

Female Fertility Preservation

(Artificial Ovary)

Ovarian follicles

- Peak number of oocytes, 6.8×10^6 , occurs at 5 months gestation
- After this point, there is no further proliferation of germ cells and a progressive atresia occurs
- At birth, $1-2 \times 10^6$
- At puberty there are only 300,000 left.
- ~300-500 will develop into mature oocytes, while the rest will become atretic.
- At 51 years of age, average age of natural menopause in developed countries, ~1000 cells left.
- In healthy women; ~37.5 years of age:
- Accelerated atresia of oocytes begins
- Increase in FSH.
- As atresia continues both the number and quality of oocytes fall
- Rate of aneuploidy increases.
- Greater risk of spontaneous abortion
- Premature ovarian failure (POF), menopause before the age of 40 years or hypergonadotropic amenorrhea, occurs in up to 0.9% of women.
- The doctrine of age-dependent follicle depletion has been challenged, presence of ovarian stem cells in mice (2009), in human, the cells called oogonial stem cells (OSCs), spontaneously generated apparently normal immature oocytes when cultured in the lab.

Fertility preservation

➤ Ovarian insufficiency

- Premature ovarian failure (**POF**)
- Poor ovarian response (**POR**)
- Advanced maternal age (**AMA**)

➤ Social reasons

- Age-related infertility
- A woman's **most fertile** years are in her **20s** and early **30s**, when the ovaries still contain a large number of healthy eggs.
- For the **10 to 15 years before menopause**, despite a woman having regular monthly periods, **ovarian function declines**.

➤ Cancer treatment

- **Gonadotoxic** treatments /radiotherapy

➤ Diseases

- In patients with **benign** diseases like systemic **lupus** erythematosus before cytotoxic therapy
- In patients with **endometriosis** before surgery
- In patients with **genetic predispositions** which can lead to a premature ovarian failure
- **Autoimmune** diseases
- **Mutations** in genes implicated in the regulation of ovarian function and **development, chromosomal and developmental abnormalities**
- **Infectious** diseases

Options for Fertility Preservation

- Embryo Cryopreservation

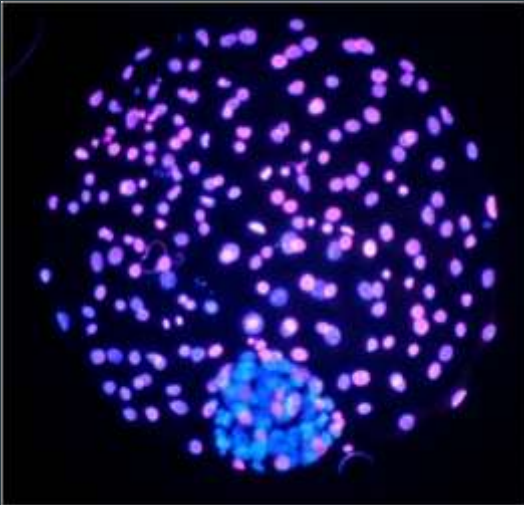
- **Primary** modality for fertility preservation, **since the 1980s**
- **No effect** on **miscarriage, implantation rates, or live birth**
- **Disadvantage** : **ovarian stimulation** medications
- **Ovarian stimulation** is a **particular concern** for patients with **hormonal sensitive tumors** such as **breast cancer**

- Oocyte Cryopreservation

- Ovarian Tissue Cryopreservation

Cryopreservation

Embryo



Oocyte



Ovarian tissue



Ovarian Tissue Cryopreservation

- **Advantages**

- Can be performed in **prepubertal girls** and **adolescents**
- Can be performed at **any point** in the **menstrual cycle**
- The potential to save **large numbers** of oocytes
- May allow for spontaneous **pregnancy** ,**without IVF** or **ovarian stimulation**
- **Eliminate** the risk of **exposure to gonadotropins** during **IVM** or in follicle maturation (**IFM**) of oocytes

- **Disadvantages**

- The need for **surgery** (typically by laparoscopy)
- Risk of **graft failure**
- **Oocyte quality** may be **compromised**
- Possibility of **contamination** of ovarian **tissue** by **malignant cells**
- **Limited success** ,utilizing aspirated **immature oocytes**

Fertility preservation

(Intervention in cancer treatment)

- Medical
 - Surgical
- } **Combination** therapy
- **Third-party** reproduction

- **Medical** treatments:
 - Suppress ovarian function during chemotherapy
 - Radiation, pelvic shielding
- **Surgical**:
 - Biopsy; surgical removal of ovarian cortex
 - Surgical repositioning of the ovaries out of the pelvis (**oophoropexy**)
- **Third party**:
 - Use of either **oocytes donated** by another individual
 - Gestational carriers** (“surrogates”) to carry a pregnancy

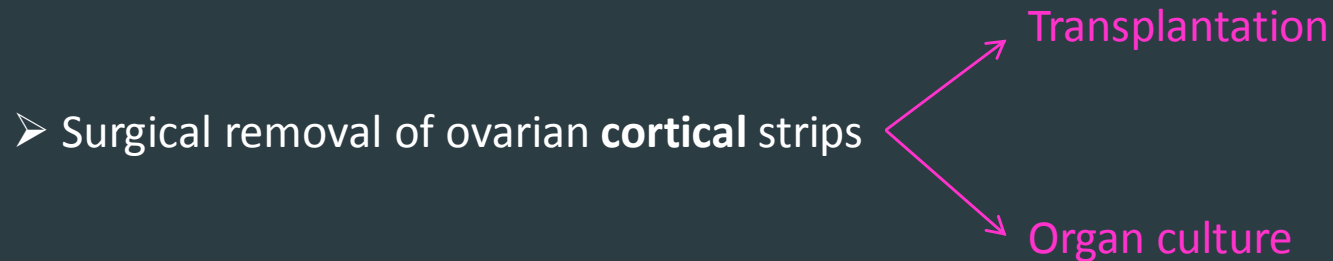
Medical approach

- Ovarian **suppression** with GnRH analogs or antagonists
- **Aromatase** inhibitor (anastrozole, letrozole)
- **Antiapoptotic** agents
- Estrogenic **receptor antagonist** (tamoxifen, clomifene)

Surgical approach

Oophoropexy

Radiation exposure could be diminished by **relocating** the **ovaries** outside, the radiation field



Three options for **re-implantation** of the **ovarian tissue**:

- **Auto-transplantation** orthotopically or heterotopically
- **Xenograft** transplantation
- **Cryopreservation** of the **whole ovary** with vascular transplantation after thawing
- **Auto-transplantation** of **cryopreserved ovarian tissue** is the **only** tissue banking approach that eventually led to the **live birth**

Organ Culture

- Culture of intact ovarian cortical strips
 - **Two**-dimensional
 - **Three**-dimensional
- **Retains** the organizational **structure** of the ovarian tissue
- Maintains the **interactions** between the **follicle** and **surrounding stromal cells**
- Support **growth** of human **primordial** follicles to the **secondary** follicle
- Support **follicle survival** for **up to 4 weeks**
- Main **challenge**, is **preventing atresia** due to **ischemia** in the **interior** of the **tissue**, since there is **no possibility** of **revascularization** in the **in vitro environment**
- Varying the **size** and **geometry** of the cultured **tissue pieces** or slices improve **follicle survival**

In Follicle Maturation

- Culture of **isolated follicles**,
 - Individual **monitoring** and **tracking** of each **follicle**
 - Followed by **IVM**
- Isolation { **Mechanical**
Enzymatic

To date, **no meiotically competent oocytes** have been **produced**.

I) **Two-dimensional**; Referred to as an **attached follicle approach**, proliferating **granulosa cells attach** and **migrate** onto a **two-dimensional** surface.

II) **Three-dimensional**; **follicles do not attach** to a substrate and the growth occurs radially therefore are able to **maintain** their **three-dimensional architecture**

- **Both approaches** have produced **live murine** offspring
- While successful in the **mouse**, two-dimensional systems have not been able to support normal follicle development in bovine, ovine, or human systems

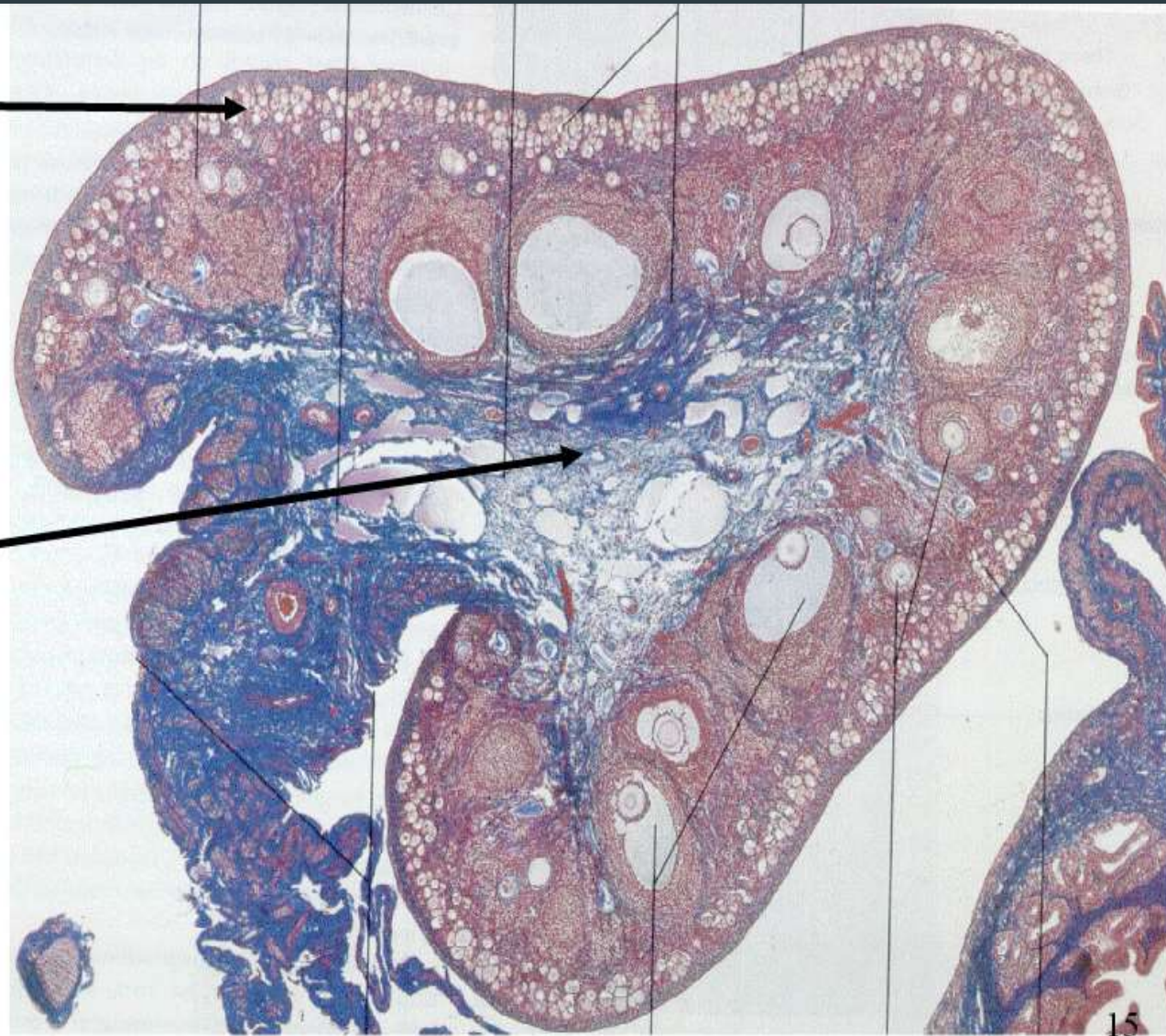
Ovarian Tissue Section

Cortex

Contains 90% of
the follicular reserve
(primordial follicles)

