

# Spermatozoa and oocytes induced *in vitro* from ESCs and iPSCs

Renier Myburgh  
Journal Club (05.03.2013)



# *Breakthrough of the Year 2012*



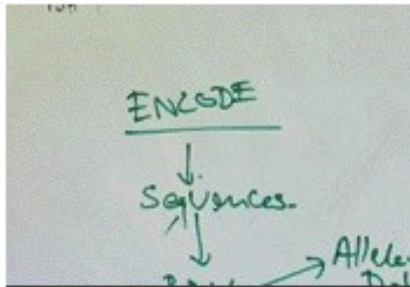
**Denisovan Genome**



**Genome Engineering**



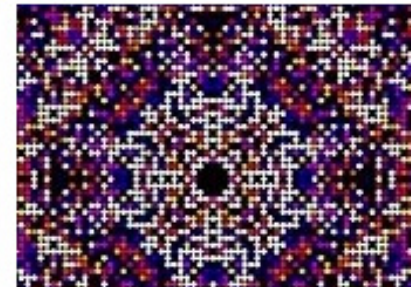
**Neutrino Mixing Angle**



**ENCODE**



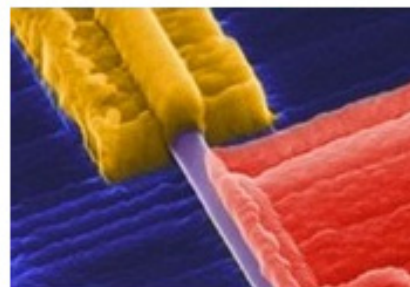
**Curiosity Landing**



**X-ray Laser Advances**



**Controlling Bionics**

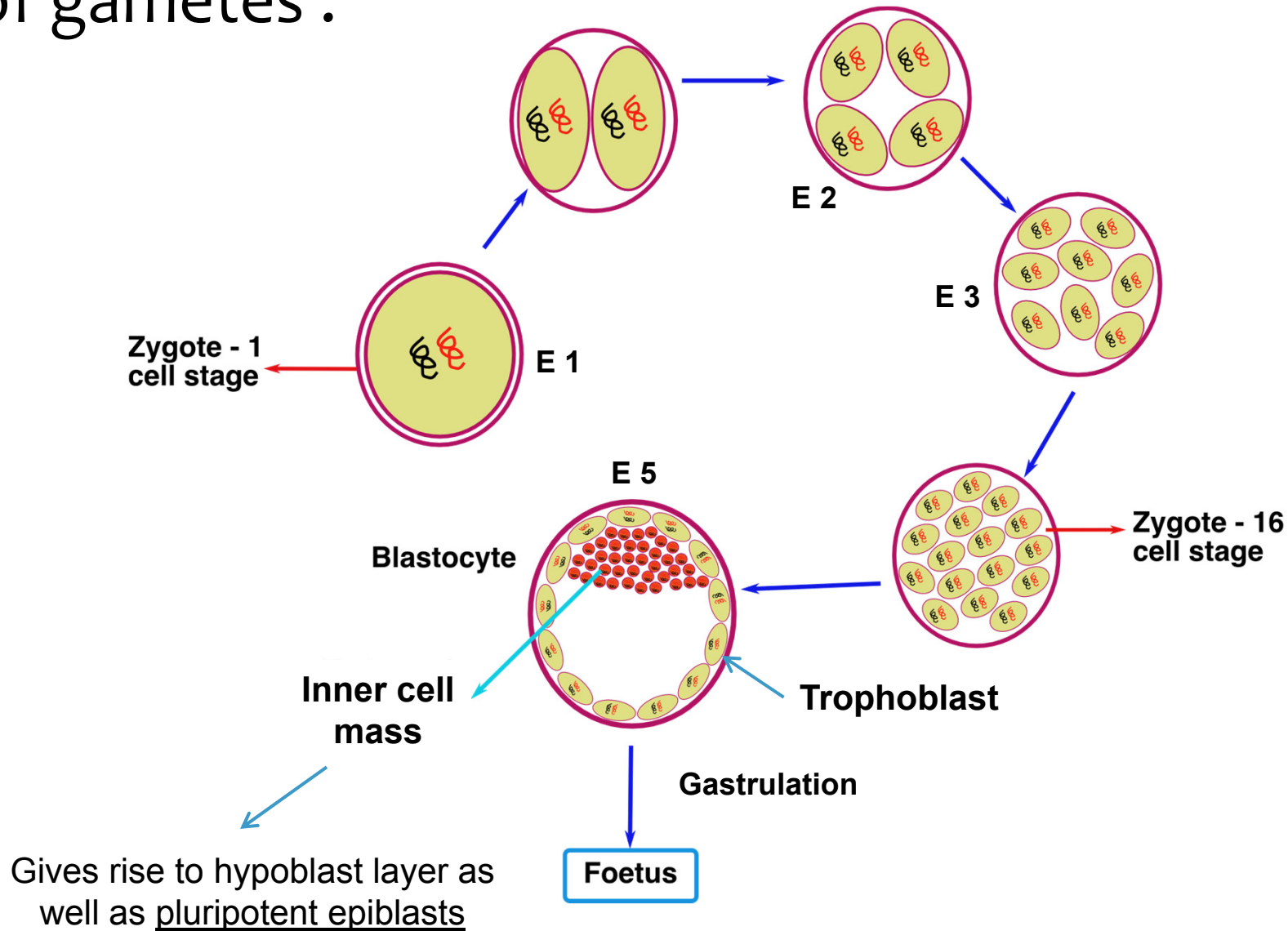


**Majorana Fermions**



**Eggs from Stem Cells**

# Embryogenesis & origin of gametes :



# Germ cell generation *in vivo* in mice:

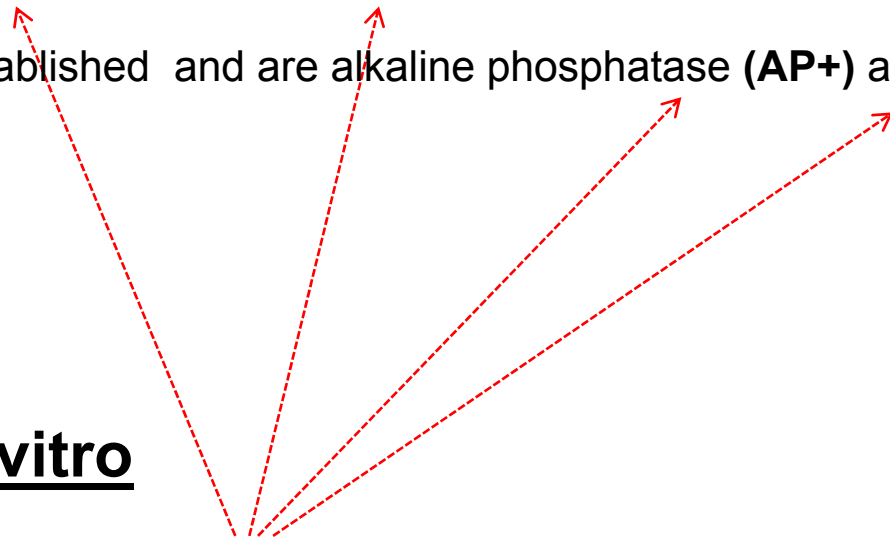
**E5.5-E6** germ cell fate induced in **epiblasts** by signals from bone morphogenetic protein 4 (**Bmp4**)

Early epiblast cells express **Blimp1** (Prdm1) and **Prdm14** in response to **Bmp4**

E7.5 primordial germ cells (**PGC**) are established and are alkaline phosphatase (**AP+**) and **Dppa3**

## Objective: Reproduce in vitro

ESC or iPSCs → PGC-like cell origins of sperm and oocytes



## Previous attempts :

- Generate gametes or PGCs *in vitro* from ESCs and EpiSCs where the cells were differentiated spontaneously under undefined conditions
- ESCs resulted in obtaining PGCs at very low efficiency 0,1%. The induced PGCs have not shown to be able to produce healthy offspring.
- EpiSCs express Blimp1 under self-renewing conditions but still in the presence of BMP4 low frequency (1.52%) of PGCs were obtained. The function of the obtained PGCs have not been demonstrated *in vivo*

(Nayernia et al., 2006 & Hayashi and Surani. 2009)

# Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells

Katsuhiko Hayashi,<sup>1,3</sup> Hiroshi Ohta,<sup>1,3</sup> Kazuki Kurimoto,<sup>1,3</sup> Shinya Aramaki,<sup>1</sup> and Mitinori Saitou<sup>1,2,3,\*</sup>

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DOI 10.1016/j.cell.2011.06.052

Aim : ESC or iPSCs <sup>1</sup> → EpiLCs <sup>2</sup> → PGC-like cell: origins of sperm

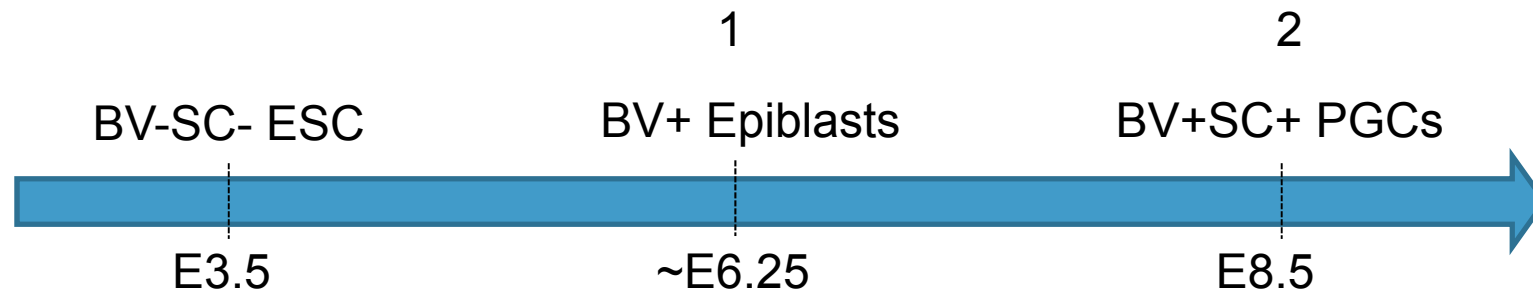
Define conditions for **a two step differentiation** where **ESC** and **iPSCs** with naive pluripotency can be **induced** into **pregastrulating** (E5.5 - E6.0) epiblast-like cells (EpiLCs), in turn **induced** into **PGC-like cells** which should contribute to **spermatogenesis**.

**BVSC transgenic mice:** Source of ESC are E3.5 preimplantation blastocyst

(Blimp1-mVenus-Stella-ECFP) **BVSC**

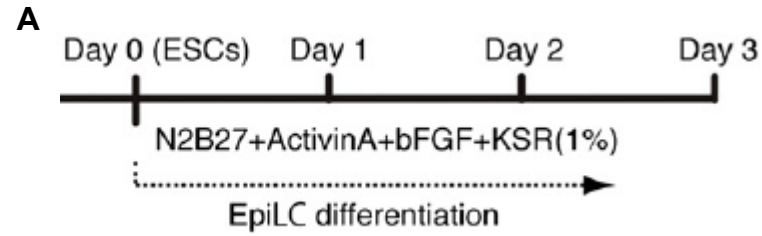
Allows for **marking** of **Blimp1** expression in **precursors** of PGCs at/and before E6.25 (**EpiLCs**)

