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**, **$1\text{-}2 \times 10^6$**
- 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***

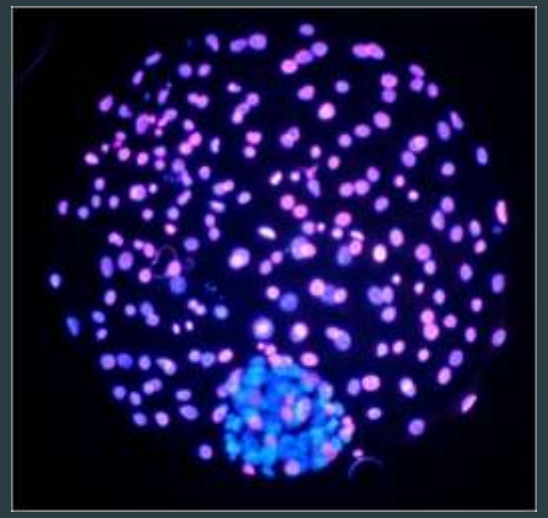
- **Primary** modality for fertility preservation, **since the 1980s**
- **No effect** on **miscarriage**, **implantation rates,** or **live birth**
- **Disadvantage** : **ovarian stimulation** medications
- **Ovarian stimulation** is a **particular concern** for patients with **hormonal sensitive tumors**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

- ***Oocyte Cryopreservation***

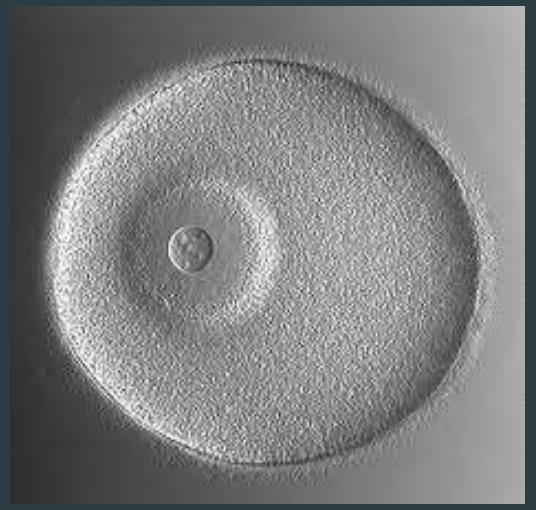
- ***Ovarian Tissue Cryopreservation***

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

➢ Surgical removal of ovarian **cortical** strips → Transplantation / Organ culture

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**; f**ollicles 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

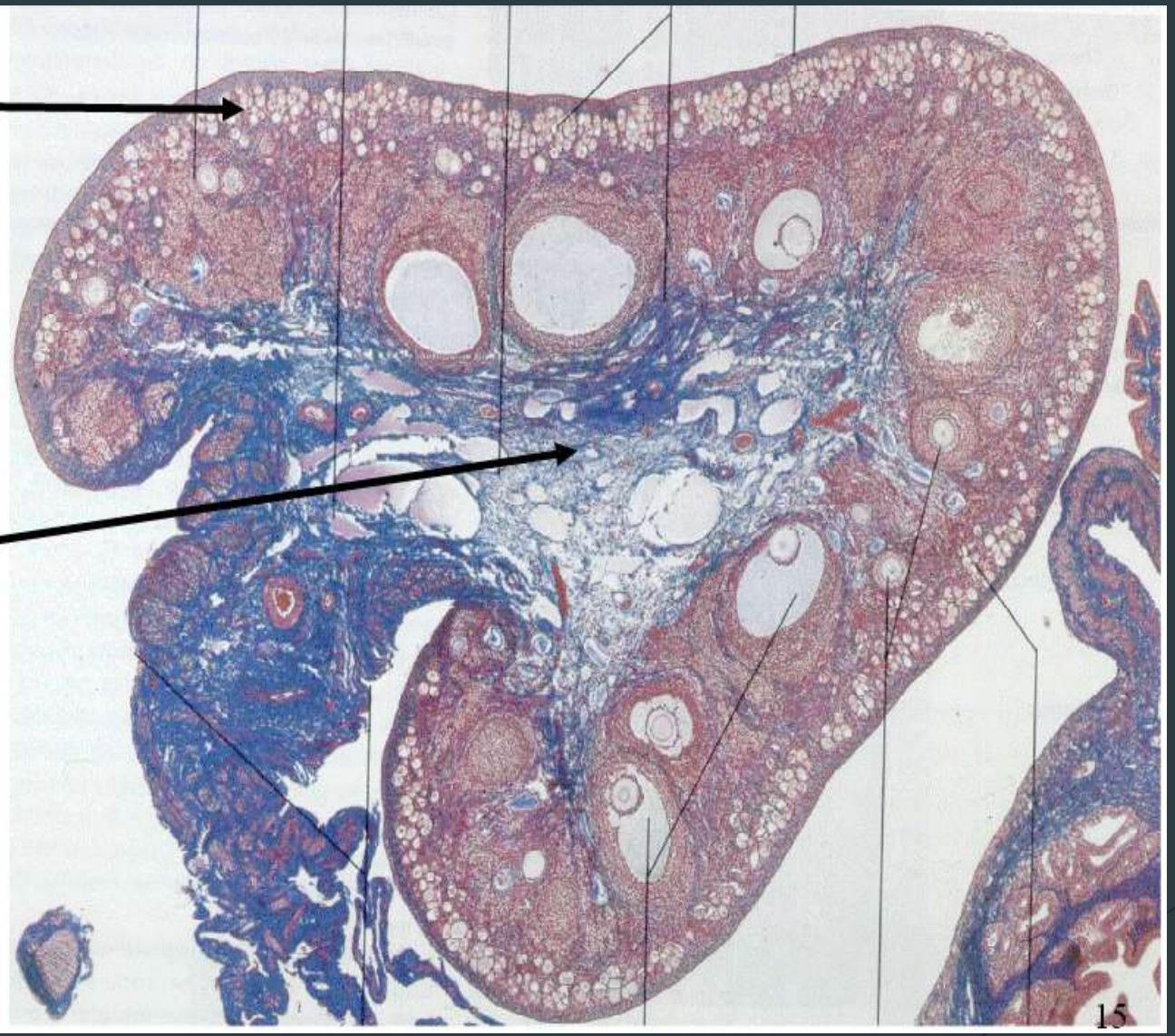
Ovarian Tissue Section

Cortex

Contains 90% of the follicular reserve (primordial follicles)