Medulla

Contains the
growing follicles



Cryopreservation of ovarian tissue

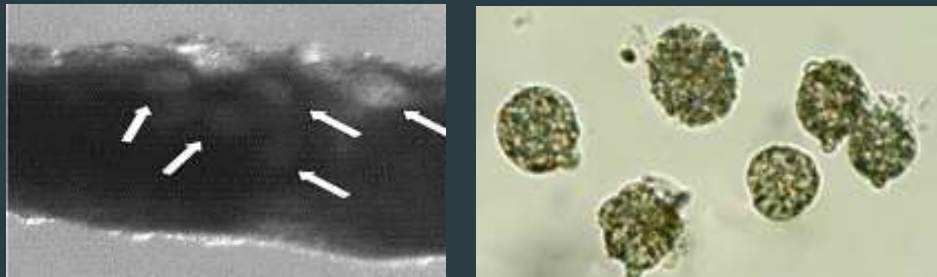
➤ Cortical ovarian biopsy

Transplantation



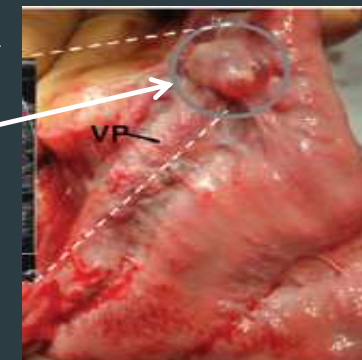
Risk of mamlignant cell
transmission

Culture and follicle isolation



Avoid transmission
of malignant cells

➤ Whole ovary transplantation (live birth in sheep; 2014)



Orthotopic Transplantation

- IF OVARY **PRESENT**: Decortication and suture of ovarian fragments



(Donnez, 2006)

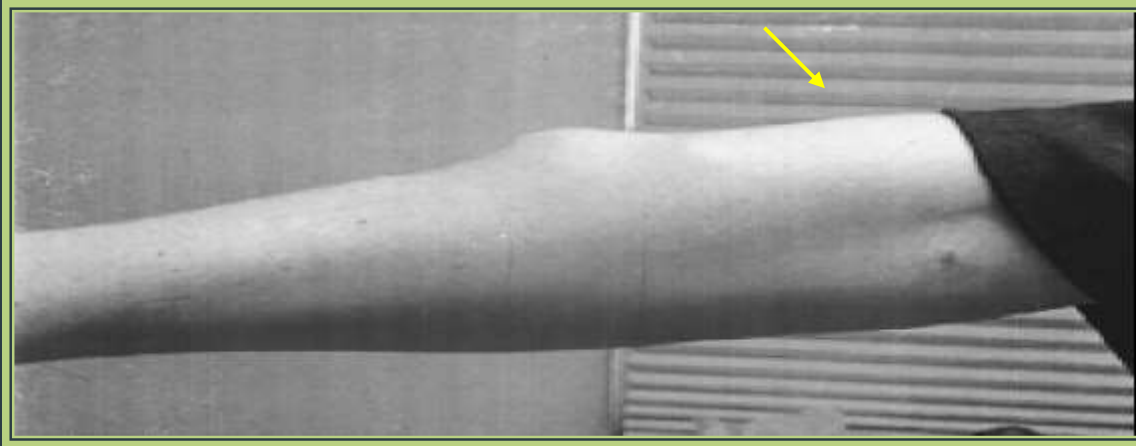
- IF OVARY **ABSENT**: Peritoneal window



(Donnez, 2012)

Heterotopic Transplantation

- Subcutaneous tissue of forearm



(Oktay, 2003)

- Subcutaneous tissue of abdominal wall

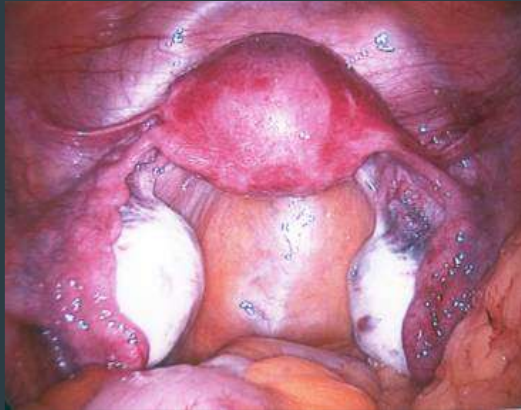


(Kim, 2009)

Ovarian Transplantation

CORTEX SLICES

ORTHOTOPIC TRANSPLANT



RESUMPTION OF CYCLICITY

NATURAL CONCEPTION

80-90% ENDOCRINE FUNCTION RESUMPTION

NO CHEMO BEFORE CRYO

Endocrine function resumption: 3.5 to 4.5 mths

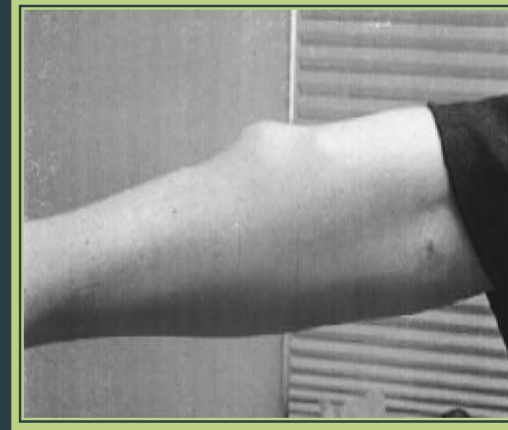
Longevity of the ovarian graft: > 4 yrs

CHEMO BEFORE CRYO

Endocrine function resumption : 5.5 to 6.5 mths

Longevity of the ovarian graft : < 2 yrs

HETEROTOPIC TRANSPLANT



EGG RETRIEVAL

IN VITRO FERTILIZATION

- ▶ **Long-term ovarian activity** in women who underwent ovarian tissue cryopreservation **before the age of 22** years, followed by **re-implantation some years later**.
- ▶ Ovarian function was **restored** for a period of **6–7 years**
- ▶ Whether **ovarian tissue freezing at a young age followed by re-implantation at menopause** could indeed be the **anti-aging therapy** of the future
- ▶ The **first successful ovarian tissue transplantation** resulting in a **live birth** occurred in **2004**; resulting in **>130 live births globally**.

Whole Ovarian Transplantation

- ▶ Whole-ovarian transplantation enables **immediate revascularization** with **blood vessel anastomosis**, significantly reducing the risk of ischemic injury
- ▶ **Reduce the risk of ischemic damage**
- ▶ **Whole ovary cryopreservation** and transplantation have been **successfully** achieved in several experimental **animal studies**
- ▶ Moreover, in cancer patients who lack sufficient time for an IVF cycle prior to chemotherapy or radiation therapy, **immature oocyte collection** may be a promising alternative.
- ▶ Although many researchers aim to achieve improved outcomes by combining IVM of oocytes and vitrification, **no live births have been reported from an IVM program in patients with cancer**
- ▶ **Oocyte cryopreservation** with **IVM** is still considered an **experimental** technique

Whole ovary cryopreservation and transplantation (Campbell 2014)

- ▶ Ovary and 10–15 cm of the ovarian vascular pedicle was carefully dissected

- ▶ Freezing medium:

Leibovitz (L-15) + 10% FCS + 1.5 mol/l DMSO + 0.1 mol/l sucrose

Perfused with the cryopreservation media for 60 min at a rate of 0.5 ml/ min using a syringe driven perfusion pump

- ▶ Warming solution :

L-15 + 10% FCS + 1 mol/l DMSO

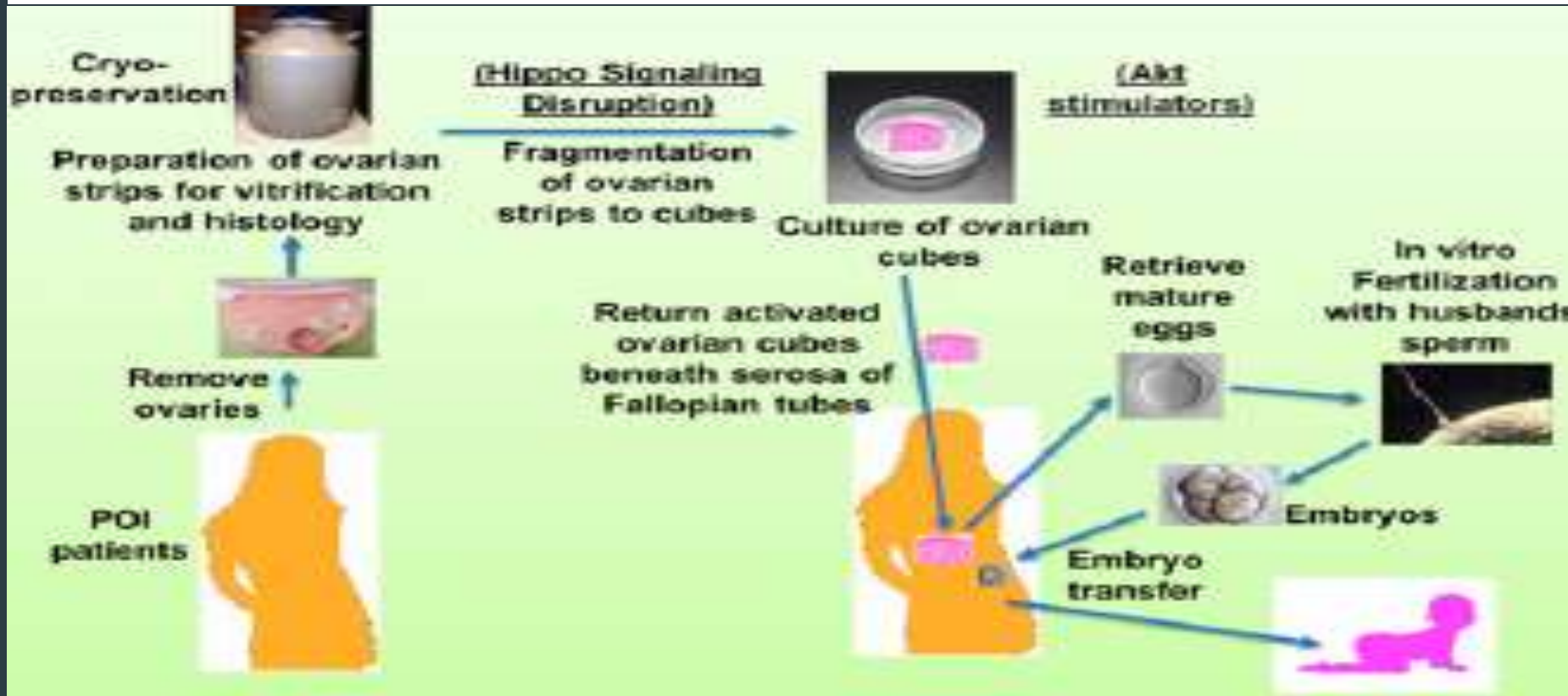
L-15 + 10% FCS + 0.5 mol/l DMSO

L-15 + 10% FCS

Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment

2013

Kazuhiro Kawamura^{a,b,1,2}, Yuan Cheng^{c,1}, Nao Suzuki^a, Masashi Deguchi^c, Yorino Sato^{a,c}, Seido Takae^{a,c}, Chi-hong Ho^c, Nanami Kawamura^{b,d}, Midori Tamura^a, Shu Hashimoto^e, Yodo Sugishita^a, Yoshiharu Morimoto^e, Yoshihiko Hosoi^f, Nobuhito Yoshioka^a, Bunpei Ishizuka^{d,2}, and Aaron J. Hsueh^{c,2}



- Hippo and Akt signaling pathways regulate follicle growth.
- Removing ovaries from infertile patients, followed by fragmentation to disrupt Hippo signaling and drug treatment to stimulate Akt signaling.
- After grafting ovarian tissues back to patients, they found rapid follicle growth in some patients and successfully retrieved mature eggs. After IVF and ET, a live birth is now reported.