**Specifically** illuminates **Blimp1-** and **Stella-**positive **PGCs** after E8.5



# Results: 1

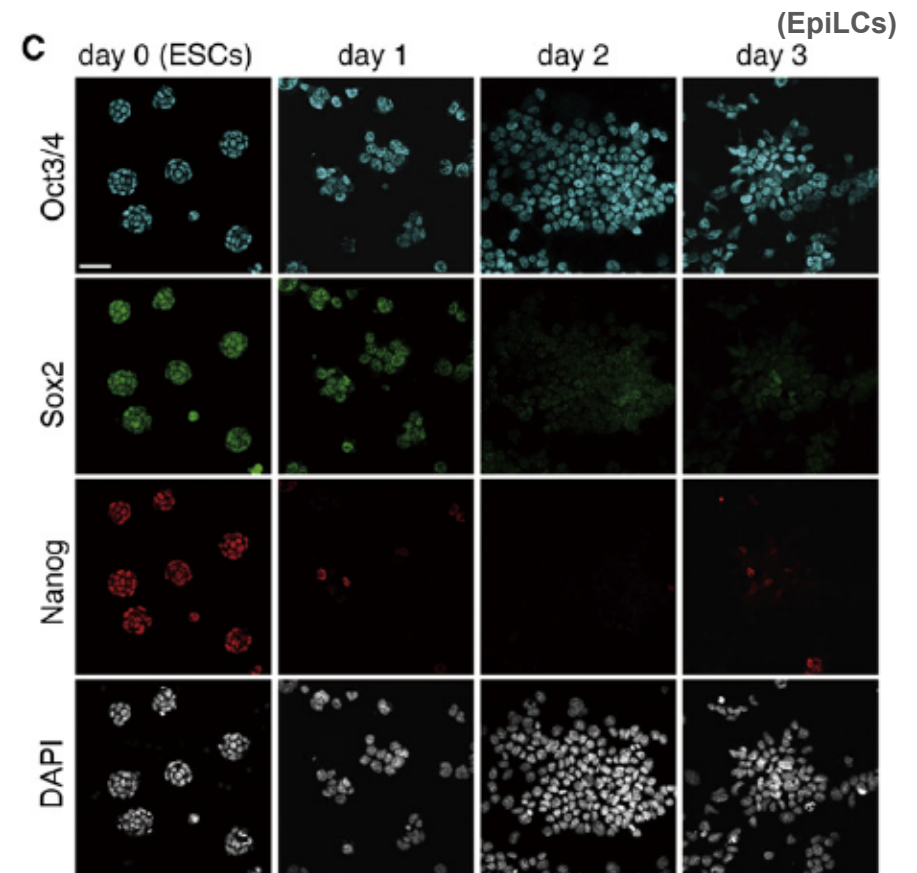
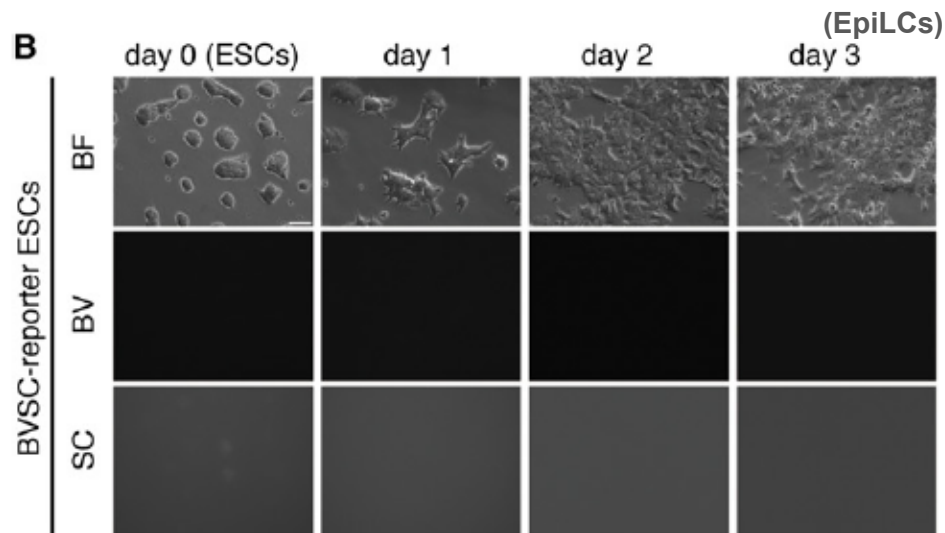
## i) EpiLC induction from ESCs:



Dome shaped

Flattened

Immunofluorescence



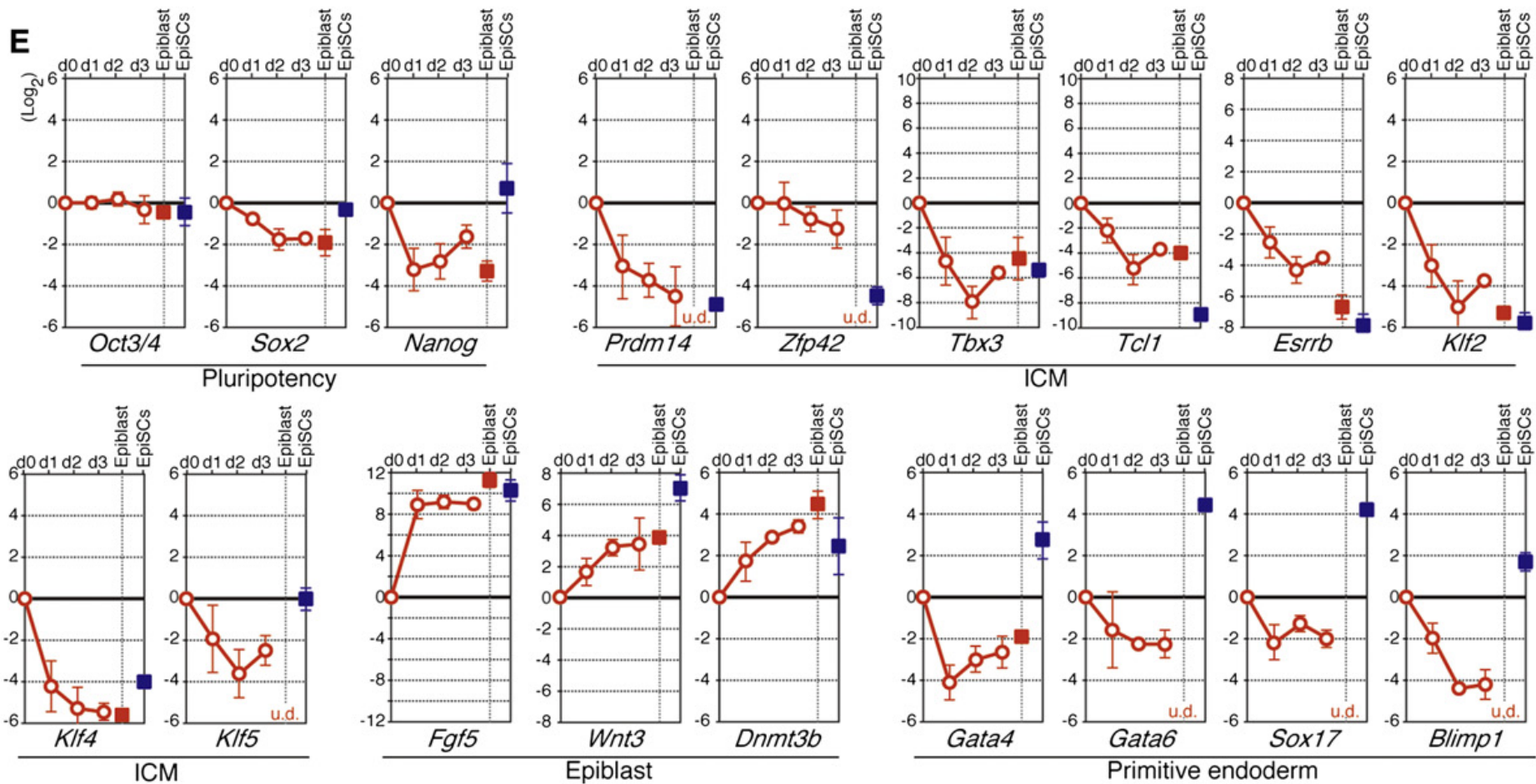
*ECS derived from E3.5 blastocysts bearing *Blimp1-mVenus-stella-ECFP* (BVSC) transgenes*



# EpiLC induction from ESCs: 1

QPCR of gene expression profiles of EpiLCs, Epiblasts (E5.75) and EpiSCs

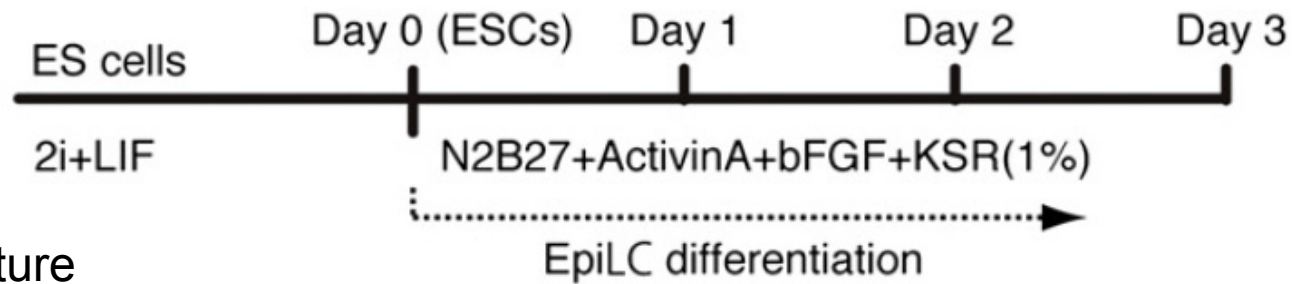
**EpiLCs similar to pregastrulating epiblasts and distinct from EpiSCs**



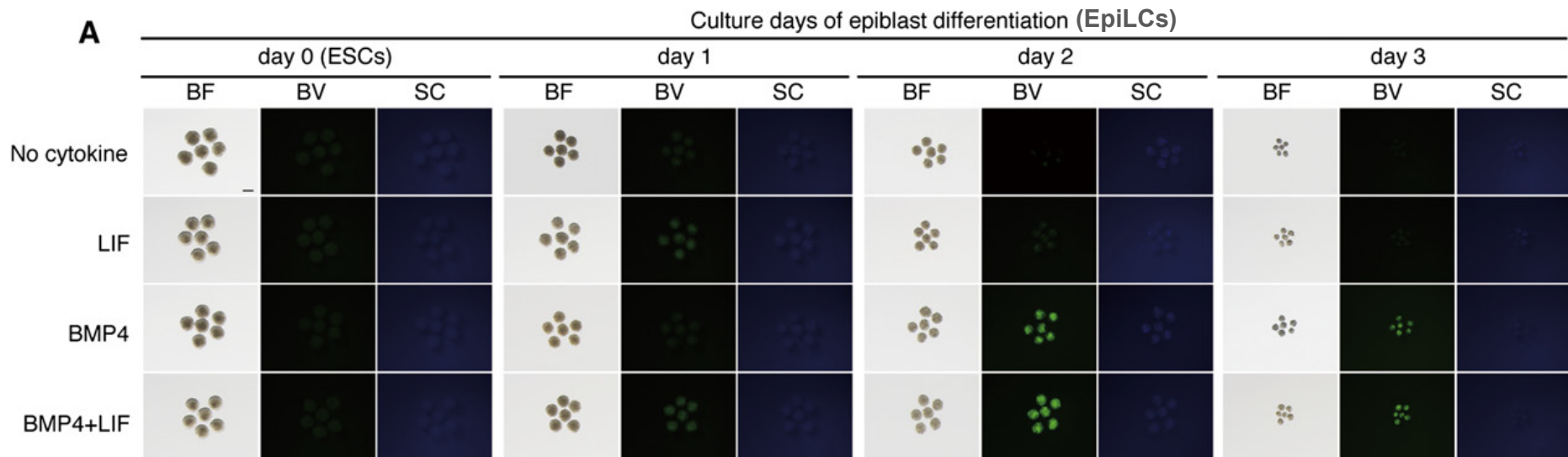
## When to induce PGCLCs from EpiLCs? 1 → 2

Condition when the cells can respond optimally to Bmp4

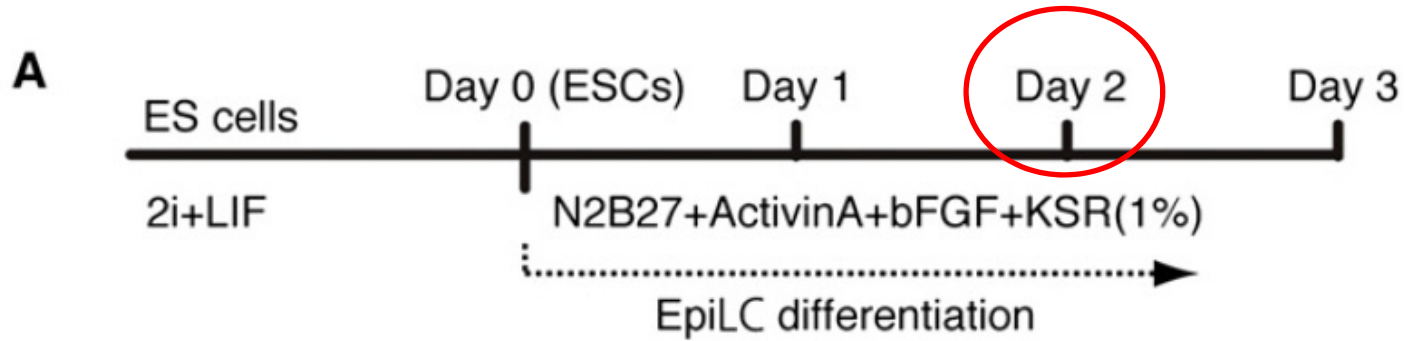
Effect of cytokines on BV (Blimp1-mVenus) partial induction GMEM + KSR15%



