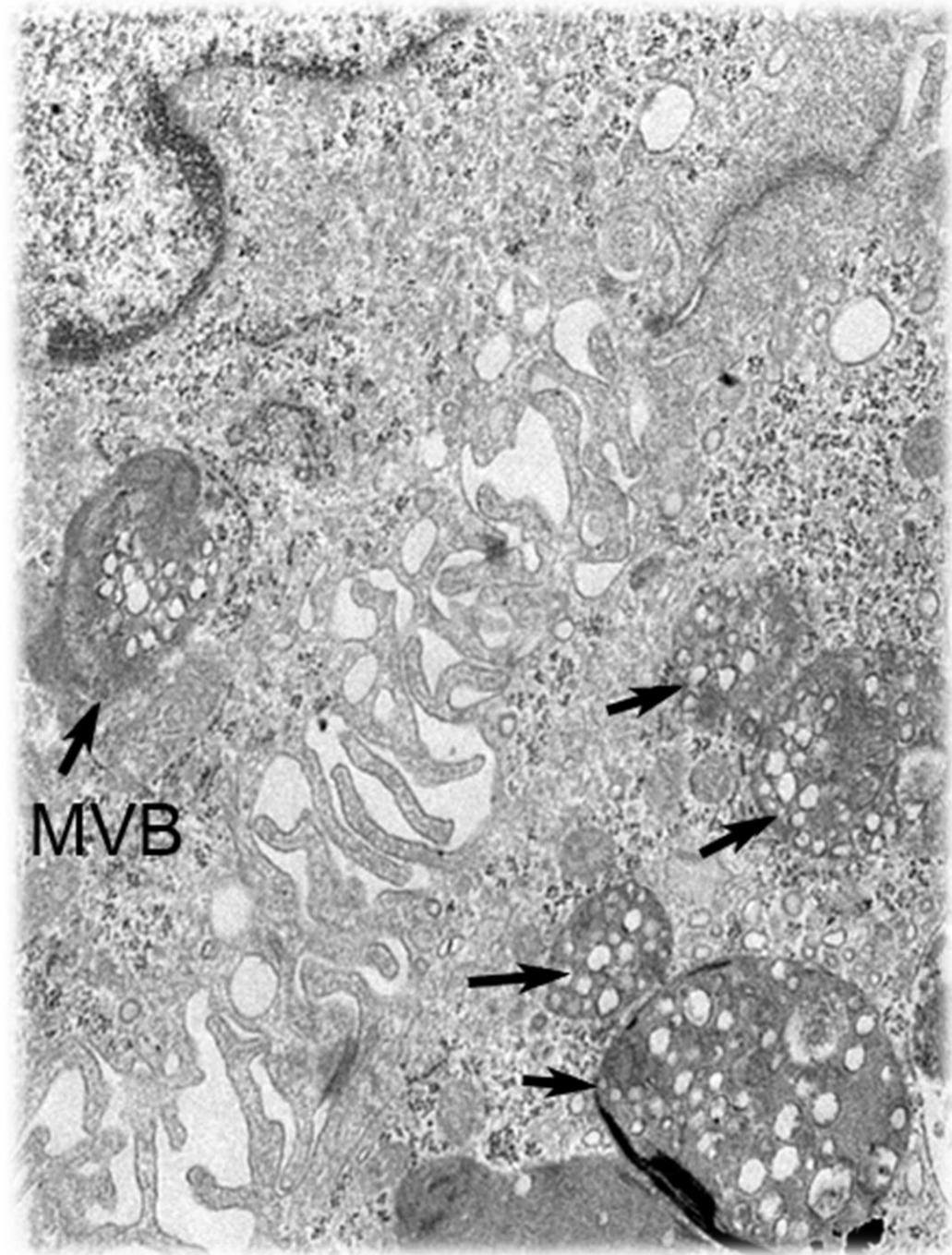


# Regulation of autophagy during embryo-uterine crosstalk

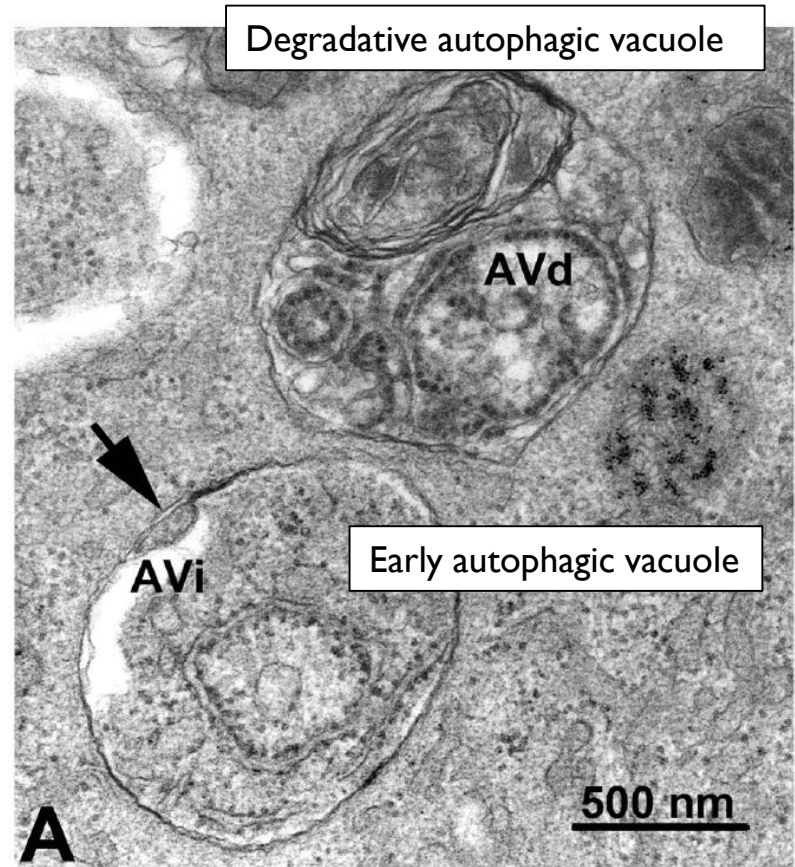
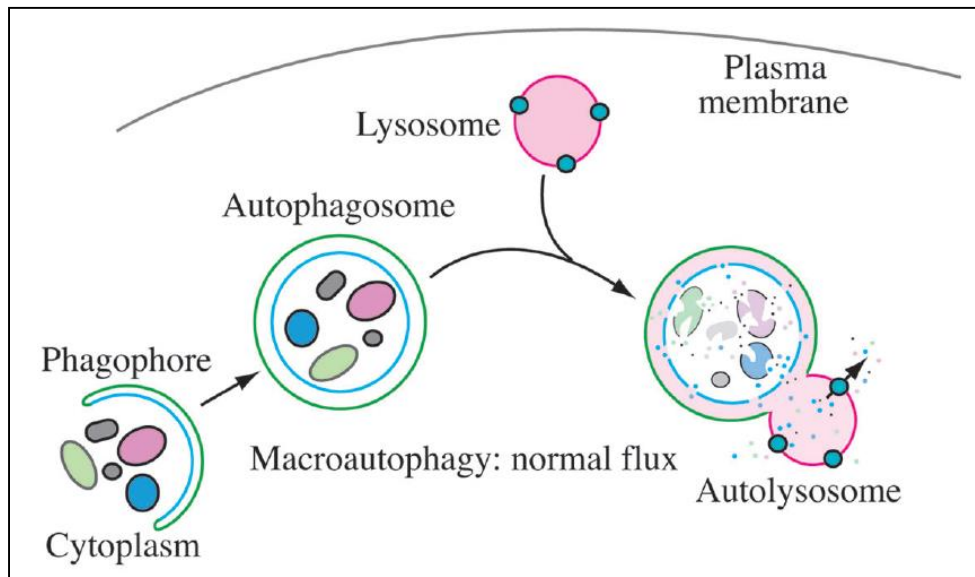
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Hyunjung (Jade) Lim  
Konkuk University, Seoul, KOREA

2015 KSDB Annual Meeting  
CHA Bio Complex, Pangyo

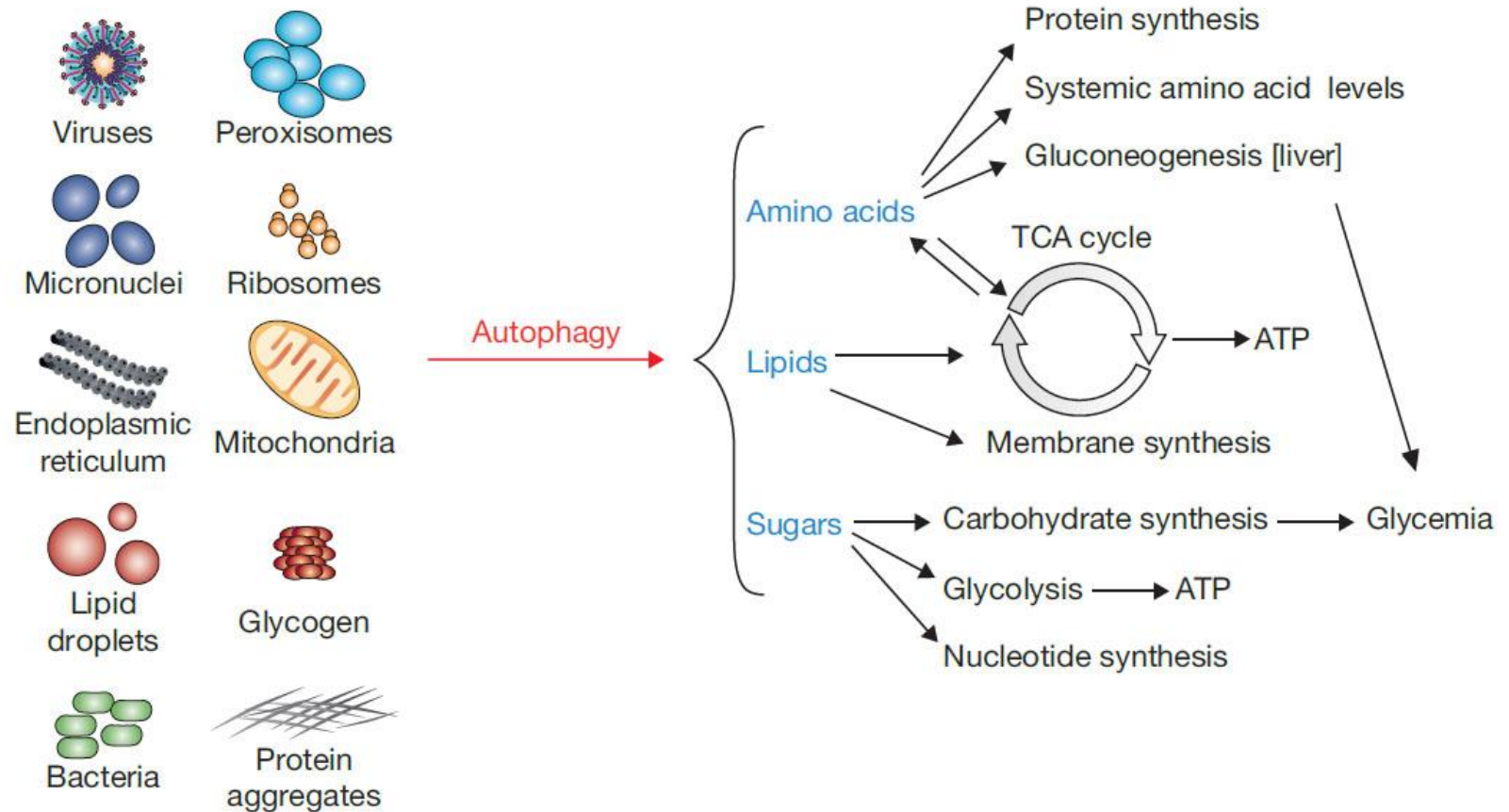


# Autophagy is a basic catabolic mechanism that involves degradation of unnecessary or dysfunctional cellular components through lysosomes



1. Formation of a phagophore
2. Expansion into a autophagosome
3. Fusion with lysosomes
4. Degradation of the contents

# The catabolic products of the intracellular structures that are targeted by autophagosomes are used to generate new macromolecules and membranes to sustain cellular homeostasis