- ▶ Hippo signaling consists of several negative growth regulators acting in a kinase cascade that ultimately phosphorylates and inactivates key Hippo signaling effectors, Yes-associated protein (YAP)/ transcriptional coactivator with PDZ-binding motif (TAZ). When Hippo signaling is disrupted, decreases in YAP phosphorylation increase nuclear levels of YAP.
- ▶ YAP acts in concert with TEAD transcriptional factors to increase downstream CCN growth factors and baculoviral inhibitors of apoptosis repeat containing (BIRC) apoptosis inhibitors. CCN proteins, in turn, stimulate cell growth, survival, and proliferation.
- ▶ Fragmentation of ovaries promoted actin polymerization and disrupted ovarian Hippo signaling, leading to increased expression of downstream growth factors, promotion of follicle growth, and the generation of mature oocytes

Hippo signaling
↓
kinase cascade
↓
phosphorylates and inactivates key Hippo signaling effectors, YAP & TAZ

Disruption of Hippo signaling
↓
Decreases in YAP phosphorylation
↓
Increase nuclear levels of YAP
↓
YAP acts in concert with TEAD transcriptional factors
↓
Increase CCN growth factors and BIRC (apoptosis inhibitors)
↓
CCN proteins, in turn, stimulate cell growth, survival, and proliferation

Anti-apoptotic Agents

- ▶ Increased apoptotic follicles were observed shortly after OTT.
- ▶ Key factors that ensure the survival of OTC-T are **revascularization** and **apoptosis prevention**.
- ▶ **Sphingosine-1-phosphate (S1P)**, an **anti-apoptotic** substance in oocytes, and ceramide play central roles in apoptosis.
- ▶ **S1P inhibits ceramide** and has been shown to **protect vitrified ovarian grafts** from ischemic reperfusion injury and promote **neo-angiogenesis** in ovarian transplants
- ▶ **S1P** did not help preserve, or increase the proliferation of, follicles, nor did it protect against DNA damage during the freezing-thawing Process
- ▶ In contrast, **Z-VAD-FMK** administration **improved follicle preservation** and follicular cell **proliferation**, also **preventing DNA damage** during the **freezing-thawing process** and **reducing apoptosis** in transplanted OT
- ▶ Administration of recombinant **AMH inhibits** the initiation of **primordial follicle recruitment**
- ▶ Recently, **co-administration of AMH and chemotherapy agents** has been shown to **protect the ovarian reserve** by **suppressing primordial follicle recruitment**
- ▶ **Co-transplantation of the graft with exogenous endothelial cells** engineered to produce AMH **in situ** significantly **decreased primordial follicle loss** in human xenotransplants

- ▶ **Malignant cells** in grafted OT may never undergo activation or may be eliminated due to the patient's **newly formed immune system** following bone marrow transplantation.
- ▶ Although heterotopic transplantation has the advantage of not requiring abdominal surgery, **all births reported** thus far have been from **orthotopic** transplants; spontaneous pregnancies are difficult to predict, and **in vitro fertilization** is required
- ▶ In **animal** studies, **offspring** are reportedly obtained from **heterotopic** transplants placed close to the cutaneous area.
- ▶ Additionally, oocytes and embryos have been obtained in humans as a result of subcutaneous transplantation.
- ▶ Although it may be controversial to classify transplants placed **intra-abdominally** into the peritoneal wall as heterotopic, **pregnancy** and **live births** have been obtained using **assisted reproduction** in this region

Reducing the risk of re-implanting malignant cells

In vitro growth and maturation of primordial follicles

- ▶ This **risk** could possibly be **reduced** by different methods:

I) In vitro growth (**IVG**) for **primordial follicles** (In vitro growth and maturation of primordial follicles)

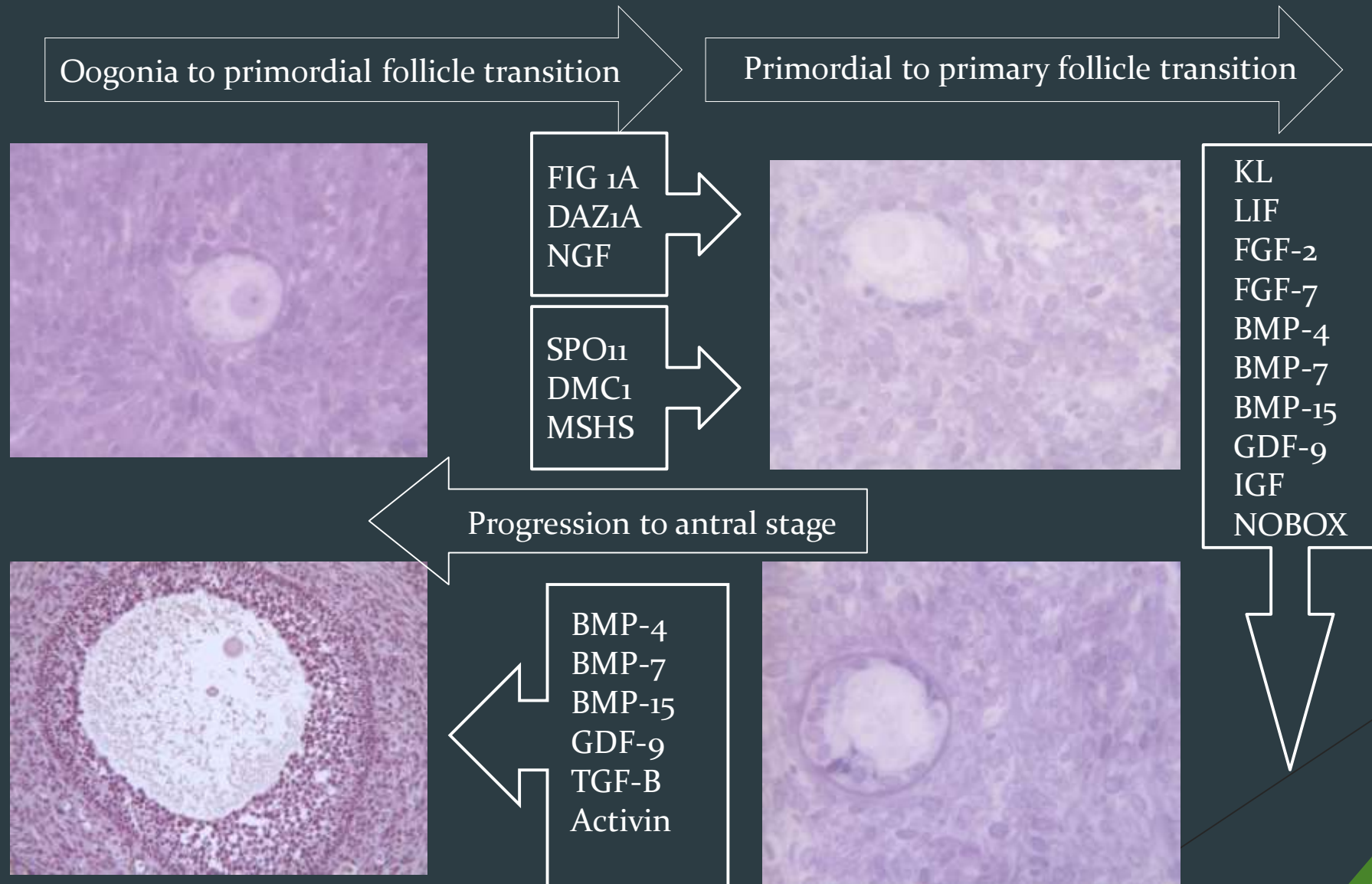
- In vitro growth (**IVG**) of **follicles** and in vitro maturation (**IVM**) of oocytes minimizes this risk.
- The aim of IVG is to accomplish the **entire follicular growth in vitro**, ending with oocytes which can be **fertilized**.
- To date, follicular IVG has led to **live births in mice only**
- A **two-step culture system** was established by Telfer et al. This system first initiates the growth of **primordial follicles** and afterwards cultures **secondary follicles** which have been **isolated** in an individual culture, allowing growth until the **preantral/early antral** stage
- **IVG of meiotically competent human oocytes** from preantral follicles has **not** been achieved .

II) Transplantation of an **artificial ovary containing isolated follicles** which are embedded in a matrix (*Artificial ovary*).

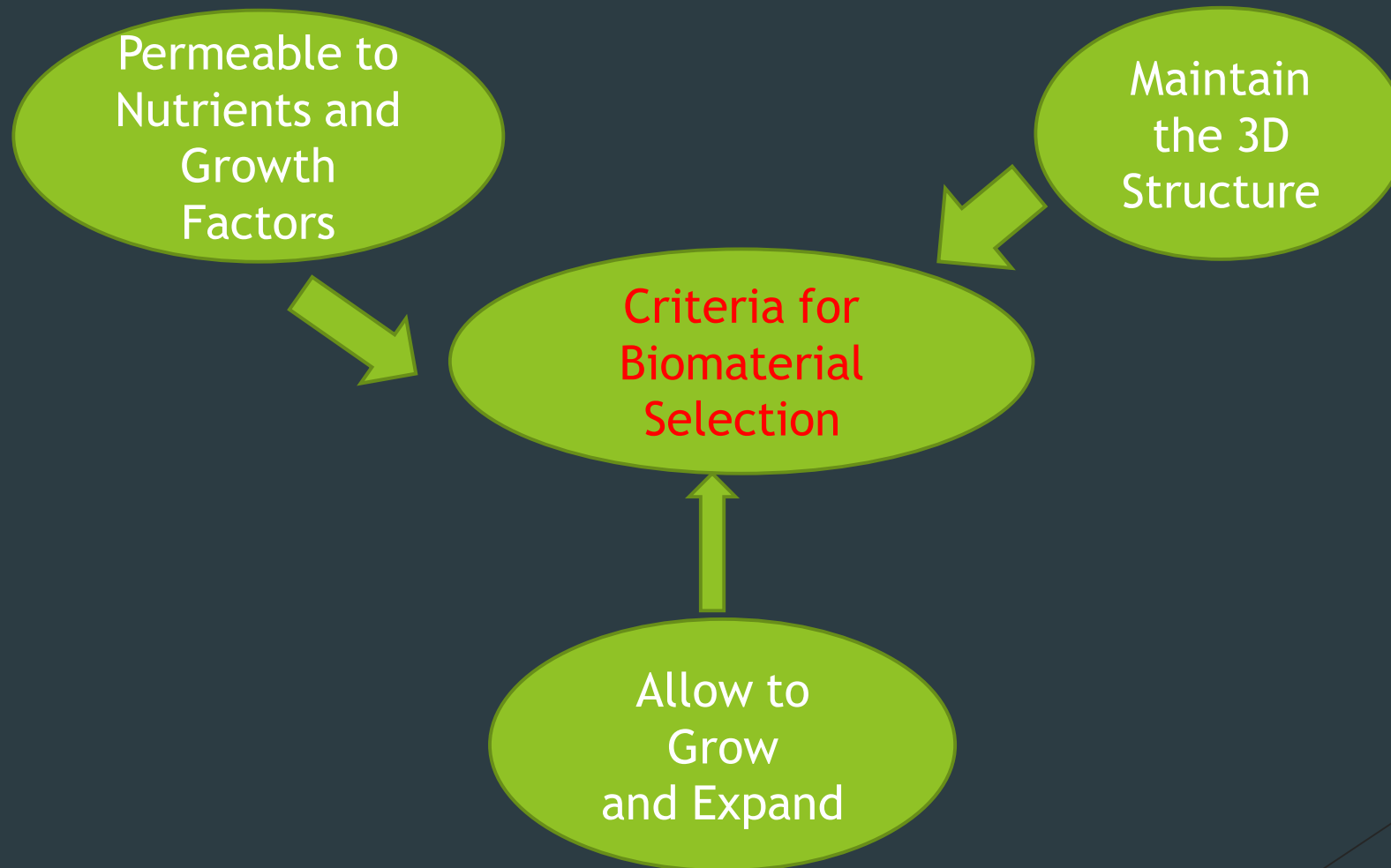
- **Stem cells** (*Stem cells*)
- **Allografting** and **xenotransplantation of ovarian tissue**