2 days of culture

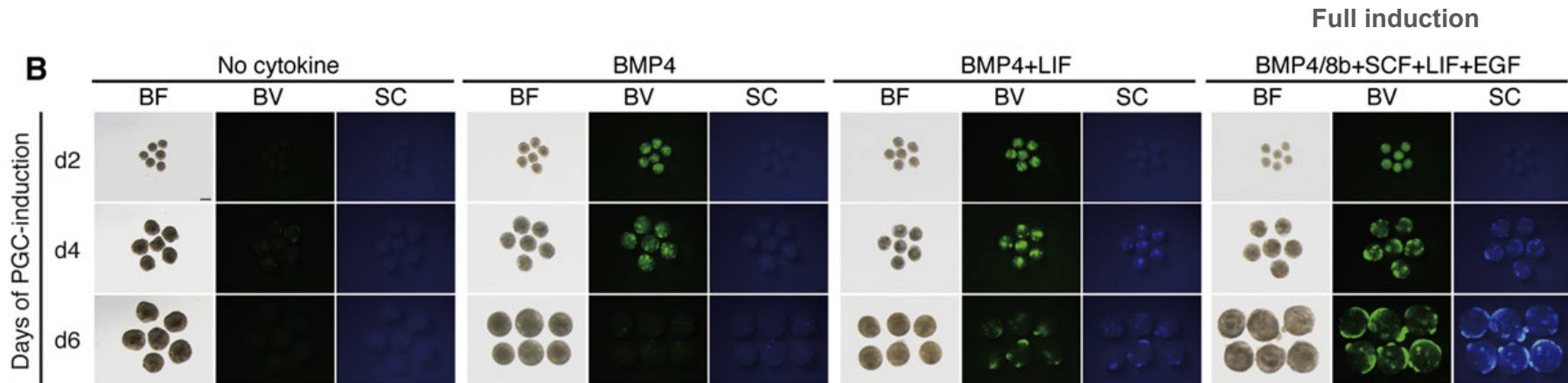


**d2 EpiLC induction to PGCLC : Bmp4/8b + SCF + LIF + EGF**



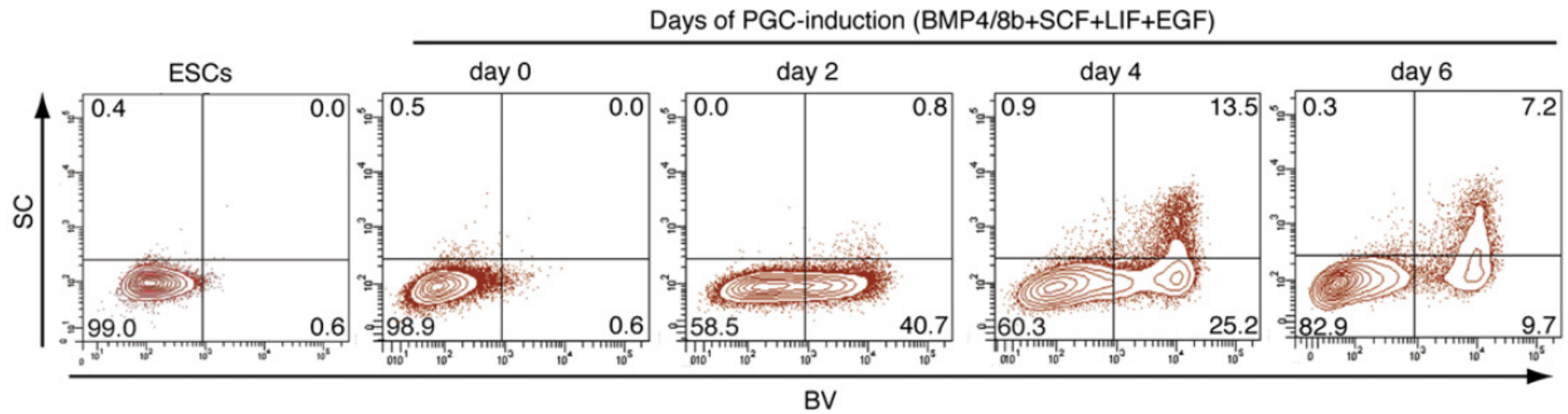
Effect of **full** induction on the **day 2** cultured **EpiLCs** from the previous slide

Look for BV & SC expression



8 days of culture in total

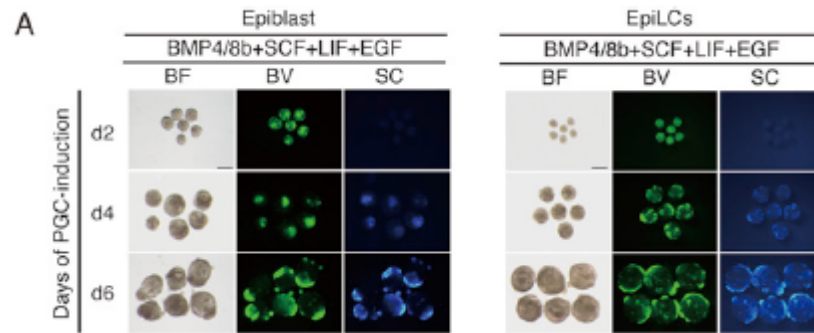
**d2 EpiLC induction to PGCLC : Bmp4/8b + SCF + LIF + EGF**



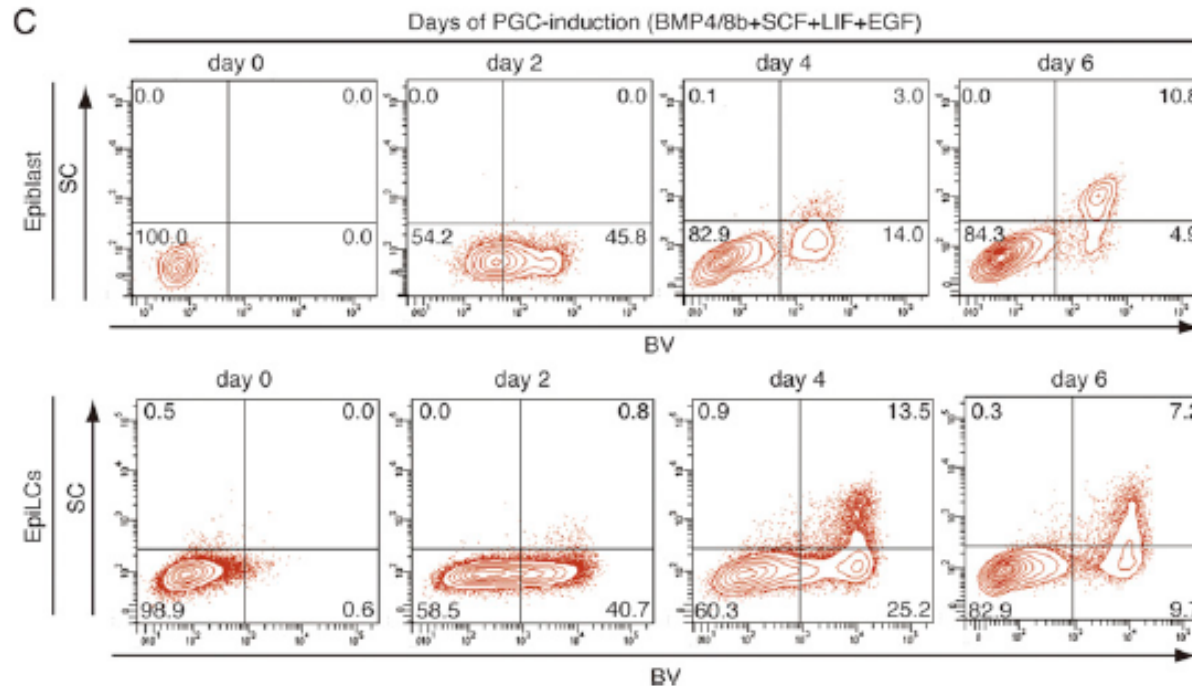
*Alkaline phosphatase expression was also confirmed at day 6*

# EpiLCs vs Epiblasts: BVSC positivity under full induction

*Supplementary  
Figures*



Both cell types have the same competence to express **Blimp1** in response to **Bmp4**



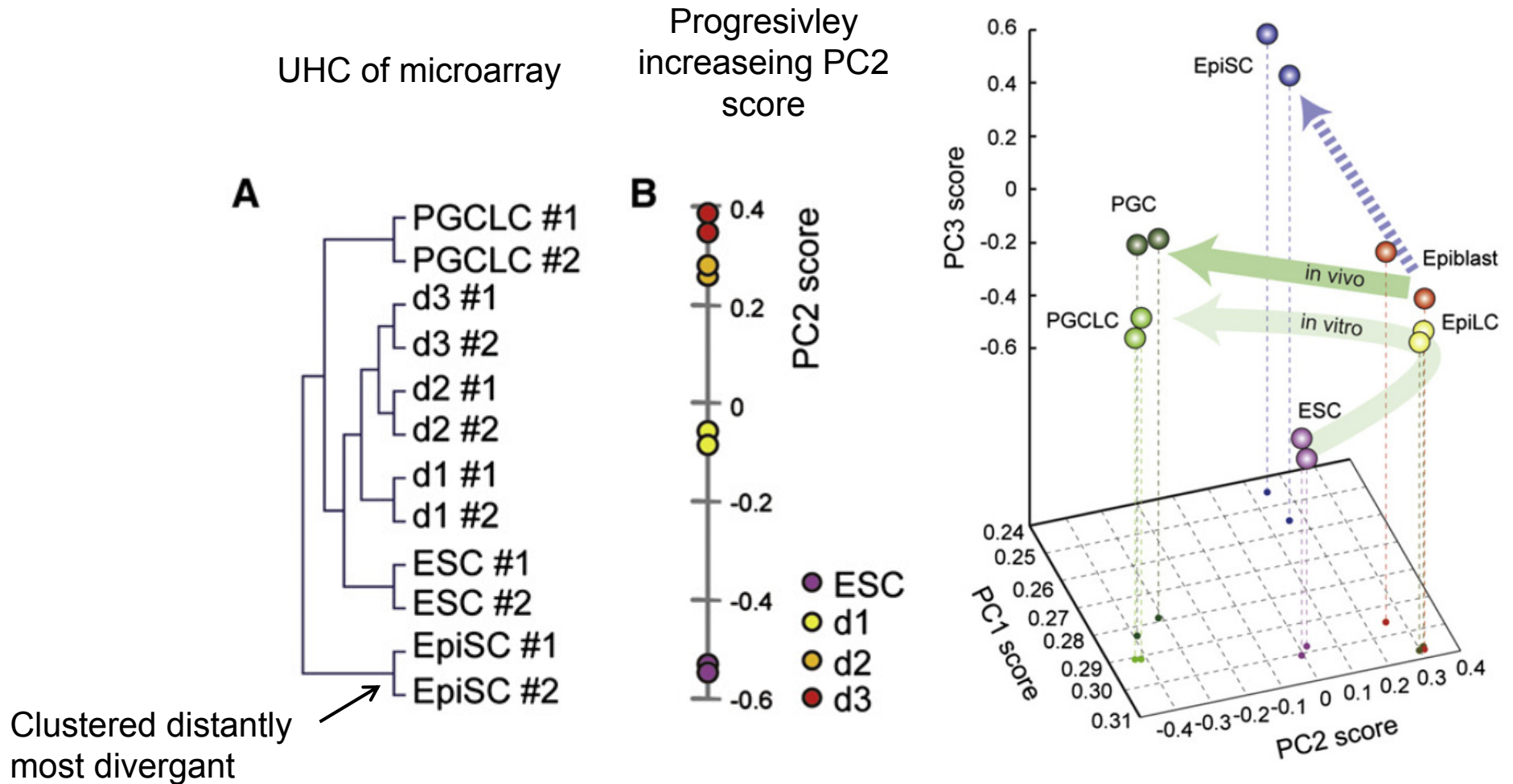
**2 day EpiLCs = natural Epiblast cells**

