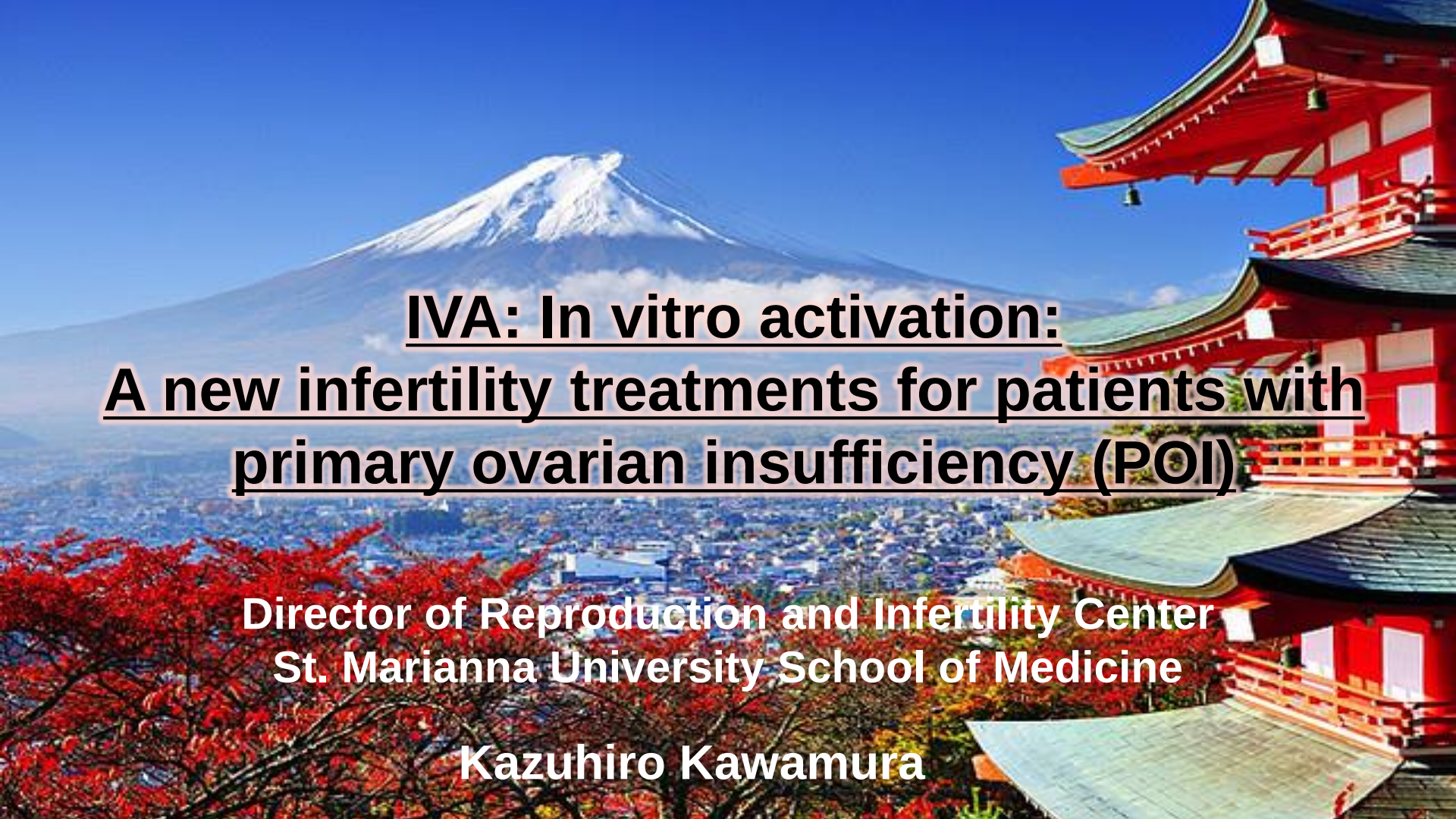

IVA: In vitro activation:
A new infertility treatments for patients with primary ovarian insufficiency (POI)

Director of Reproduction and Infertility Center
St. Marianna University School of Medicine

Kazuhiro Kawamura

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

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

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.

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…**

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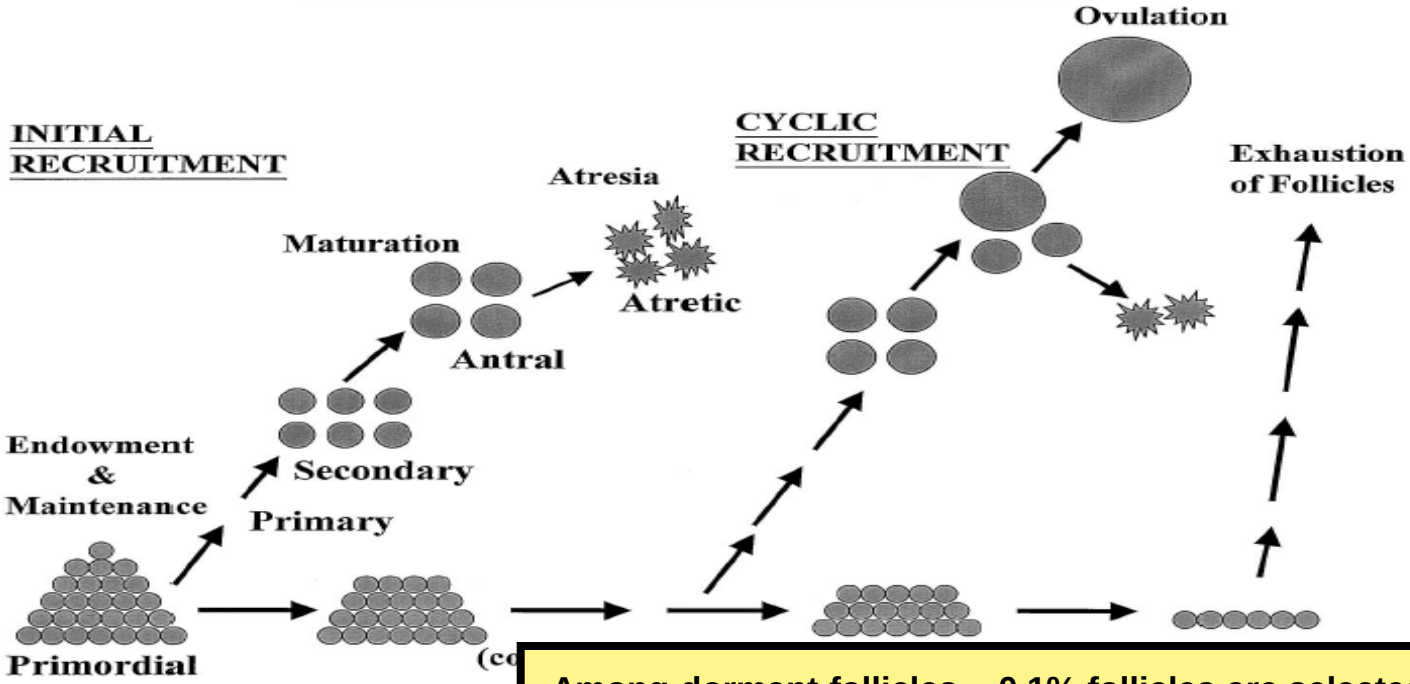
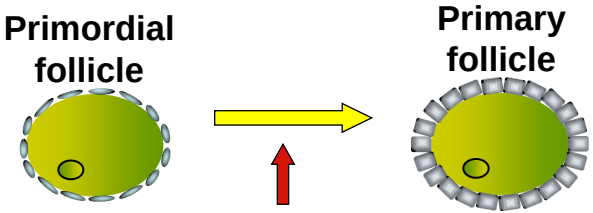
Life History of Ovarian Follicles

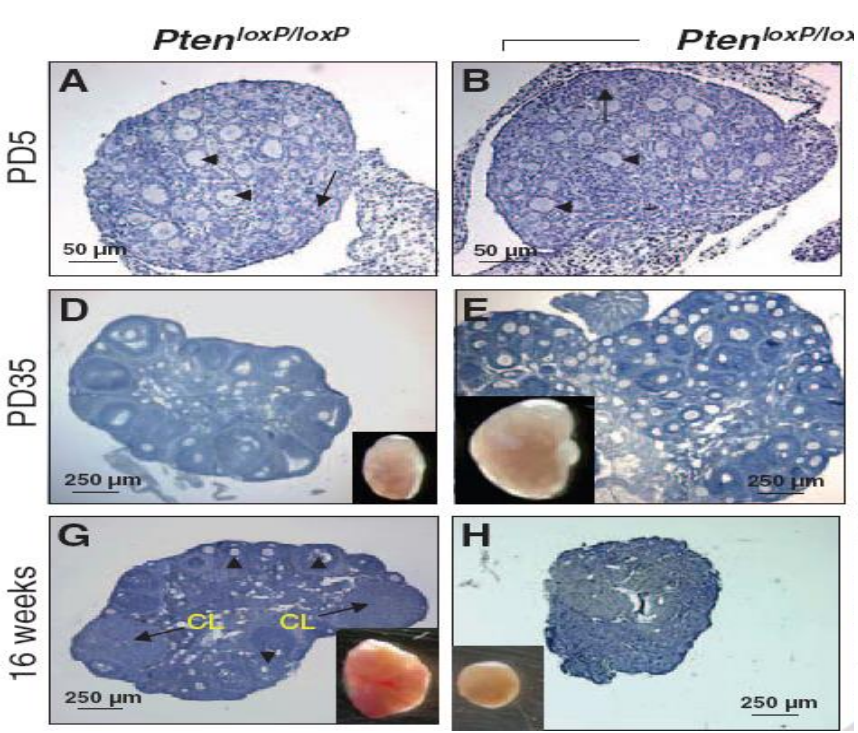




Among dormant follicles, ~0.1% follicles are selected to activate. Because POI has < 1,000 residual follicles, these growth factors can activate only 1 follicle.

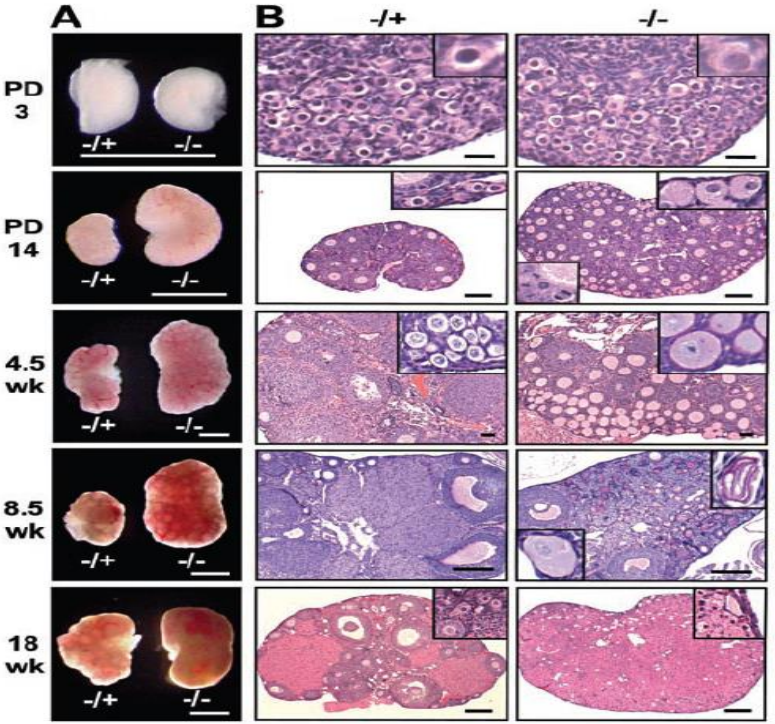
We focused on intracellular signaling system Involving in the activation.

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



Reddy et al. Science, 2008

PTEN null mice

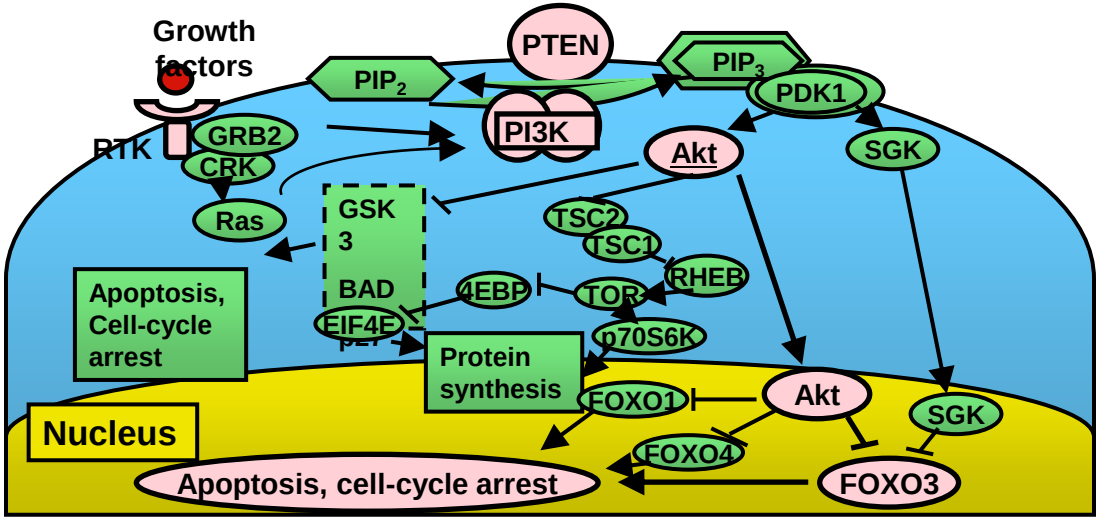


Castrillon et al. Science, 2003

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.