Medulla

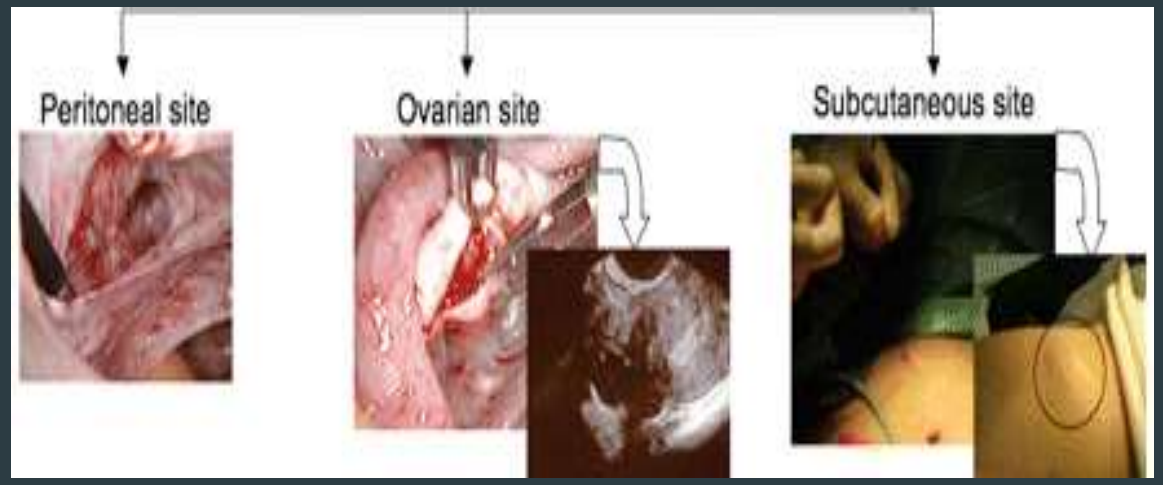
Contains the growing follicles



Cryopreservation of ovarian tissue

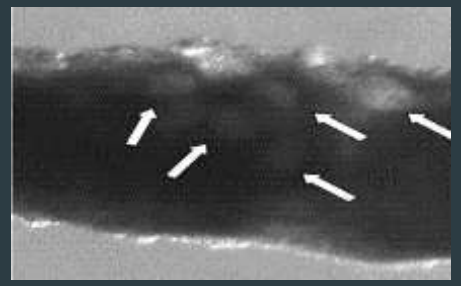
- **Cortical ovarian biopsy**

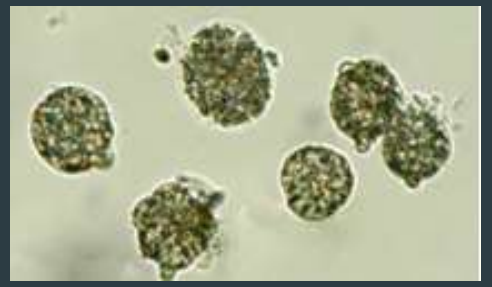
Transplantation



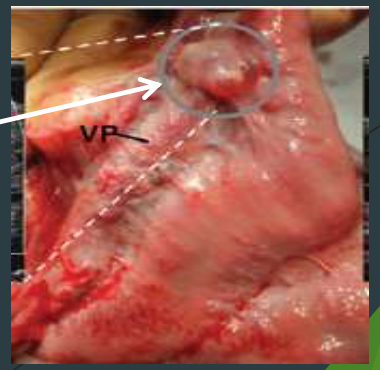
Risk of mamlignant cell transmission

Culture and follicle isolation





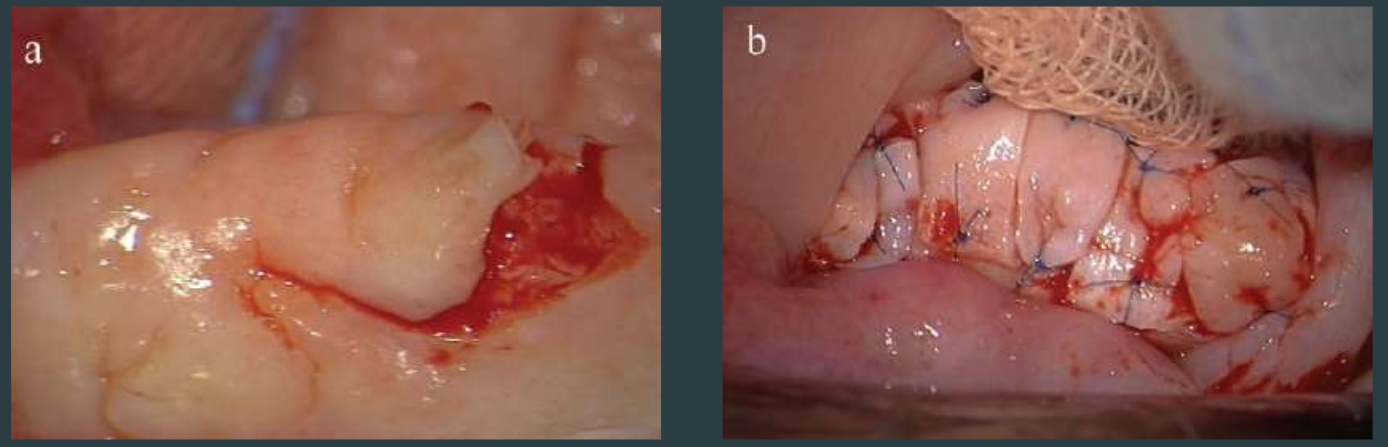
Avoid transmission of malignant cells



- **Whole ovary transplantation**
  (live birth in sheep; 2014)

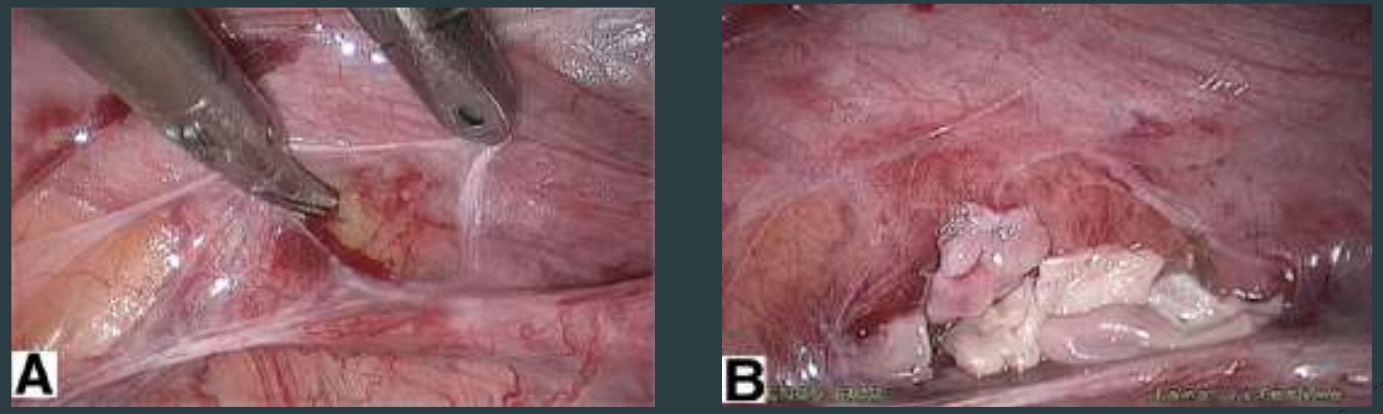
Orthotopic Transplantation

- IF OVARY PRESENT: Decortication and suture of ovarian fragments



(Donnez, 2006)

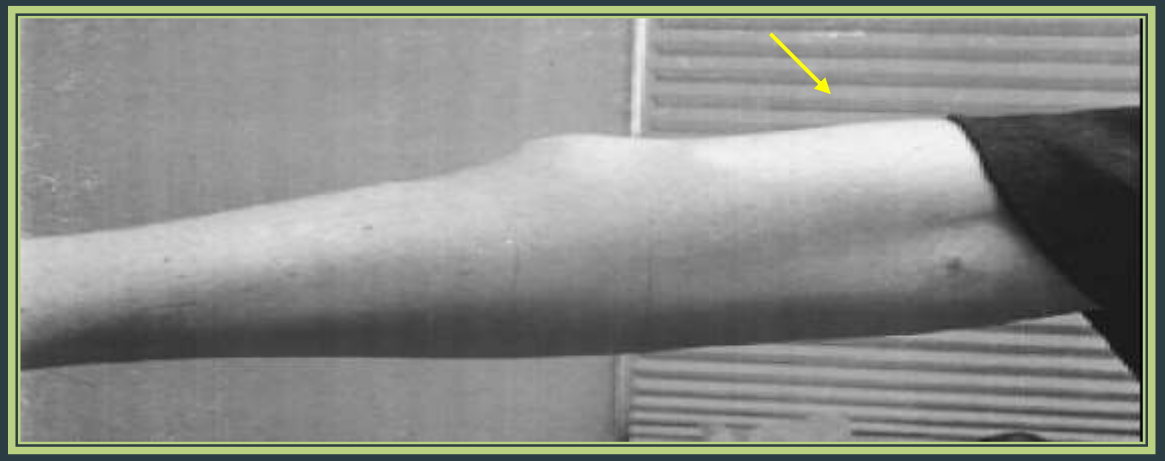
- IF OVARY ABSENT: Peritoneal window



(Donnez, 2012)