## **Are PGCLCs really similar to PGCs?**

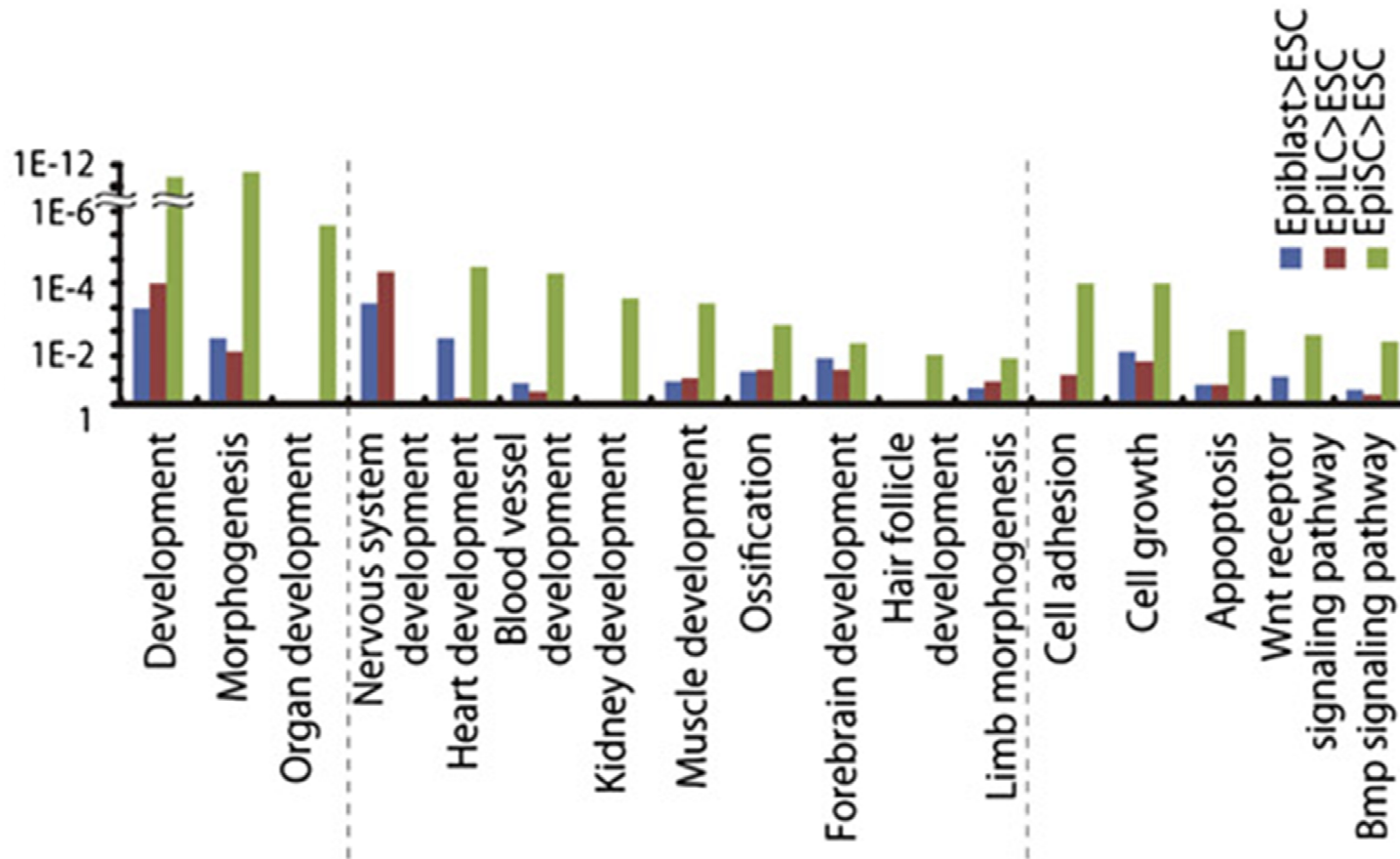
- Transcription profiles
- Epigenetic reprogramming
- Cellular dynamics

# Global transcription profiles during PGCLC induction from EpiLCs are analogous to PGC specification from epiblasts.

- Total RNA from ESCs, d1/2/3 EpiLCs, EpiSCs, Epiblasts, BVSC(+) PGCLCs & stella-EGFP(+) PGCs



## Global transcription profiles:



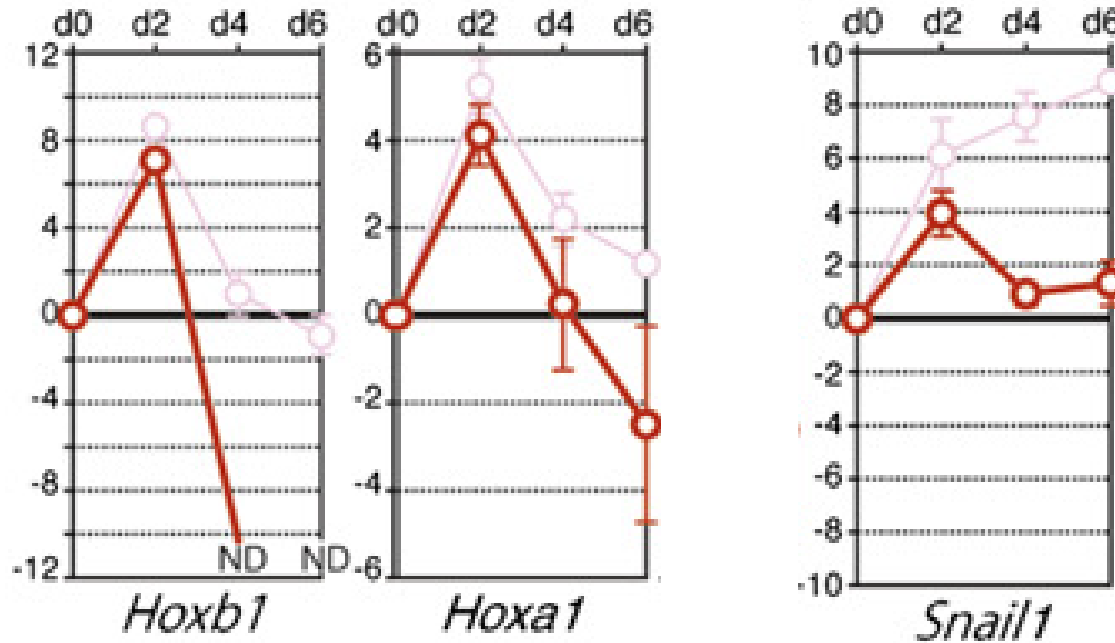
EpISCs upregulate more genes associated with development of different organ systems

The d2 EpiLC showed similar expression profiles to Epiblast cells

EpISCs acquire more developmentally advanced characteristics than Epiblasts/d2EpiLCs



**PGCLCs:** Genes associated with specific differentiation of **non-germline cell types**



Pink lines  
BV- cells

Red lines  
BV+ SC+

*Snail* (*Snail1*) gene is expressed during palate development in mice

*Murray et al., 2007*  
*Development and disease*

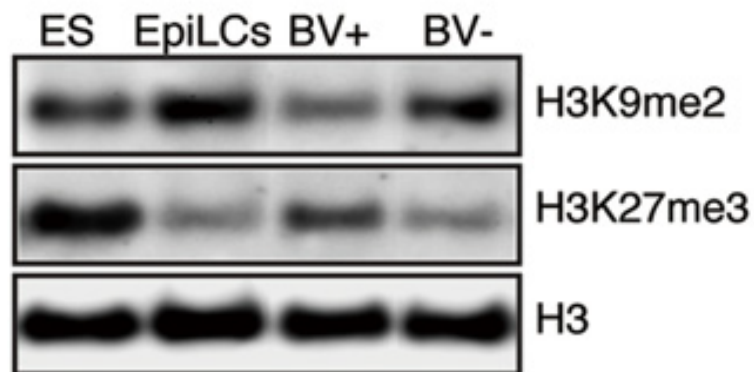
Hind brain development

## Epigenetic profiles of PGCLCs: DNA methylation markers

H3K9me2 = di-methylation of lysine 9 on histone (H3)

H3K27me3 = tri-methylation of lysine 27 on histone (H3)

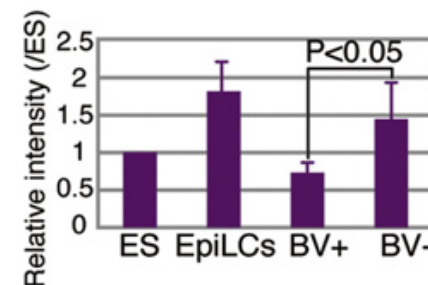
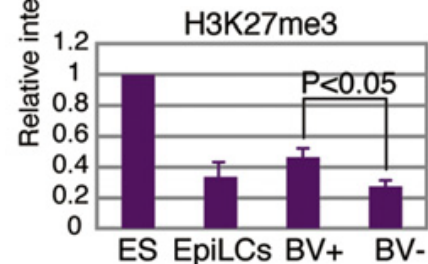
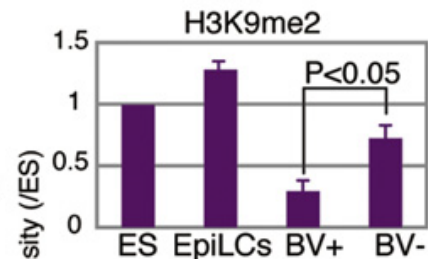
5mC = methylated form of DNA base cytosine



Western blot analysis



Dot blot analysis 5mC

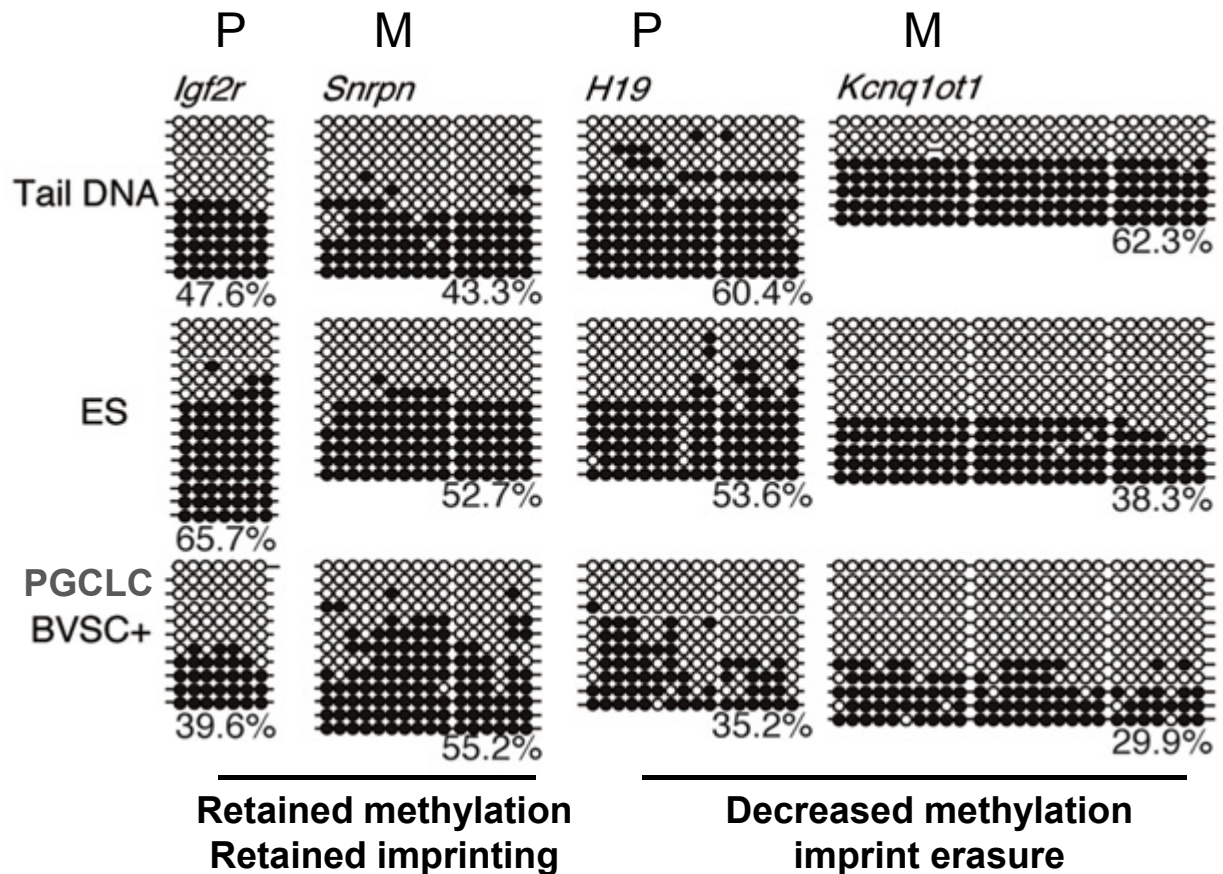


Histone modification and 5mC changes during PGCLC induction *in vitro* = PGC formation *in vivo*