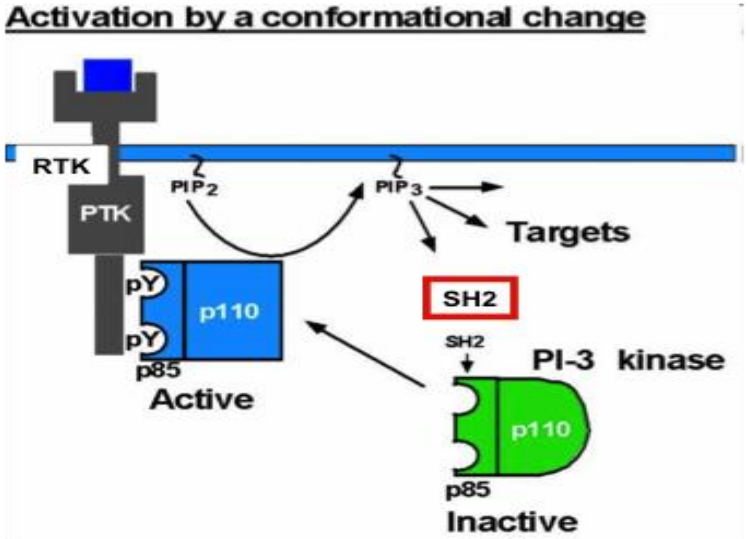
Is it possible to activate residual dormant follicles in POI patients artificially by transient PTEN suppression and/or PI3K activation using drugs?

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

Pups

IVF-ET

Adult ovariectomized

FSH treatment

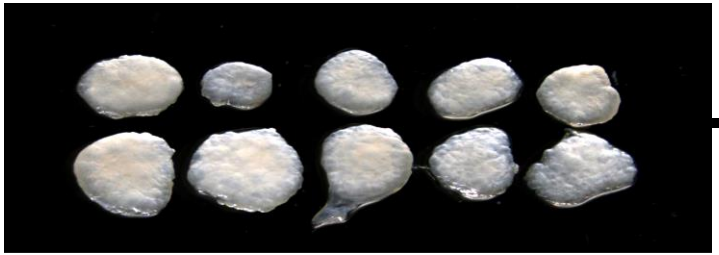
Histological analyses

Epigenetic analyses

Mature oocytes

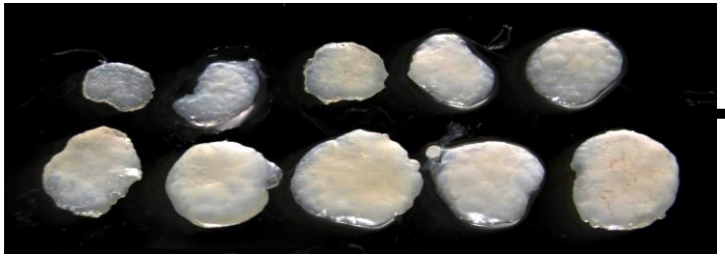
hCG treatment
Oocyte retrieval

In vitro activation (IVA) - in vivo transplantation



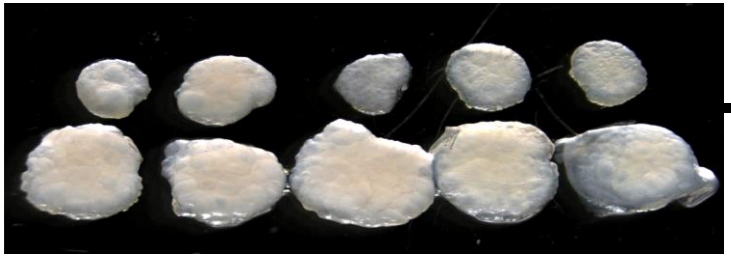
control

PTEN inhibitor



control

PI3K activator

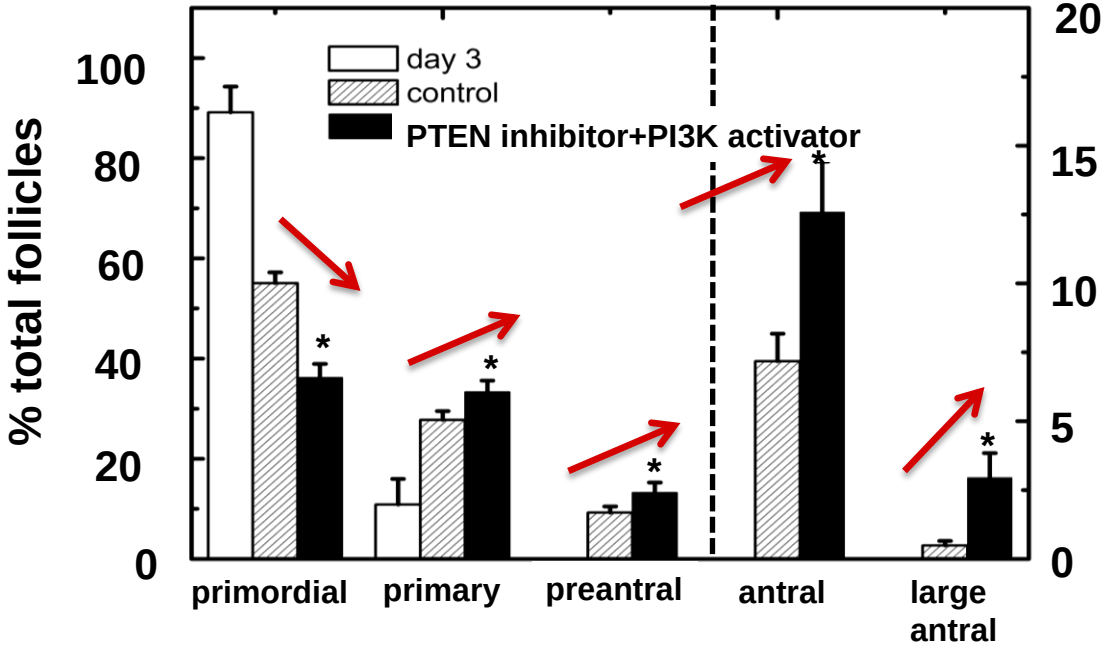


control

PTEN inhibitor
+
PI3K activator

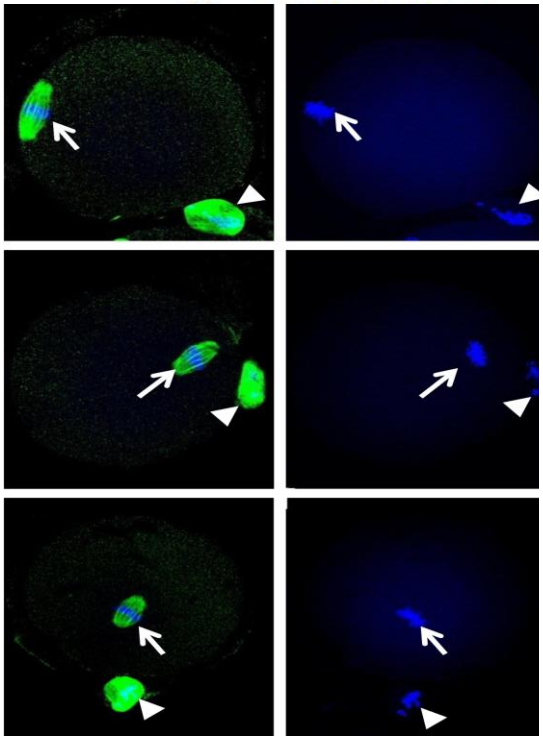
Changes in ovarian size at day 14 after transplantaion of D3 ovaries treated with PTEN inhibitor and/or PI3K activator beneath kidney capsule of host mice.

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

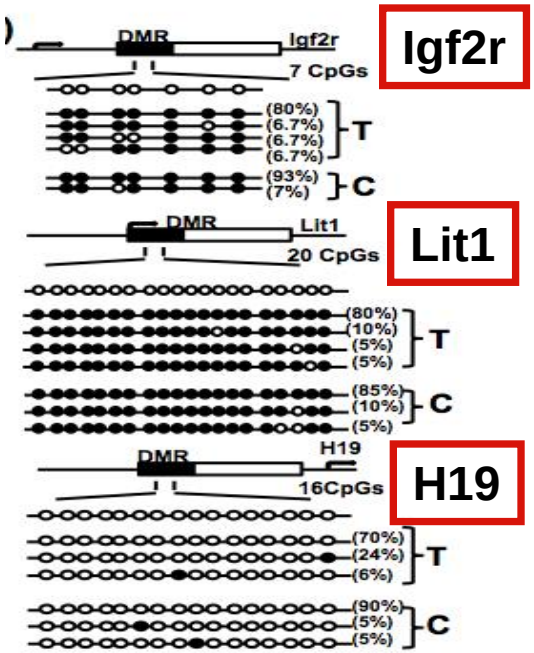


Follicular dynamics at day 14 after transplantaion of activated ovaries beneath kidney capsule of host mice.

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

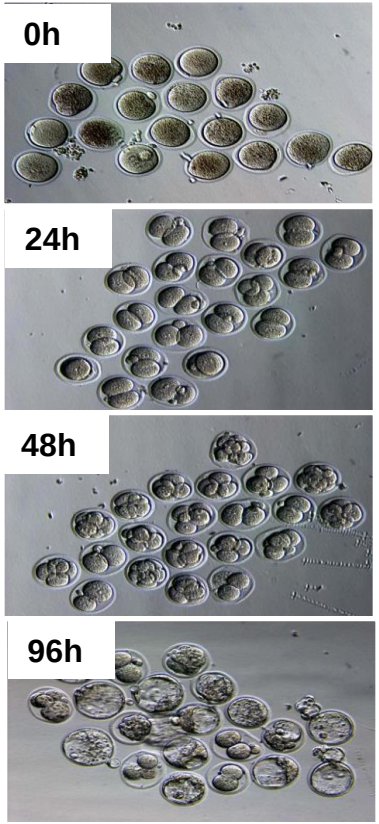


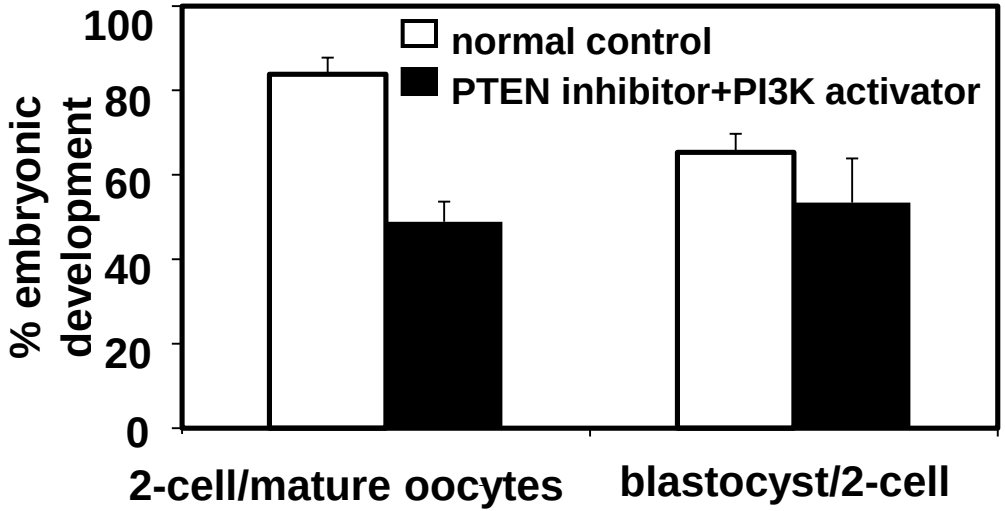
β-tubulin staining



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).