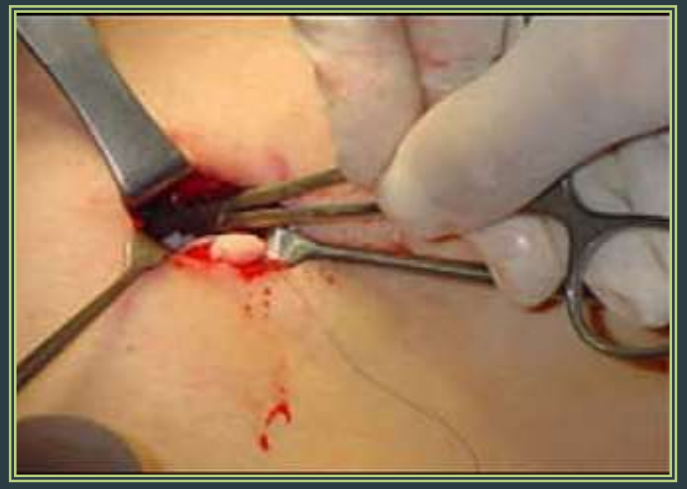
Heterotopic Transplantation

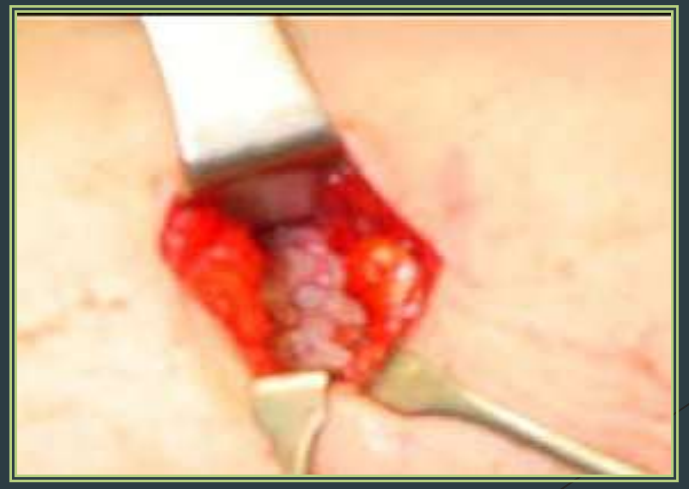
- **Subcutaneous tissue of forearm**



(Oktay, 2003)

- **Subcutaneous tissue of abdominal wall**



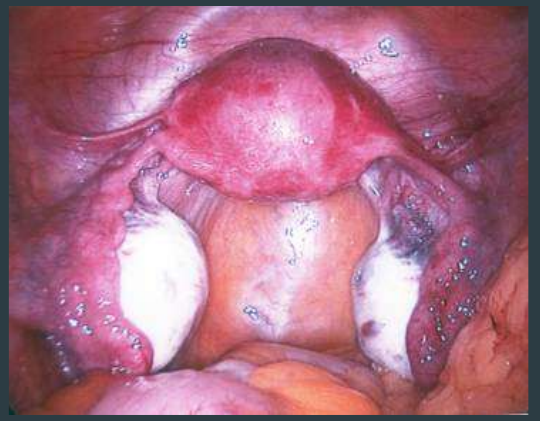


(Kim, 2009)