**Genomic imprinting:** Imprinting occurs during embryogenesis to allow monoallelic gene expression due to methylation

Bisulfite sequence analysis  
 5mC methylated regions: ○ unmethylated  
 ● methylated

In germ line cells the imprint can be erased and then re-established according to the sex of the individual



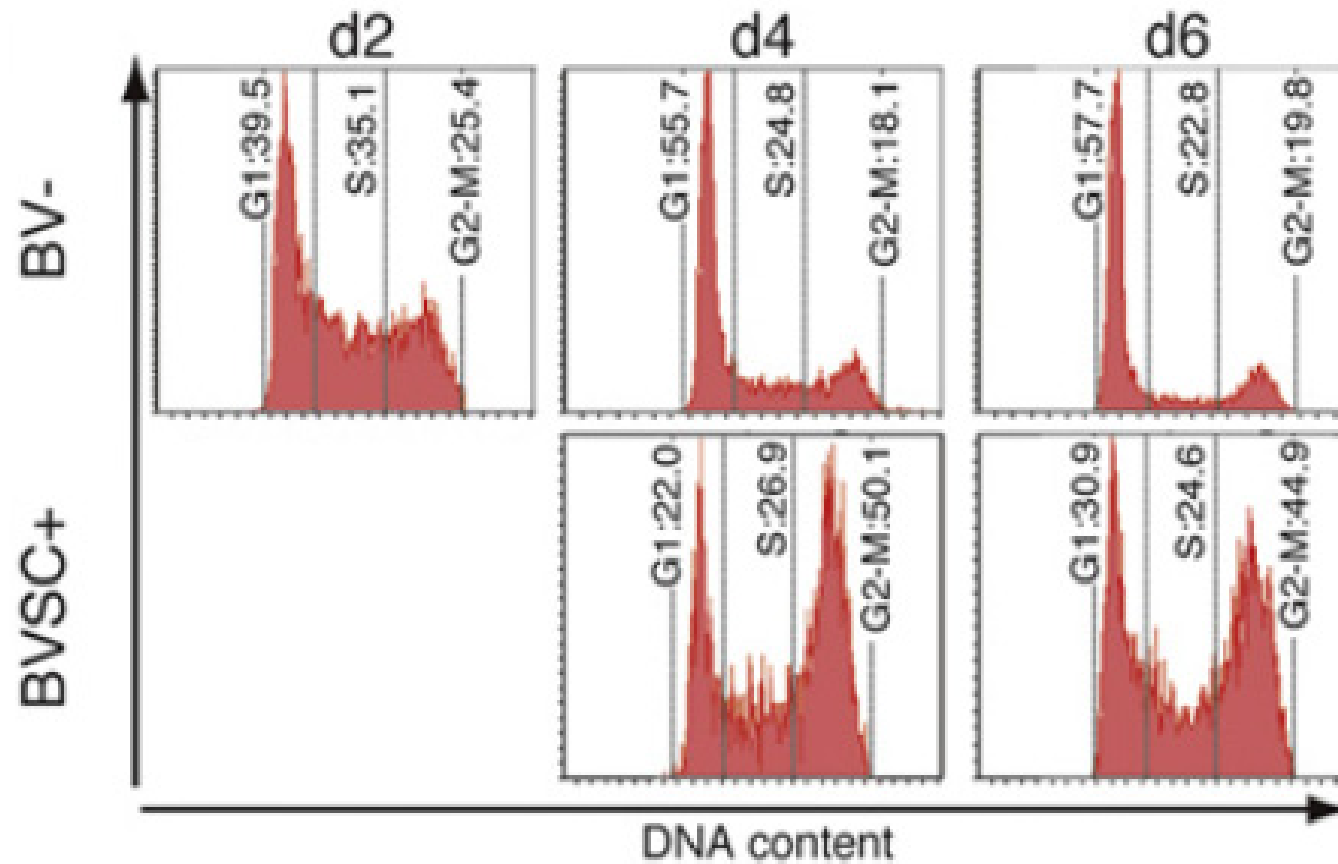
Global decrease of 5mC

Relative maintenance of imprinting in PGCLCs

Consistent with that of PGCs

*Lee et al., 2002*

# Cellular dynamics: FACS analysis of the cell cycle states during PGCLC induction



BVSC + Enrichment in G2 phase, slower growth = PGCs

BV- cells have similar profiles to cycling somatic cells

## Spermatogenesis and offspring derived from PGCLCs:

- This is tested by transplanting the PGCLCs into seminiferous tubules
- $W/W^v$  mice are viable but sterile, germ cell deficient

### Procedure:

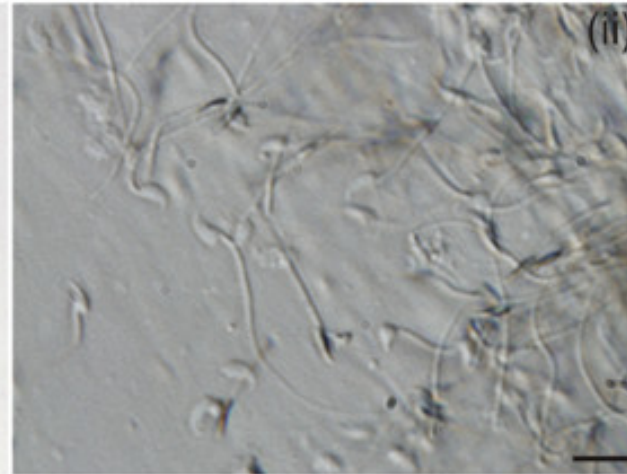
- PGCLCs were induced for 6d (*Bmp4/8b* + *SCF* + *LIF* + *EGF*)
- FACS sorting of BV+
- Transplantation of BV+ FACS sorted cells and nonsorted cells from aggregates
- ( $10^4$  cells) per testis
- Evaluation after 10 weeks

# PGCLCs normal spermatogenesis:

- 50% of testes with BV(+) transplanted PGCLCs had seminiferous tubules with proper spermatogenesis, nonsorted cells 100% teratoma

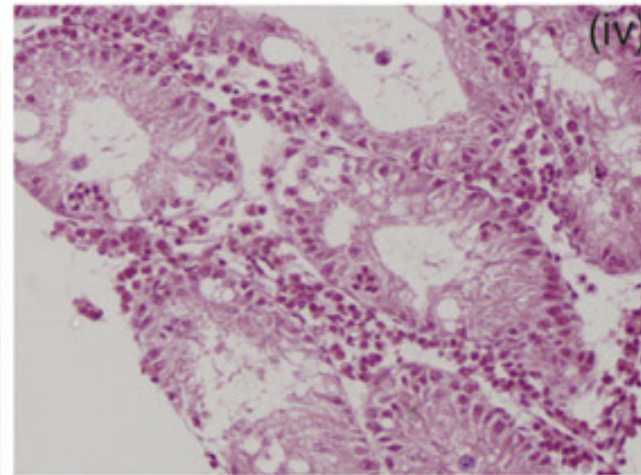
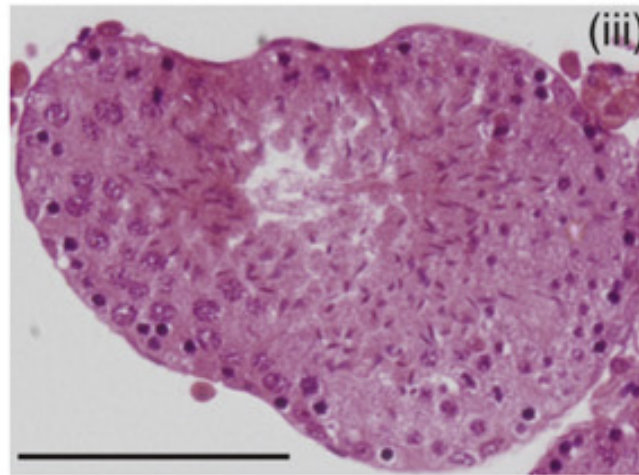
Thin tubules contained only Sertoli cells