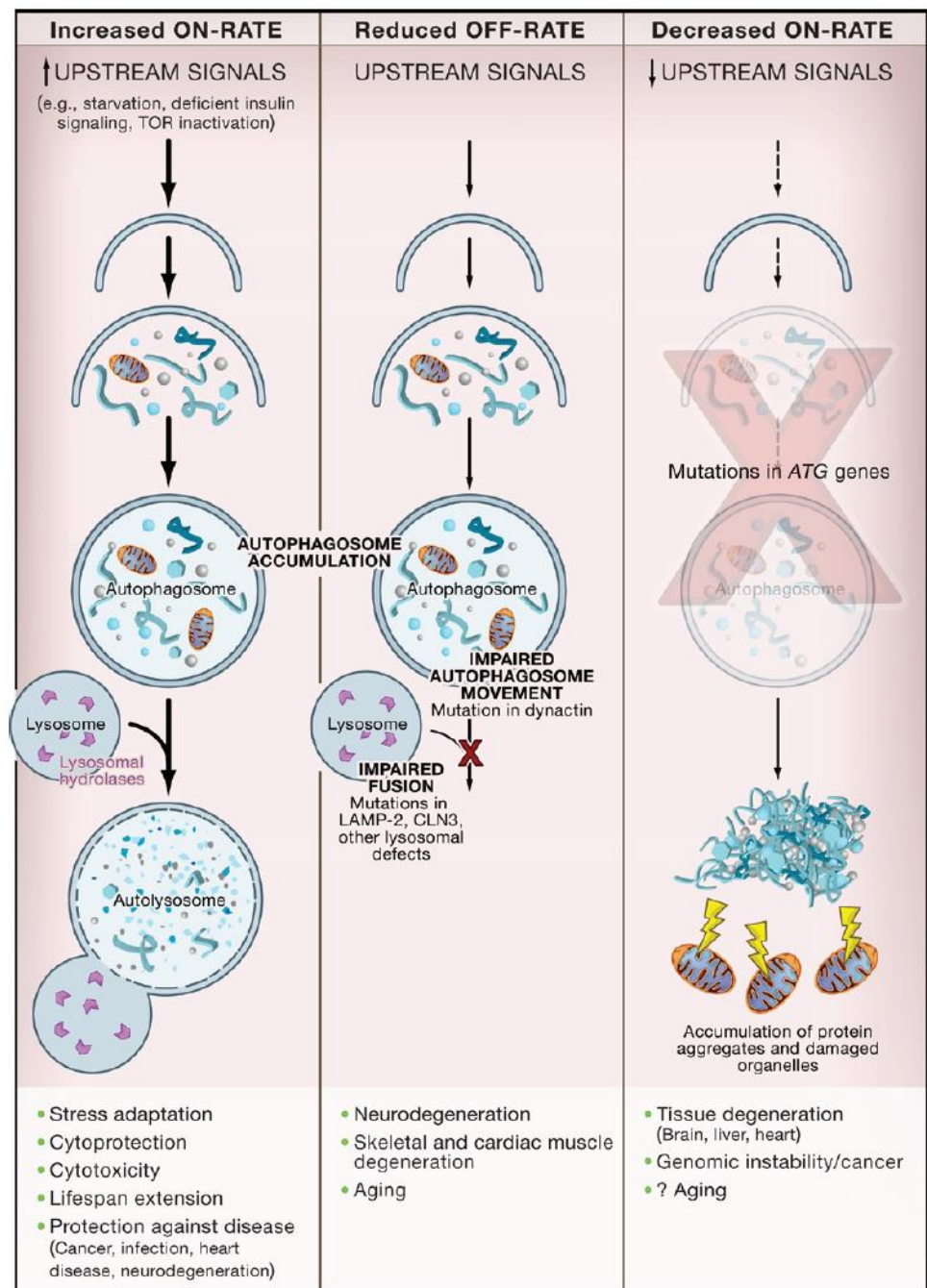
# “Suboptimal environments” of diverse sorts are indicated as inducers of autophagy

## Induced autophagy

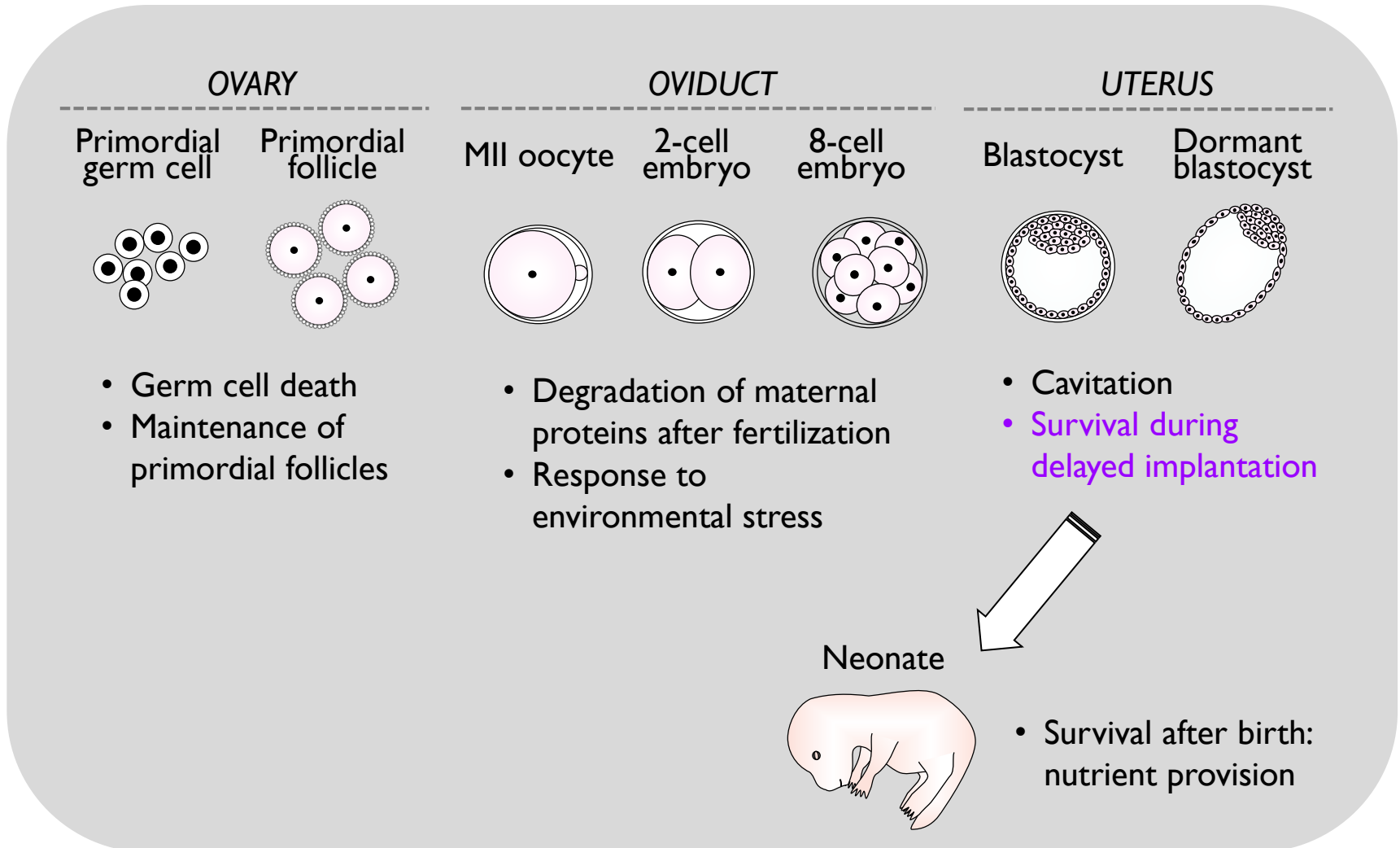
- Starvation, deficient insulin signaling, mTOR inactivation, etc.
- Stress adaptation
- Cytotoxicity
- Lifespan extension
- Protection against disease

## Defective autophagy

- Neurodegeneration
- Aging
- Muscle degeneration



# Potential roles for autophagy in female reproduction and embryonic development in mice



# Pregnancy in Mice

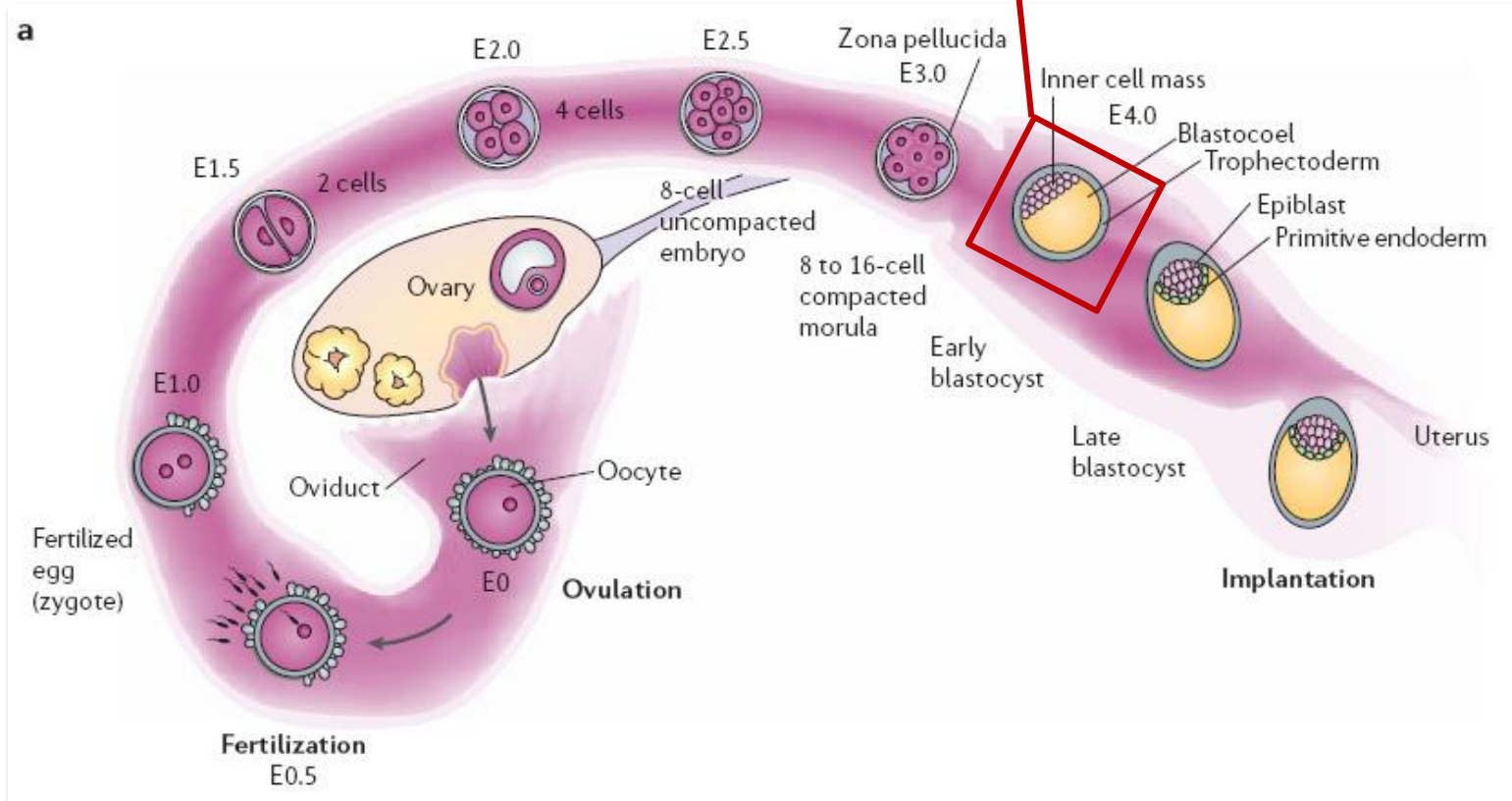
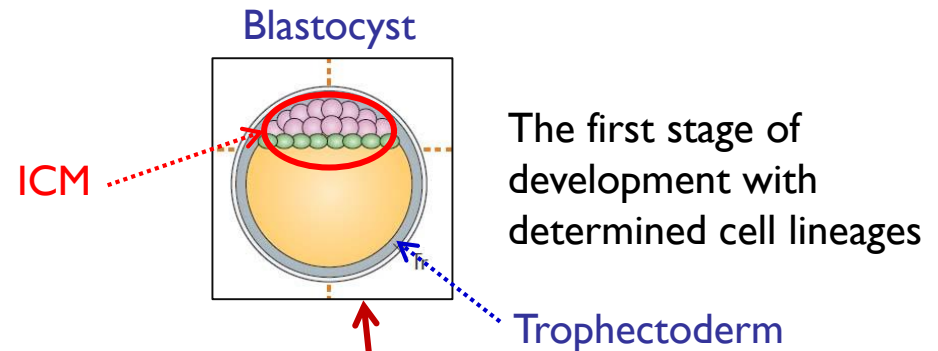
Vaginal plug (+): Day 1