IN VIVO FACTORS INVOLVED IN FOLLICLE DEVELOPMENT

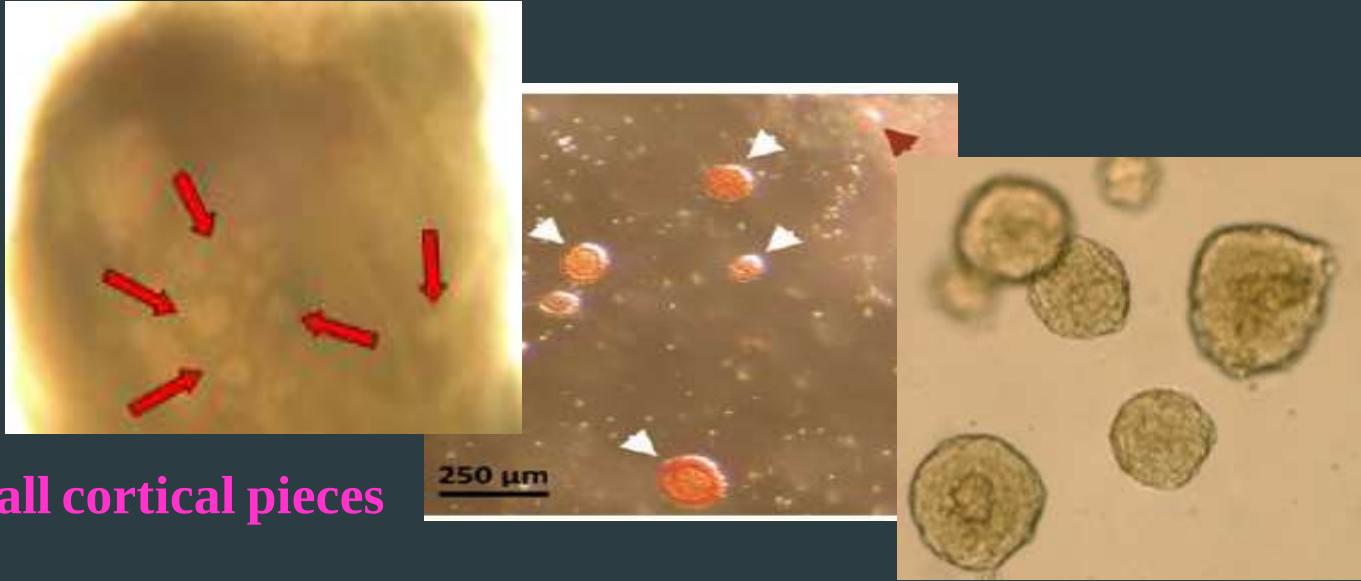
Oktem, 2008 Picton, 2008



3D System for follicle culture



Invitro human follicle culture options



➤ Culture of small cortical pieces

Cortical pieces culture supports human follicle activation and growth to the secondary stage

[Hovatta \(1999\)](#), [Wright \(1999\)](#), [Telfer \(2008\)](#)

- Technically easy
- Cellular interactions intact
- Monitoring during culture impossible
- Culture of empty specimens-possible
- Not for low follicular numbers
- Ovarian stroma inhibitors remain
- Difficult exchanging of O_2 and nutrients
- Risk of transmission of malignant cells

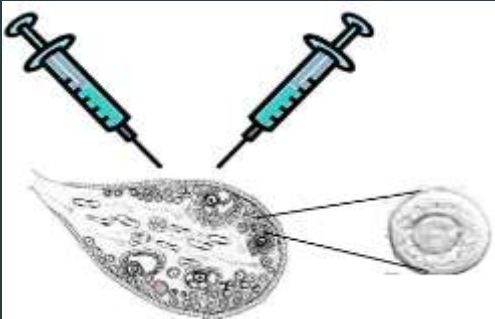
➤ Isolation and culture of growing follicles

([Hovatta 1997](#), [Abir 1999](#), 2001, [Xu 2009](#), [Amorim 2009](#))

- Technically difficult
- Cellular interactions distorted
- Monitoring during culture possible
- Culture of identifiable follicles
- Suitable for low follicular numbers
- Ovarian stroma inhibitors removed

Follicle Isolation Methods

Mechanical Isolation



- Suitable for large follicles in dense tissue
- Integrity basal lamina and theca
- High viability after isolation and culture
- High survival
- Slow and time consuming
- Laborious for dense stroma
- not suitable for small follicles

Telfer 2008

Enzymatic Isolation



- Fast and Easy
- Produce many follicles
- Suitable for small follicles
- Suitable for dense stroma
- Damage to theca cells
- Damage to basal lamina
- Poor survival

Dolman 2006: Liberase blendzyme

Rice 2008: Collagenase + DNase

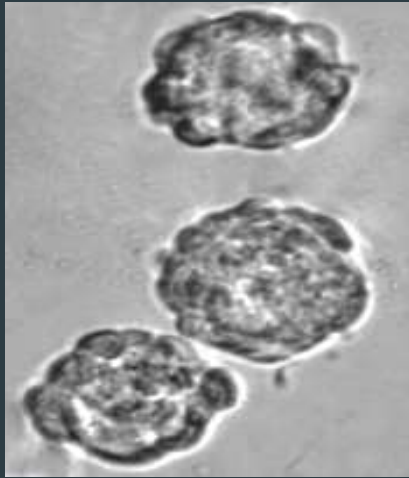
Amorim 2009 : Collagenase

Xu 2009: Collagenase + DNase

Kristensen 2011: Liberase TM+ Collagenase

Vanacker 2013 , Amorim 2013: Liberase DH

Isolated Follicle Culture



2D



GC migration
Stress on gap junction
Premature oocyte extrusion



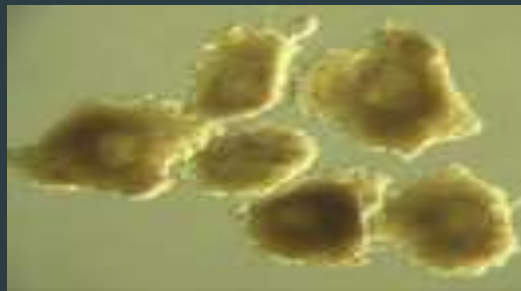
3D



GC growth in all
directions
Less stress on gap
junction
Oocyte remains enclosed



TWO STEP

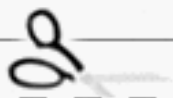


Tissue Engineering Gels for Follicle Culture

Table 2

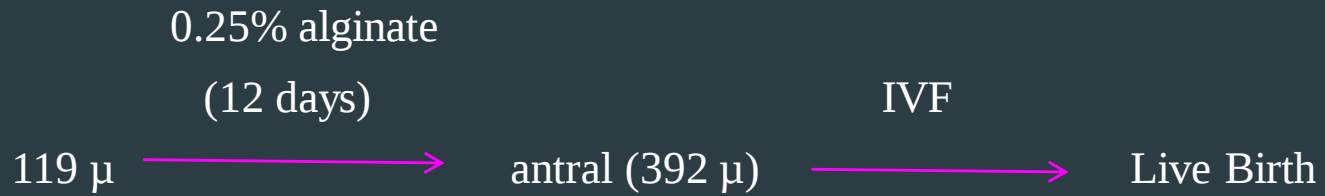
Overview of Candidate Tissue Engineering Gels for Use in Follicle Culture

Material	Origin	Method of Gelation	Method of Digestion	Interactions with Cells
Polyethylene glycol (PEG)	Synthetic	Photocrosslinking, covalent crosslinking via chemical reaction	Dependent on gelation method, many methods not safe for cells	None
Collagen	Natural (animal)	Temperature	Collagenase	Binds integrins
Matrigel	Natural (animal)	Temperature	Collagenase	Binds integrins
Agarose	Natural (plant)	Temperature	Agarase	None
Alginate	Natural (plant)	Ionic crosslinking in presence of divalent cations (i.e., Ca^{2+})	Alginate lyase or EGTA	None



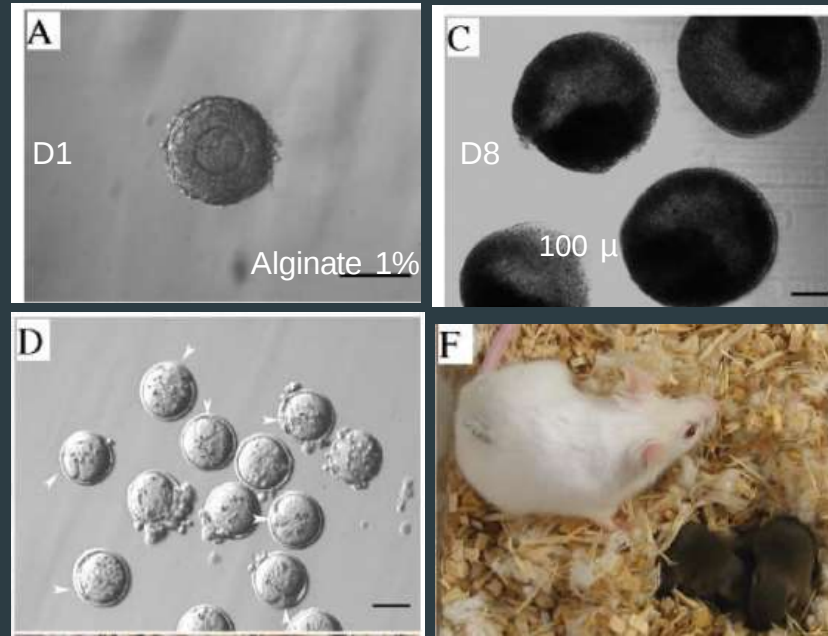
	Advantages	Disadvantages	
Collagen	✓Maintenance of the 3D architecture of the follicle (because of Mechanical properties of this biomaterial) ✓Transparency	• Preparation has not yet been standardized and vary from time to time and from lab to lab; • Submitting follicles to dramatic changes in temperature • Submitting follicles to damage when they are released from gel	Hovatta 1999, Abir 1999, Abir 2001
Alginate	✓Possibility of determining the pores size of the alginate matrix ✓easily releasing follicle by a calcium-chelating agent	• not degradable therefore not suitable for long-term culture	Amorim 2009 (human, 1%) Xu 2009 (human, 0.5%) Hornick 2012 (primate, 2%)
Fibrin - Alginate	✓Higher meiotically competent oocytes (Shikanov 2009) ✓ Degradable during culture and suitable for long-term culture	• Defects in spindle formation, • Chromosome alignment, • Failure to extrude PB (Mainigi et al., 2011)	Shikanov 2009 (mouse, 5%), Xu 2011 (Baboon, 0.5%) Xu 2013 (primate, 0.25%), Amorim 2013 (human ovarian cells)
Hyaluronan	✓optical transparency ✓molding into different shapes ✓ Quickly degraded with enzymes ✓Higher GVBD	• Lower MII	Desai 2012 (mouse, 3mg/ml)
Syntethic hydrogel (PEG & PVA)	✓degrade in response to proteases creating space for follicle expansion ✓oocytes are capable of resuming meiosis and reach the MII phase		Shikanov et al., 2011

3 Dimensional Follicle Development Studies

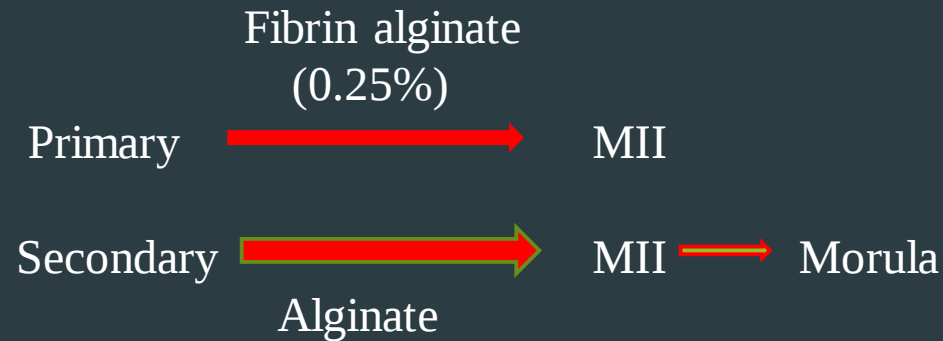
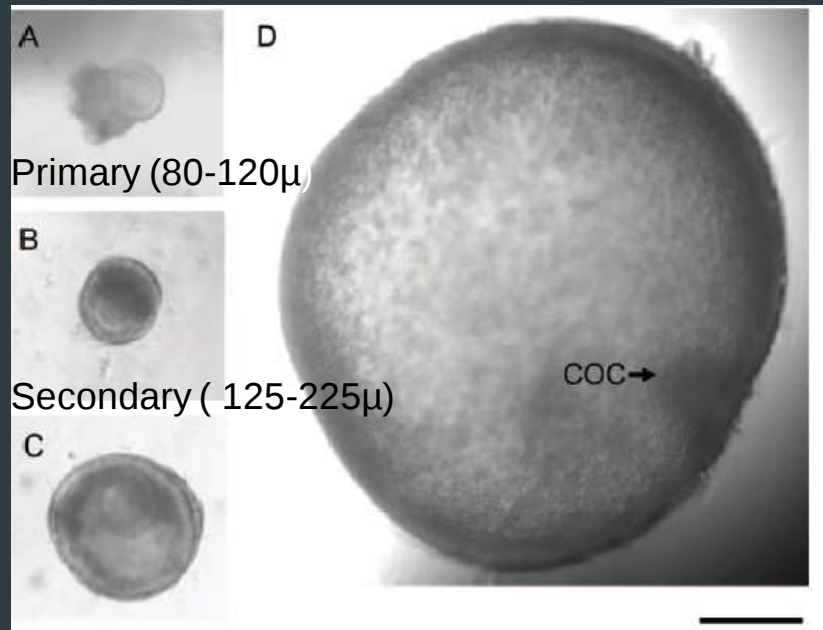


- Theca cell proliferation and steroid production were hindered in follicles cultured in 1.5% alginate.
- IVF and embryo culture revealed that oocytes obtained from **0.25%** alginate retained the **highest developmental** competence.