Dark central sections = spermiation



Sperm

Histological Analysis



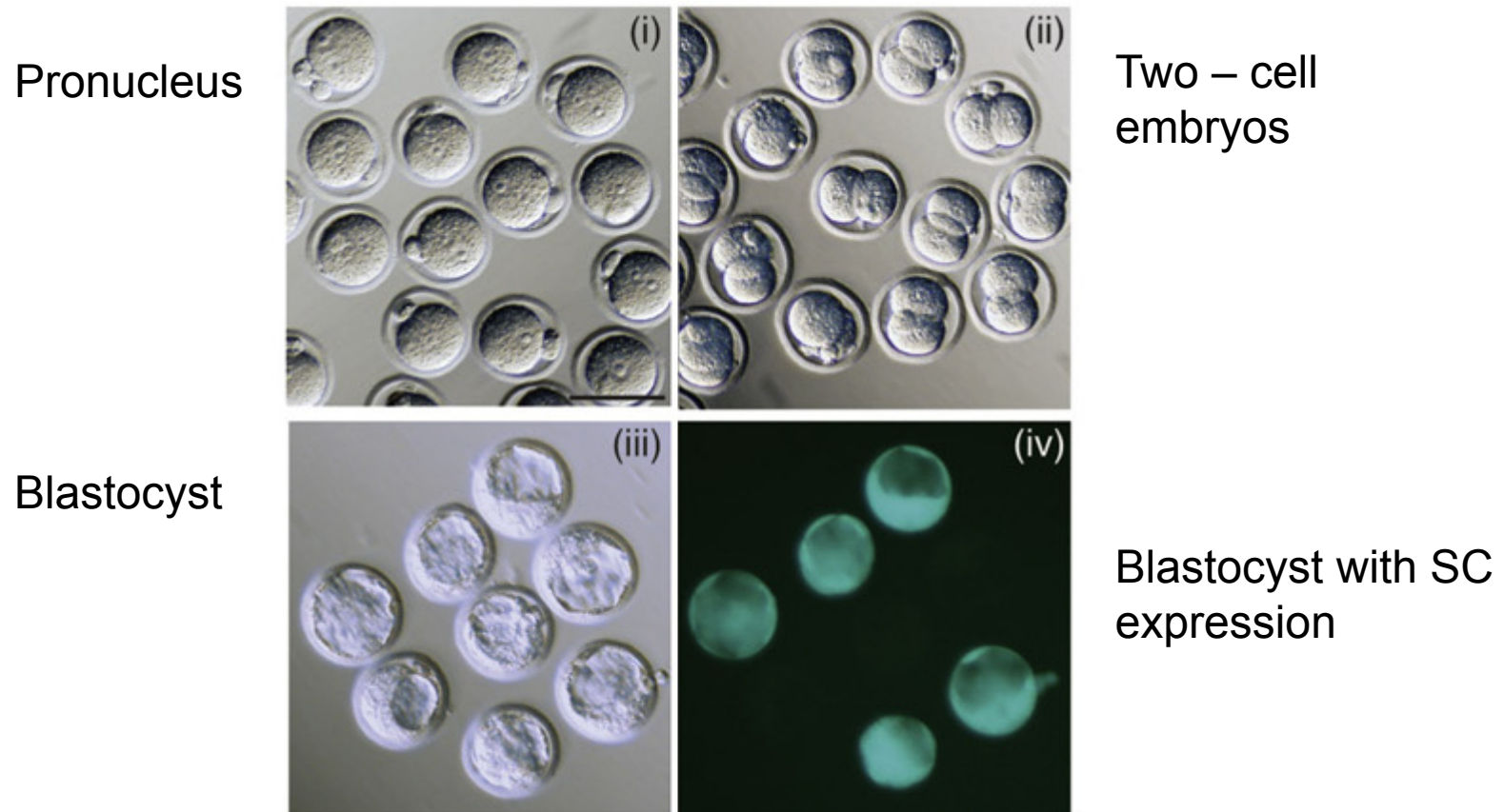
Spermatogenesis

No spermatogenesis

Hematoxylin & Eosin stain

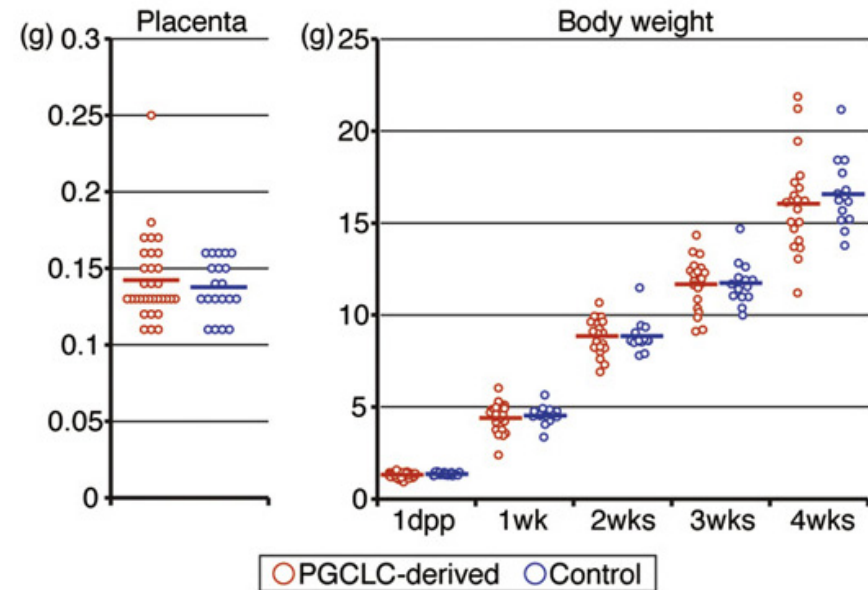
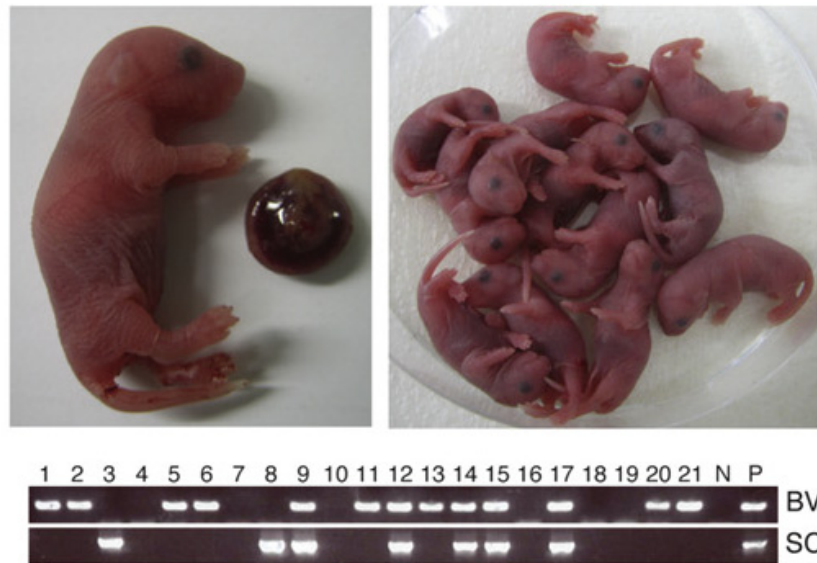
## Oocyte fertilization with PGCLCs derived sperm: ICSI

- Performed intracytoplasmic sperm injection (ICSI)
- Resulting zygotes developed normally and blastocysts showed SC expression



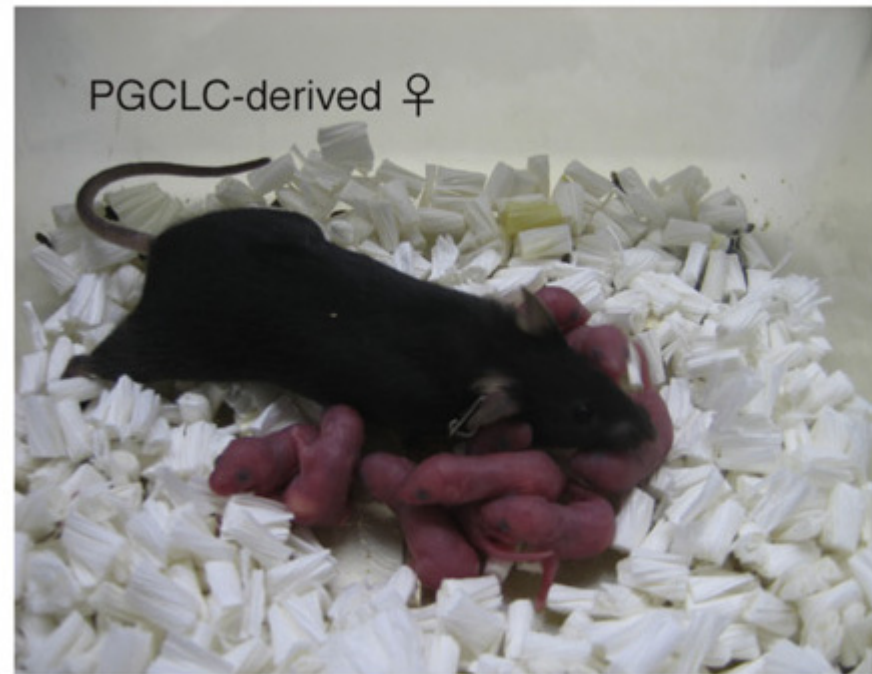
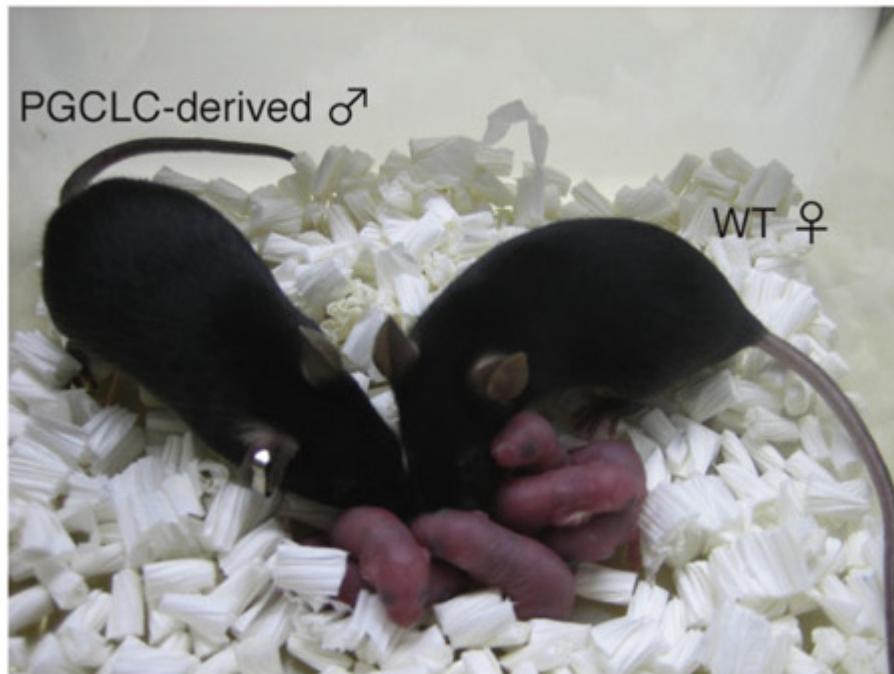
## PGCLC derived offspring:

- Embryos at blastocyst stage were transplanted to foster mothers
- 5 out of 21 had both BV and SC transgenes 23% = haploid donor spermatozoa



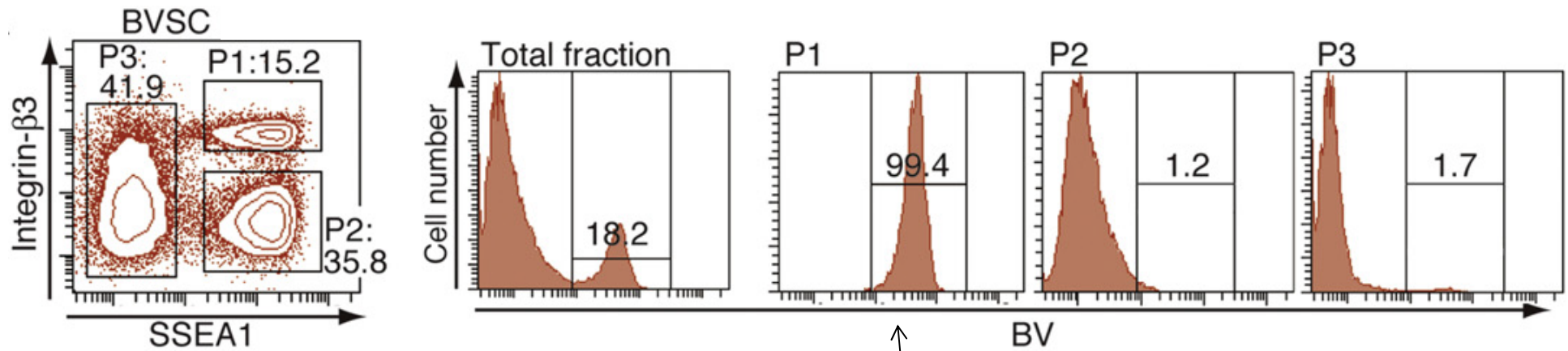


**PGCLC derived offspring are fertile:**



## Identification of surface markers to isolate a pure population of PGCLCs:

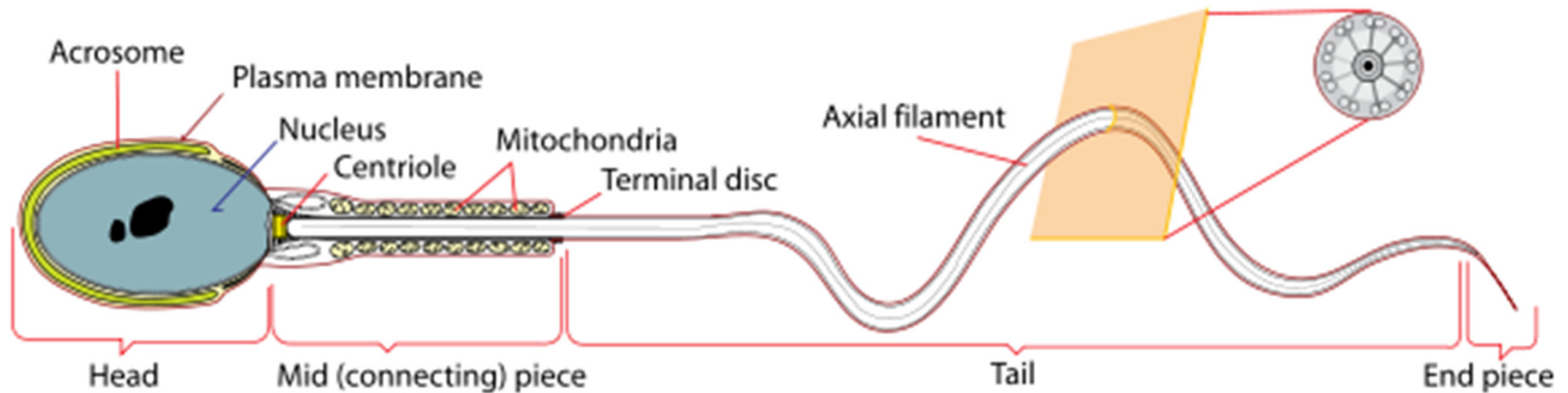
- Essential for isolation of PGCLCs with no transgenic reporters
- Determine which markers define BV(+) population (BV- = teratoma)
- FACS sorting of PGCLC 6d aggregates SSEA1 and integrin-b3