Ovarian Transplantation

CORTEX SLICES

ORTHOTOPIC TRANSPLANT



RESUMPTION OF CYCLICITY

NATURAL CONCEPTION

HETEROTOPIC TRANSPLANT

EGG RETRIEVAL

IN VITRO FERTILIZATION

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**

- **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

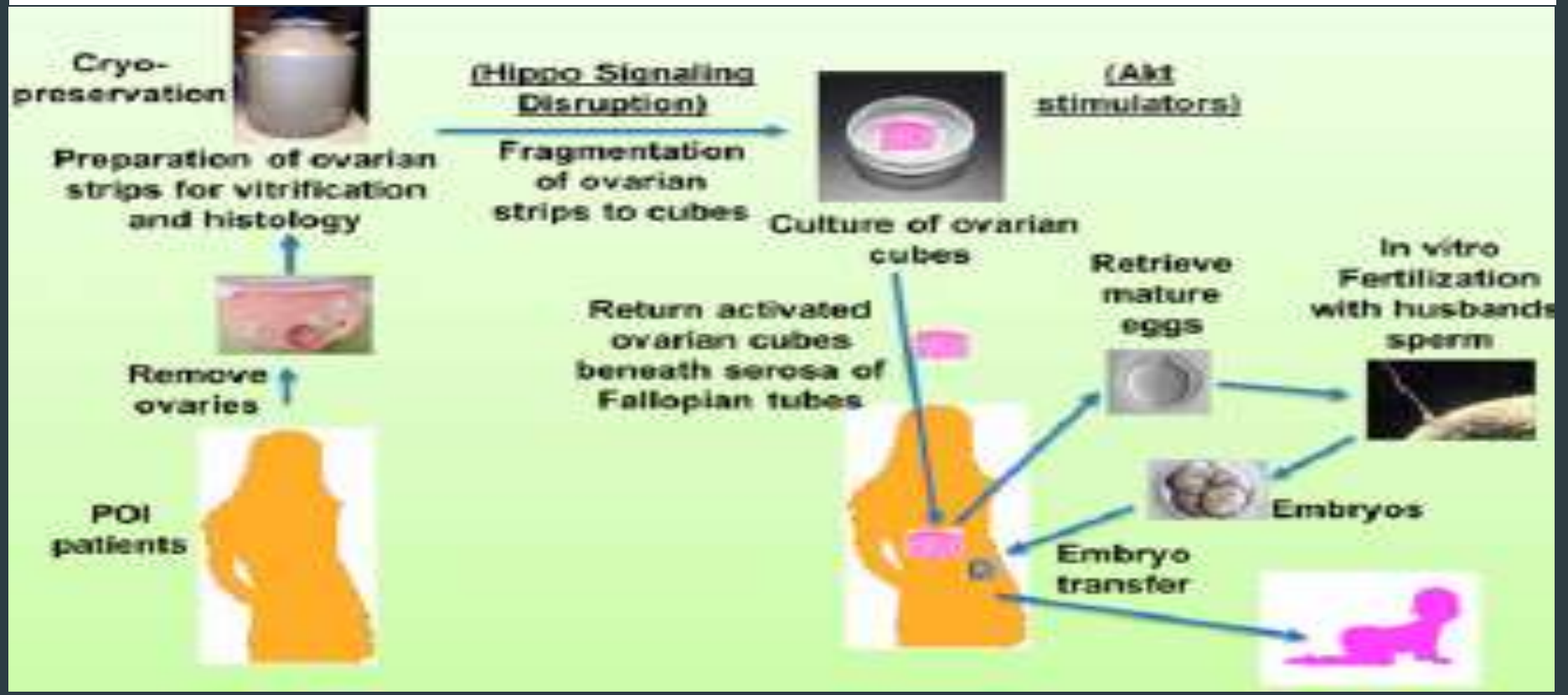
  L-15 + 10% FCS + 0.5 mol/l DMSO

  L-15 + 10% FCS

2013

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

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**

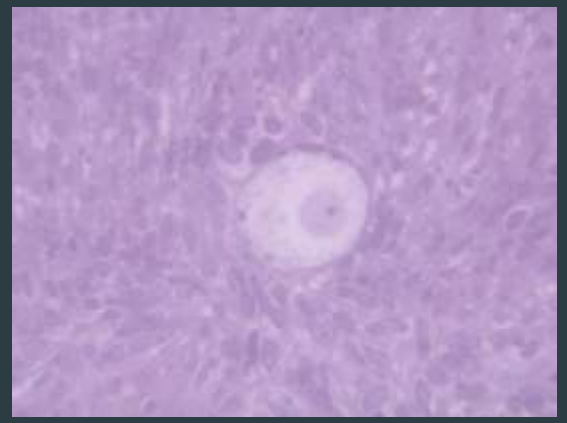
IN VIVO FACTORS INVOLVED IN FOLLICLE DEVELOPMENT

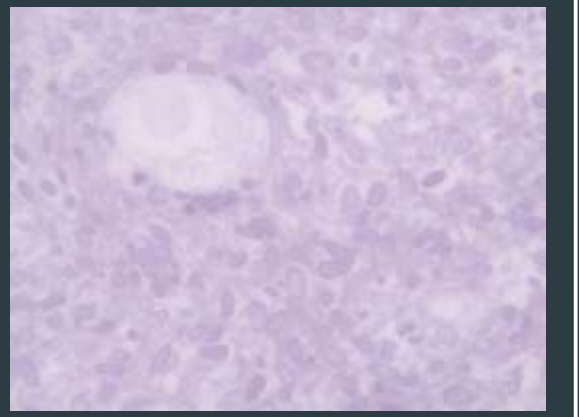
Oktem, 2008 Picton, 2008

Oogonia to primordial follicle transition

FIG 1A
DAZ1A
NGF

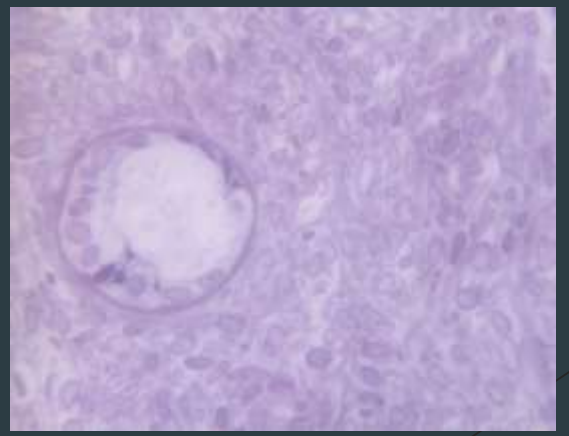
SPO11
DMC1
MSHS





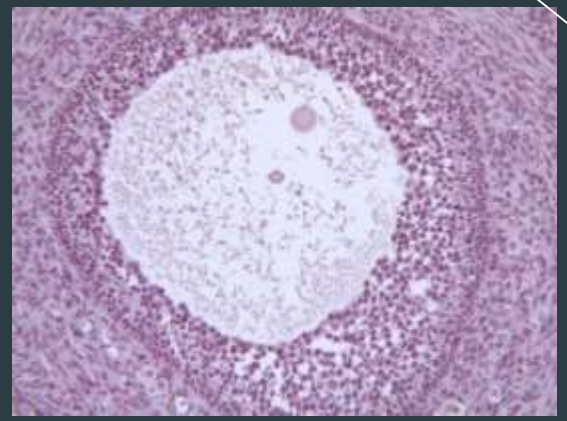
Primordial to primary follicle transition

KL
LIF
FGF-2
FGF-7
BMP-4
BMP-7
BMP-15
GDF-9
IGF
NOBOX

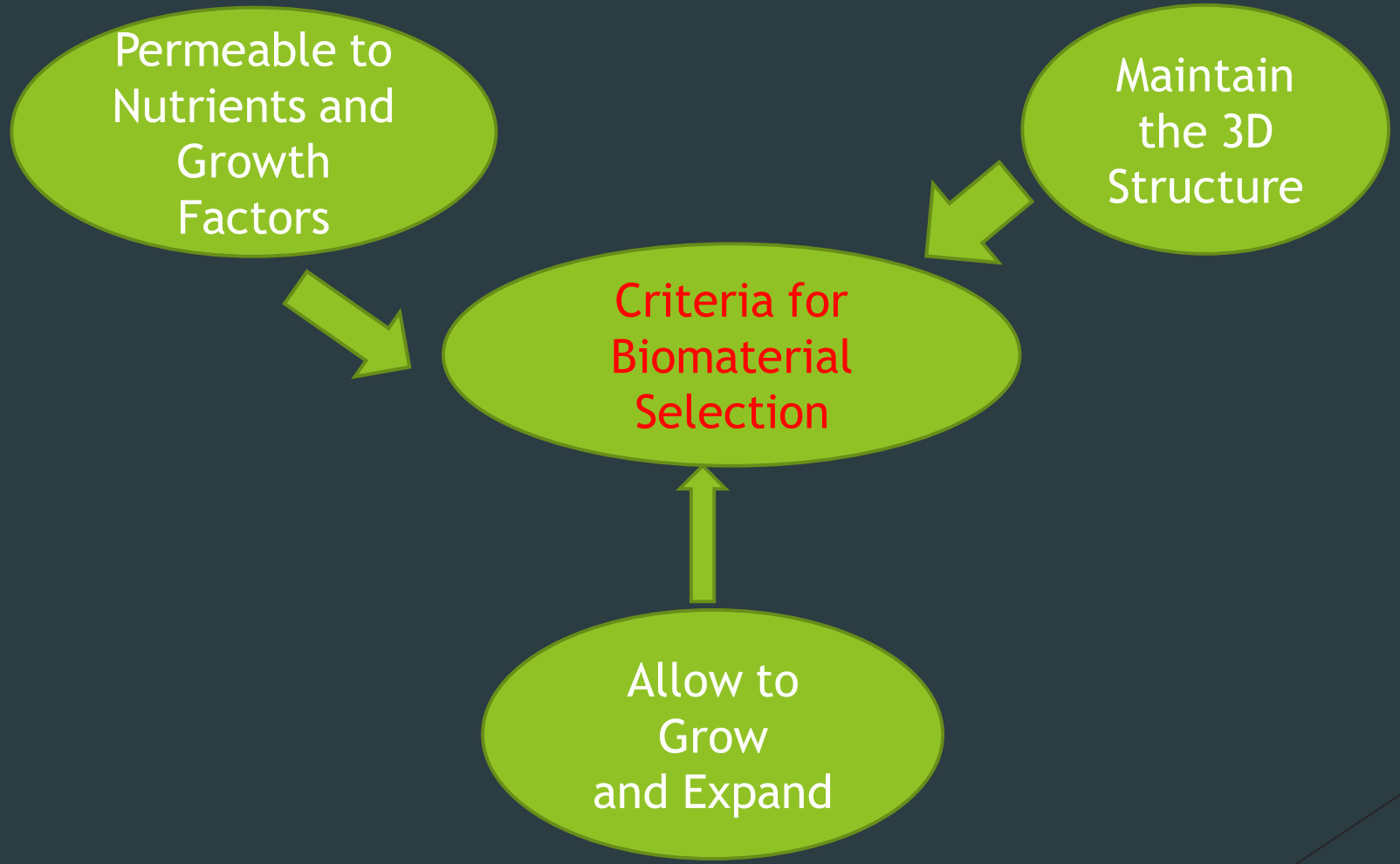


Progression to antral stage

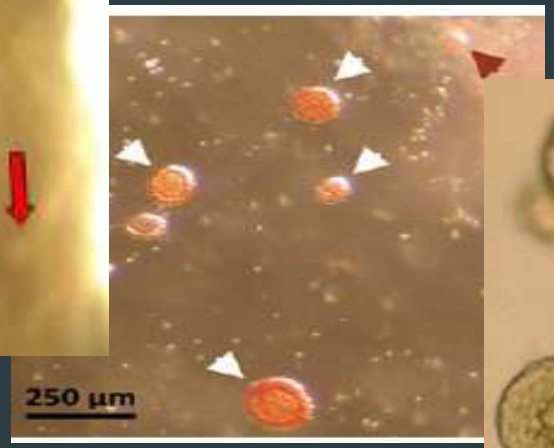
BMP-4
BMP-7
BMP-15
GDF-9
TGF-B
Activin

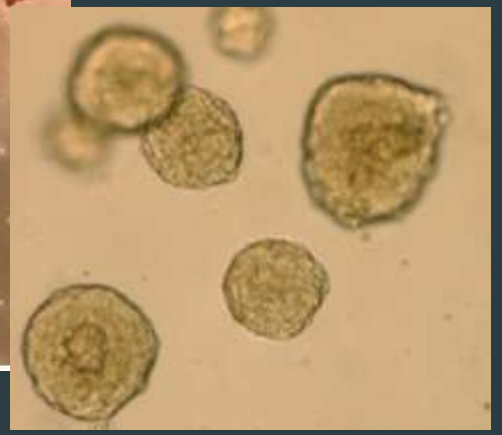


3D System for follicle culture



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

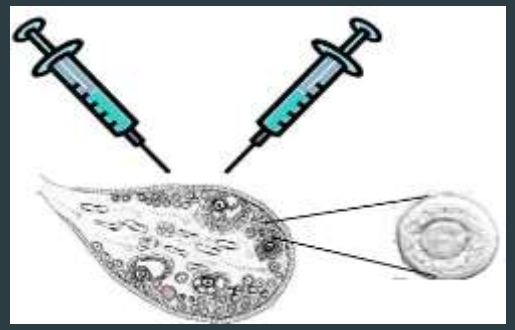
(Hovatta 1997 , Abir 1999 , 2001 , Xu 2009, Amorim 2009)