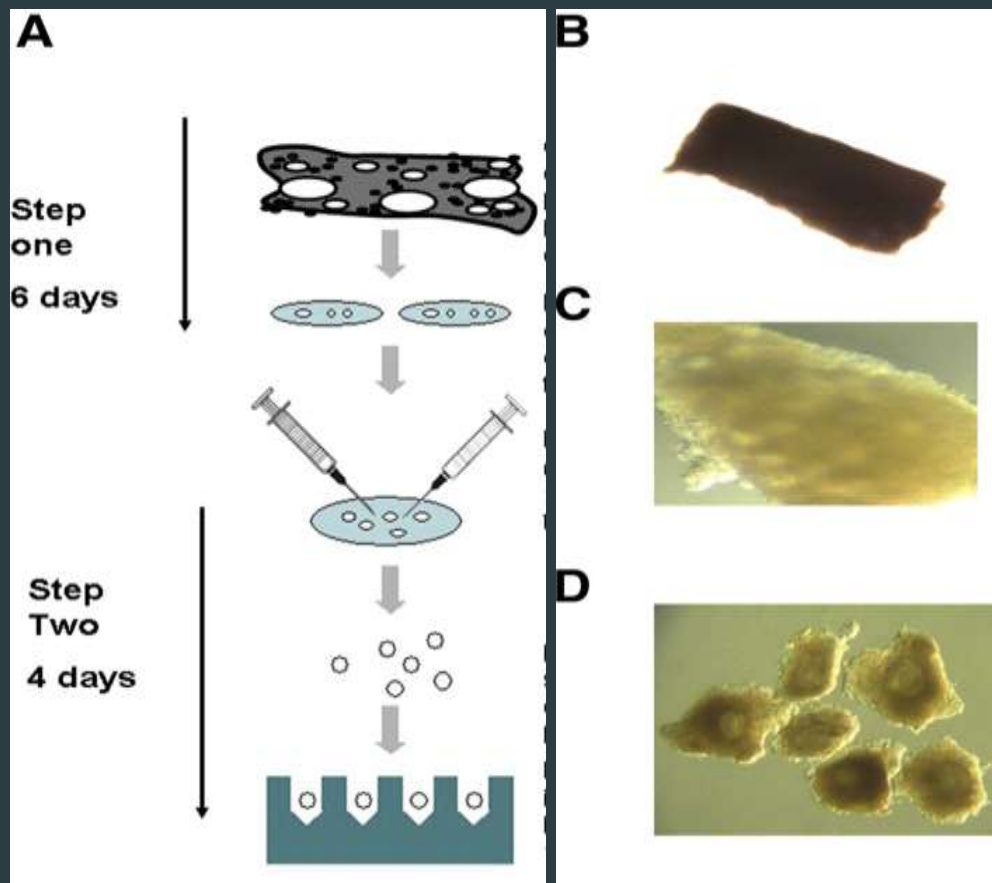




Alginate hydrogel-based **3D** *in vitro* culture of follicles permits **normal growth** and development of **follicles** and oocytes.



Fibrin–alginate improves **macaque primary follicle** development during encapsulated 3D culture in terms of growth, steroidogenesis and can be cultured **to the antral stage** to provide mature oocytes, they represent an additional source of follicles for in vitro follicle maturation.



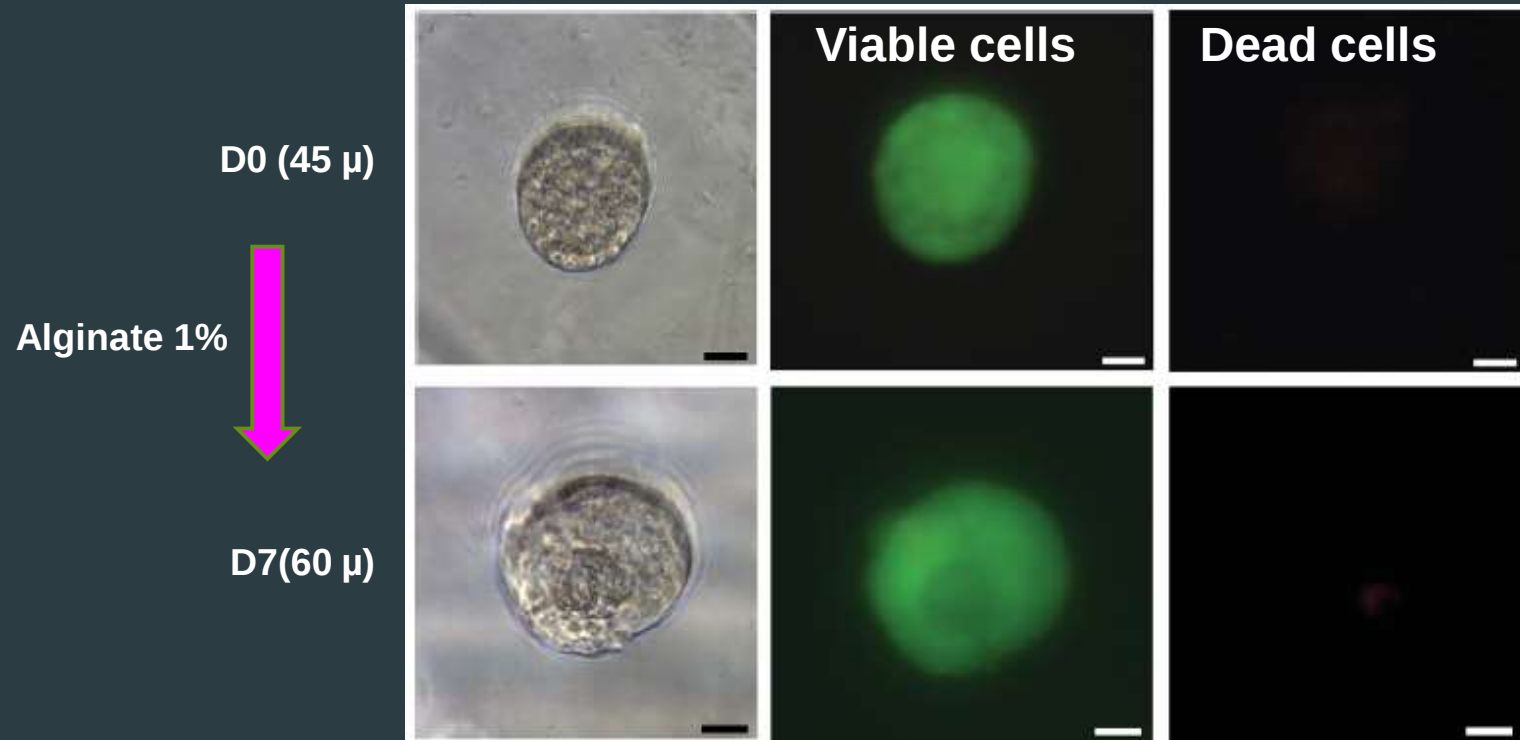
Ovarian tissue

6 days

Preantral follicle isolation
(66-132 μ m) and culture
(In presence of activin)

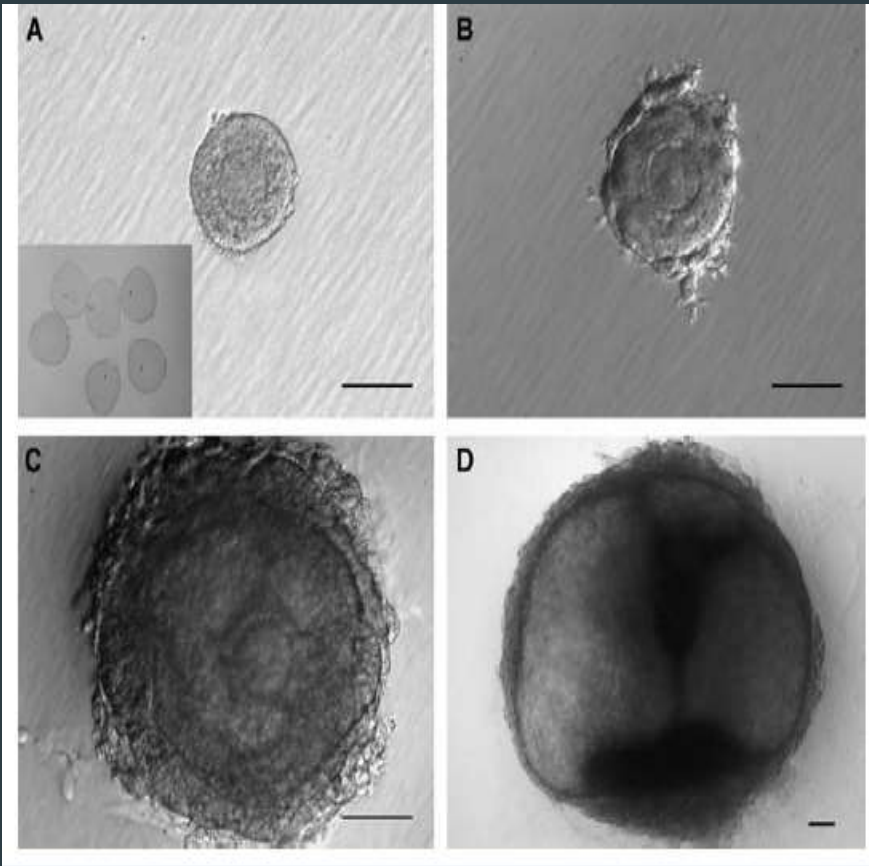
4 days

Antral follicle

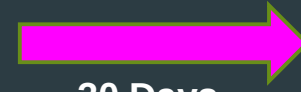


Follicular **viability assessment** by **calcein-AM** and **ethidium homodimer**:

- Cell-permeant calcein-AM is cleaved by esterases in live cells, producing calcein, which is well retained within live cells, generating intense, uniform green fluorescence.
- Ethidium homodimer-I enters cells with damaged membranes, binds to DNA with high affinity, resulting in a 40-fold enhancement of fluorescence, producing bright red fluorescence in dead cells.