99% of cells in P1 BV positive

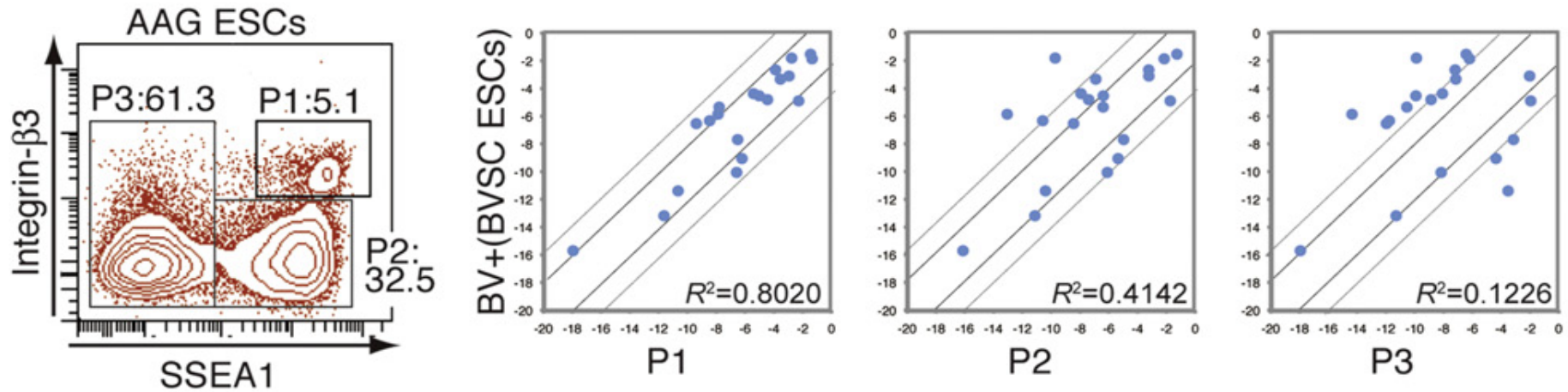
## Induction of ESC carrying Acro/Act-EGFP transgenes:

- double eGFP transgenic mouse
- eGFP expressed by ubiquitous b-actin promoter and specific acrosin promoter
- All germ cells including haploid cells and spermatozoa are GFP+
- Acrosin is a digestive enzyme that acts as a protease and released from the acrosome helps sperm penetrate the egg



## Induction of ESC from Acro/Act-EGFP (AAG) mice to PGCLCs:

- d6 aggregates were FACS sorted for SSEA1 and Integrin- $\beta$ 3



- Expression of 20 different genes were compared:

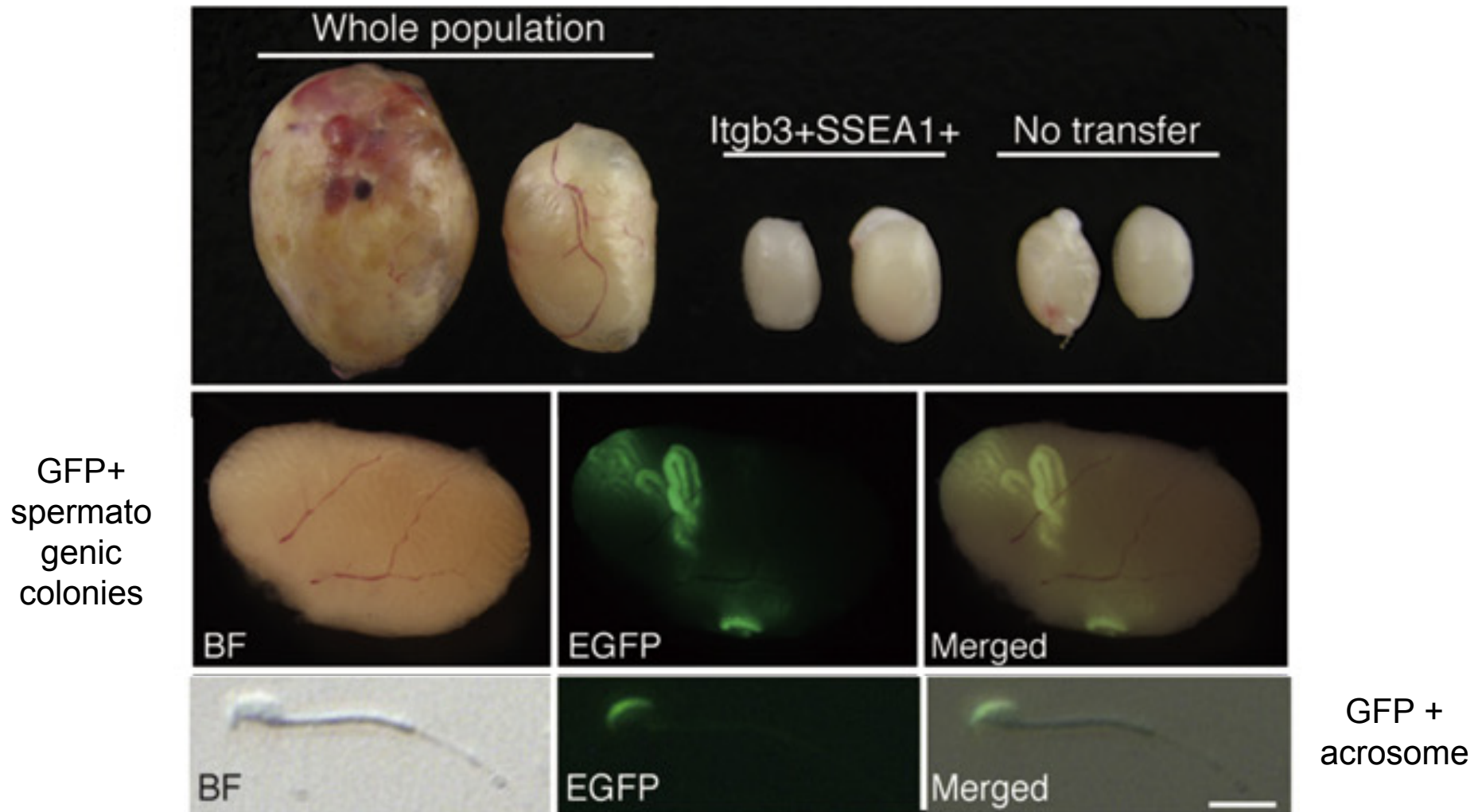
P1 (AAG\_ESC derived PGCLCs) vs. P1 (BVSC\_ESC derived PGCLCs)

## Do AAG\_PGCLCs (P1) cells contribute to spermatogenesis:

Transplant P1 cells and whole pop. into seminiferous tubules of W/W<sup>v</sup> mice

Teratomas in all testes for whole pop.

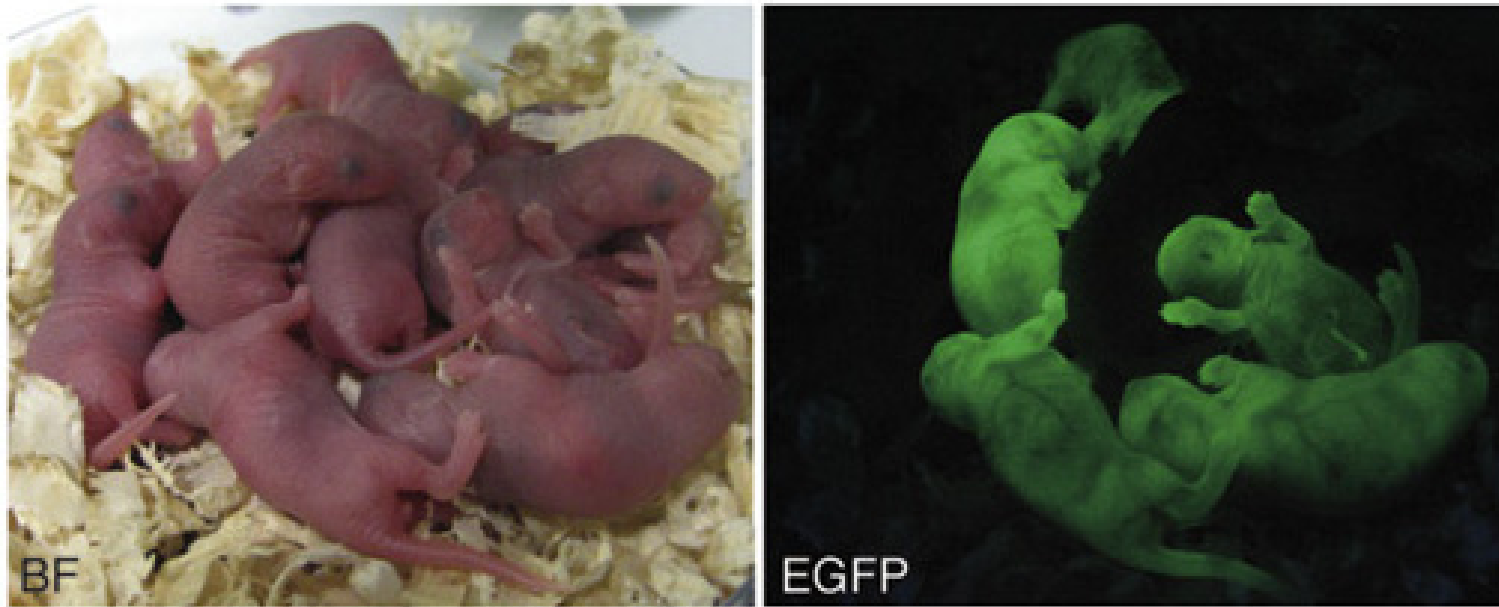
5 out of 6 testes with P1 cells = spermatogenesis



## AAG\_PGCLCs derived sperm produce viable offspring:

- Oocytes were fertilized via (ICSI) and embryos transferred to foster mothers

50% GFP+



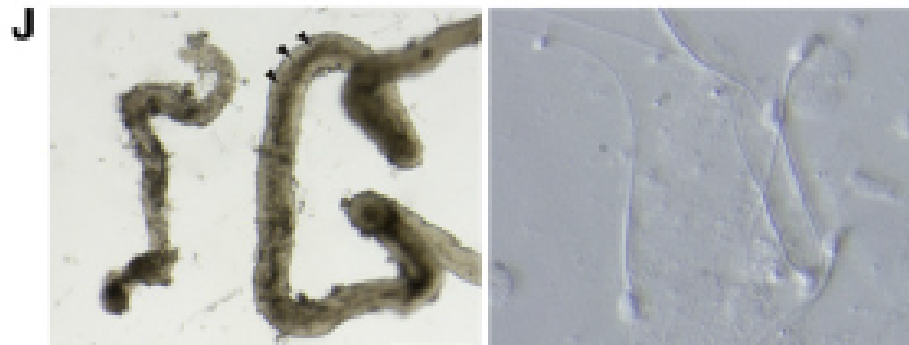
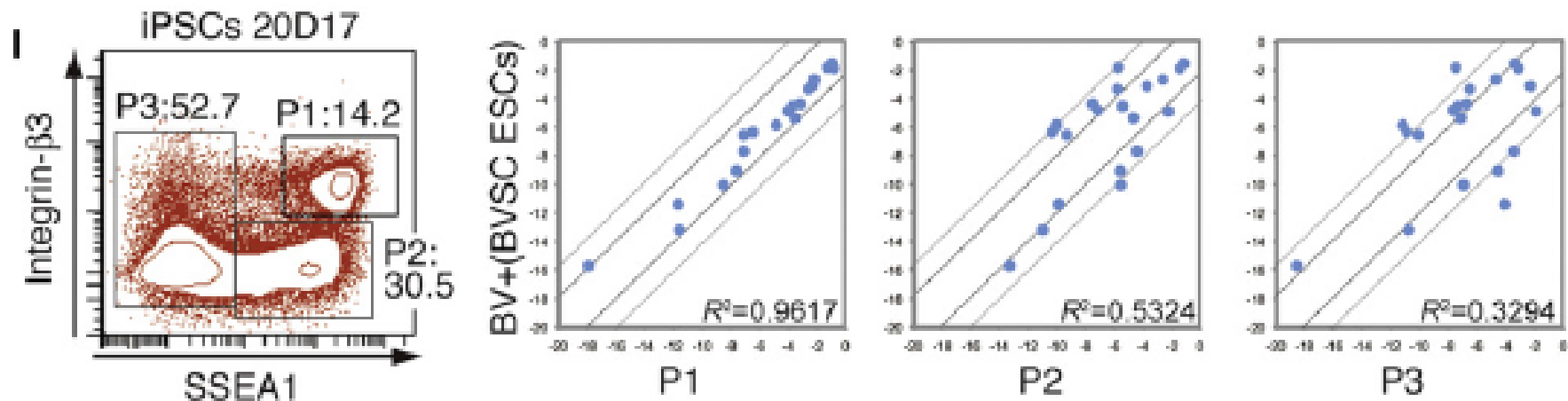
**Conclusion:** SSEA1 and Integrin- $\beta$ 3 selection purifies PGCLCs induced from ESCs regardless of the presence of relevant transgenic markers such as Blimp1 and Stella (BVSC)