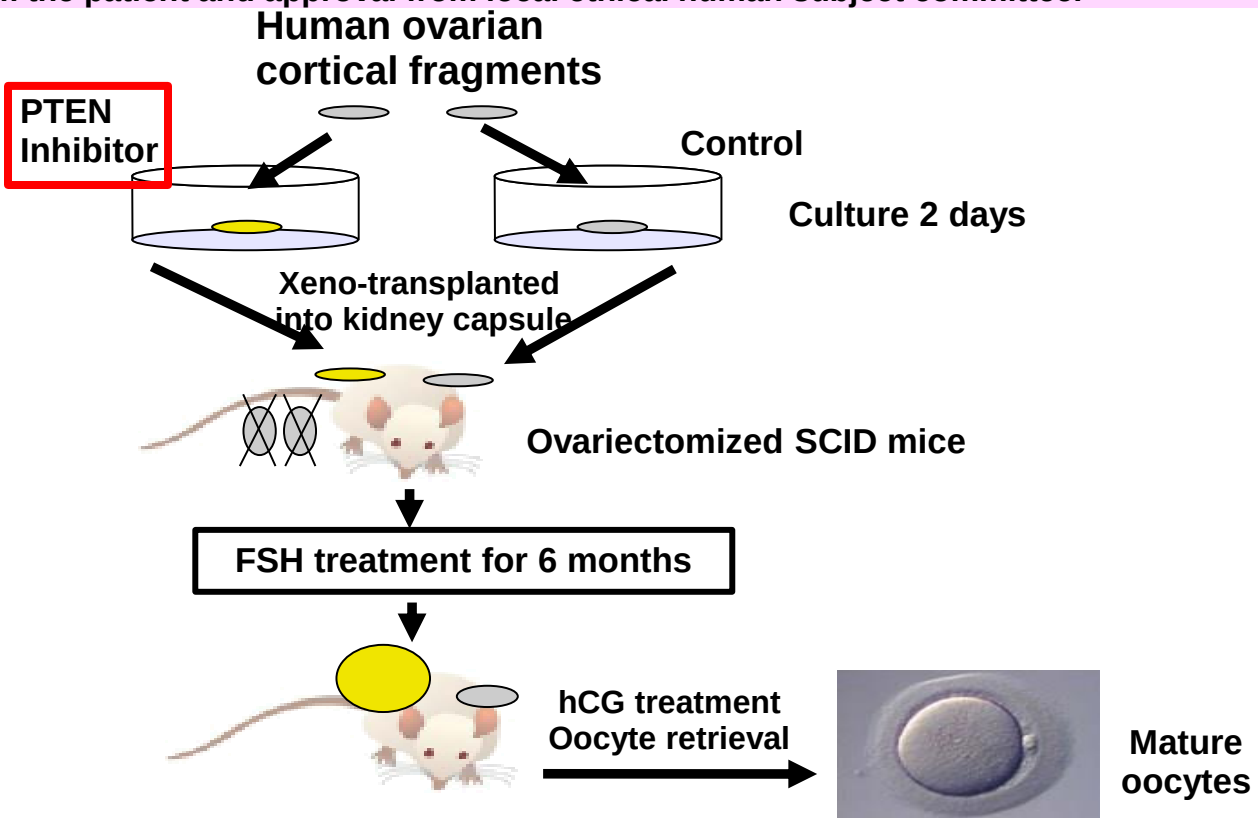
In vitro activation (IVA) - in vivo transplantation
-- early embryonic development of retrieved mature oocyte after IVF and healthy pups after embryo transfer



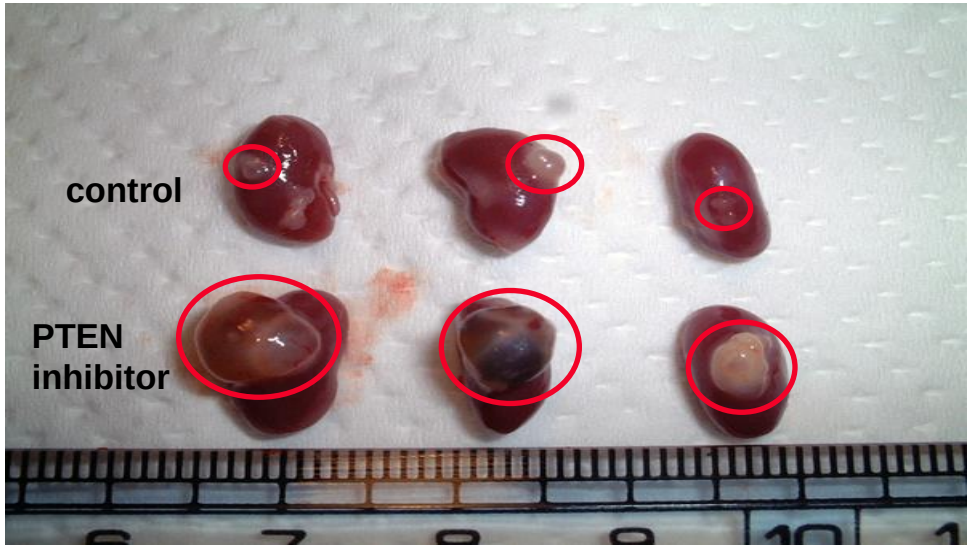


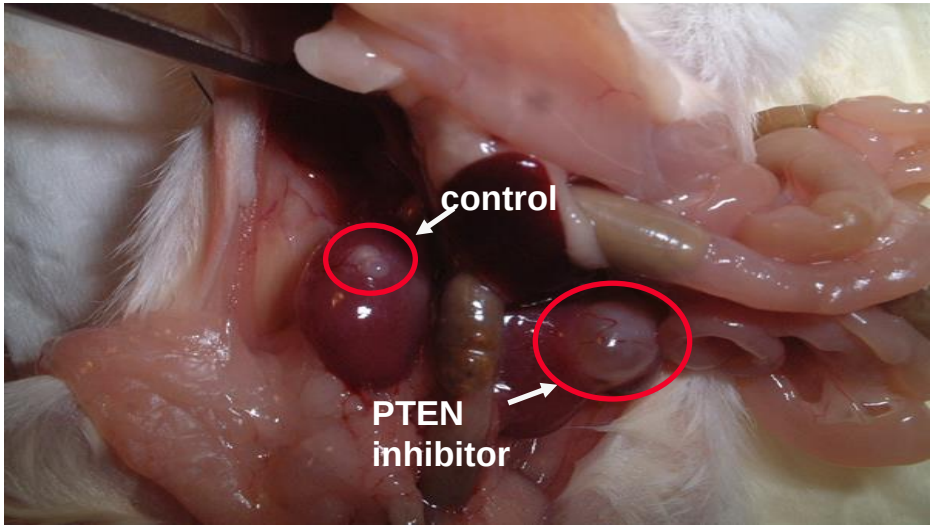
Xeno-transplantation of human ovarian fragments to activate dormant follicles: IVA, in vitro activation

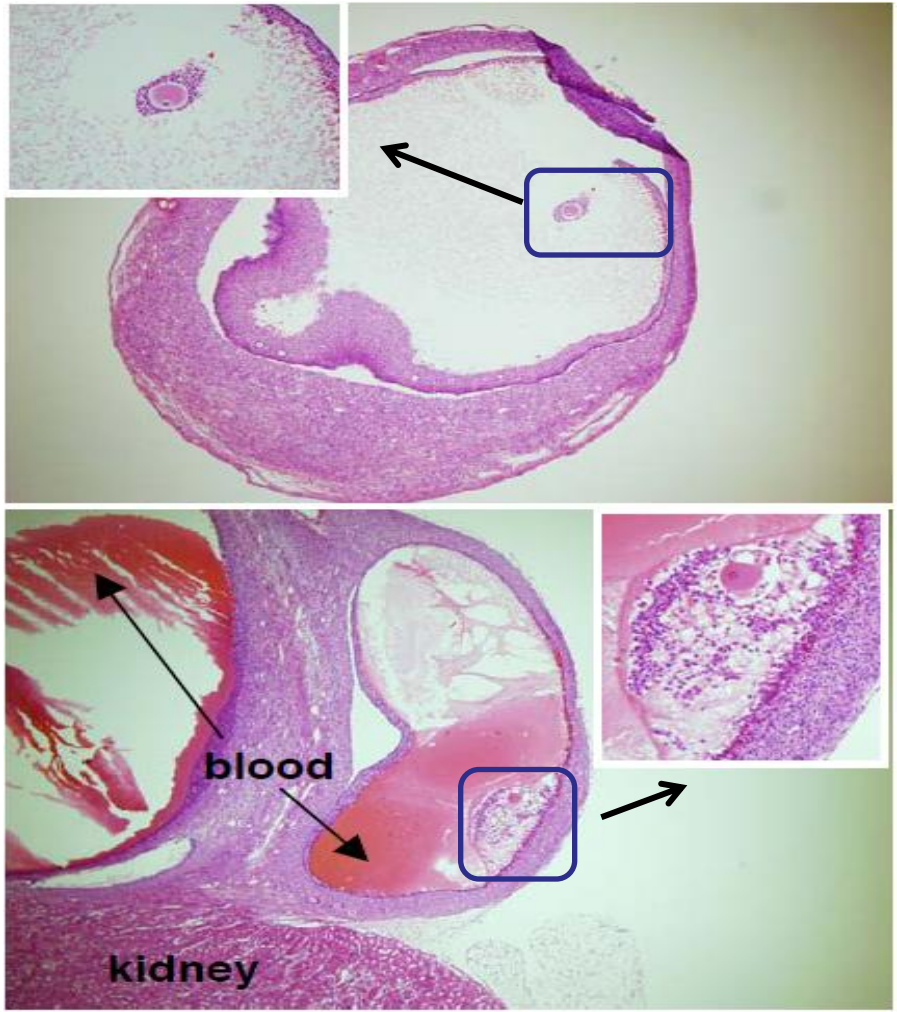
Ovarian cortical fragments were obtained from patients with benign ovarian tumor with informed consent from the patient and approval from local ethical human subject committee.



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**

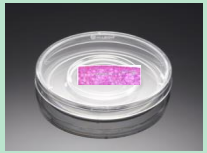
1. Future studies for IVA

Clinical application of IVA for POI patients

Histological analyses

Cryo-preservation by vitrification

Fragmentation of ovarian strips to cubes



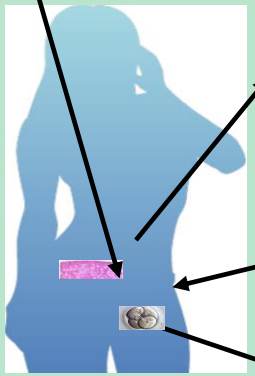
IVA: In Vitro Activation

Preparation of ovarian cortical strips for freezing

Culture of ovarian cubes with PI3K activators

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

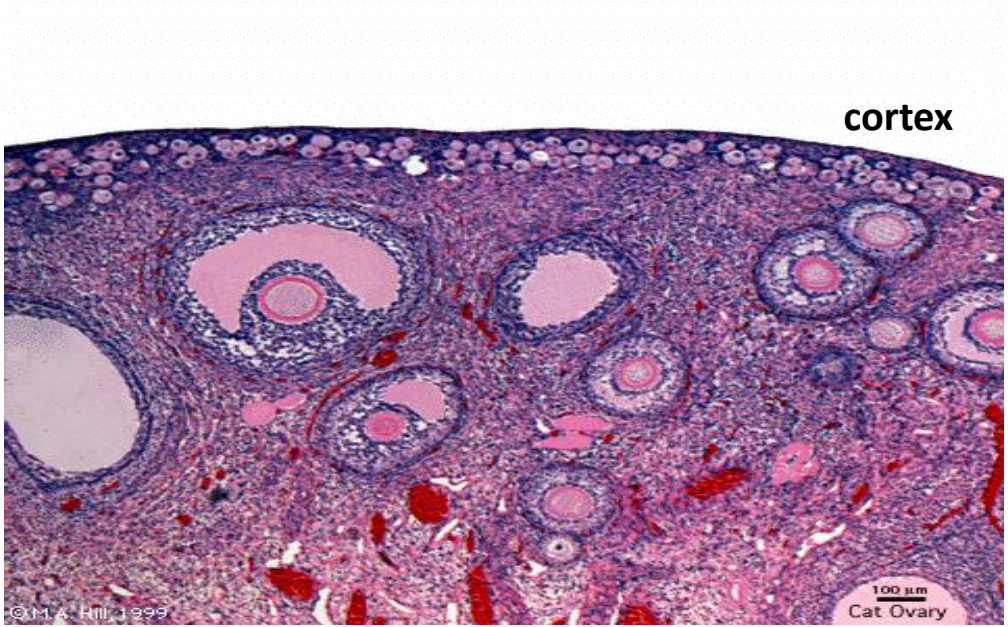
83 of POI patients (37.4 ± 4.9 years of age)