135 μ

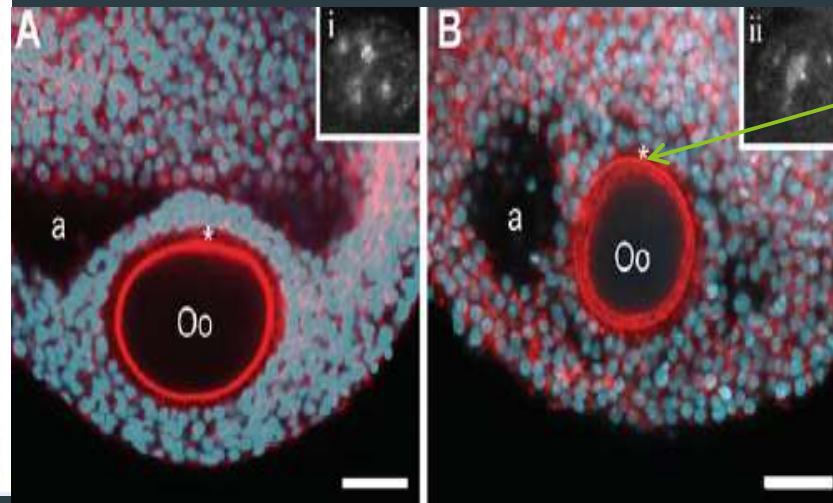


1489 μ

30 Days
Alginate 1%

Fresh

follicle in alginate
for 14 days



TZP

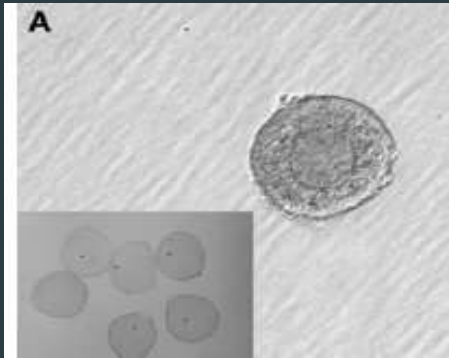
Comparison between follicle development in alginate



Alginate 0.25% (12days)
(xu 2006)

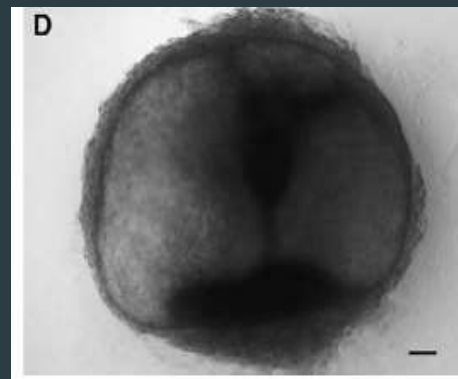
MOUSE

Alginate 1% (8 days)
(xu 2006)



Alginate 0.25% (30 days)
(xu 2013)

PRIMATE



Alginate 1% (30 days)
(xu 2009)

HUMAN

Rescue of already existing follicles

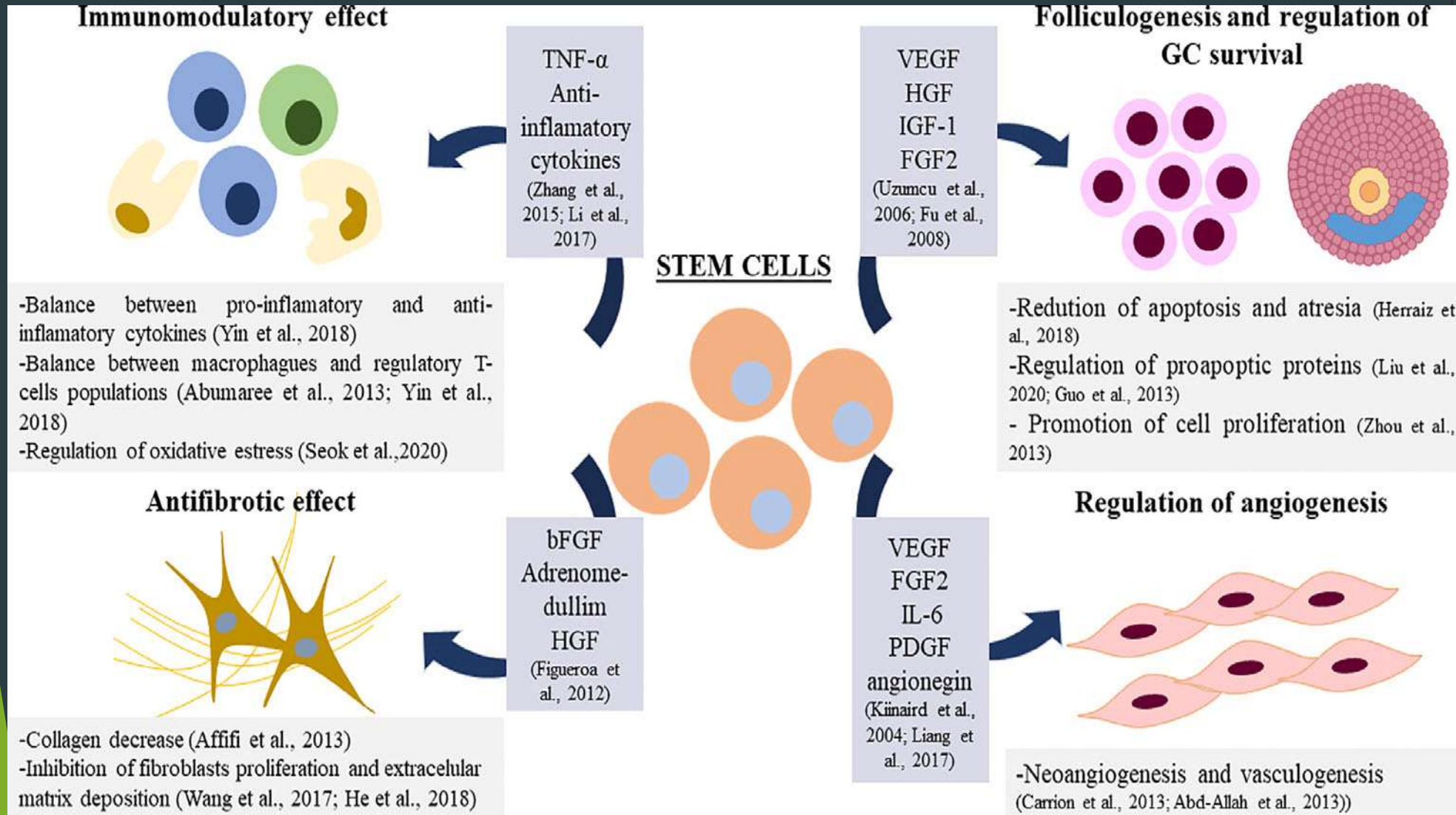
- ▶ Inhibition of molecular pathways by IVA
- ▶ Tissue mechanical fragmentation,
- ▶ Stem cell administration,
- ▶ PRP ovarian injection

- ▶ The **ASCOT (autologous stem cell ovarian transplantation)** technique, involving **infusion of stem cells in the ovarian artery**, which has recently been shown to be **successful** in **low-responder** patients, has now shown it can **achieve pregnancy** in a **woman** with premature ovarian failure (**POF**)
- ▶ **Less invasive** option consisting of **mobilising the stem cells**, and allowing them to **reach the ovaries through the bloodstream** directly
- ▶ **Ovarian follicle development** was achieved with some patients **re-starting menstruation**,
- ▶ **Embryos** were obtained in 2 out of the 10 participants, and even **one pregnancy** through the ASCOT technique
- ▶ More about the **ASCOT technique** development: **3 babies and 6 pregnancies** achieved so far in low-responder patients
- ▶ Transplanting bone marrow-derived stem cells (**BMDSC**) into the **ovarian artery**
- ▶ **Stem cells** mobilized, **extracted from peripheral blood** and **implanted back into the ovary** in order to **reverse the ageing process** and **activate the dormant follicles**.
- ▶ This technique has **improved ovarian function** biomarkers in **81% of low responder** patients

Proposed mechanisms for stem cell therapy

- ▶ SCs (BMDSCs) secrete **soluble factors** and **chemokines** to **regulate angiogenesis, apoptosis, the immune system, and fibrosis** in the ovary
- ▶ **Increase of vascularization** in the ovarian niche, **improve the healing process**
- ▶ **BMDSCs produce** Vascular Endothelial Growth Factor (**VEGF**), Platelet Derived Growth Factor (**PDGF**), Fibroblast Growth Factor-2 (**FGF2**) and Interleukine-6 (**IL-6**) **promote arteriogenesis** in vitro and in vivo
- ▶ In ovarian tissue, **angiogenin** produced by BMDSCs has been reported to play a **positive role** in **angiogenesis** after transplantation
- ▶ **coculture** with BMDSCs **decreases** the levels of the **proapoptotic proteins P21** and **BAX** and **increase** the levels of the **proto-oncogene c-myc** in GCs
- ▶ Different **cytokines** present in the BMDSCs culture medium— **VEGF, HGF, IGF-1**—**are able to decrease apoptosis** of **granulosa cells** in vitro and in vivo and **promote** their **proliferation**
- ▶ the **balance** between different populations of **immune cells** or between **pro-inflammatory** and **anti-inflammatory cytokines** mediated by BMDSCs could underlie this immunomodulatory effect.
- ▶ MSCs have been reported to have **immunoregulatory properties** in the ovarian niche by **regulating populations of macrophages, regulatory T lymphocytes, and associated cytokines**
- ▶ **TNF-alpha** has also been associated with the immunoregulatory function of human MSCs in the ovary
- ▶ In fact, **BMSCs** may **inhibit fibroblasts proliferation** and **decrease** the level of **extracellular matrix deposition**.
- ▶ This **antifibrotic** effect has been associated with certain soluble factors such as **HGF, adrenomedullin** and Basic Fibroblast Growth Factor
- ▶ (bFGF)

Proposed mechanisms for Stem Cell Therapy in ovarian damage



PRP approach

- ▶ **Intraovarian** injection of **autologous PRP** has been recently proposed as an alternative to restore ovarian function in **POI** women.
- ▶ This approach is also based on the **paracrine signaling**, as PRP is a concentrate composed by **platelet-enclosed growth factors**, which could promote **tissue healing, angiogenesis** and **cell growth**
- ▶ Treatment resulted in **restoration of menses**, with presence of **ovarian follicles**
- ▶ Increased in ovarian **vascularization**, promoting follicle development increasing **follicular cell proliferation** and survival
- ▶ **Ovarian and endocrine positive effects** and **live births** have been also reported in several series of patients with impaired ovarian function such as **POR** and **POI women**
- ▶ **Intraovarian administration of PRP** was able to induce an **increase in serum AMH** and a decrease in serum FSH, sufficient to permit **oocyte retrieval 2 months after treatment**
- ▶ **PRP injection**
 - **Improve ovarian reserve biomarkers**, as **AMH** and **AFC increased**
 - **Menstrual** remained stable
 - **FSH and LH** levels were **reduced** and remained **stable**.