➢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

Collagenase & DNase

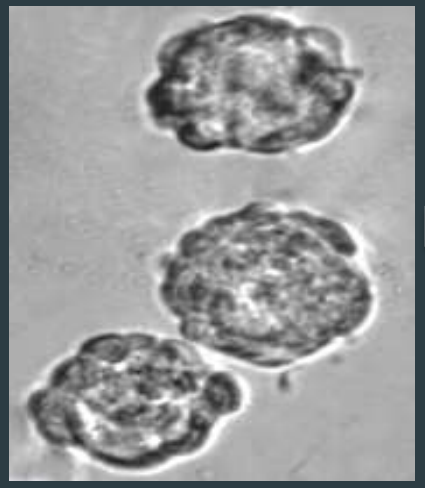
Liberase

- **Fast and Easy**
- **Produce many follicles**
- **Suitable for small follicles**
- **Suitable for dence stroma**

- **Damage to theca cells**
- **Damage to basal lamina**
- **Poor survival**

Dolman 2006: Liberaze blendenzyme
Rice 2008: Collagenzae + DNase
Amorim 2009 : Collagenaze
Xu 2009: Collagenaze + DNase
Kristensen 2011: Liberaze TM+ Collagenzae
Vanacker 2013 , Amorim 2013: Liberaze 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

Desai, 2010

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

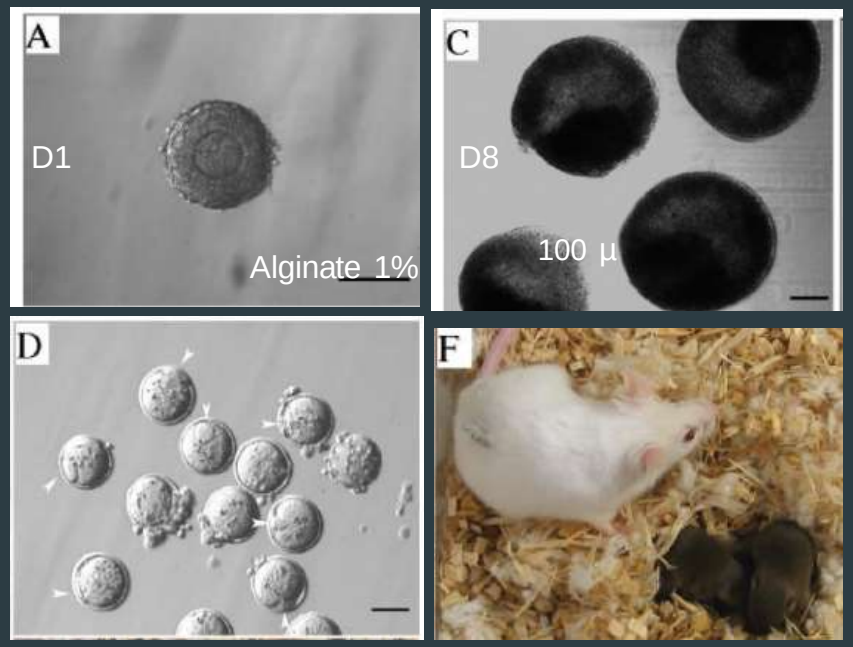
0.25% alginate

(12 days)

IVF

119 µ → antral (392 µ) → Live Birth

- 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.



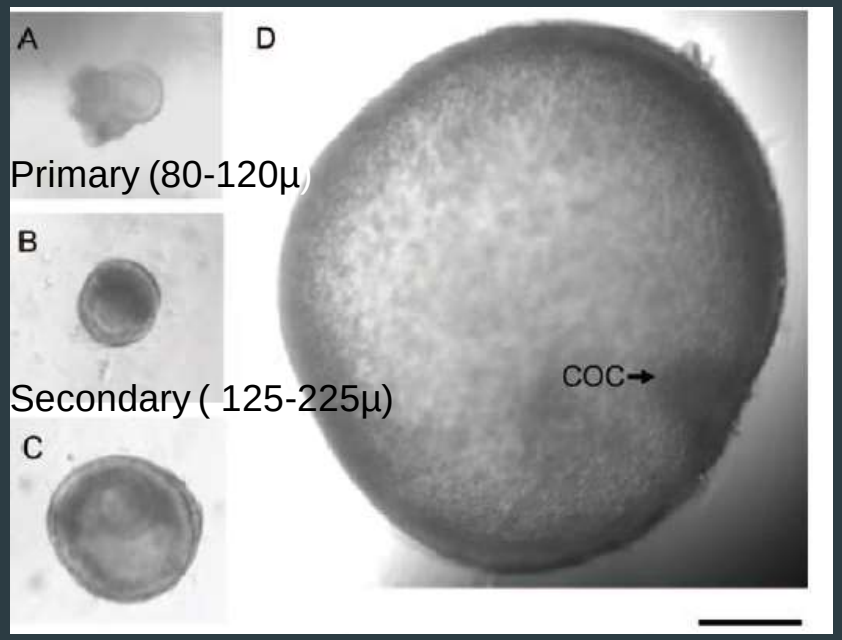
1% alginate
(8 days)

150 µ ⟶ antral (350µ) ⟶ Live Birth

IVF

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

2013



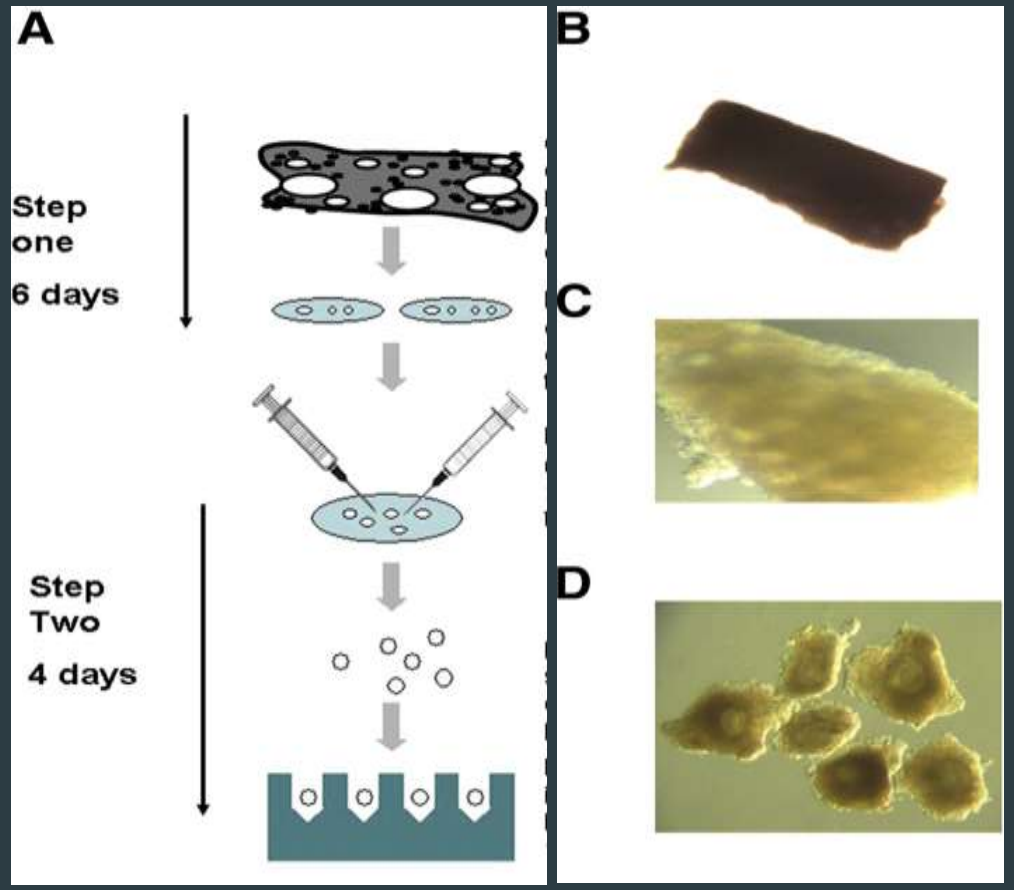
Fibrin alginate (0.25%)

