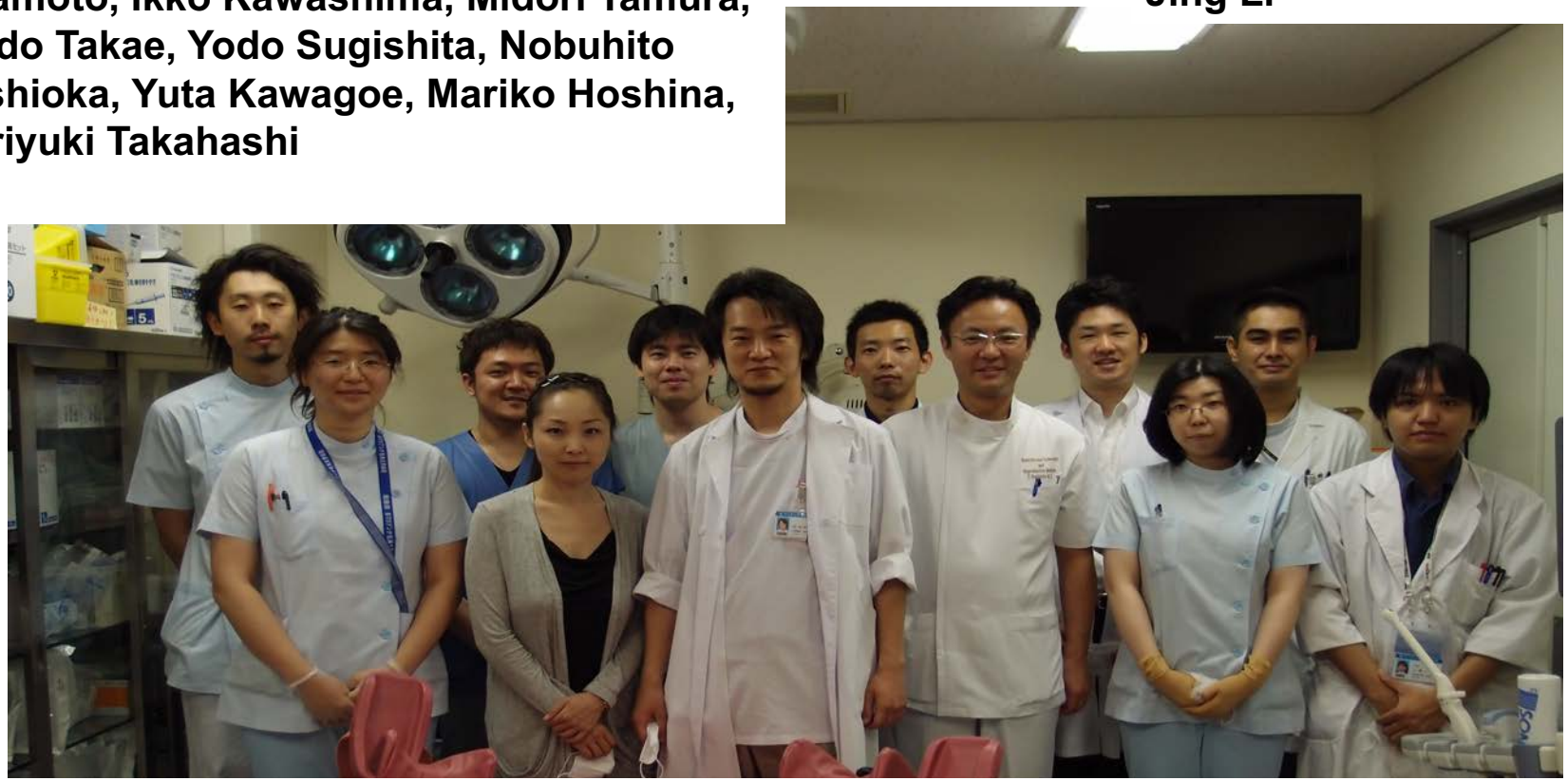

Questions

For questions, comments, and collaborations, please feel free contact me via email.

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Thank you for your kind attention.