Blastocyst formation: Day 4

Implantation: Day 4.5

Placentation: Day 10

Gestation length: 20 days

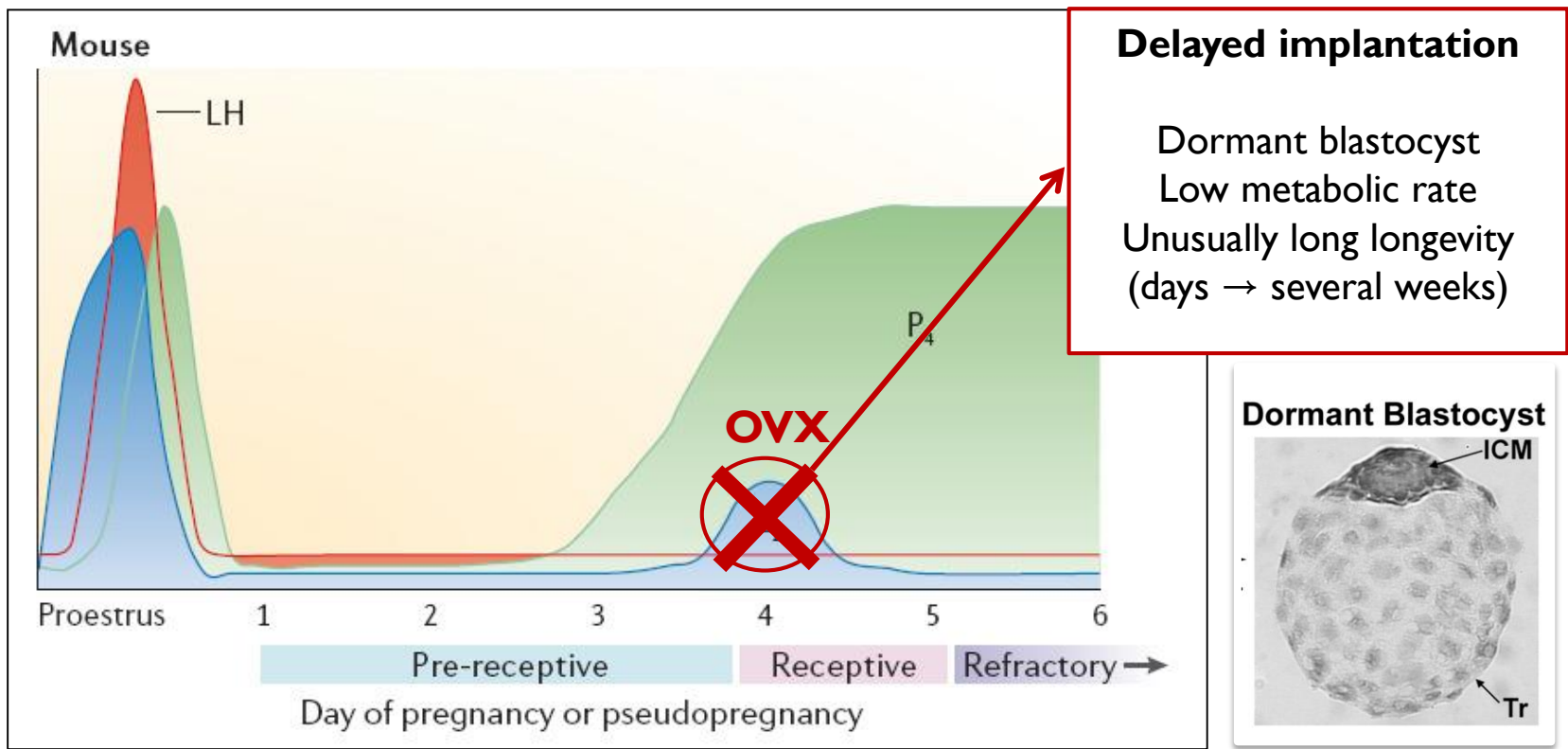


# Endocrinology of embryo implantation in mice

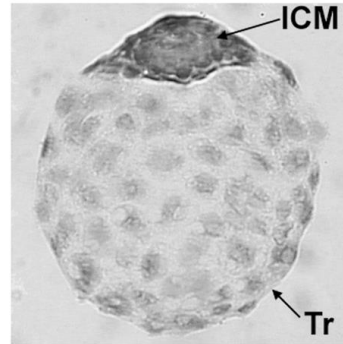
Hormones of implantation:

**Progesterone (P<sub>4</sub>)** – maintenance of pregnancy

**Estrogen (E<sub>2</sub>)** – uterine receptivity & blastocyst activation



*Mouse*  
Dormant  
blastocyst

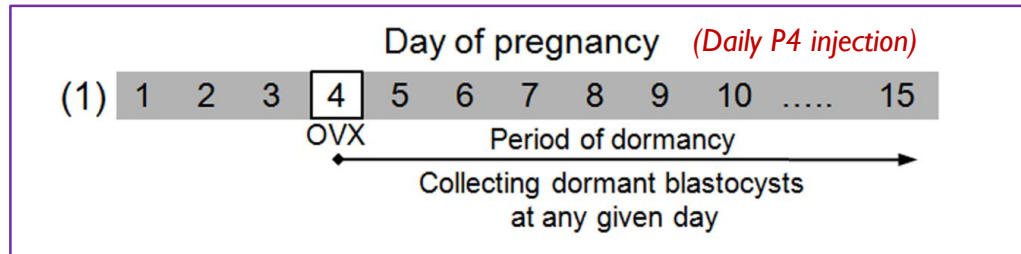


- 1) Is autophagy turned on in dormant blastocysts during delayed implantation?
- 2) Is autophagy required for the prolonged survival of dormant blastocysts during delayed implantation?



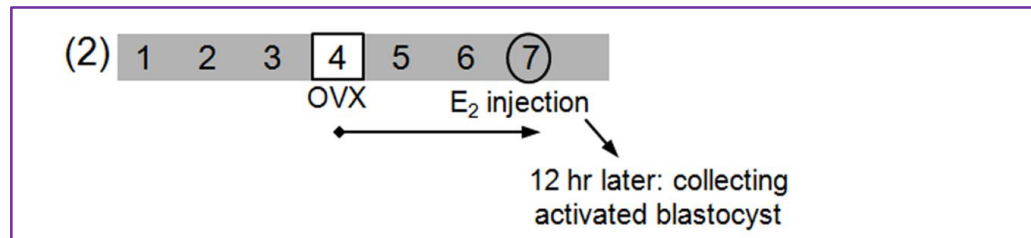
# Experimental schemes: Experimentally induced delayed implantation

## Ovariectomy and the initiation of delayed implantation

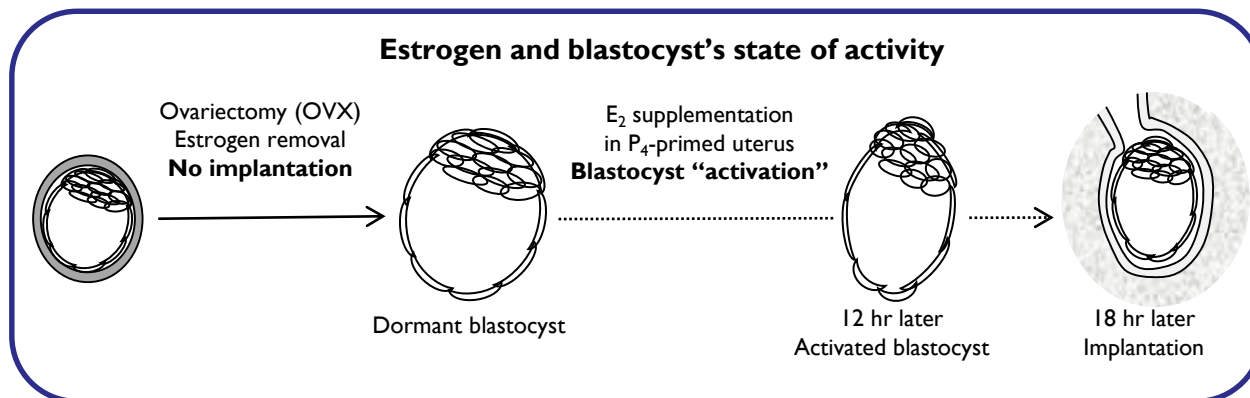


Dormant blastocysts  
Short dormancy: 3.5 - 4.5  
Long dormancy: 9.5 <

## E2 injection and the initiation of implantation



Activated blastocysts  
12-14 h after E<sub>2</sub> injection

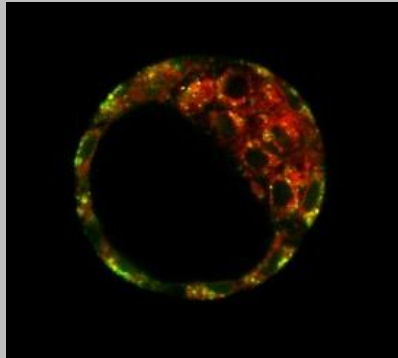


## GFP-LC3 transgenic mouse

Mizushima et al. (2004) *MCB* 15:1101-1111

- Transgene: rat LC3(Atg8) fused to GFP
- Ubiquitously expressing the transgene
- Used for in vivo observation of autophagy

Day 4 morning blastocyst

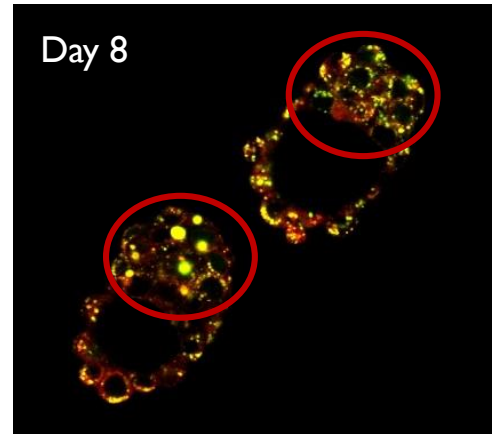


GFP-LC3: autophagy

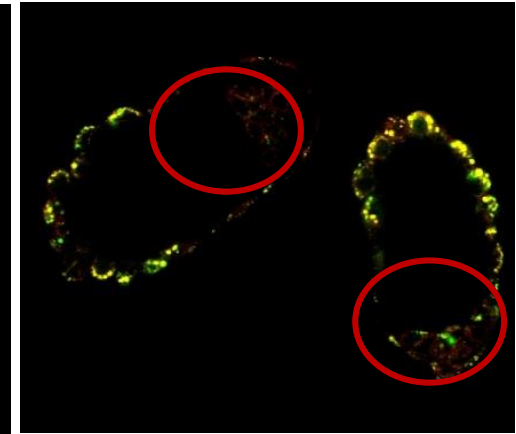
LysoTracker Red: acidified lysosome

Autophagolysosomes (green + red)