- ▶ **Imatinib** is a c-Abl **tyrosine kinase inhibitor** which was found to **protect ovarian follicles** in mice
- ▶ **Tamoxifen**, a selective **estrogen receptor modulator** is well known in the field of treating estrogen-sensitive cancers like breast cancer. **Tamoxifen** was found to **reduce follicle loss** and oocyte
- ▶ Fragmentation
- ▶ **Stabilizing** the anti- Mullerian hormone (**AMH**) level, **increasing** insulin-like growth factor (**IGF-1**) and **counteracting** oxidative stress mediating **apoptosis**
- ▶ Immune modulator **AS101** **prevents follicle activation** by **inhibiting** the **PI3K/PTEN/Akt signaling** pathway
- ▶ **Melatonin, ghrelin** and **mTOR inhibitors** have also been found to **inhibit accelerated activation of primordial** follicles
- ▶ A combination of **melatonin, vitamin E, hyaluronan**, and vascular endothelial growth factor A (**VEGF-A**) increased neovascularization and **reduced apoptosis**
- ▶ **Two pregnancies and one live birth** have been reported after **transplantation of ovarian tissue with a decellularized extracellular matrix (ECM)**

Future trends

Alternative sources of follicles & oocytes

- ▶ **Stem cells** or induced pluripotent stem cells (**iPSCs**) could serve as robust **alternative source** of **ovarian follicles**
- ▶ Pluripotent epiblast-like primordial germ cells (**PGCs**) and undergoes **sexually dimorphic development**, generating **spermatozoa** in **males** and **oocytes** in **females**.
- ▶ **Saitou's team** showed that embryonic stem cells (**ESCs**) and **iPSCs** from **male (XY) mice** could be **transformed** into **epiblast-like cells**, and then **differentiate** into **primordial germ cell-like cells**, with normal **spermatogenic** function and **offspring** production
- ▶ **Hayashi et al.** applied a strategy of '**reconstituted ovaries**', where they **aggregated female PGCs derived from ESCs** with embryonic gonadal somatic cells
- ▶ **Derived PGC-like cells** then **underwent oogenesis** and reached the fully grown germinal vesicle stage 4 weeks and 4 days after transplantation. These **PGC-derived oocytes** were **fertilized** and yielded a **live birth** rate of 3.9%, compared with 12.9% with wild-type ones.
- ▶ **Similar results** were obtained with **female iPSCs** transformed into **fully functional oocytes** which, after growing *in vivo* in reconstituted ovaries, resulted in **fertile offspring** in mice

► Oogonial stem cells (OSCs) in the adult mammalian ovary

- In 2012, **White** *et al.* reported that **OSCs** could be purified from adult **human and mouse ovaries** by **DDX4** antibody-based FACS, and that these isolated OSCs could form **oocytes** after *in vitro* manipulation.
- Since it is **not yet possible to differentiate human ESCs into oocytes**

► Physiological restoration of ovarian endocrine function

- An **immunoisolating** device with **encapsulated ovarian tissue** for restoration of ovarian endocrine function.
- The **TheraCyte** device, for example, **shields implanted ovarian tissue** from the **host immune system** without hindering the exchange of nutrients

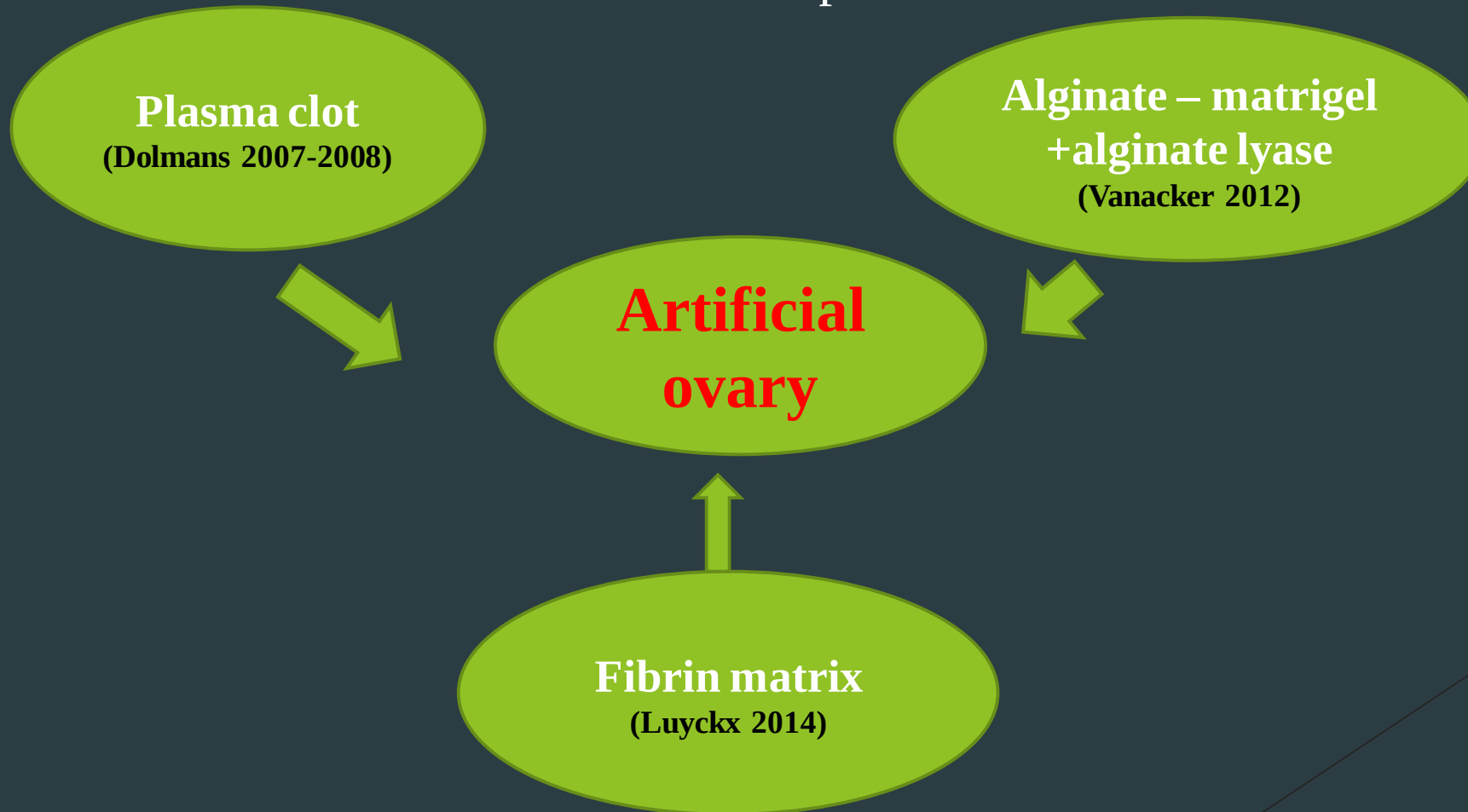
► Reproductive toxicology

- ‘**repro-on-a-chip**’ concept allows **preclinical toxicity screening** in human tissues. The system **mimics** the complexity of *in vivo* human physiology
- Potential for **personalized** medicine.

ARTIFICIAL OVARY

Artificial ovary

- Follicles back to their natural environment
- Restoration of ovarian function
- Natural conception



Bioengineering a transplantable artificial ovary

- ▶ Main objectives of an **artificial ovary**
 - i) Safely **transplant isolated primordial** and **primary** follicles, avoiding inadvertent contamination by malignant cells
 - ii) To **support follicle survival** and development **after transplantation**, ensuring **secretion** of **sex hormones** and **production** of fertilizable **mature oocytes**.
- ▶ Inactivating the enzymatic digestion process every 30 min and **filtering the suspension**, so that **fully isolated follicles** would **not be continuously exposed** to **enzymes**, which could **damage their basement membrane** and result in their death
- ▶ This **filtration-digestion step** was repeated
- ▶ As the **goal of the artificial ovary is to avoid reintroduction of malignant cells** that could potentially reside in transplanted ovarian tissue
- ▶ **Washing** step whereby **isolated follicles** were **transferred three times** from one **medium droplet** to another
- ▶ **Integration of ovarian stromal cells into the artificial ovary** might therefore **re-establish natural communication** between follicles and surrounding stromal cells

- ▶ Human ovarian **preantral follicles** in autologous **plasma clots** and **xenografted** them to immunodeficient mice for **1 week or 5 months**.
- ▶ After **short-term** grafting, the follicles were able to grow to the **secondary stage**, while after **long-term** transplantation, **antral follicles** were also found in the grafts (Dolmans *et al.* 2007, 2008).
- ▶ In spite of these promising results, **plasma clots** have an **inconsistent composition** and **degrade rapidly after grafting**, which can lead to **follicle loss** and variable outcomes.
- ▶ **Pups** were only obtained in **fibrin-VEGF clots**
- ▶ A **functional transplantable artificial ovary** should be able to **restore both endocrine and reproductive functions** after grafting
- ▶ **5 months after xenografting**, **human follicles** were able to **reach the antral stage**.

- ▶ Sustainable engraftment, **natural pregnancy** and the **birth of healthy pups** after **intraovarian microinjection of isolated exogenous follicles** in a chemotherapy-induced POF mouse model
- ▶ To prepare **acellular ovarian scaffolds**, porcine **ovaries** were **diced** then processed using a **series of enzymatic and detergent washes** to **remove immunogenic material**. **Trypsin and EDTA** were used in tandem to **disrupt cell adhesions to the ECM** prior to treatments with **Triton X-100** and **sodium deoxycholate**, which act to **permeabilize cell membranes**
- ▶ Once the tissues were completely **decellularized** they were **frozen, lyophilized** and **milled** into a **powder**
- ▶ **OECM provides mechanical support**, maintains **normal cell morphology**, promotes cell **proliferation** and **steroidogenesis**.
- ▶ OECM can **sequester hormones and growth factors** within the **follicle niche** to **facilitate paracrine and endocrine signaling**.
- ▶ Effects of decellularization on ECM retention, a subset of the most highly expressed **OECM proteins: Collagen I, Collagen IV, laminin and fibronectin**.

A bioprinted ovary to fight sterility

- ▶ Scientists **identify** and **map** the **location** of **structural proteins** in a pig ovary, allowing them to **create** a **bioink** to **bioprint** the **functional human ovaries**.

Bioprinted ovary to restore fertility and hormone production

- Dr. **Laronda**, one of the scientists leading the project, explains: “*The **structural proteins from a pig ovary are the same type of proteins found in humans**, giving us an abundant source for a more complex **bioink** for 3D printing an ovary for human use.*- **Artificial ovary** would be **able** to **respond to natural ovulation signals**, allowing pregnancy to occur.

Transplantable artificial ovary

- ▶ Ovarian biopsy
 - immediately cryopreserved in its entirety
 - isolate preantral follicles that would also be cryopreserved
- ▶ After cancer remission, a second biopsy of ovarian tissue would be collected in order to isolate the required ovarian cells.
- ▶ Indeed, while chemotherapy can affect oocyte survival and development, it does not seem to have a negative impact on ovarian cells
- ▶ Autotransplant cryopreserved isolated preantral follicles and freshly isolated ovarian cells back to the patient, it is necessary to use an appropriate scaffold that
 - encapsulate, protect and maintain the 3D structure of the follicles
 - able to degrade to allow follicle development, cell migration and proliferation and vessel formation
- ▶ Polymers
 - Natural Alginate, Collagen, Fibrin, Plasma clot, Decellularized ovarian ECM
 - Synthetic Poly(ethylene glycol)

► Natural polymers

- **Biocompatible** and **biodegradable**,
- Presence of **biofunctional molecules**, they usually show **superior interaction with cells**
- **Cellular adhesion, migration, proliferation** and **differentiation**
- **Lack sufficient mechanical strength**, which can make them **difficult to handle**
- **Controlling their degradation rate** may prove **challenging**.

► Synthetic polymers

- **Biocompatible**
- Their **mechanical properties** (**degradation rate, porosity** and **elasticity**) can be tailored
- **Essential molecules for cell adhesion, bioactive compounds** can be **integrated** to stimulate cellular adhesion and hence proliferation and differentiation
- **Control of degradation**
- Can **degrade** into products that may be **toxic** at high concentrations

- ▶ Grafting **isolated murine preantral follicles** inside these different matrices, they obtained **pups** only from **fibrin-VEGF** clots
- ▶ **Platelet lysate** is known to be a source of **growth factors**, including **angiogenic cytokines**, such as **VEGF**, **PDGF** and **FGF**
- ▶ **Laronda et al.** assembled **scaffolds** from **decellularized bovine** and **human ovarian tissue** to graft primary murine ovarian cells

- ▶ **ECM scaffold** is **obtained** by **decellularizing** the tissue by **physical, chemical**, or combinative methods.
- ▶ The **type** of decellularization **agents** and **duration** of exposure have effect on structure and biocompatibility of the ECM scaffold
- ▶ **Human** and **bovine** ovarian tissue
 - Sodium dodecyl sulfate (**SDS**)
 - **SDS-Triton**,
 - **Sodium lauryl ester sulfate (SLE)**
- ▶ **SDS**
 - **Ionic surfactant**
 - **Successfully eliminates cells'** nuclear materials
 - **Can be harmful** to the structural of **ECM** and **signaling proteins**
 - It is a **cytotoxic agent** and **irremovable** due to its **ionic nature**
- ▶ **Triton X-100** is a **nonionic surfactant**
 - Liu et al. decreased SDS exposure time and decellularized ovarian tissue by **SDS-Triton** protocol
 - Elimination of residual DNA was lower than SDS alone.