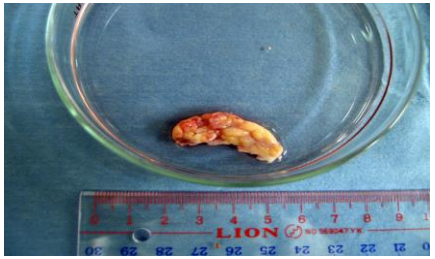
Duration of amenorrhea: 5.7 ± 3.5 years

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

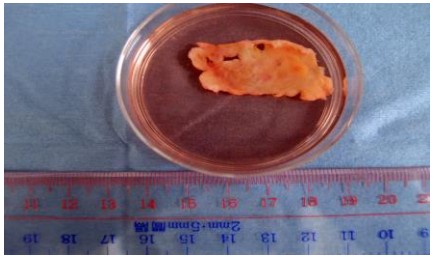
ovariectomy

Localization of early follicles in ovarian cortex

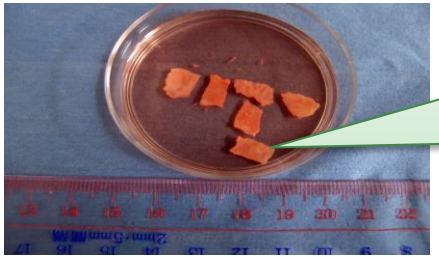




Before dissection of medulla

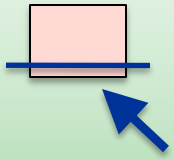


After dissection of medulla



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

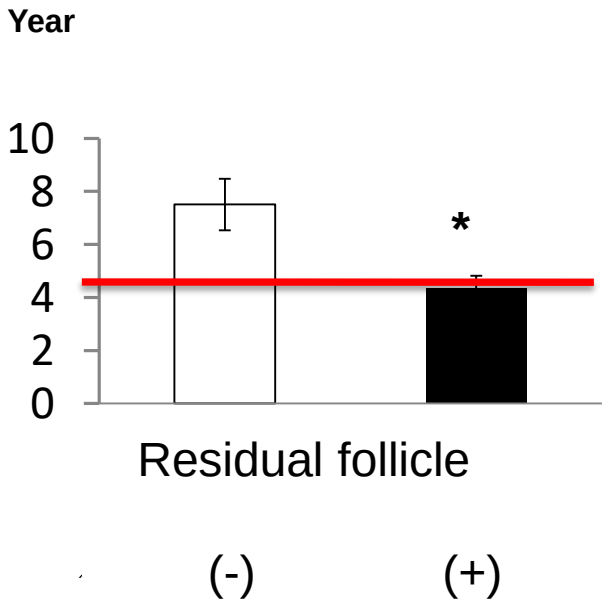
- Use 10% of volume of each ovarian stripe to detect residual follicles.

Predictive factors for presence of residual follicles

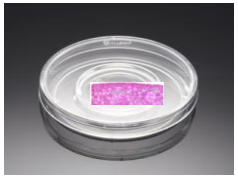
Histological analyses

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

Mean duration of amenorrhea



Fragmentation of ovarian strips to cubes

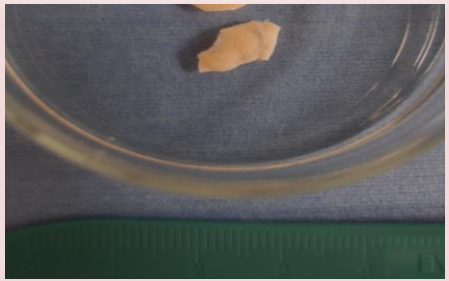


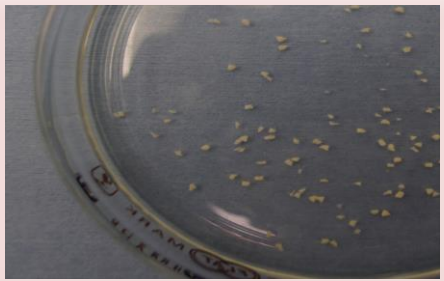
In Vitro Activation

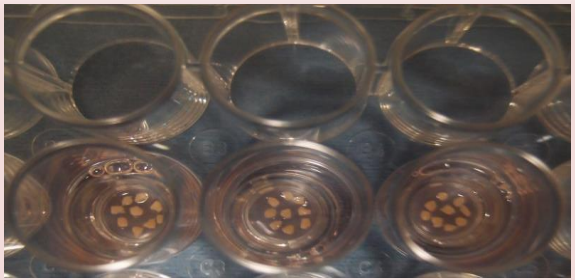
Culture of ovarian cubes

- 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

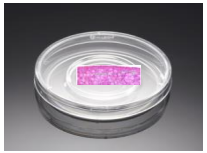






- Before auto-transplantation, wash cultured ovarian cubes by warmed culture media alone to avoid to introduce reagents inside of body.

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



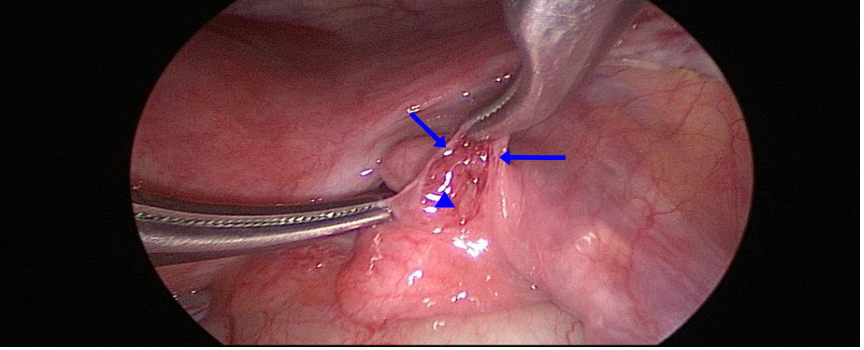
In Vitro Activation

Culture of ovarian cubes

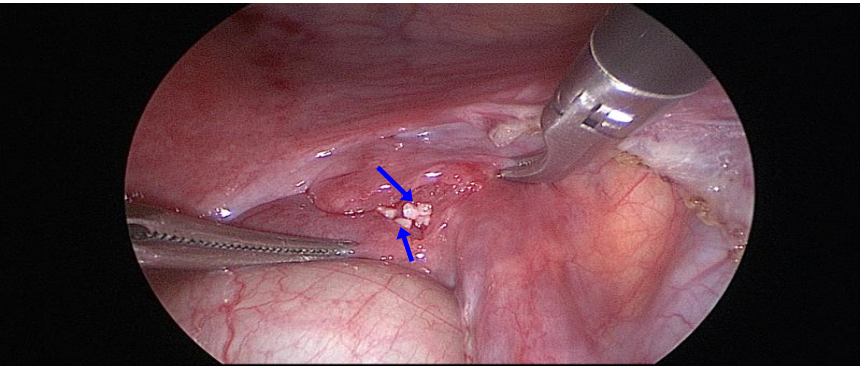
Auto-transplantation of activated ovarian cubes

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

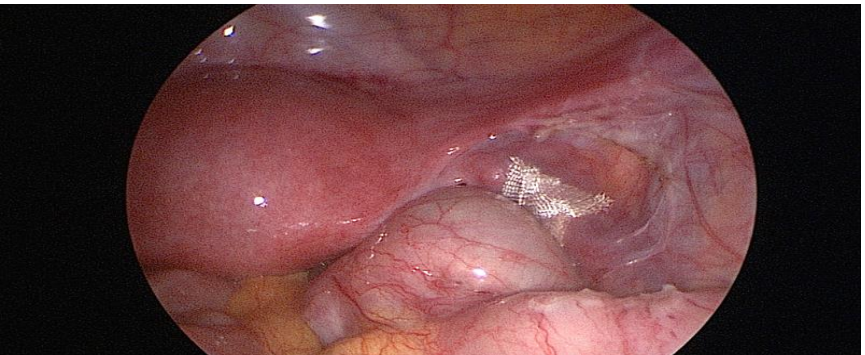
Auto-transplantation



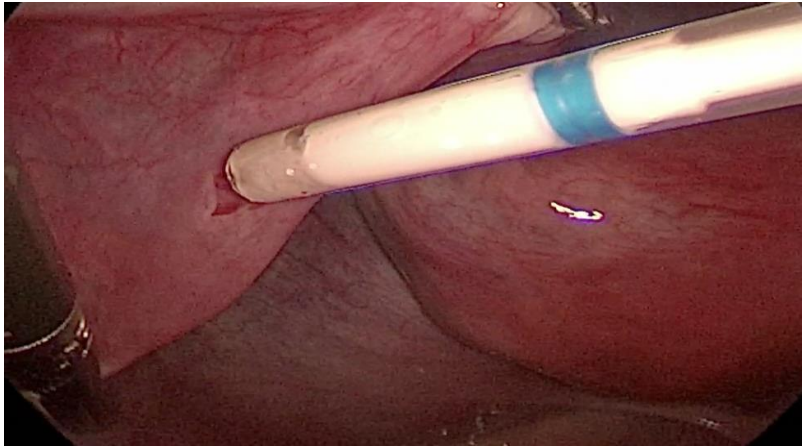
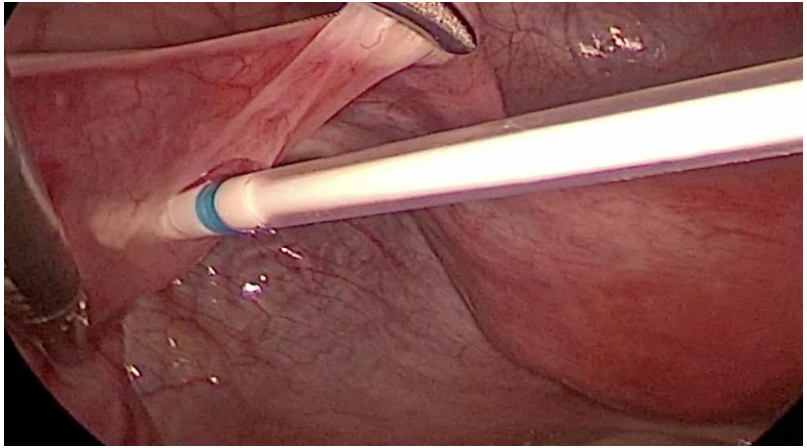
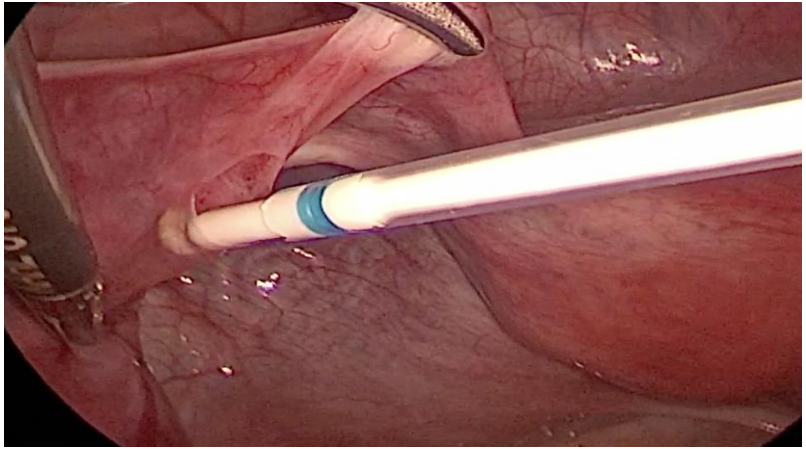
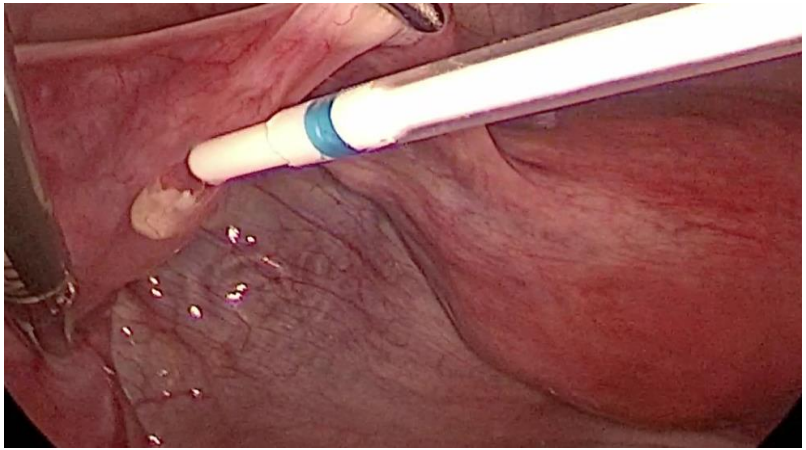
Cutting the serosa and making a pouch between serosa (arrows) and Fallopian tube (arrowhead).



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



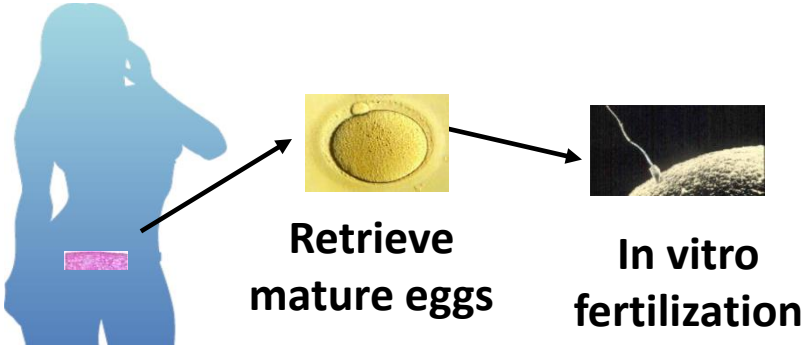
Wound was covered by an oxidized regeneration cellulose to avoid cube loss from the graft site.