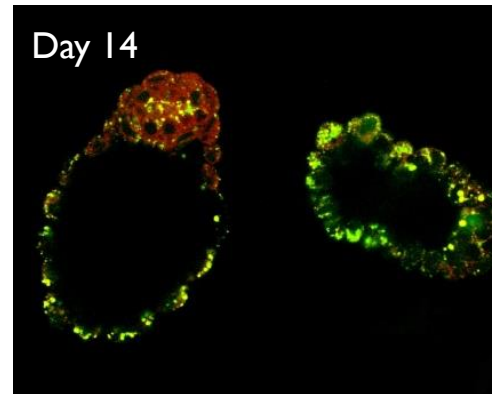
Dormant blastocyst



Activated blastocyst



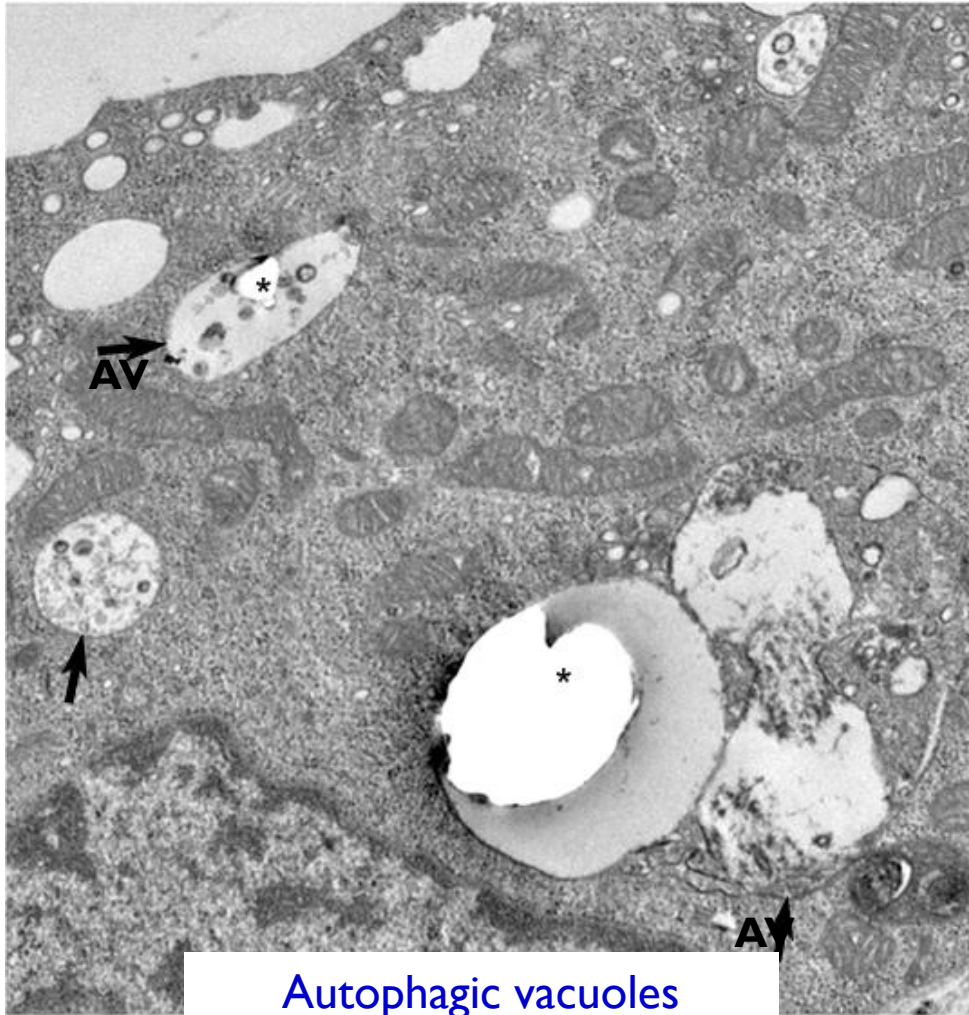
Day 14



- Dormant blastocysts showed increased numbers of GFP-LC3 puncta in both inner cell mass (ICM - red ovals) and trophectoderm compared to day 4 normal blastocyst
- After blastocyst activation (E2 injection), GFP-LC3 puncta seem to disappear from ICM

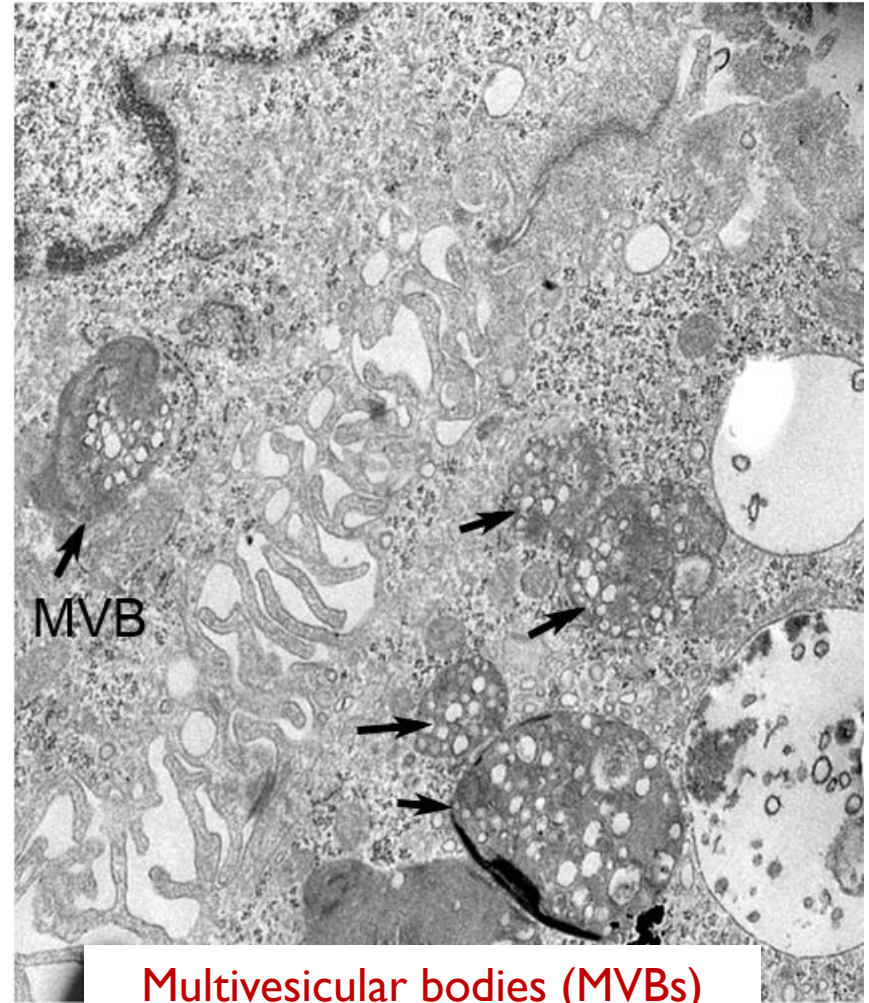
# Ultrastructure (TEM)

Day 14 dormant blastocyst



Autophagic vacuoles  
increased in trophoblast

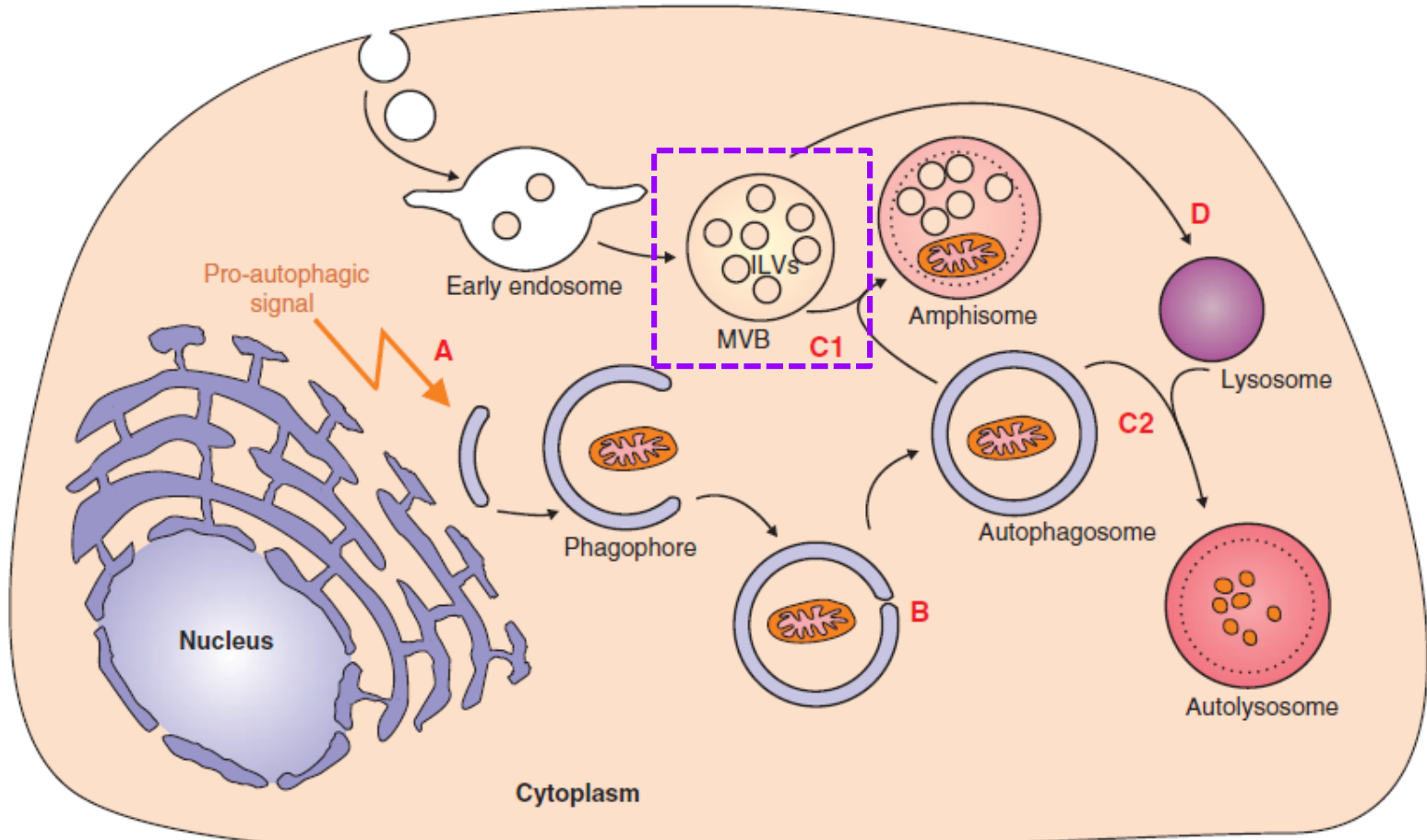
Day 14 activated blastocyst



Multivesicular bodies (MVBs)  
increased in trophoblast

# What's MVBs got to do with autophagy?

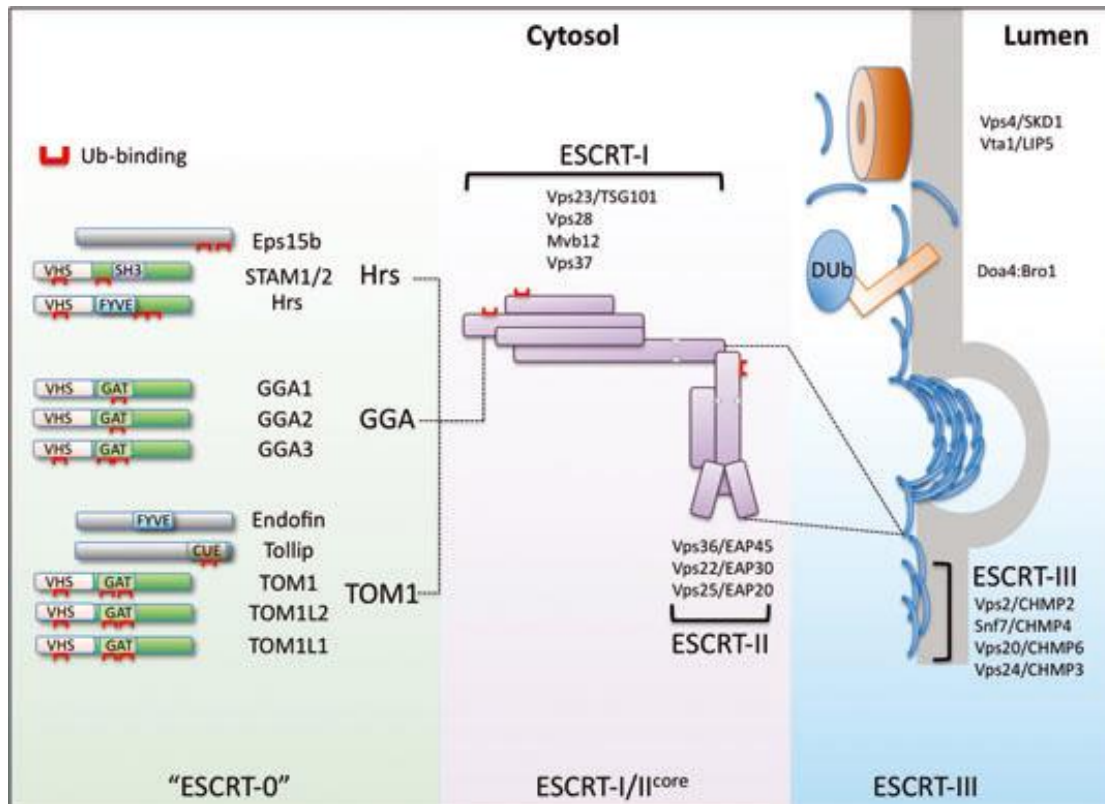
- Efficient autophagic flux -



MVBs are necessary to allow an efficient autophagic degradation  
Ubiquitin is a sufficient sorting signal for MVB/lysosomal pathway