Primary → MII

Secondary → MII → Morula

Alginate

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.



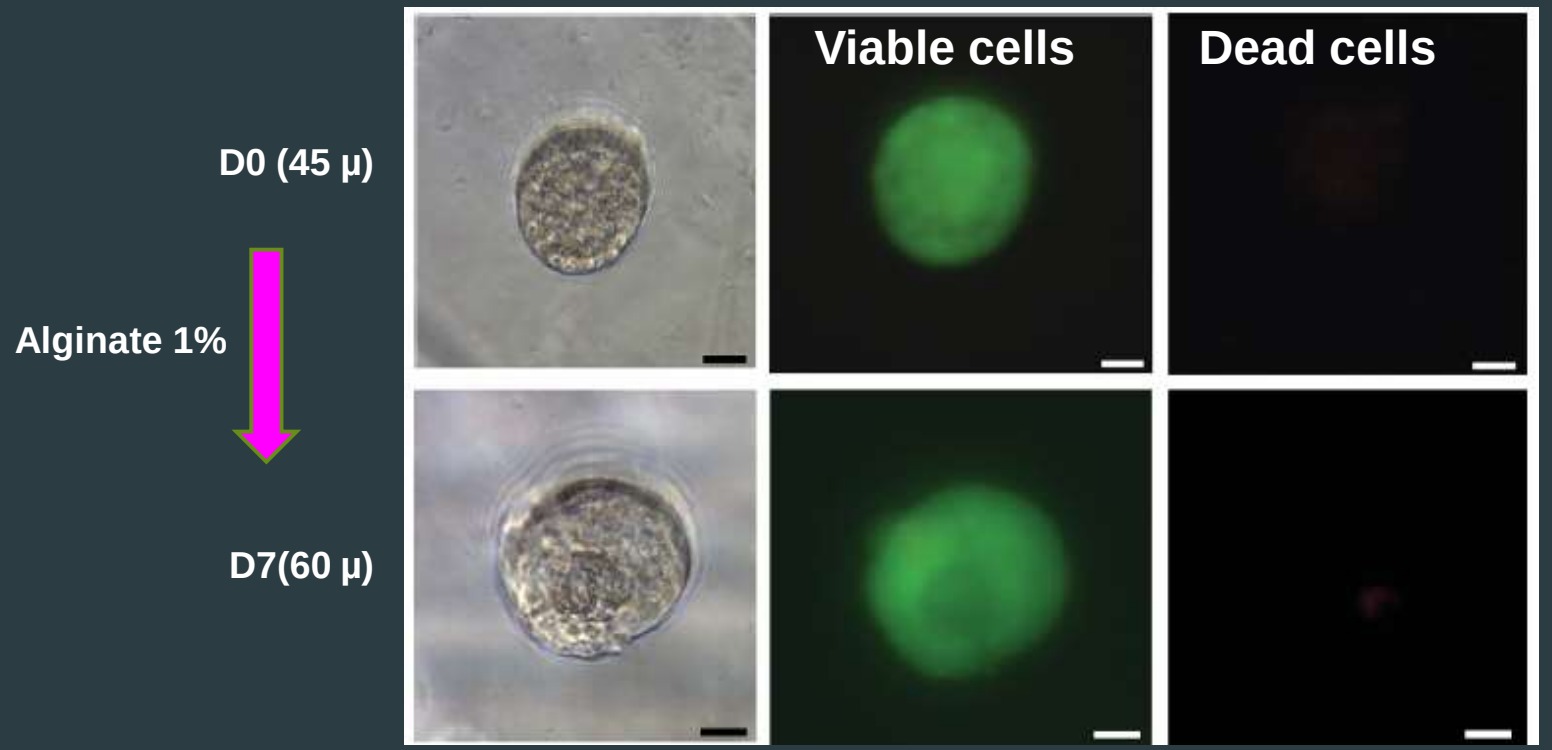
Ovarian tissue

6 days

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

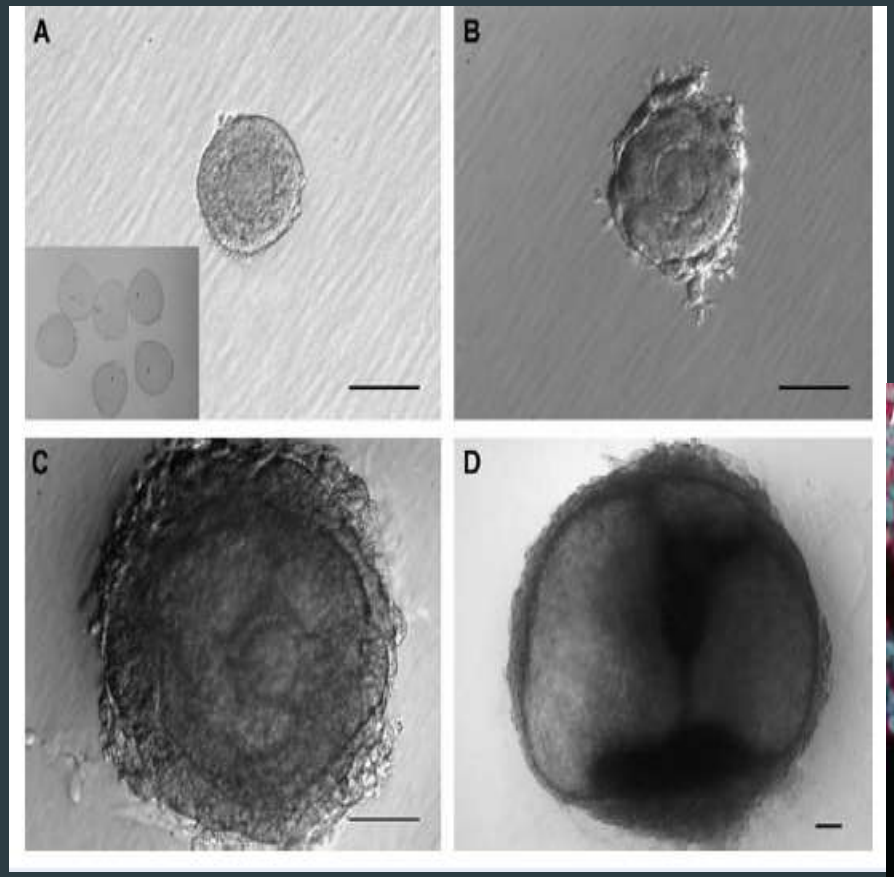
4 days

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.