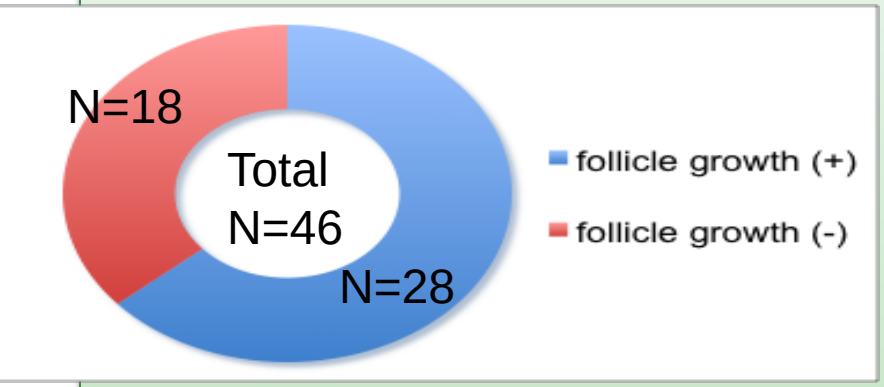
- Monitor follicle growth weekly to biweekly: transvaginal ultrasound + serum estrogen and gonadotropin levels.

- 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.



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

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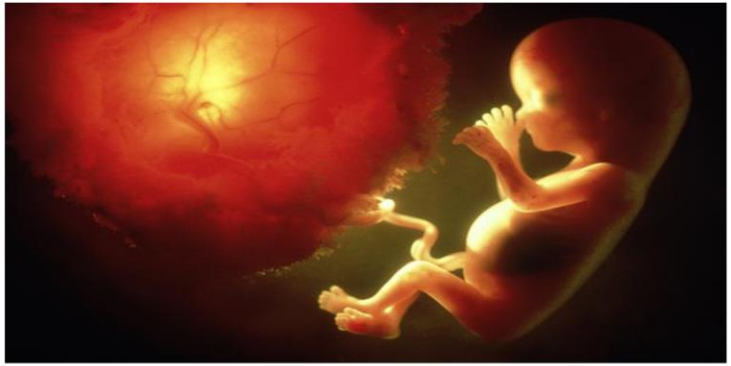
Japanese baby raises hopes for post-menopause births



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

nature.com Sitemap

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

A previously infertile woman has given birth to a healthy baby after undergoing a procedure that involved removing her ovaries and stimulating them in the lab to produce ...

Current issue

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Results

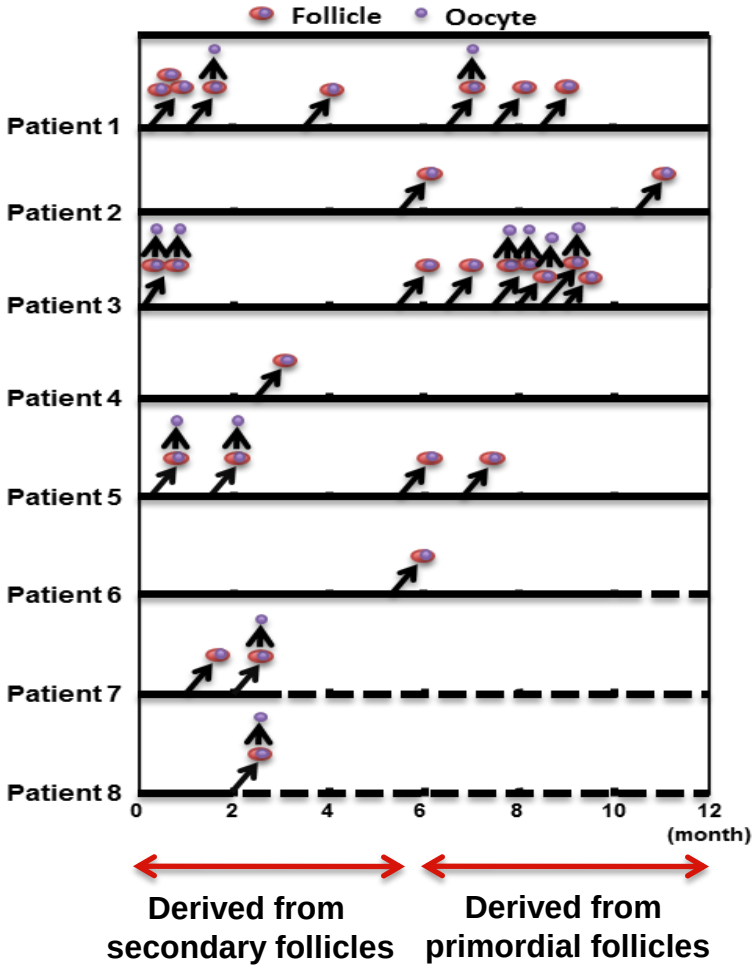
Reproducibility of IVA was already confirmed by China and Spain groups under our guidance.

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。

Temporal follicle growth in transplanted ovaries

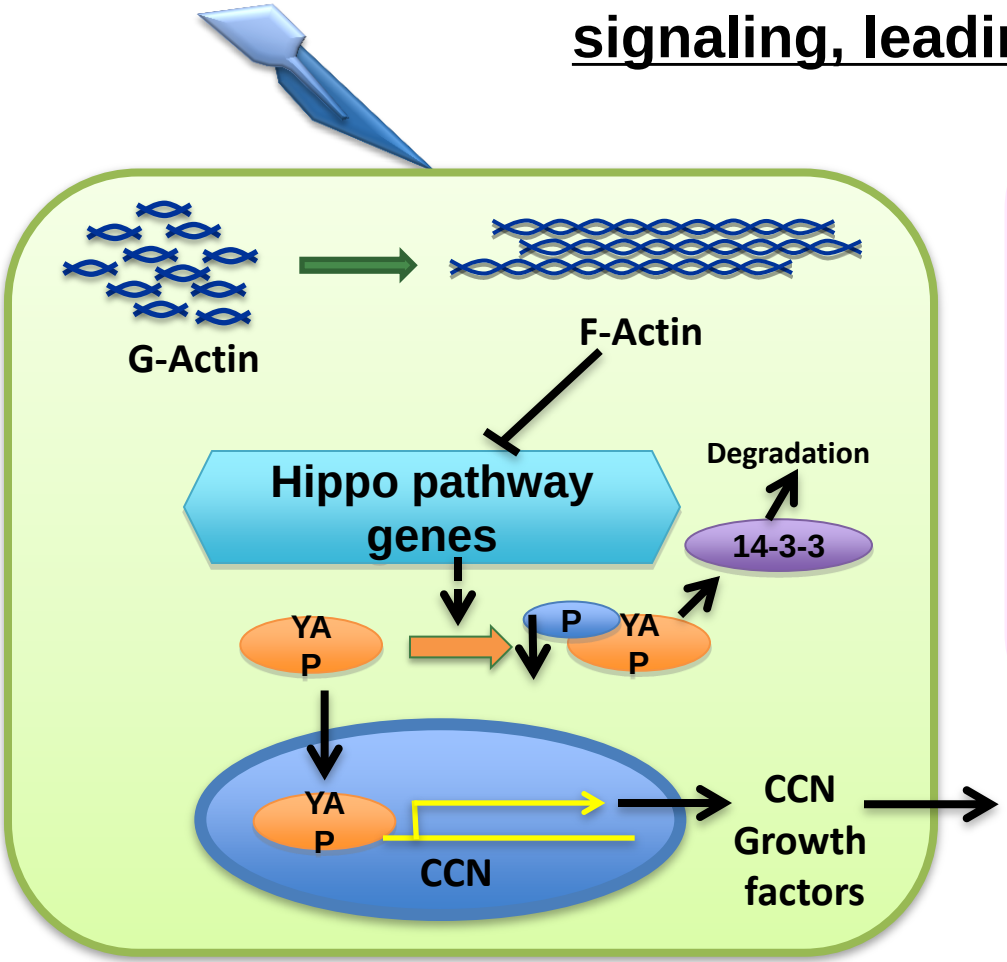


Follicle growth from primordial to preovulatory stage takes more than 6 months.

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 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 expression of downstream CCN growth factors.

Secretion of CCN growth factors stimulated follicle growth.

Secondary follicle growth

Kawamura et al PNAS 2013

Hsueh, Kawamura et al Endocr Rev 2015

Contents

1. Background of POI

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

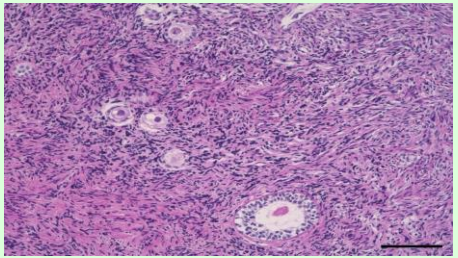
1. Clinical application of IVA

1. **Future studies for IVA**

Future studies

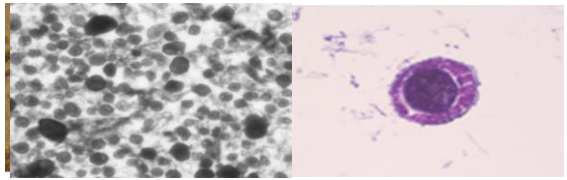
・IVA requires two times of laparoscopic surgeries.

→ **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?

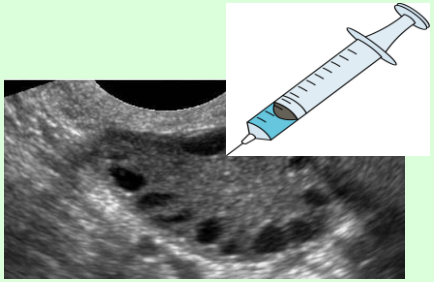


Recent progress in less invasive IVA

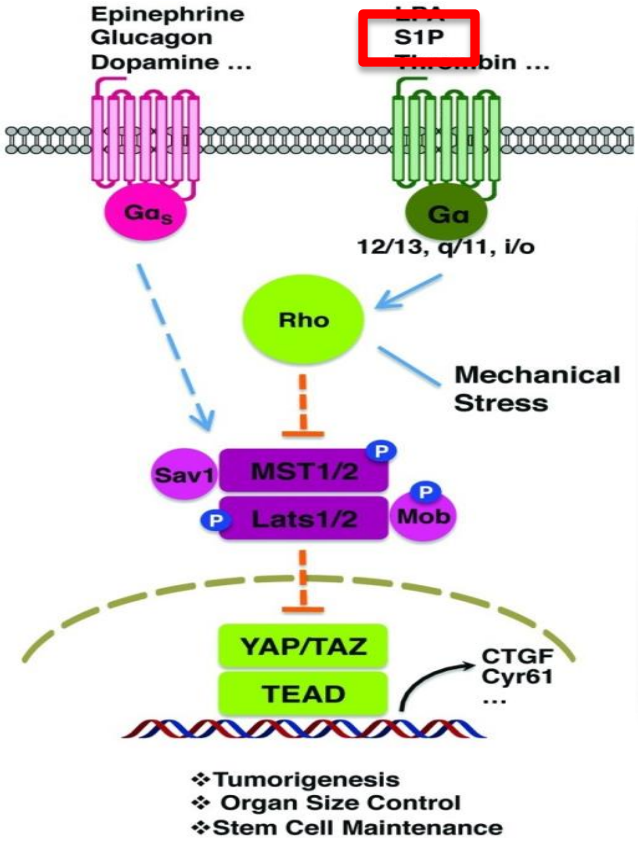
- Regular IVA needs two times of laparoscopic surgeries.

→ 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.



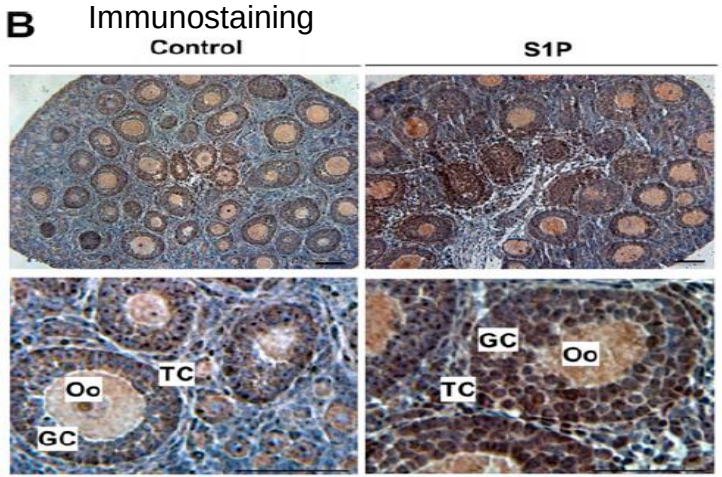
Candidate molecule: Sphingosine 1-phosphate (S1P)

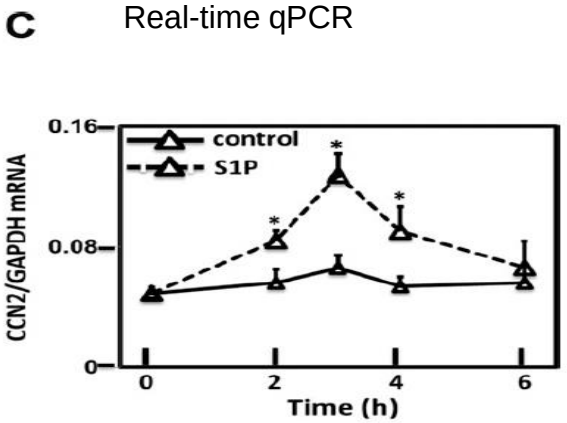


S1P is a bioactive sphingolipid, acting on GPCR to suppress Hippo signaling.