# Multivesicular body (MVB): Characteristics and visualization



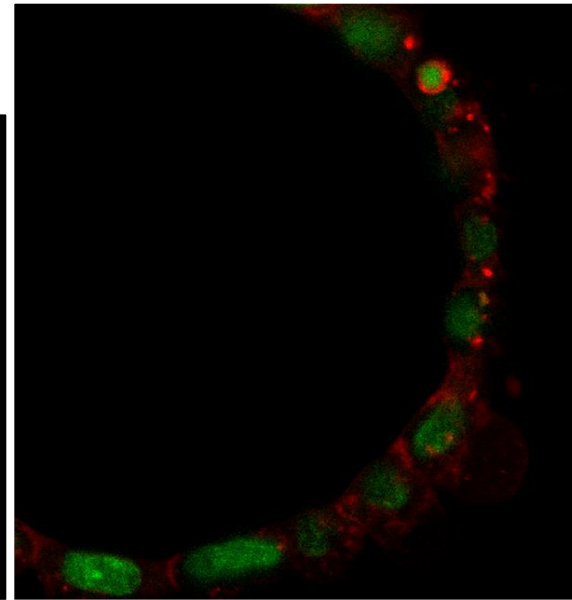
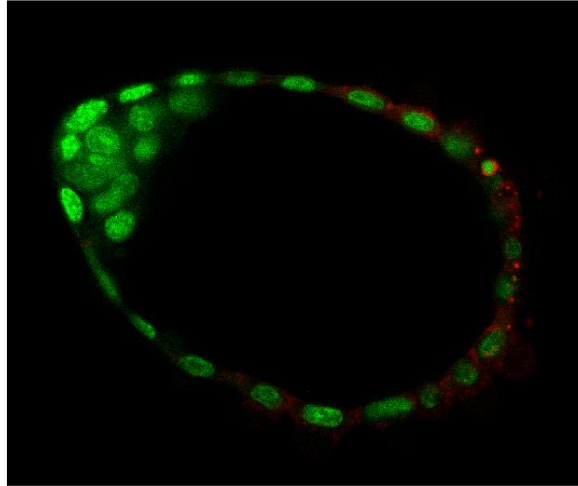
Brookhart Shields & Piper (2011)  
 How Ubiquitin Functions with ESCRTs.  
 Traffic12: 1306–1317

- **ESCRT complexes 0, I, II, and III**
- Ubiquitinated proteins are sent to MVBs for sorting and degradation
- **Tsg101** (a component of the ESCRT-I complex), **CD63**, lysobisphosphatidic acid (**LBPA**), **Di-I** dye

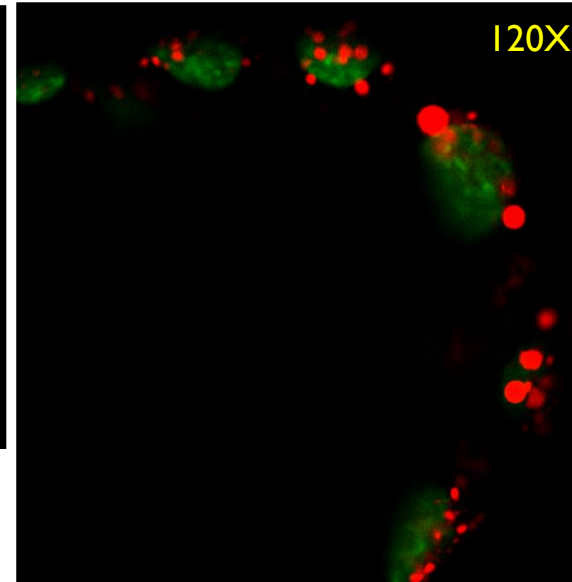
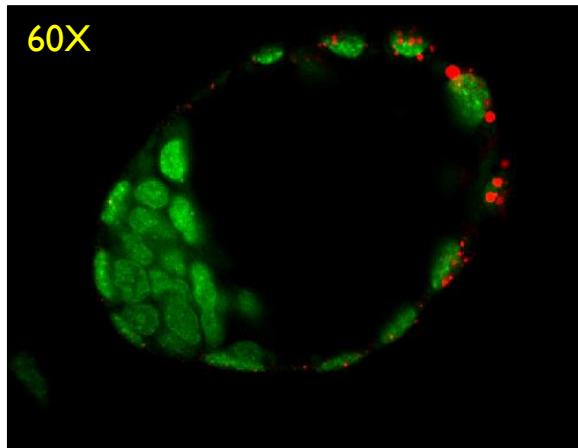
## MVB formation increases in activated blastocysts:

More Dil-positive puncta are observed in activated blastocysts under live imaging

Day 7  
Dormant  
blastocyst



Day 7  
Activated  
blastocyst

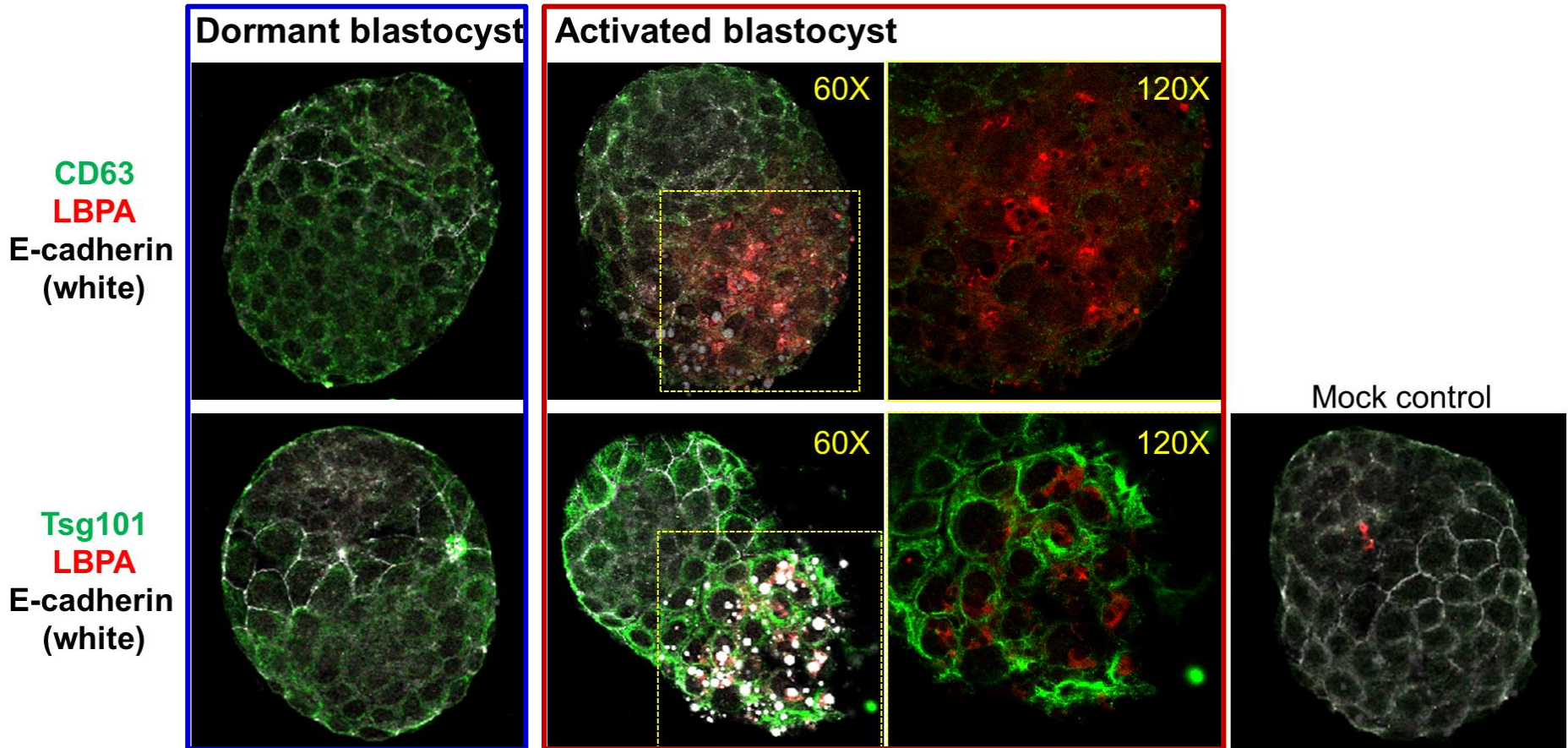


Di-I (multivesicular body)  
SYTO green (DNA)

## MVB formation increases in activated blastocysts:

Expression of **LBPA** prominently increased in the trophoctoderm of activated blastocysts.

Expression of E-cadherin, which is a marker of epithelial cells, was shown in the shape of blebs of various sizes accumulated at the mural trophoctoderm of some activated blastocysts.



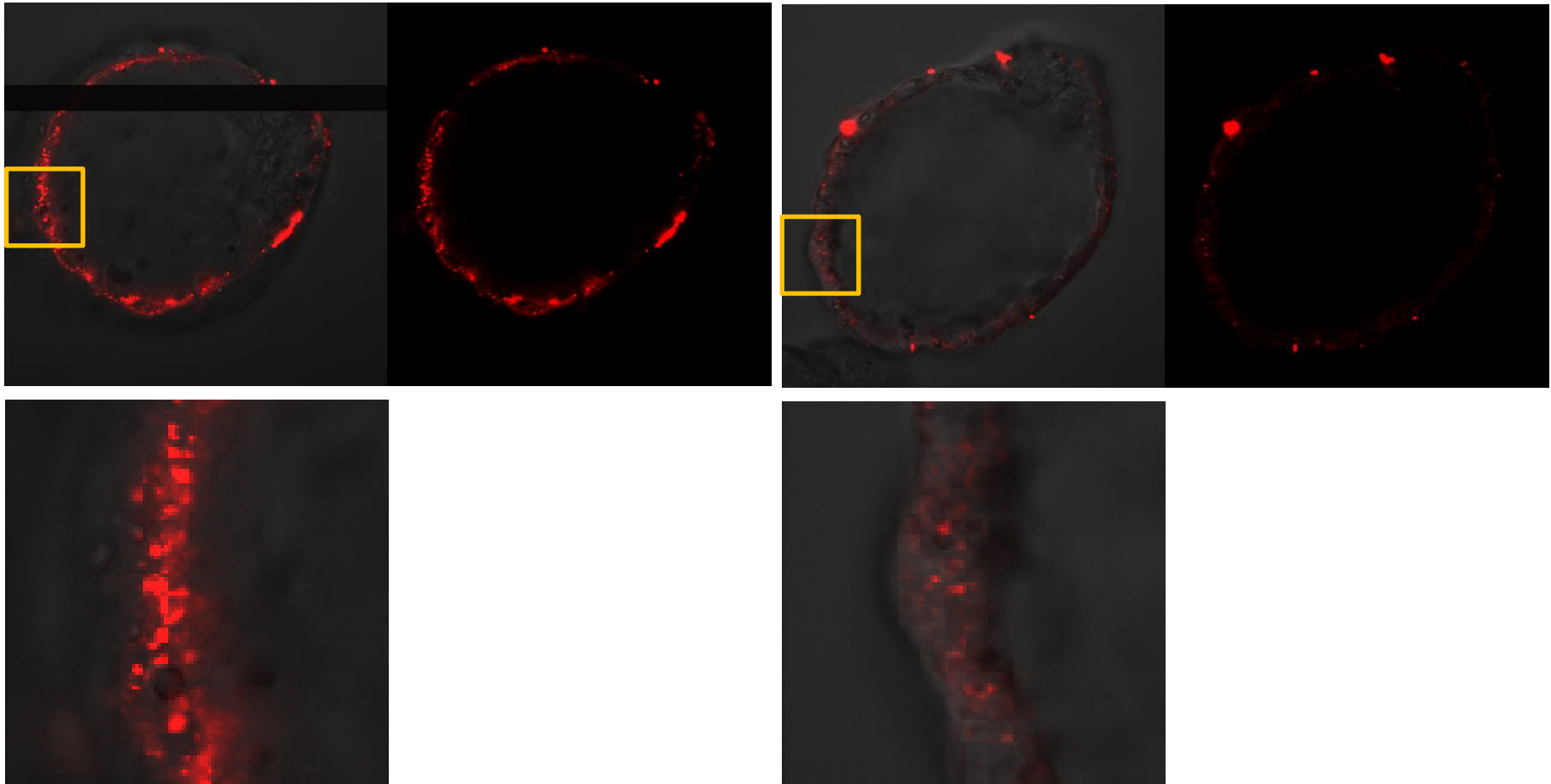
## 3-MA treatment decreases MVB formation in activated blastocysts:

\* 3-methyladenine (3-MA): an inhibitor of PI3 kinase (autophagy inhibitor)

*Dil live imaging of day 18 activated blastocysts*

E2 12.5 h (vehicle)

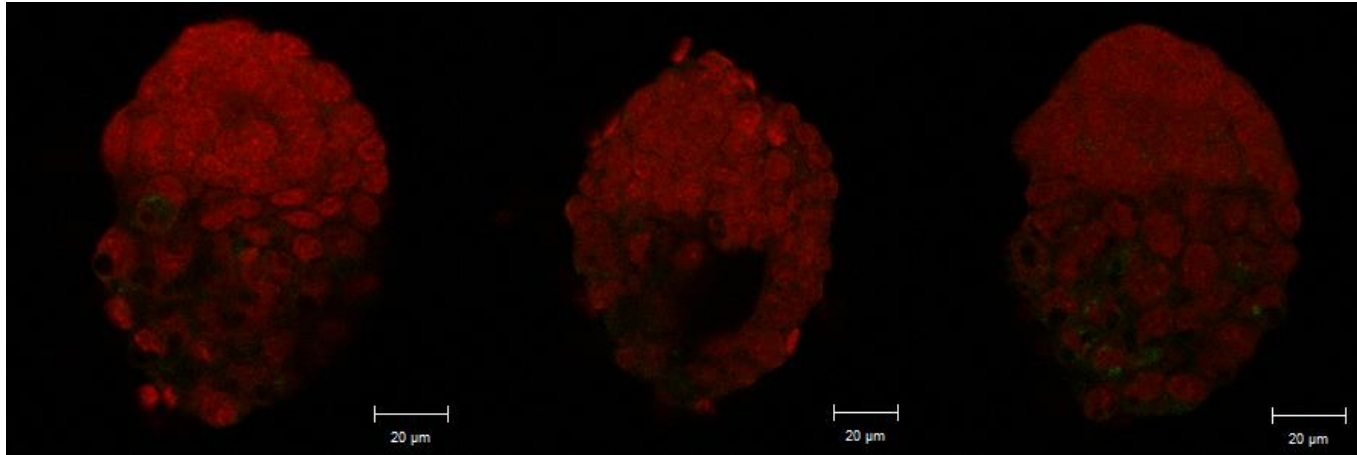
E2 12.5 h (3-MA)



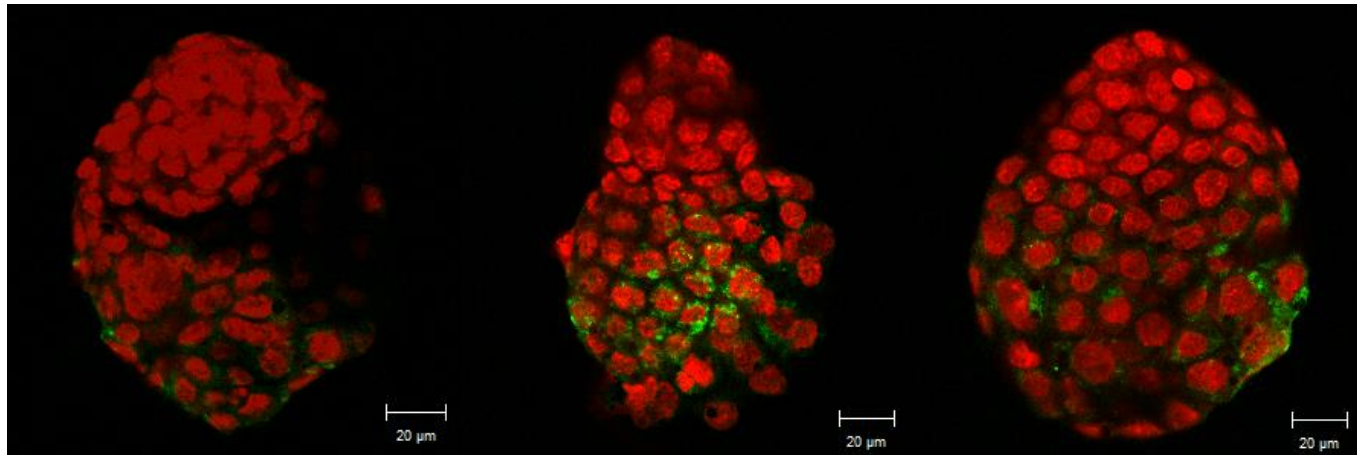


3-MA treatment decreases MVB formation and increases ubiquitin accumulation in activated blastocysts:

PBS *Ubiquitin* staining in day 8 activated blastocyst: E2 14 h



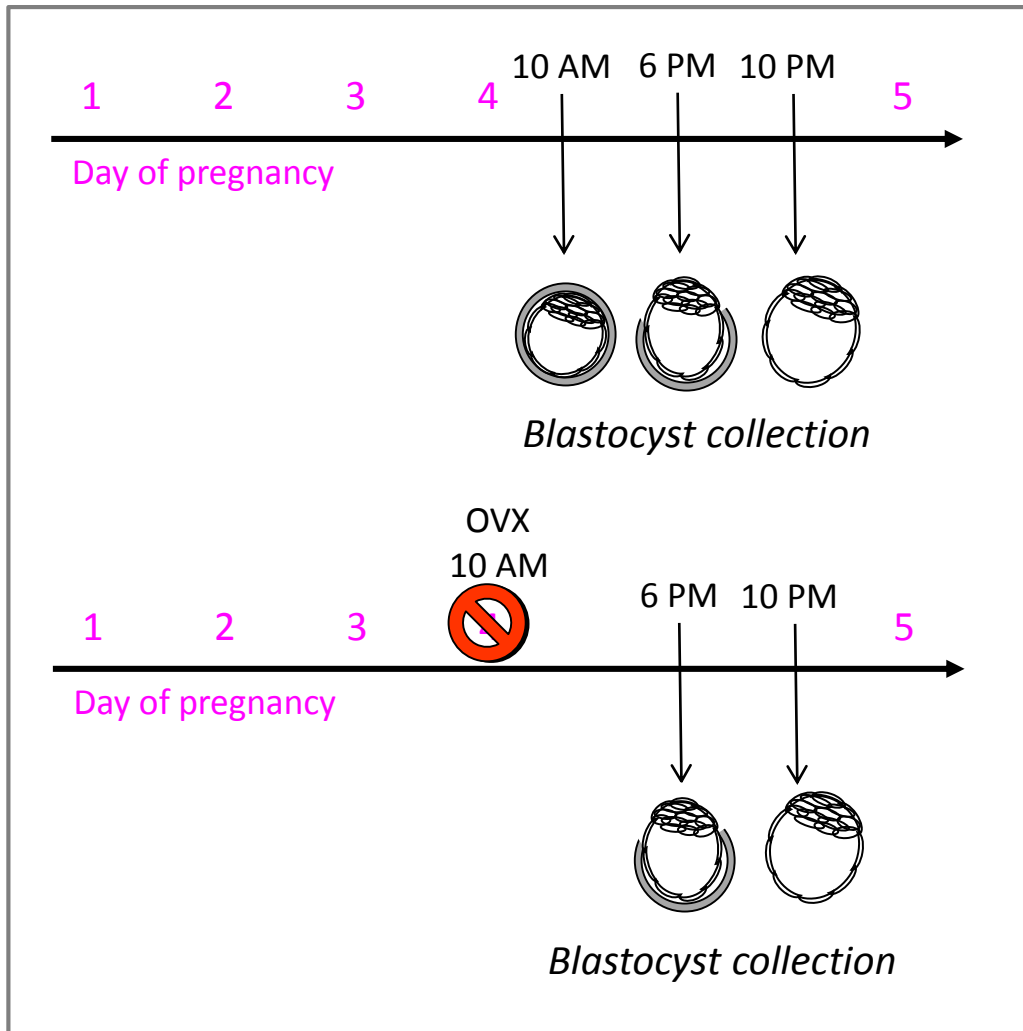
3-MA



- In dormant blastocysts, not many MVBs are visible
- Activation of blastocysts for implantation increases the number of MVBs and exosomal release
- Inhibition of autophagy in dormant blastocysts decreases the number of MVBs in the trophoctoderm after activation of implantation

MVBs are required for efficient autophagic degradation in dormant/activated blastocysts during delayed implantation (쓰레기 제거)