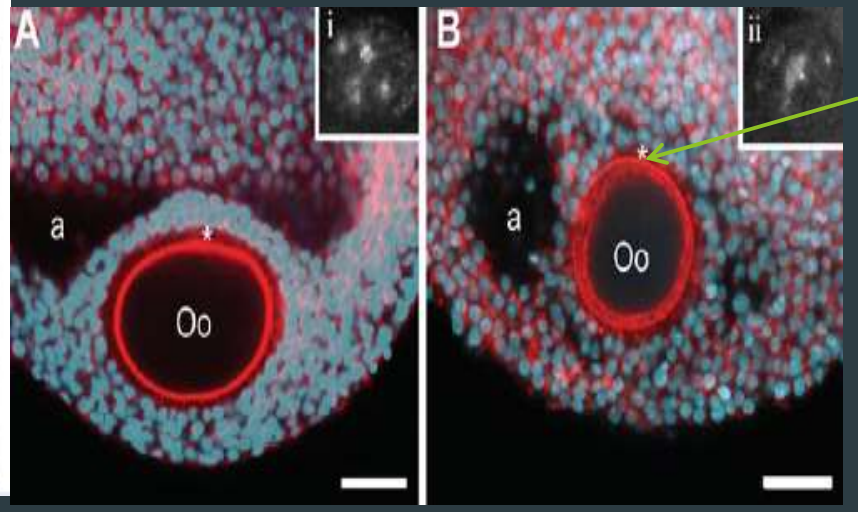


135 µ → 1489 µ

30 Days
Alginate 1%

Fresh

follicle in alginate
for 14 days

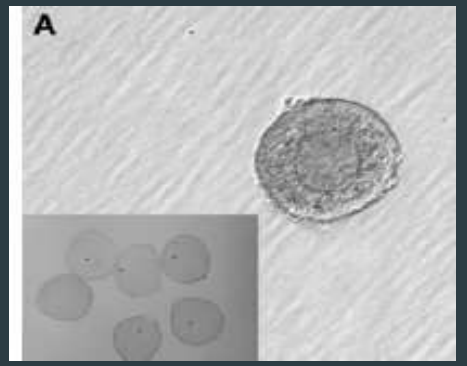


Comparison between follicle development in alginate

Alginate 0.25% (12days)
(xu 2006)

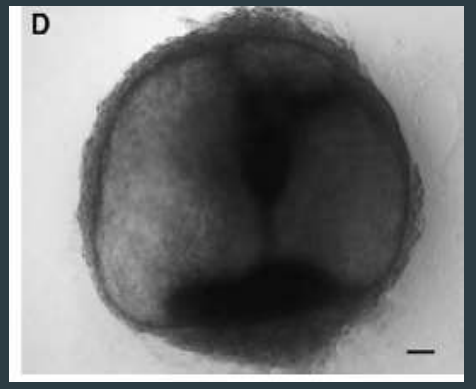
MOUSE

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

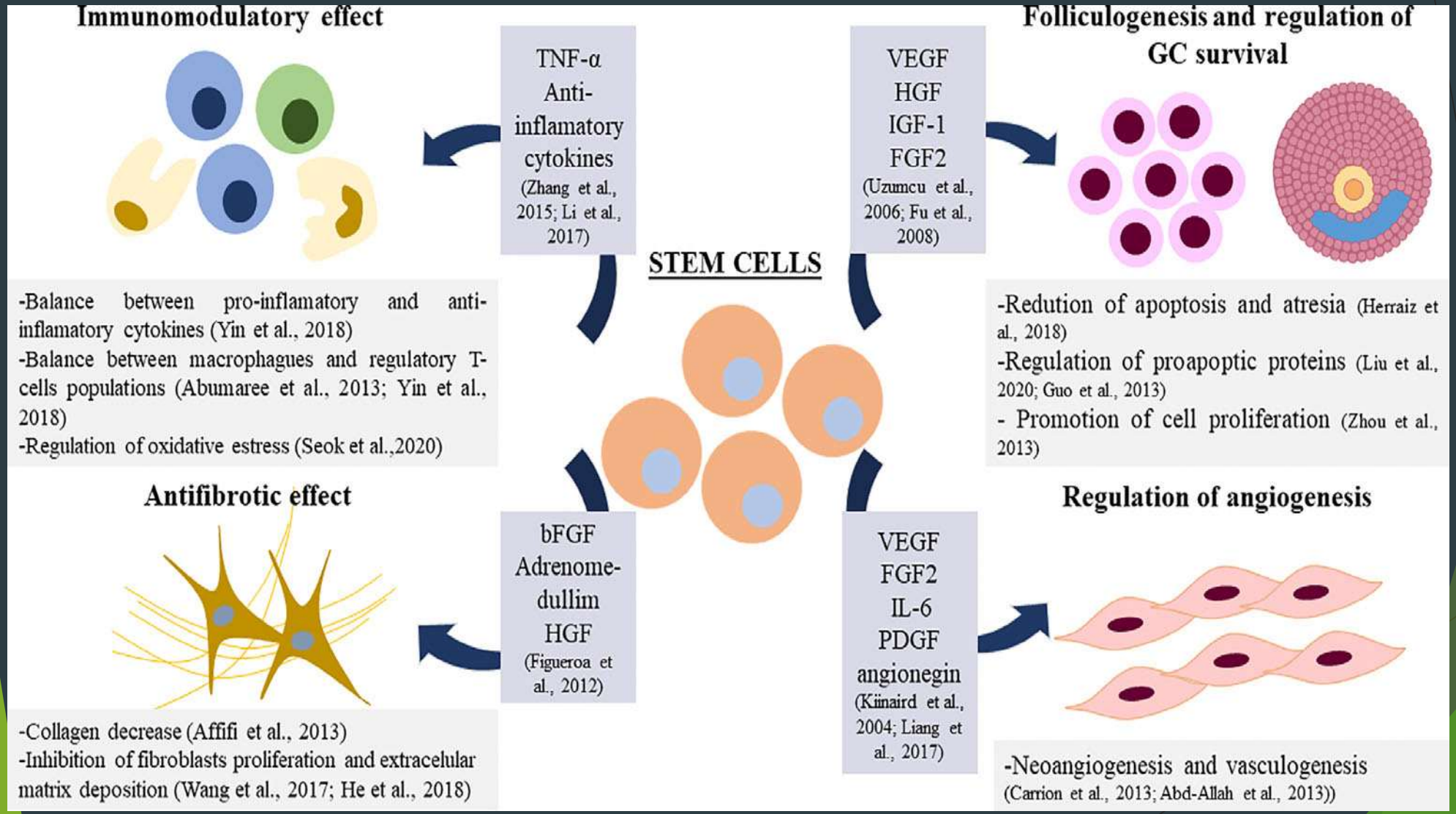
- Inhibition of molecular pathways by IVA
- Tissue mechanical fragmentation,
- Stem cell administration,
- PRP ovarian injection

- 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)** secret **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, adrenomedullim 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. T**amoxifen** 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**