- ▶ **Ammonium hydroxide** is a decellularizing agent commonly used in **combination with SDS**.
 - Ammonium **retains glycosaminoglycans (GAGs) and viscoelasticity** of the **decellularized tissue** by counteracting the negative charge of collagen
- ▶ **SDS-Triton-Ammonium** group showed **less residual DNA** content with **higher cytobiocompatibility** for follicles when compared with other groups

- ▶ Multilayer cell spheroids were then produced by forming **G cell spheroids** in **AggreWell** (STEMCELL Technologies, Vancouver, Canada).
- ▶ This procedure was followed by **Matrigel (M) coating** and/or **T cell loading onto the spheroids** to mimic the native ovarian follicle

Culture in a 3D channel network hydrogel

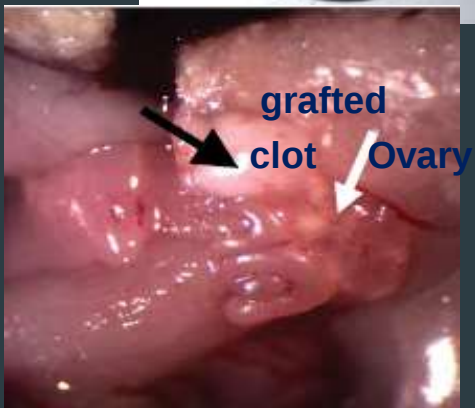
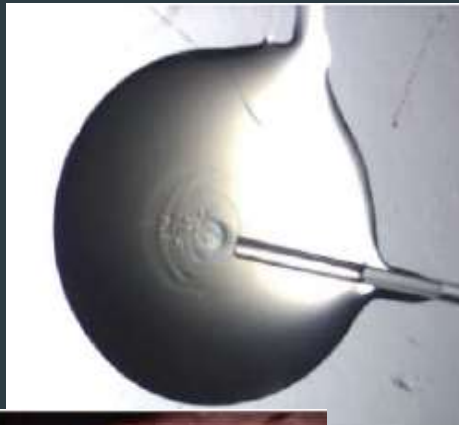
- ▶ Blood circulation is the major route used to deliver systemic hormone therapy; therefore, highly interconnected, **dense channel networks** were generated to overcome the 200- μm diffusion limit of a **3D hydrogel (vascularized hydrogel: VH)** with the culture of the **artificial follicles**.
- ▶ A **chamber** was cast by pouring polydimethylsiloxane (**PDMS**) into a **3D printed mold** to act as a container for the **VH** in a perfusion setting, with connection to a pump.
- ▶ Using the **principle of a cotton candy machine**, threads of dense, thermo-responsive poly(N-isopropylacrylamide) (**PNIPAM**) **fibers** were produced by **spinning** and then placed into the **PDMS chamber**.
- ▶ Next, to create a **hydrogel material**, a **gelatin solution was mixed with G(M) T spheroids**, poured to **cover the fiber threads** in the chamber, and subsequently subjected to **enzyme-crosslinked gelation**. Last, the **PNIPAM fibers were dissolved** at **37°C** to form **perfusable channel networks** in the gelatin gel.
- ▶ This **VH** served as a **tunable** in vitro and in vivo culture platform for the cell spheroids by continuously **perfusing culture medium** into **channel networks**. A **dynamic culture condition**, established with a medium **flow rate** of **20 $\mu\text{l}/\text{min}$** using a **peristaltic pump**,

- ▶ To create the **ovary**, the researchers formed **honeycombs** of **theca cells**, donated by reproductive-age (25-46) patients at the hospital.
- ▶ After the theca cells grew into the honeycomb shape, **spherical clumps** of donated **granulosa cells** were **inserted** into the **holes** of the honeycomb **together** with **oocytes**.
- ▶ In a **couple days** the theca cells **enveloped** the granulosa and eggs, mimicking a real ovary.
- ▶ In experiments the structure was **able** to **nurture eggs** from the “early antral follicle” stage **to full maturity**.

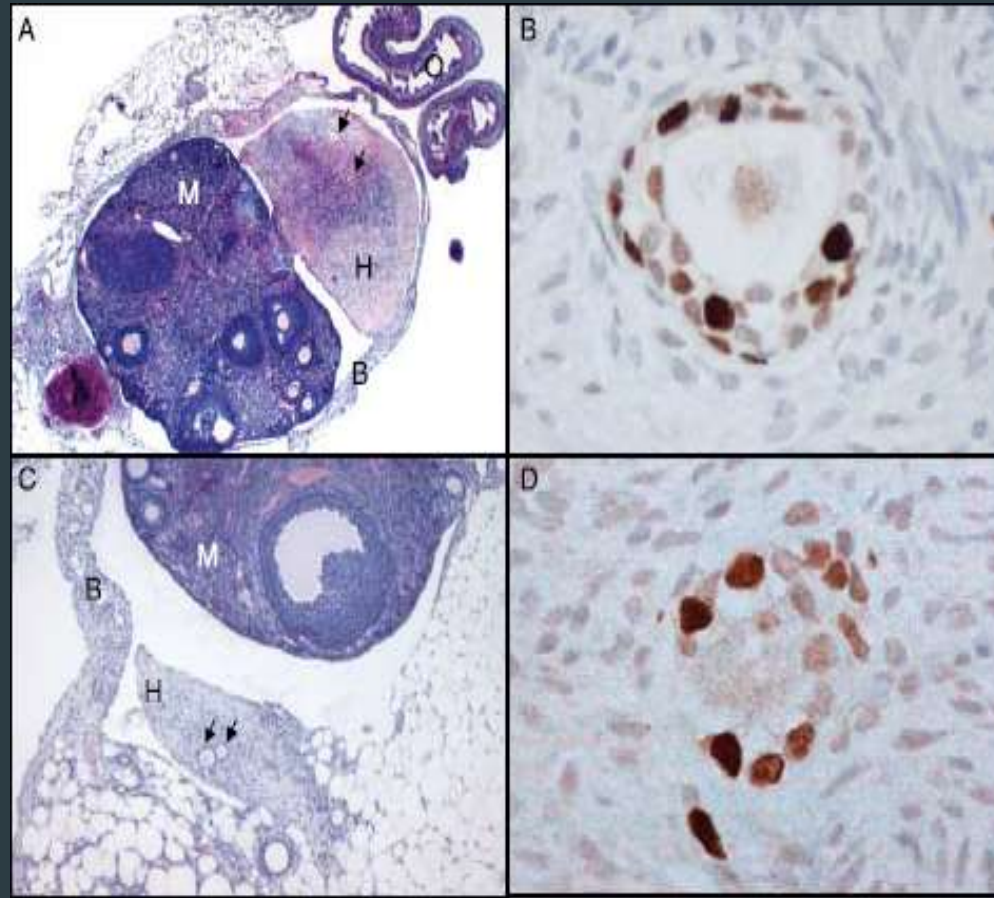
Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice

Marie-Madeleine Dolmans, Belen Martinez-Madrid, Elodie Gadisseux, Yves Guiot¹, Wu Yuan Yuan, Antoine Torre, Alessandra Camboni, Anne Van Langendonckt and Jacques Donnez

Follicular activation after transplantation of grafted fragments and isolated follicles.



Cortical fragment grafted after 7 days



Clot containing follicles grafted after 7 days

Substances for revascularization

- ▶ Ischemia is a major cause of follicular loss following transplantation, with reoxygenation taking approximately 4–5 days.
- ▶ Tissues predominantly depend on anaerobic metabolism early in the post transplantation period; the shift to aerobic metabolism occurs when oxygenation is provided by neo-vascularization.
- ▶ Following the transplantation of frozen/ thawed OT into SCID mice, approximately 28% of primordial follicles survived the procedure; the remaining follicles died due to ischemic damage
- ▶ Transplantation site could be treated with vascular endothelial growth factor (VEGF) and stromal cells enriched in CD34 cells to improve angiogenesis.
- ▶ Combined VEGF and bFGF administration induced angiogenesis, reduced apoptosis and fibrosis, and increased the survival of transplanted human OT in a rabbit model
- ▶ Treatment with melatonin, or OT incubation with hyaluronan-rich biological glue, in addition to VEGF-A and vitamin E may improve graft survival.
- ▶ Zhang et al. supplemented the freezing medium with FSH, resulting in increased revascularization and survival of ovarian grafts following vitrification in mice

- ▶ High adipose-derived stem cells (**ASCs**) concentrations have been shown to **increase** the **human vessel area over time**.
- ▶ The **ability of ASCs to stimulate human angiogenesis** through differentiation and growth factor secretion appears to **depend** on both **cell concentration** and **time**
- ▶ **ASCs** grown using a **fibrin scaffold** enhancing vascularization following transplantation of human OT. Promoting **revascularization** by combining OT with **angiogenic factors** or **pro-endothelial stem cells** is another approach

- ▶ Artificial ovary is a novel experimental technology that aims to produce mature oocytes ready for in vitro fertilization through an ex vivo multistep strategy that includes sequential in vitro cultures of ovarian tissue, follicles, and oocytes
- ▶ Although artificial ovary has been successful in producing mature oocytes only in mice
- ▶ Researchers from the Rigshospitalet in Copenhagen, Denmark, report today that they have for the first time isolated and grown human follicles to a point of "biofunctionality" on a bioengineered "decellularised" ovarian tissue
- ▶ 2019, Dr. Laronda, with three other collaborators, received a patent for creation of an artificial ovary.
- ▶ So far, she and colleagues have 3D printed an artificial ovary that they implanted into a sterile mouse that become pregnant and had live pups.
- ▶ These groundbreaking results were published in 2017 in Nature Communications.
- ▶ "The structural proteins from a pig ovary are the same type of proteins found in humans, giving us an abundant source for a more complex bio-ink for 3D printing an ovary for human use,"

- ▶ Dr .Susanne Pors from the Rigshospitalet's said: "This is the first time that **isolated human follicles** have survived in a **decellularised human scaffold**,
- ▶ This **will help the development** of an **ovary-specific bio-ink** that can be used to three-dimensionally (3-D) print artificial, **implantable human ovaries** that will allow infertile women to bear children.
- ▶ Proteomic analyses of decellularized porcine ovaries identified new **matrisome proteins** and spatial differences across and within ovarian compartments
- ▶ All tissues and organs have a specific **combination of ECM and ECM-associated proteins** — called the **matrisome** — and a specific structure and organization.
- ▶ Thus, an **organ-specific bio-ink** must **respect** several **chemical, physical, and biological constraints**
- ▶ The **ovary** is divided into two visibly distinct compartments: the **external, denser cortex**, containing the **reserve of follicles** — fluid-filled structures in which the egg matures — and the **internal medulla**, containing most of the **activated and growing follicles**
- ▶ **Identification of 42 structural proteins** that were present at significantly different levels **across ovarian depths**

Future perspective

► Artificial ovary

- A valuable alternative to restore fertility in cancer patients who cannot undergo transplantation of frozen-thawed ovarian tissue
- Toxicology studies to evaluate the impact of different drugs and chemicals on follicle survival and development
- Research tool to investigate folliculogenesis

- To determine the ovarian cortical and medullary matrisome signatures by identifying and mapping structural proteins in the two main ovarian compartments (cortical and medullar)
- Application of the ovarian structural proteins to engineer a biological scaffold capable of supporting a bank of potential eggs and hormone producing cells.
- To develop an ovary-specific bio-ink that can be used to three-dimensionally (3-D) print artificial, implantable human ovaries
- Once implanted, the artificial ovary would respond to natural cues for ovulation, enabling pregnancy, led to live birth
- Cooperation of an interdisciplinary team of researchers made up of engineers and materials scientists and gynaecologists and natural scientists

A young child with blonde hair, wearing a red long-sleeved shirt, blue jeans, and a blue backpack, is walking away from the camera down a paved path. The path is covered with fallen autumn leaves. The path is flanked by grassy areas with some dry, brown vegetation. In the background, a bright light source, possibly the sun, is visible at the end of the path, creating a lens flare effect. The overall scene is peaceful and suggests a journey or exploration.

It's just the beginning

THANK YOU