# Turning on autophagy: When do blastocysts first recognize the lack of estrogen?

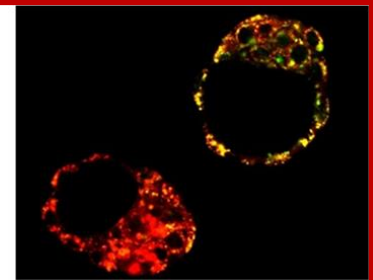


GFP-LC3 live imaging  
LysoTracker Red  
Overlay

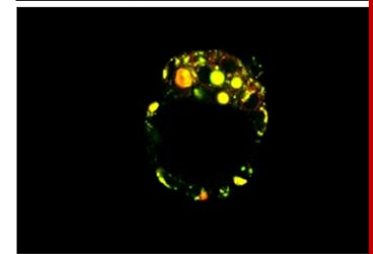
Day 4  
10 AM



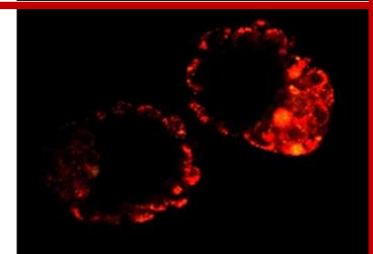
Day 4  
6 PM



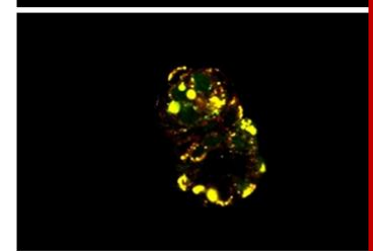
Day 4  
OVX  
6 PM



Day 4  
10 PM

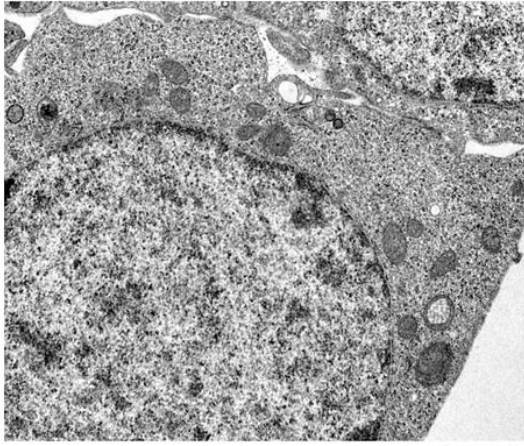


Day 4  
OVX  
10 PM

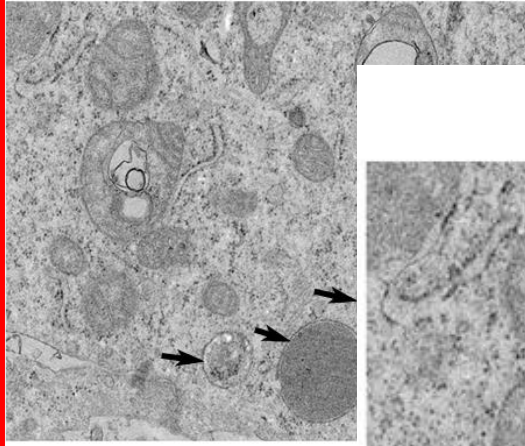


## Ultrastructure (TEM)

Day 4 6 PM

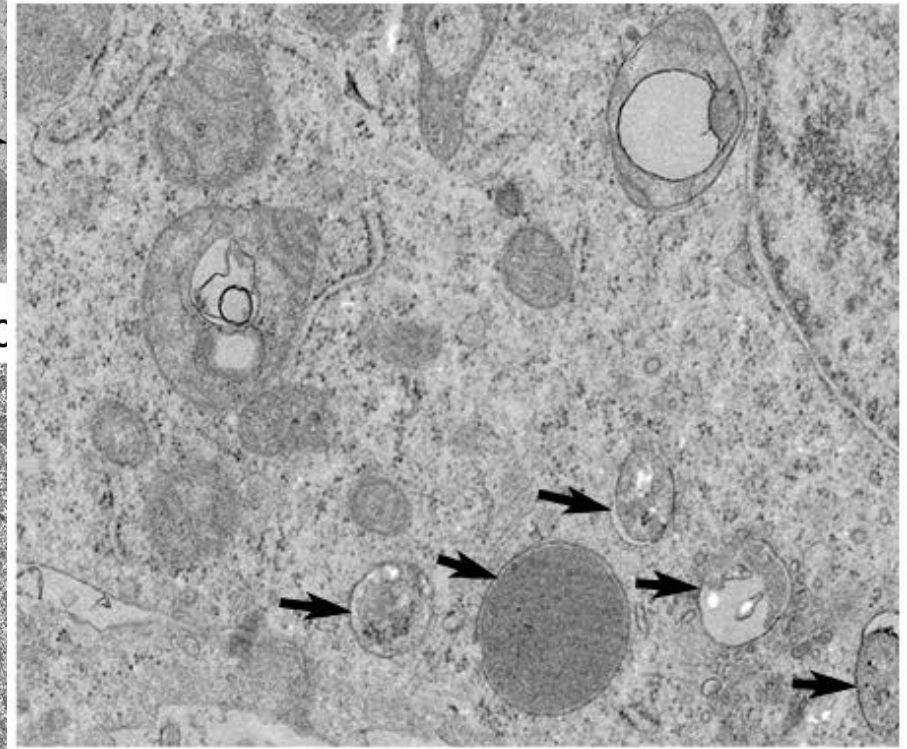


Day 4 OVX 6 PM

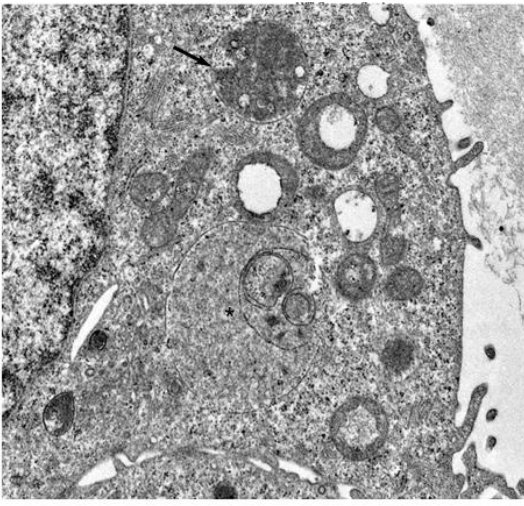


OVX

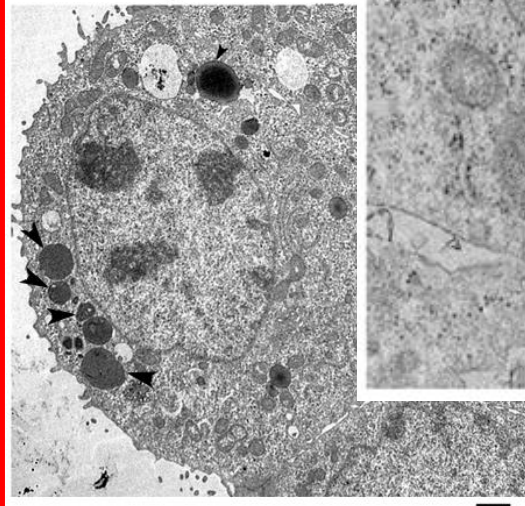
Day 4 OVX 6 PM



Day 4 10 PM



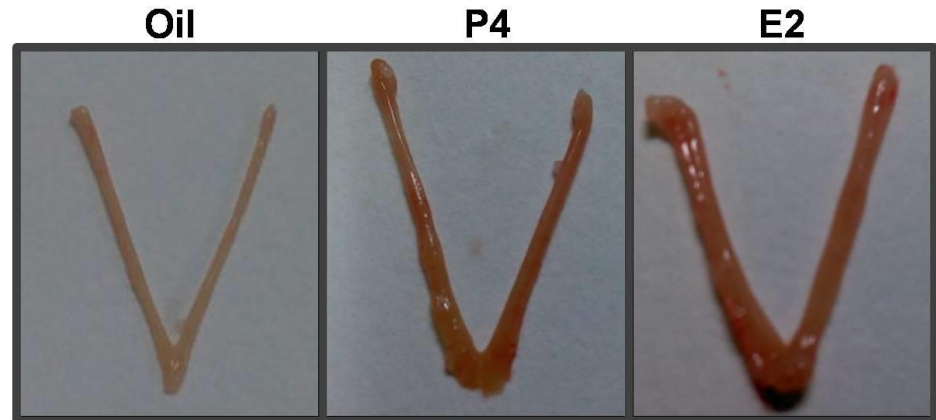
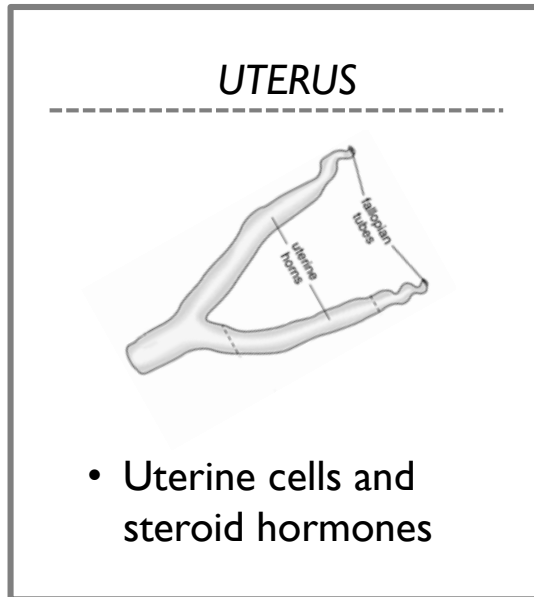
Day 4 OVX 10 PM



- Trophoblast cells of OVX blastocysts show numerous AVs as early as 6 PM (8 h post-OVX), suggesting that blastocysts recognize the absence of estrogen at this time.

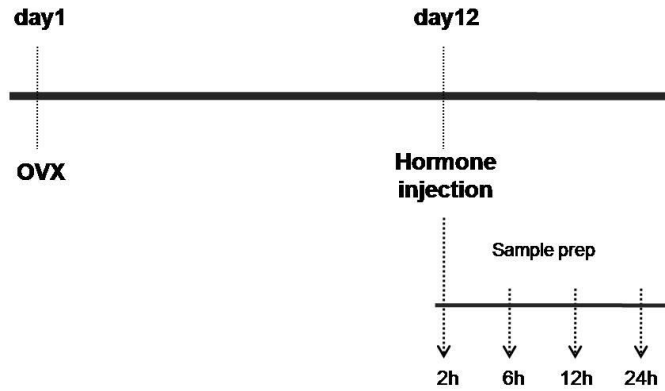


- Results: Day 4 blastocysts turn on autophagy as early as 8 h after OVX.
- Question: What role does  $E_2$  play in all this?
  - 1) Choose a right system to address this: a system which responds to  $E_2$  and  $P_4$
  - 2) Blastocysts won't do because  $E_2$  does not directly work on them



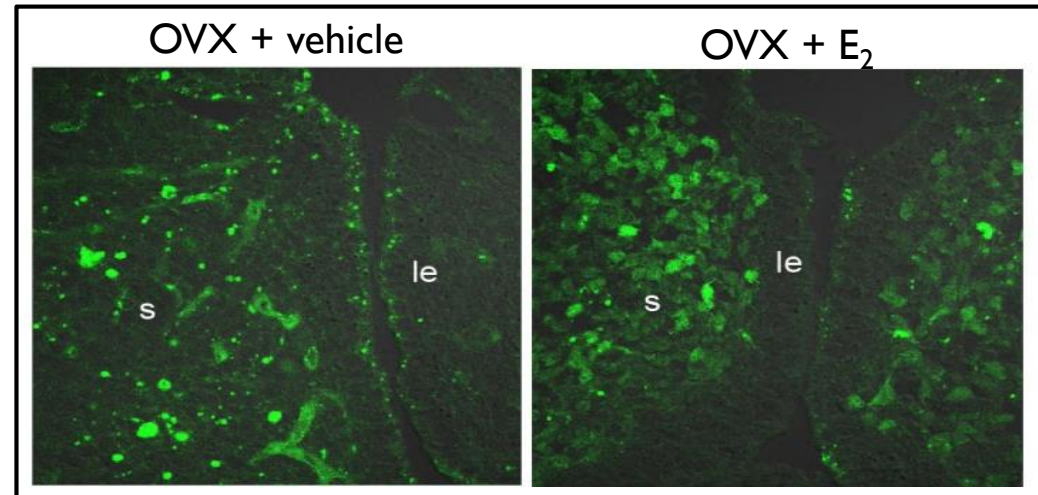
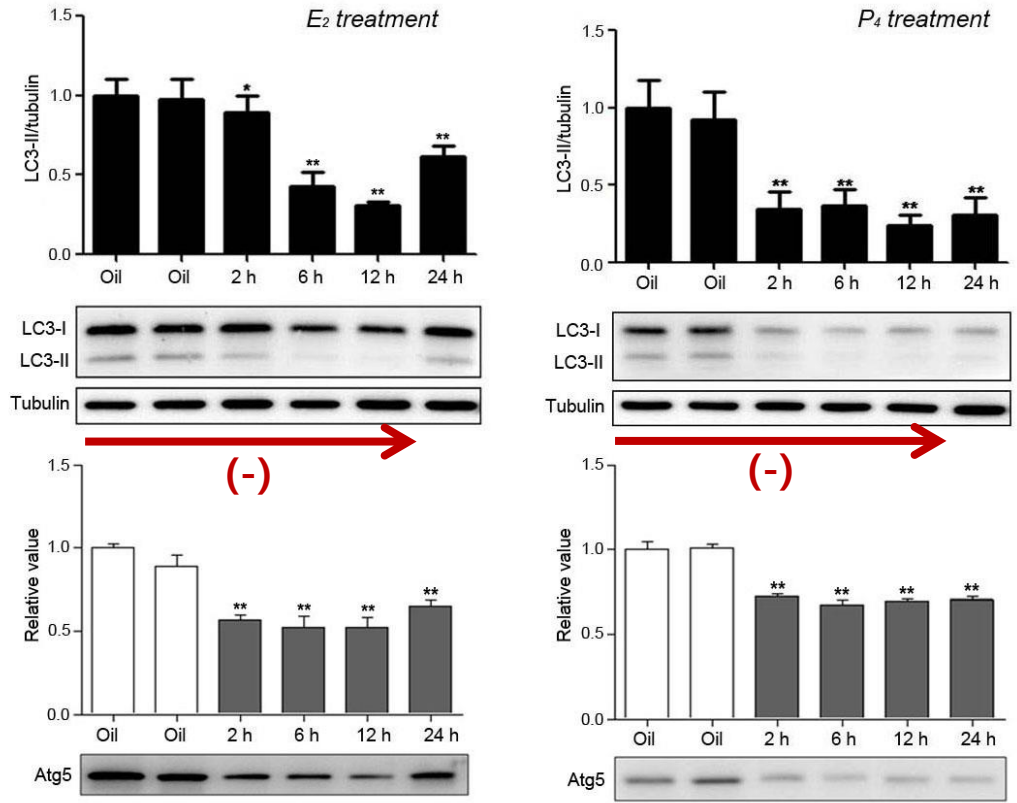
# Regulation of autophagy by E2 or P4 - Effect of a single hormone -