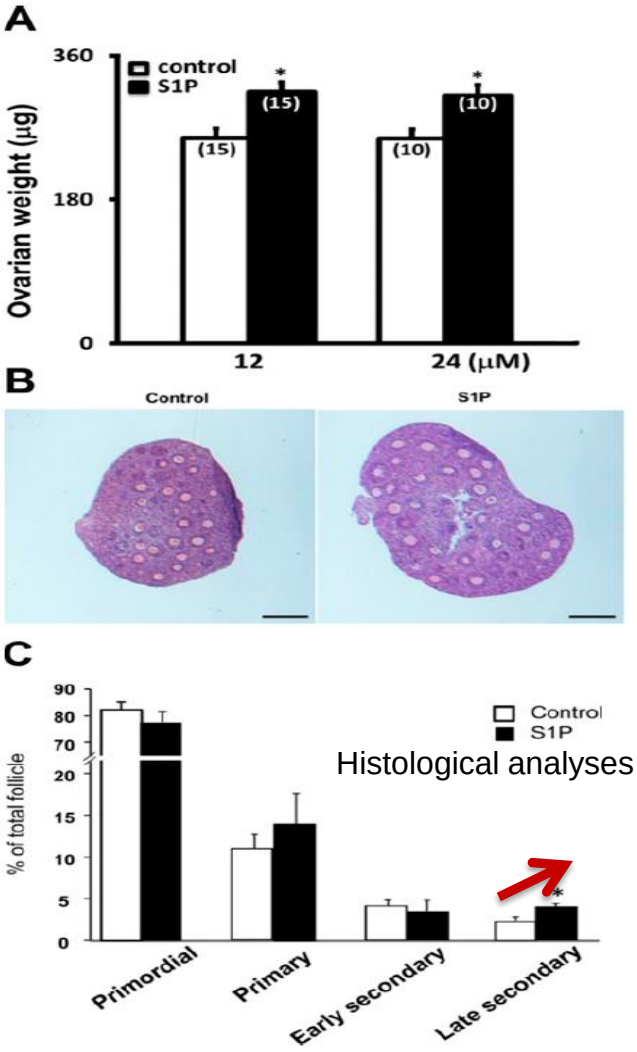
S1P is a physiological substance and exists in follicular fluid in ovaries.

Effects of S1P on disruption of Hippo signaling in D10 mouse ovarian tissue culture





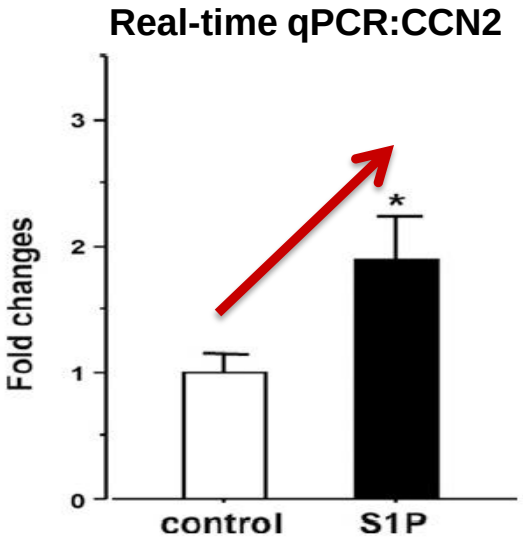
S1P stimulates nuclear translocation of YAP in granulosa cells followed by increase in expression of downstream CCN2 growth factor.



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.

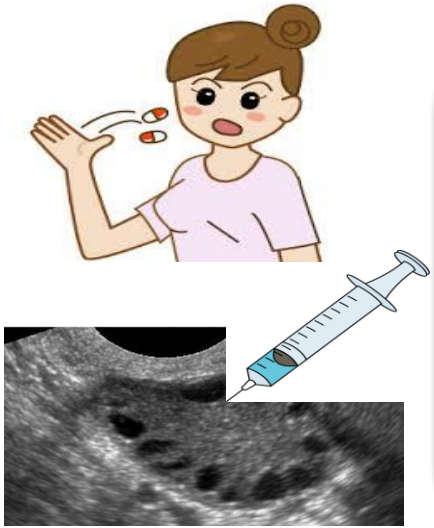
Human ovarian cortex was obtained from patients with ovarian tumor with IRB approval and informed consent.

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



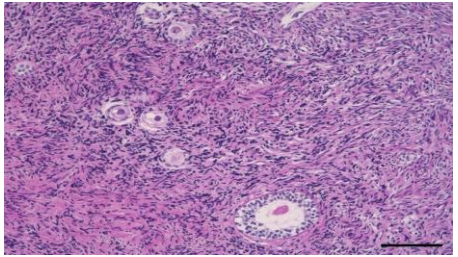
Because S1P is physiological substance existing in follicular fluid, intake or injection of S1P expects to stimulate follicular growth in POI/DOR patients including aging without severe adverse reactions.

Patent: PCT/US2013/059800

Future studies

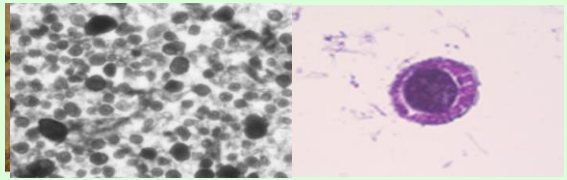
- IVA requires two times of laparoscopic surgeries.

→ **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?



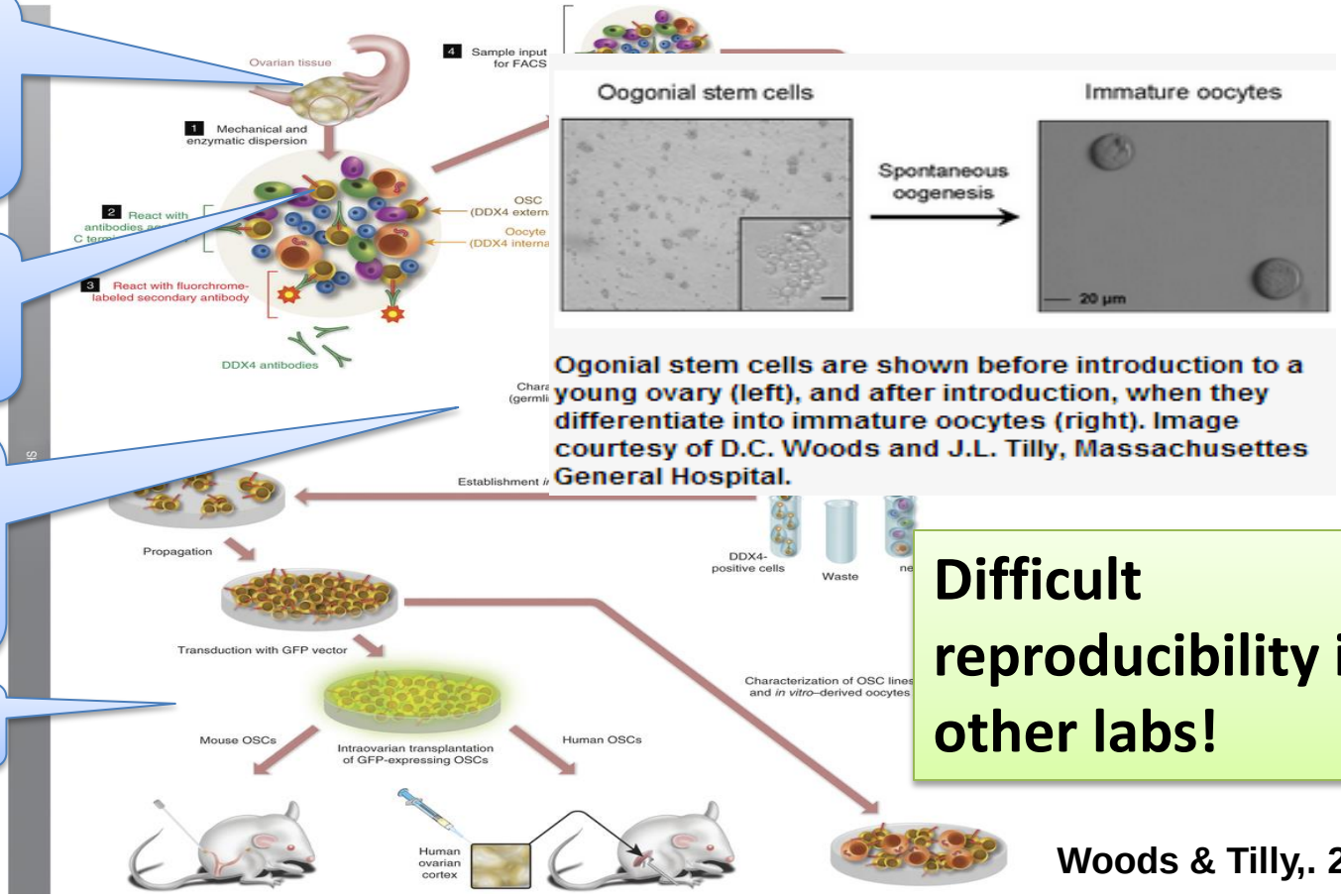
The method for isolation of oogonial stem cells by Dr. Tilly

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

Development novel method for regenerate oocytes

Human ovary

cortex

medulla

Woods and Tilly (2013) Nature protocol

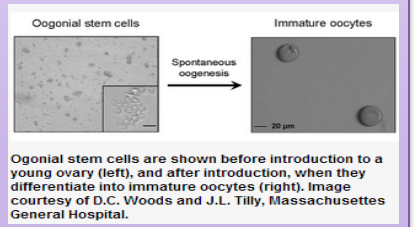
Collagenase

Tissue dispersion

DDX4 antibody

FACS DDX4(+)

Culture with 10%FBS



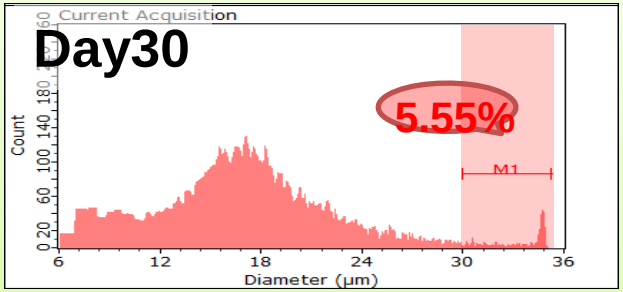
Ogonial stem cells are shown before introduction to a young ovary (left), and after introduction, when they differentiate into immature oocytes (right). Image courtesy of D.C. Woods and J.L. Tilly, Massachusettes General Hospital.