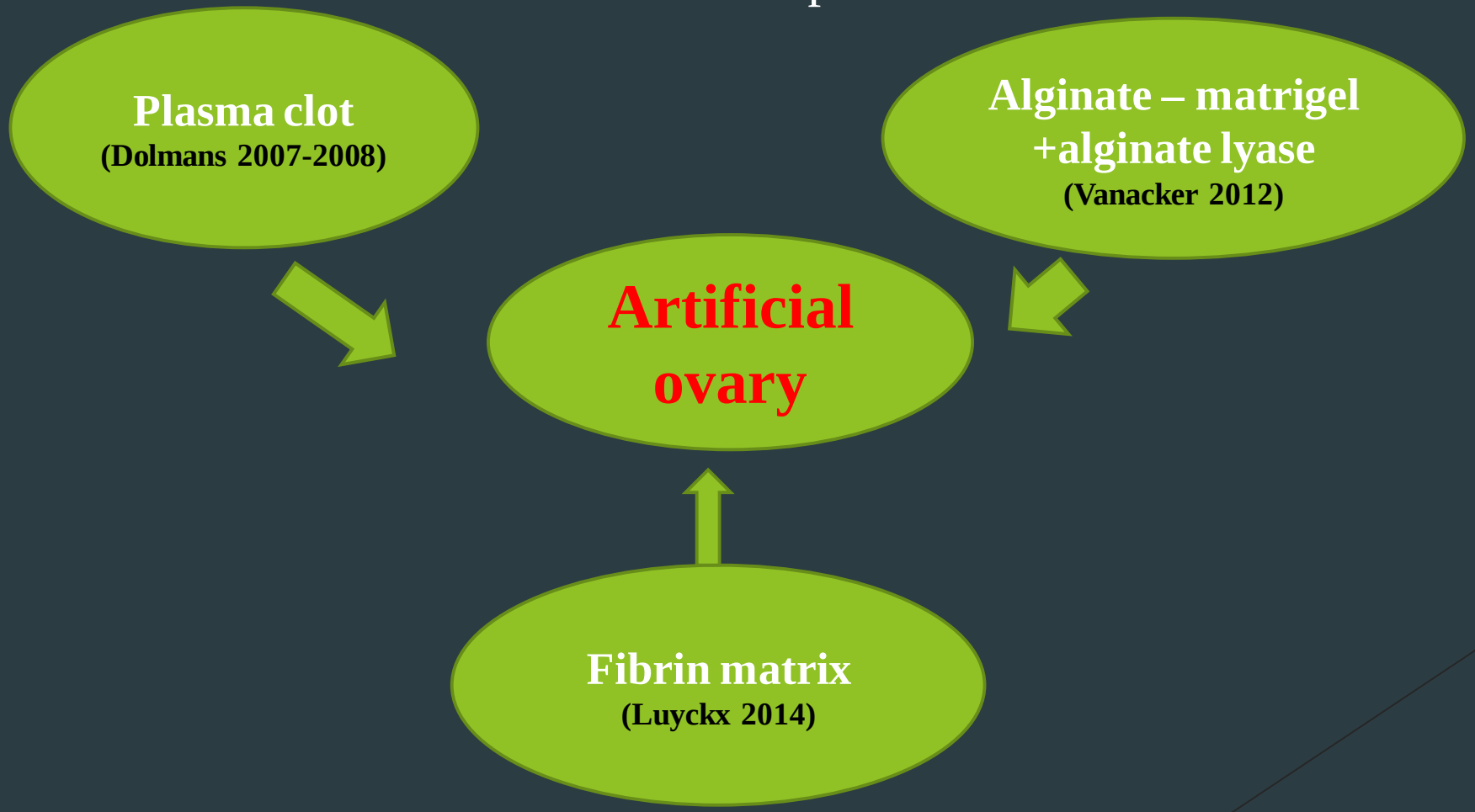
- **Physiological restoration of ovarian endocrine function**
  - An immunoisolating device with encapsulated ovarian tissue for restoration of ovarian endocrine function.
  - The **TheraCyte device**, for example, **shields implanted ovarian tissue** from the **host immune system** without hindering 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 μl/min** using a **peristaltic pump**,

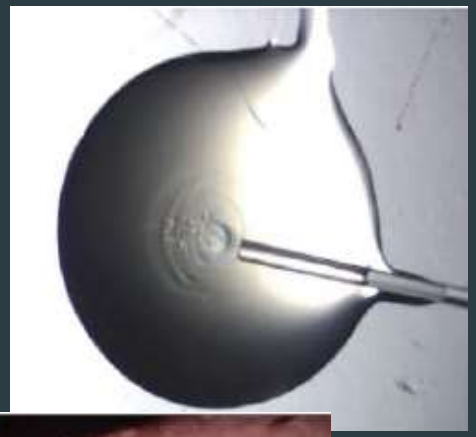
- To create the ovary, the researchers formed **honeycombs** of **theca cells**, donated by reproductive-age (25-46) patients at the hospital.
- Aft**er the theca cells grew into the honeycomb shape**, **spherical clumps** of donated **granulosa cells** were **inserted** into the **holes** of the honeycomb **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.**

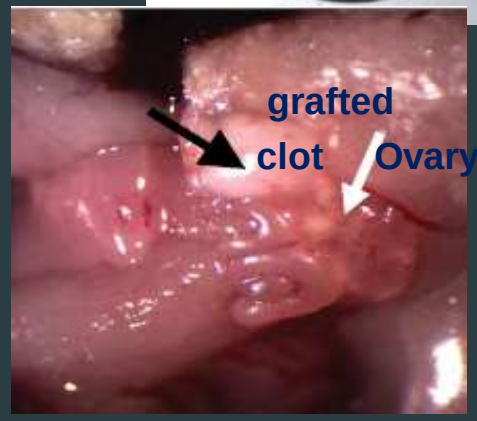
REPRODUCTION RESEARCH 2007

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

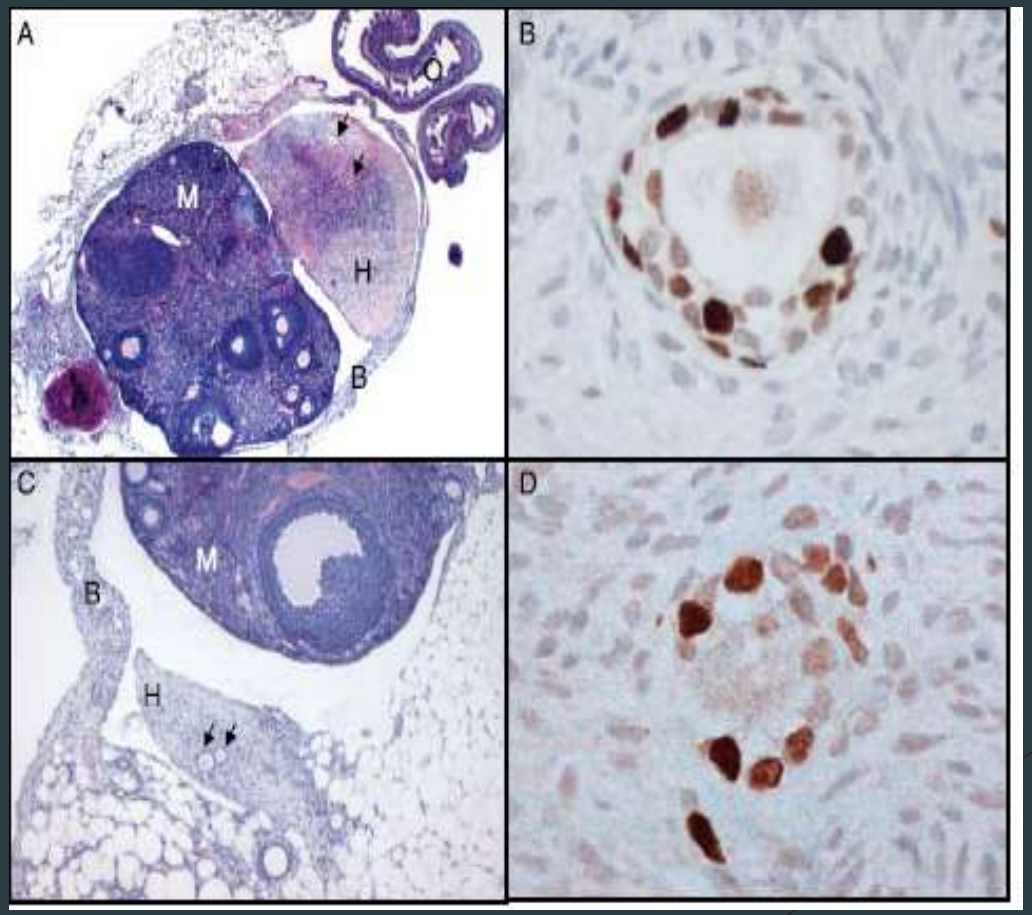
Marie-Madeleine Dolmans, Belen Martinez-Madrid, Elodie Gadisseux, Yves Guiot[1], Wu Yuan Yuan, Antoine Torre, Alessandra Camboni, Anne Van Langendonckt and Jacques Donnez

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**


It's just the beginning


THANK YOU