Ovariectomy + E2 or P4 injection  
→ whole uterine tissue preparation

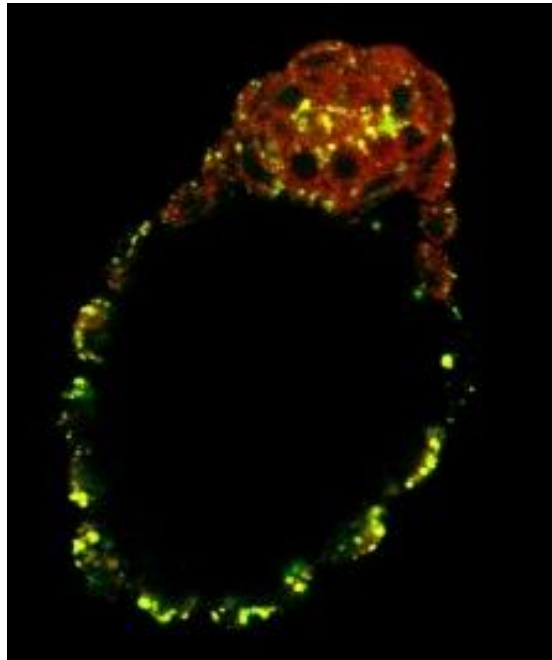


autophagy

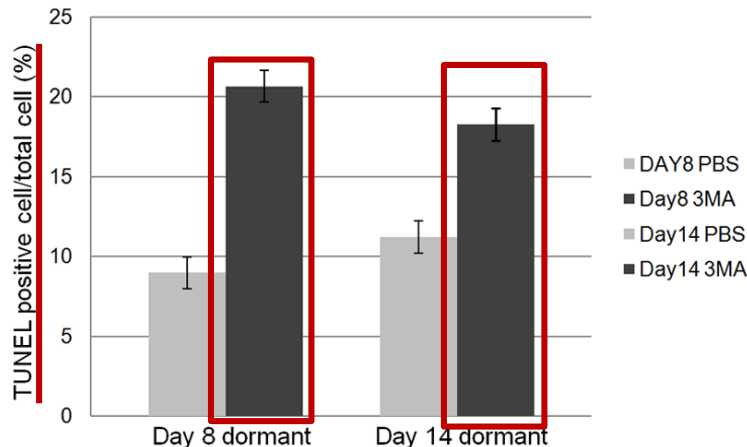
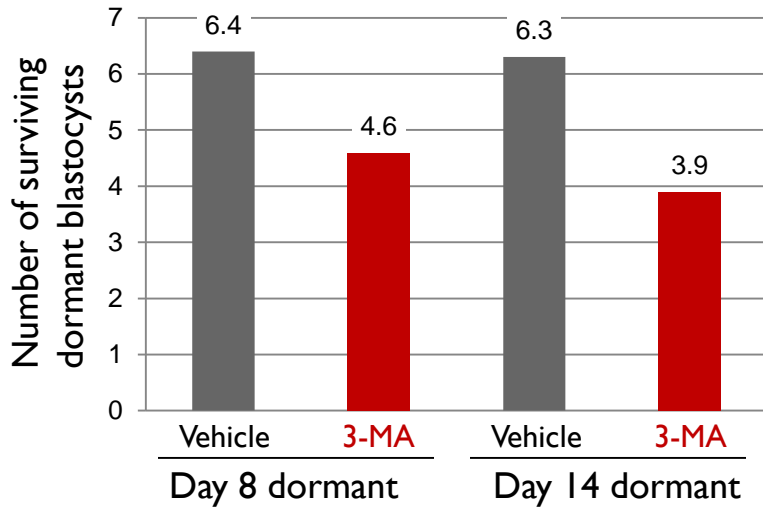
- Hormone deprivation induces autophagy in the mouse uterus (OVX)
- E<sub>2</sub> or P<sub>4</sub> suppresses autophagic activation in the uterus of OVX mice



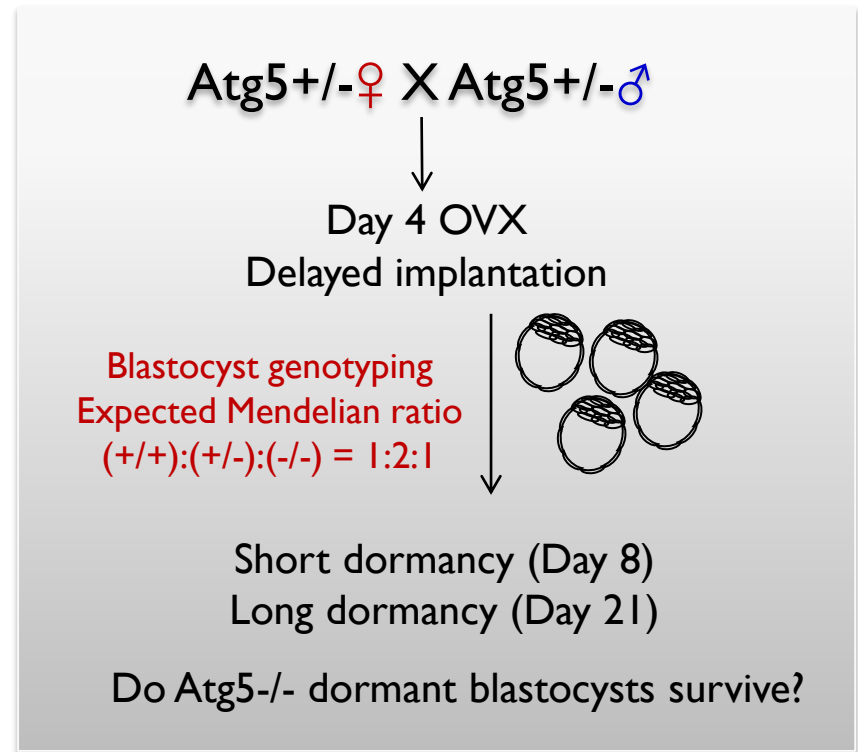
- Results: Dormant blastocysts turn on autophagy during delayed implantation.
- Question: What happens to them when autophagy is blocked?
  - 1) Pharmacological inhibitor: 3-methyladenine (3-MA)
  - 2) Atg gene deficient mouse models (*Atg5*)



# Injection of 3-MA to delayed implanting mice compromise survival of dormant blastocysts



*-The use of Atg5 deficient mice -*



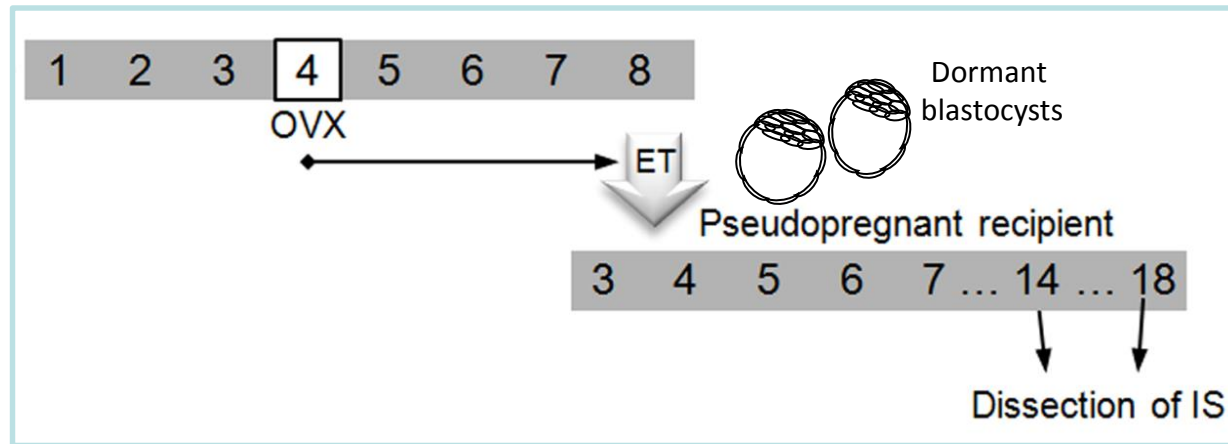
*(Ongoing)*  
*A clear tendency of decreased number of Atg5-/- embryos as the dormancy prolongs*

\* 3-methyladenine (3-MA): an inhibitor of PI3 kinase



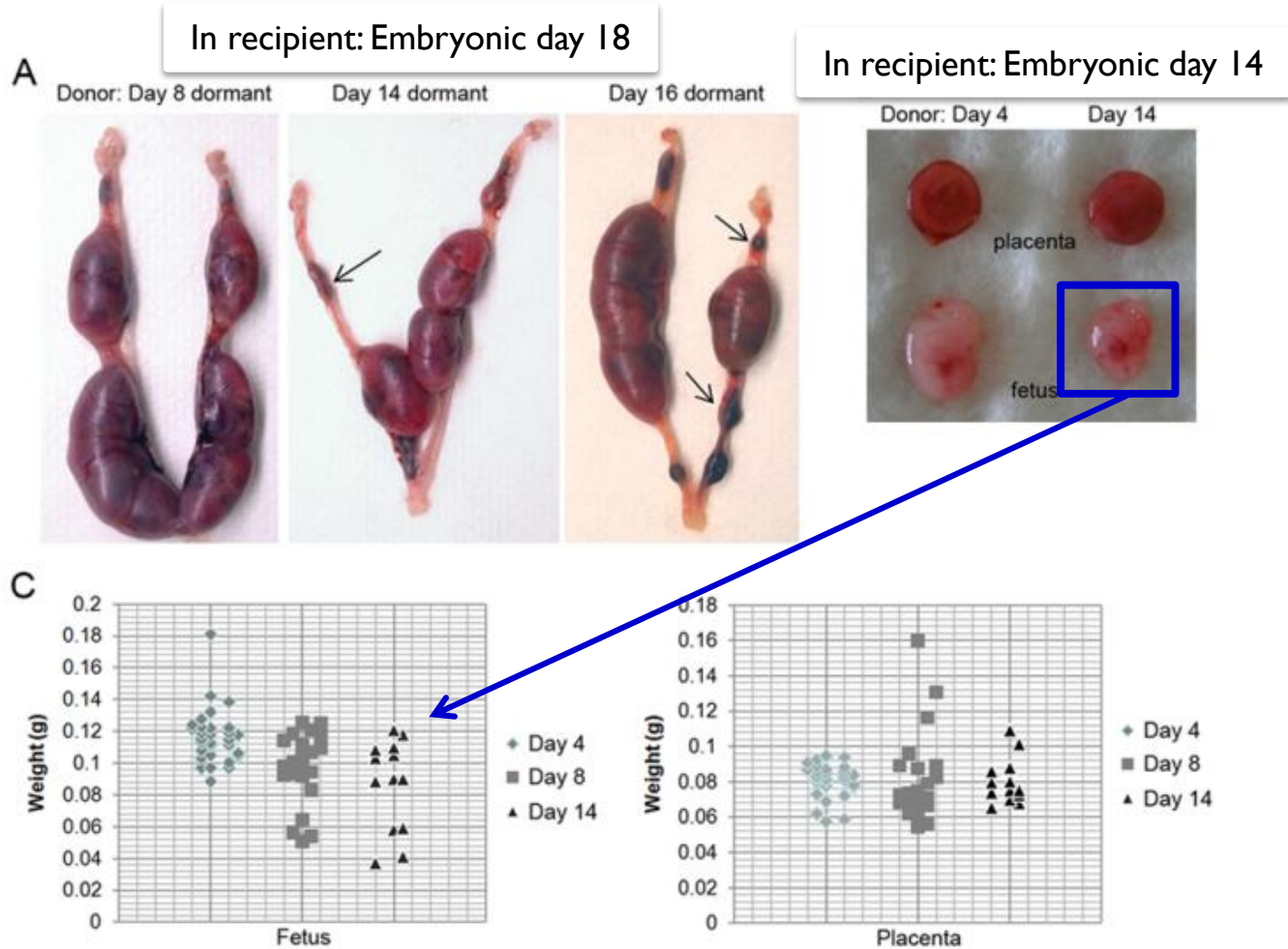
Developmental competence of activated blastocysts after prolonged dormancy:  
**Embryo transfer** to normal recipient mice on day 4 of pseudopregnancy

**Developmental competence:** normal implantation, normal postimplantation embryonic development, and normal placentation



Donor	Length of dormancy (days)	Total # of transferred blastocysts	Total # of recipient	Total # of normal IS (%)	Mean fetal weight (mg) Mean±SD	Mean placental weight (mg) Mean±SD
Day 4 normal	0	65	5	30 (46.2)	116±17	81±10
Day 8 dormant	3.5	66	6	24 (36.4)	96±22	81±25
Day 14 dormant	9.5	77	6	13 (16.9)	83±29	79±13

# Developmental competence of blastocysts is compromised with prolonged dormancy (IUGR)



- Blastocysts with longer dormancy show compromised developmental competence
- Manifestation of the intrauterine growth retardation (IUGR) phenotype