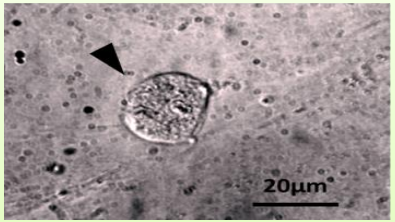
Our method

Collagenase

Tissue dispersion

Culture with 10%FBS

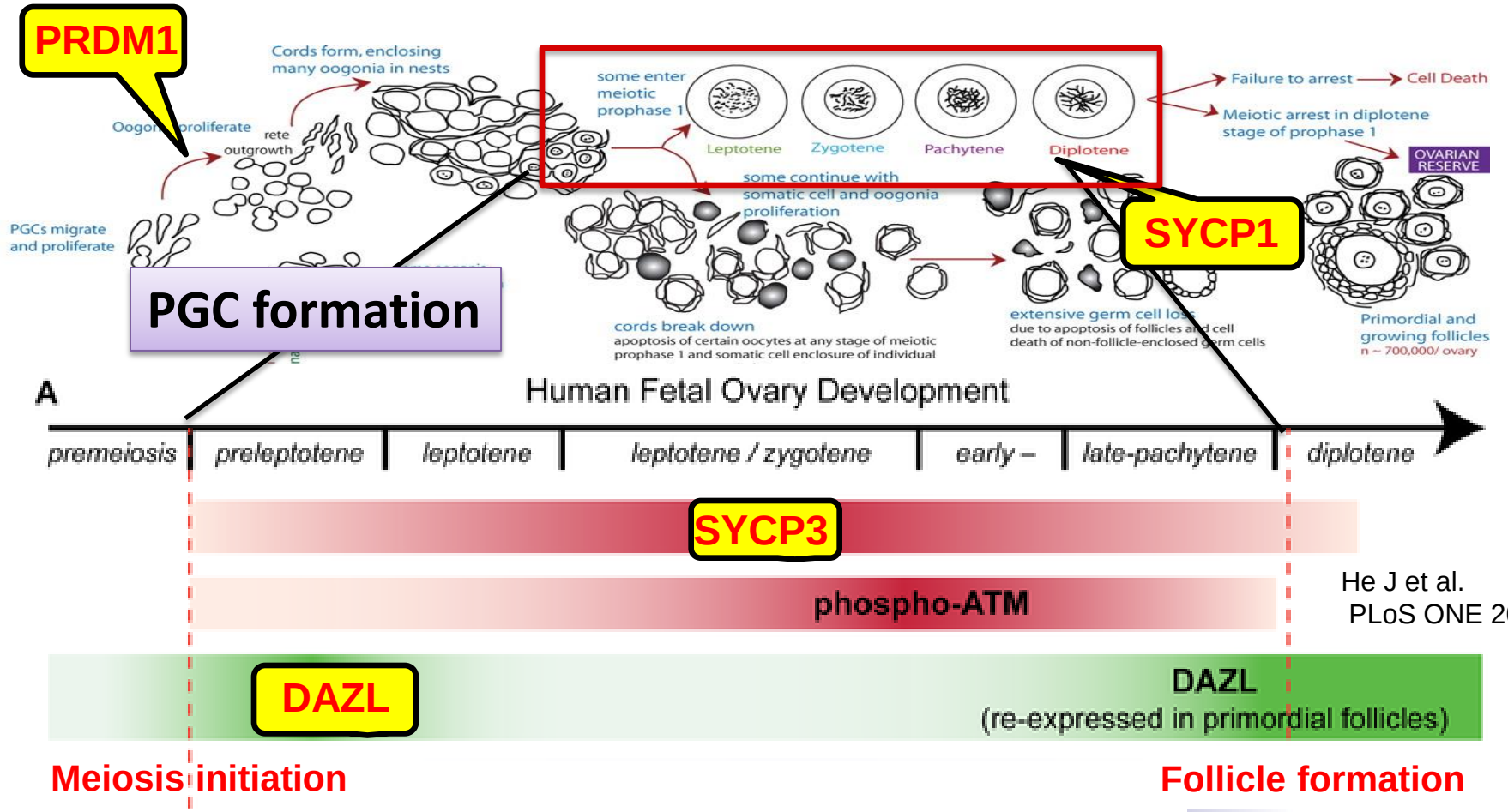




We could obtained oocyte like cells (>30 µm) after culture.

Our oocyte-like cells did not complete meiosis.

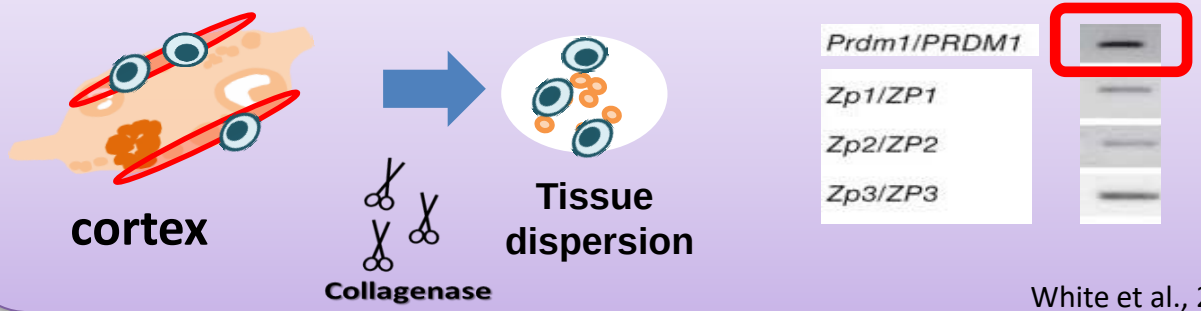
Gene expression changes during human oogenesis



He J et al.
PLoS ONE 2013

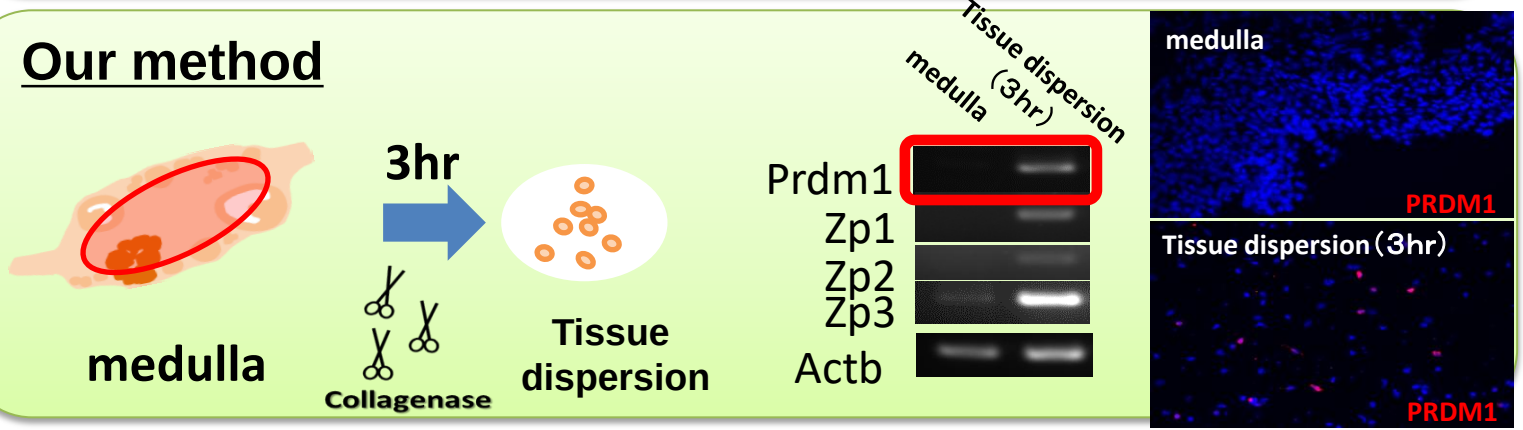
Differentiation of PRDM1 positive cells by tissue dispersion of medulla

Woods and Tilly (2013) Nature protocol



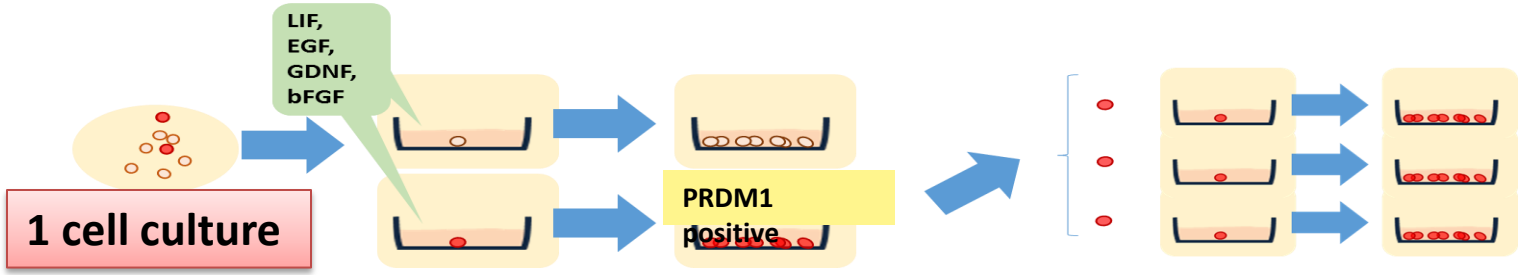
White et al., 2013 Nature Med

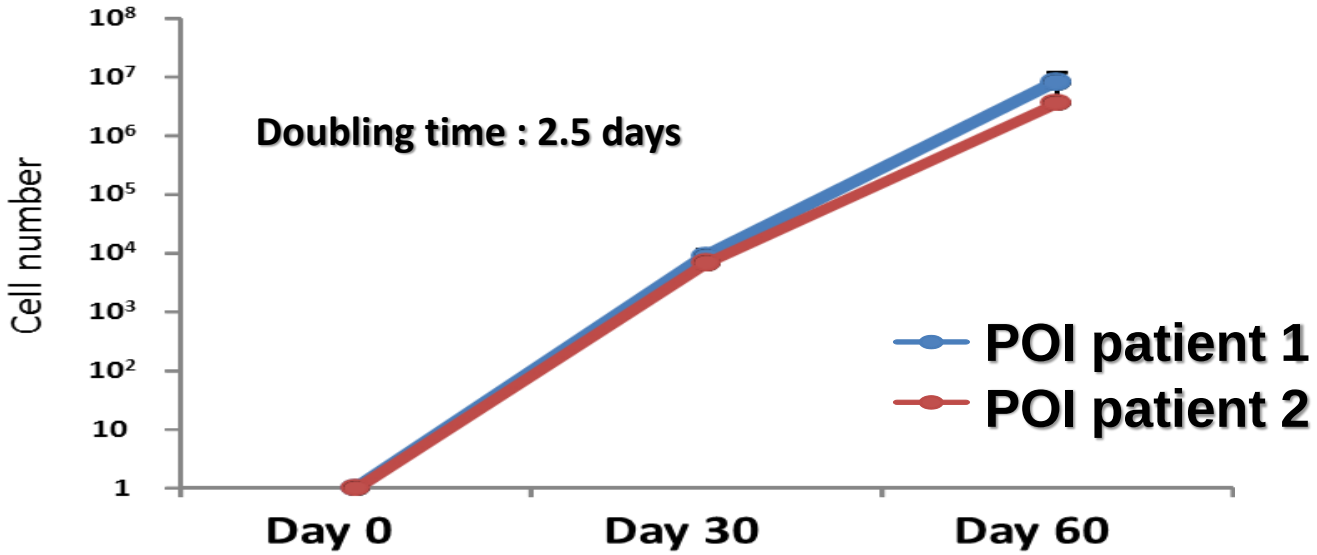
Our method



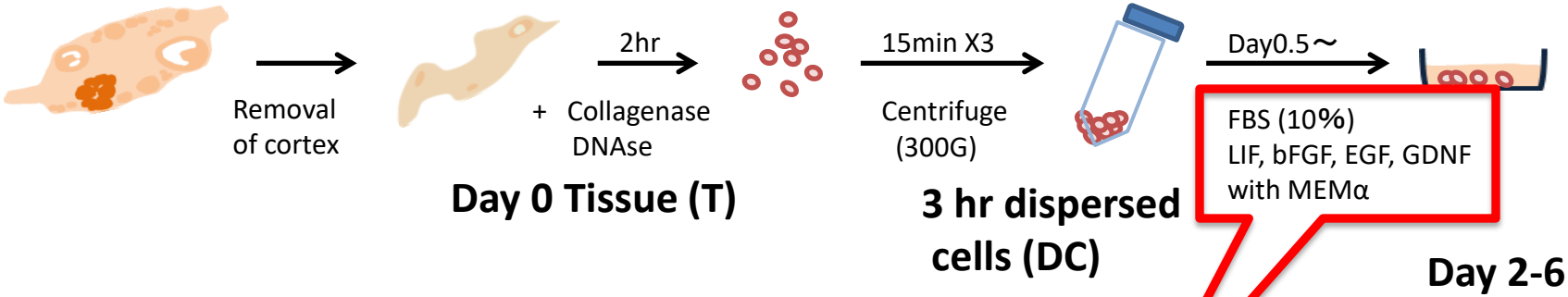
Tissue dispersion could differentiate PRDM1 positive cells from medulla.

PRDM1 positive cells have an ability for self-replication

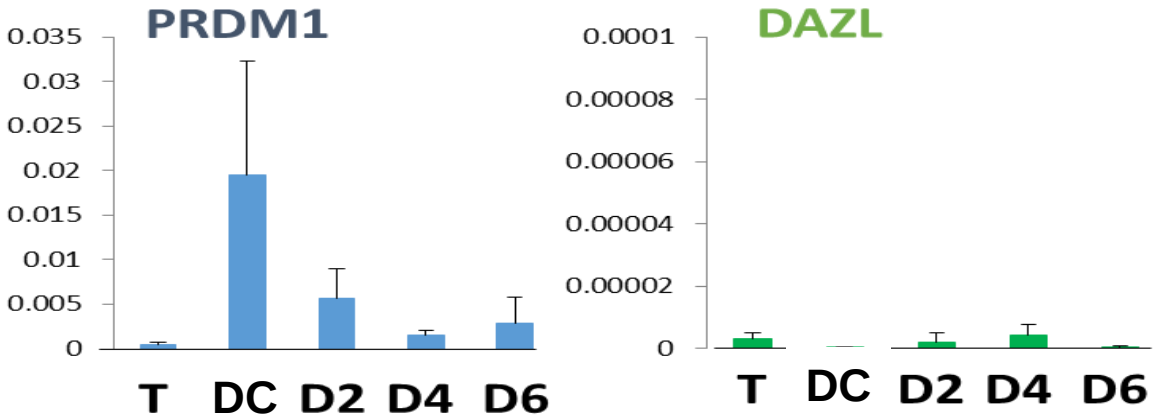




Induction of meiosis related genes during culture in PRDM1 positive cells

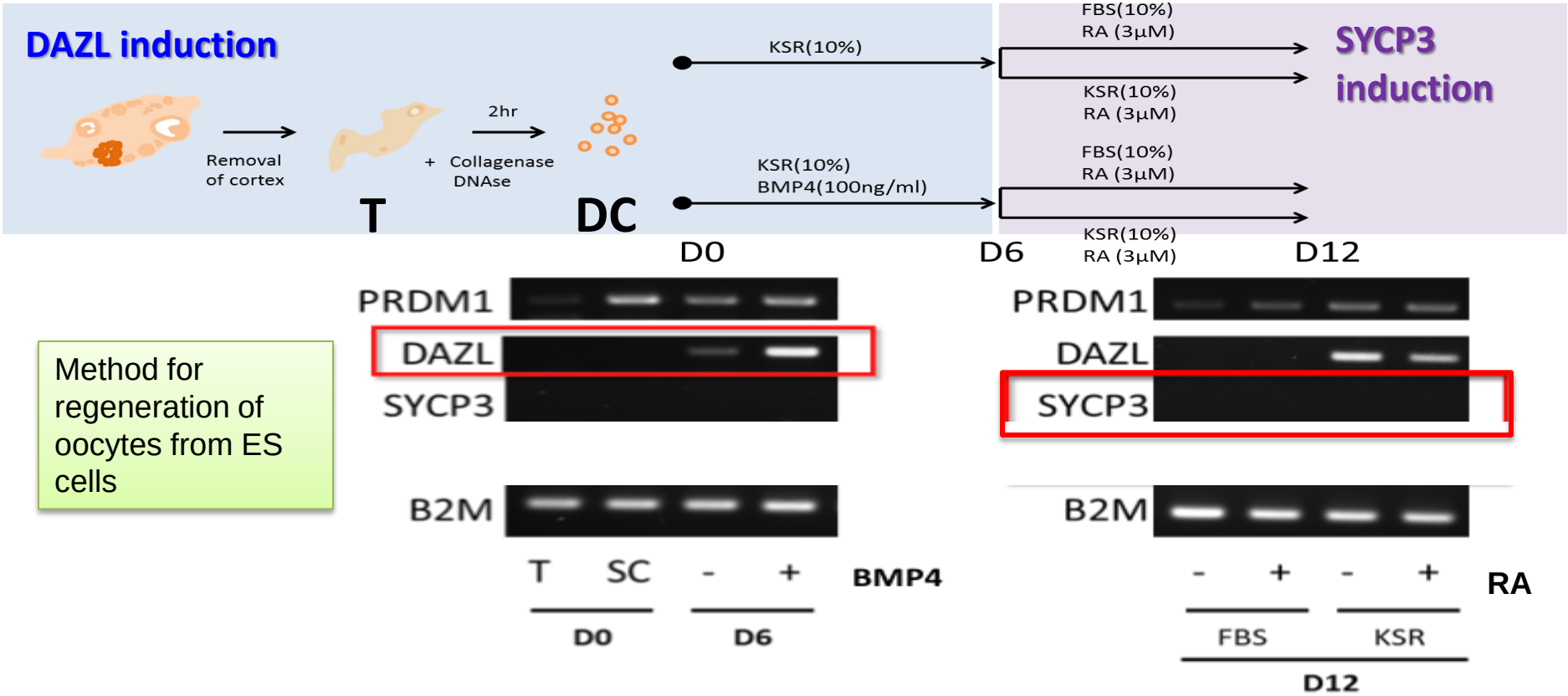


Real-time qPCR



DAZL expression could not induce following Tilly's method in our cells.

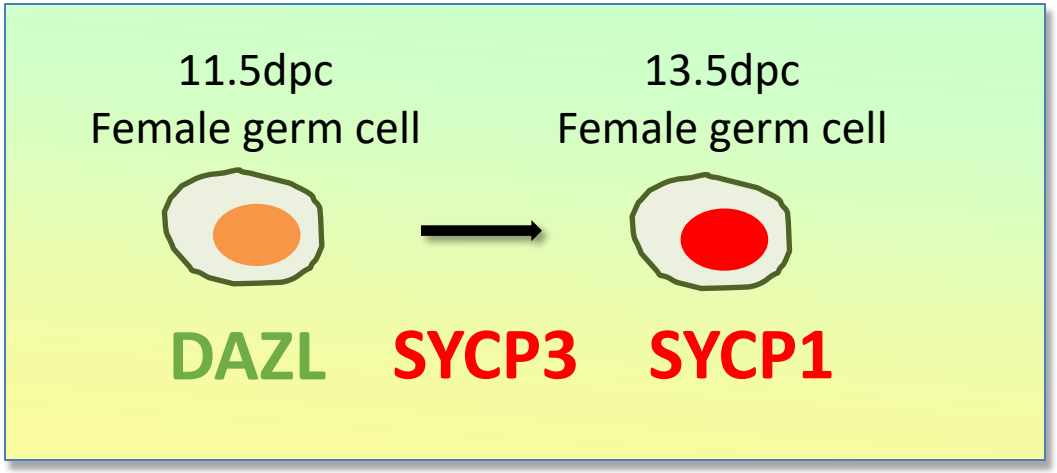
Induction of meiosis related genes during culture in PRDM1 positive cells



Method for regeneration of oocytes from ES 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



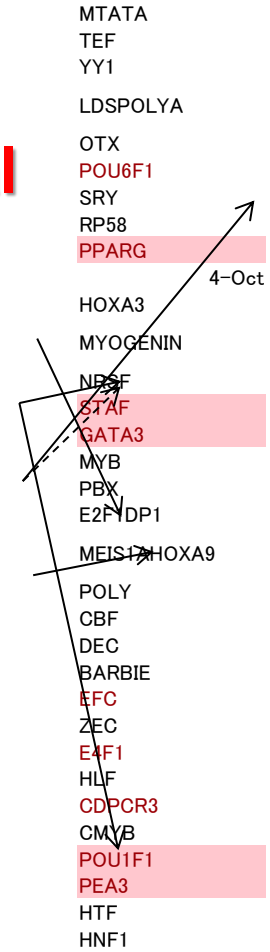
Origin → **Up regulation**

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

Results

Transcriptional factors

Factor A
Factor B
Factor C
Factor D



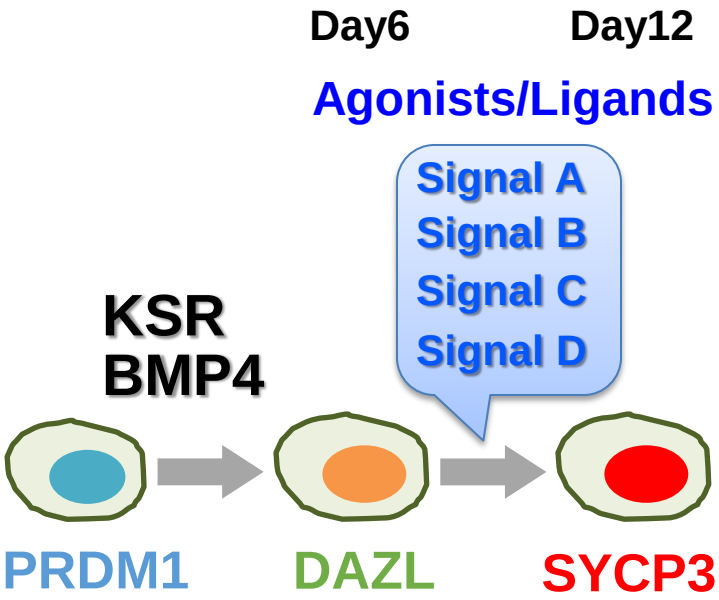
CDX
CLOX
NFAT
E2F4DP1
E2F1DP1RB
R
AFP1
ATF3
MINI20
HFH4
FOXM1
NKX62
ETS2
HNF3B
CEBPA
PADS
PR
PAX5
MEIS1BHOXA9
DR1
TAL1BETAE47
ZTA
CDPCR1
SOX
LXR_DR4
HNF3
TAL1BETAITF2
CDX2
LHX3
PAX

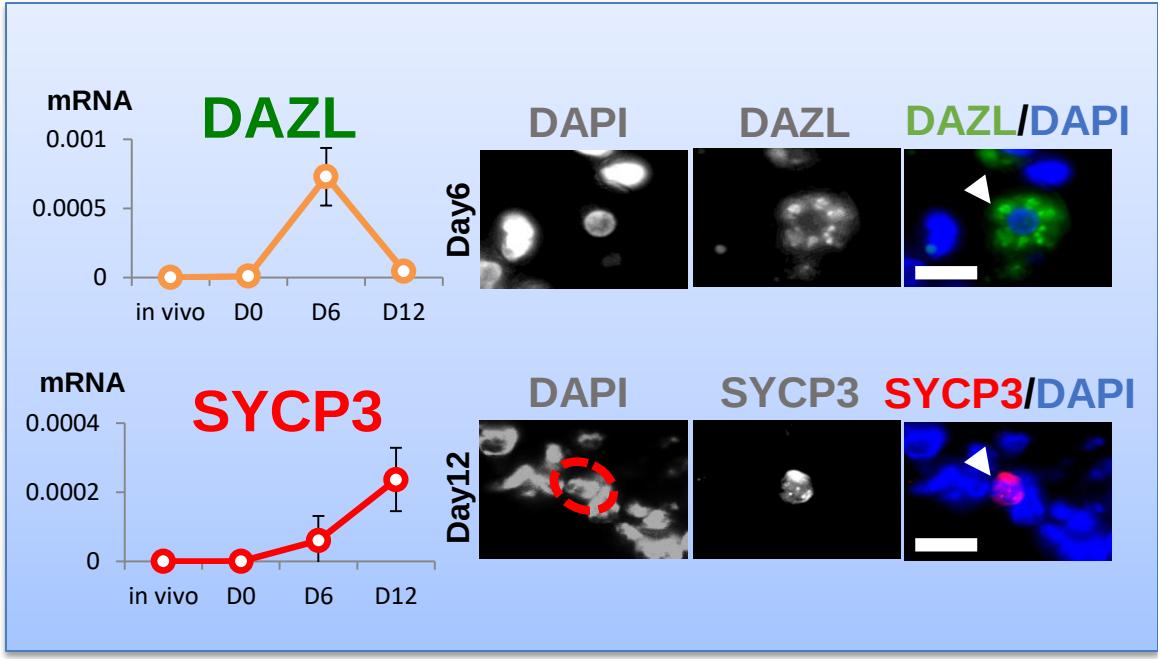
Upstream signals

Signal A
Signal B
Signal C
Signal D

Predict upstream signals based on published data base and papers

Identification of agonists and ligands to induce SYCP3 expression





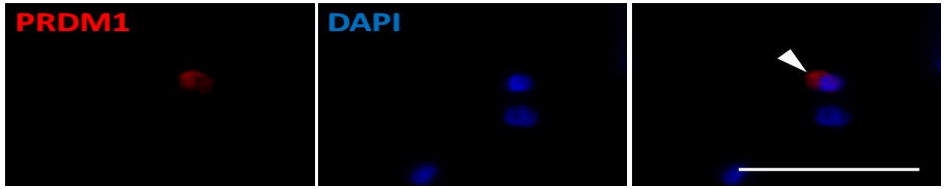
SCYP3 expression could be induced by treatment of agonists and ligands for predicted signaling systems A-D for meiosis.

Presence of PRDM1 positive cells in medulla obtained from POI patients

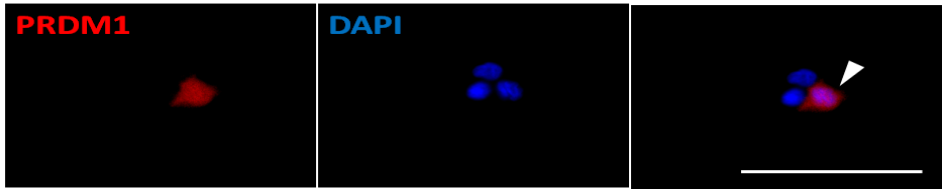
	normal	POI
PRDM1(+)	21 / 21	16 / 16
DAZL(+)	14 / 14	7 / 7
SYCP3(+)	4 / 4	NA

NA: under investigation

Normal ovary



POI ovary



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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Jing Li

Thank you for your kind attention.