## Is this possible with iPSCs:

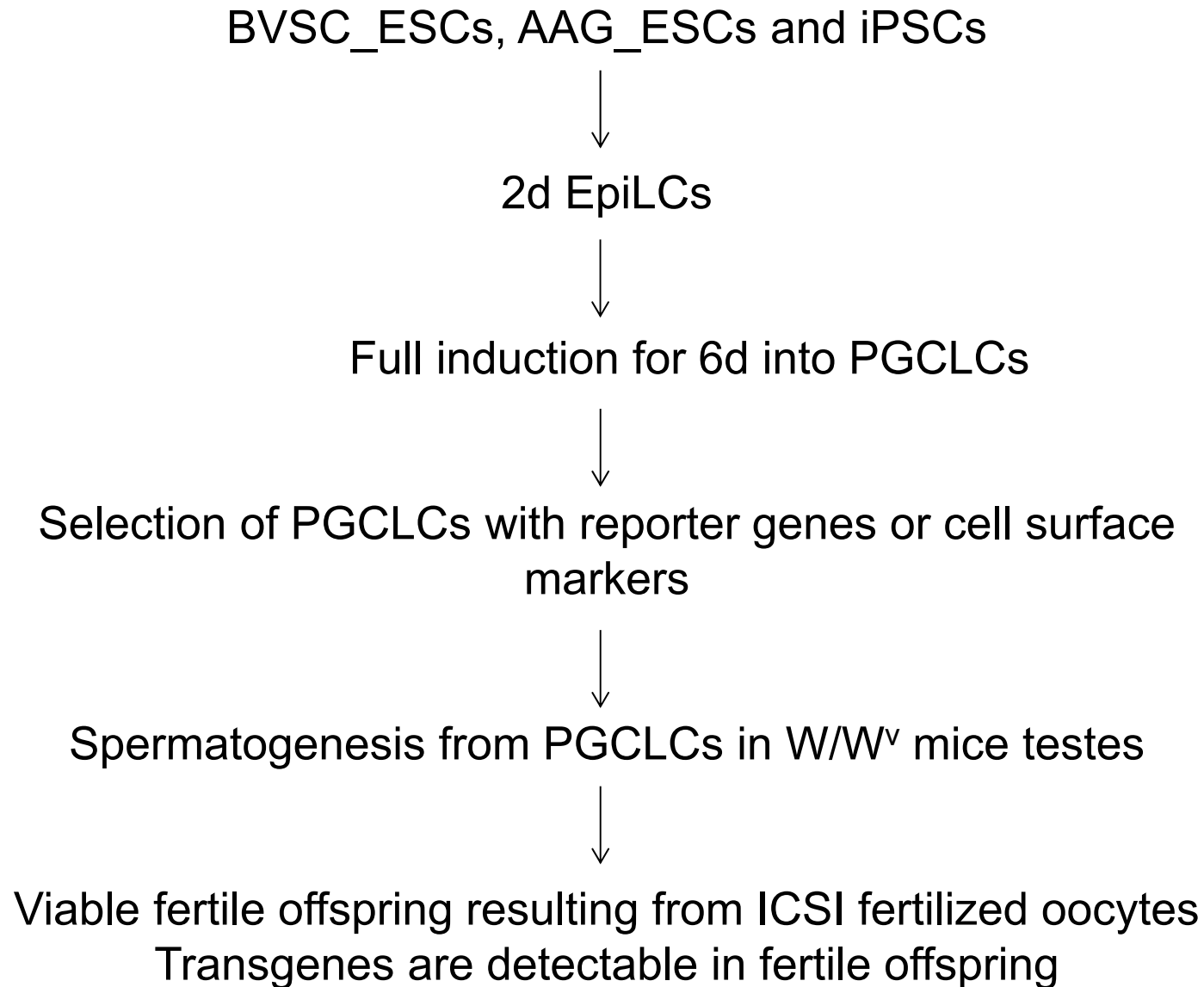
Induced PGCLCs from iPSCs using the same culturing conditions as before

FACS sorting of P1 cells

iPSC derived P1 PGCLCs gene expression correlated with BV+ PGCLCs (20genes)



## Summary:





# Offspring from Oocytes Derived from in Vitro Primordial Germ Cell-like Cells in Mice

Katsuhiko Hayashi,<sup>1,2,3\*</sup> Sugako Ogushi,<sup>1,4</sup> Kazuki Kurimoto,<sup>1,5</sup> So Shimamoto,<sup>1</sup>  
Hiroshi Ohta,<sup>1,5</sup> Mitinori Saitou<sup>1,2,5,6\*</sup>

SCIENCE VOL 338 16 NOVEMBER 2012

“This is more important than the previous paper because oocytes are far less abundant in nature than spermatozoa”

# Summary of methods :

## 1) in vitro:

Female ESC → PGCLCs → reconstituted ovaries (in vitro) = E12.5 PGCs in embryonic ovaries in vivo.

## 2.1) in vivo:

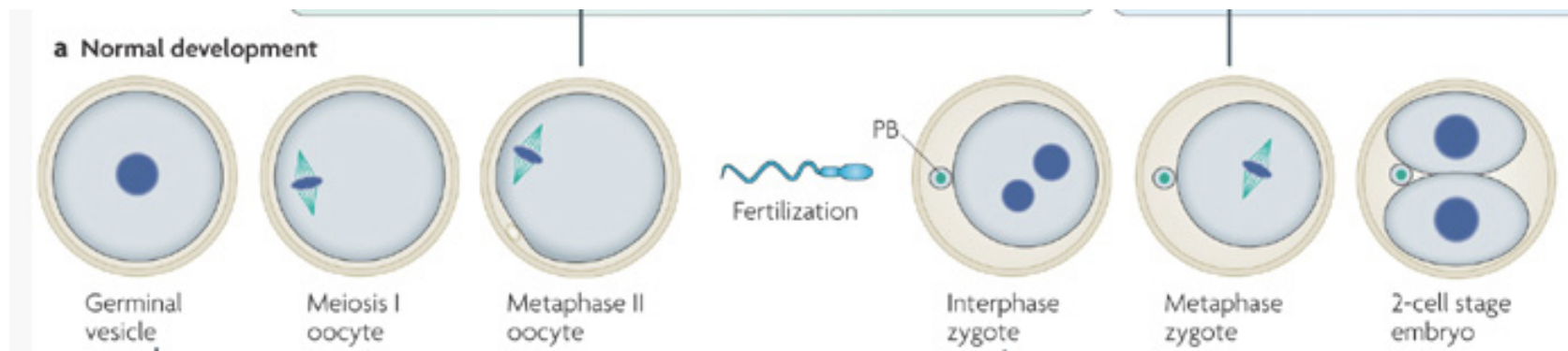
Transplanted PGCLC reconstituted ovaries to nude female mice → fully grown GV stage oocytes

## 2.2) ex vivo:

Oocytes harvested → in vitro maturation (metaphase II) → in vitro fertilization

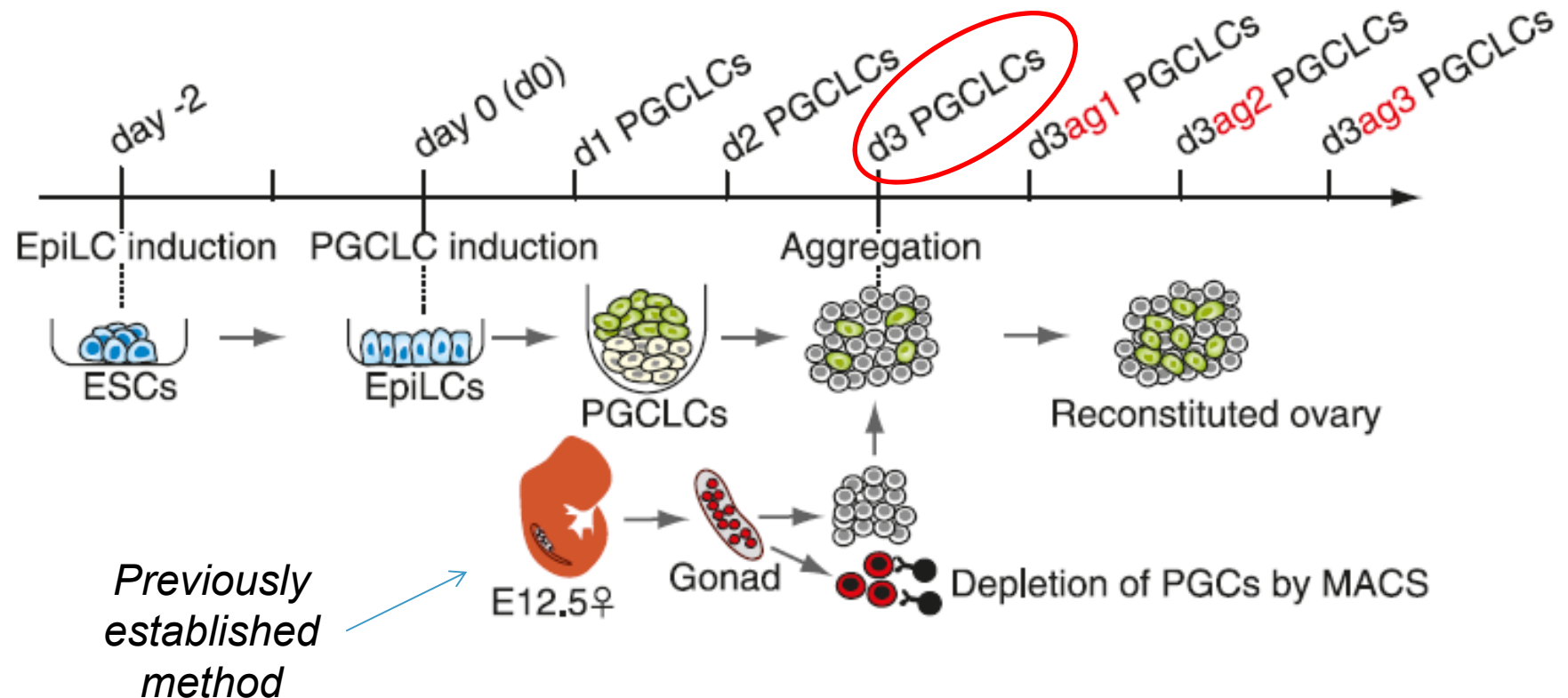
## 2.3) in vivo:

Transfer of two cell embryos to foster mother → healthy and fertile offspring



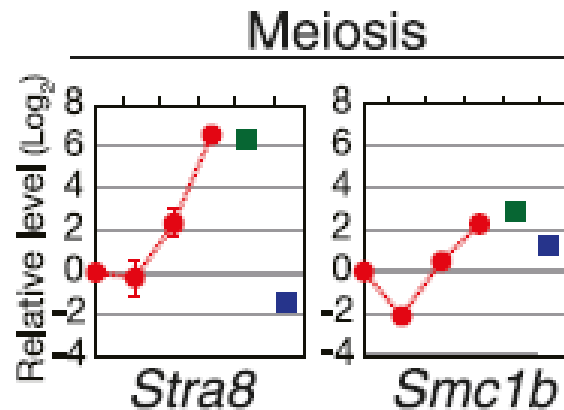
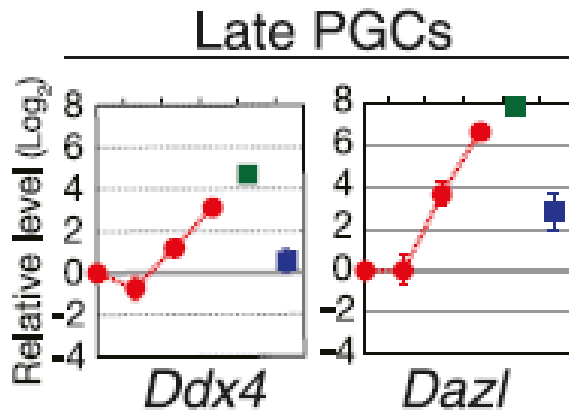
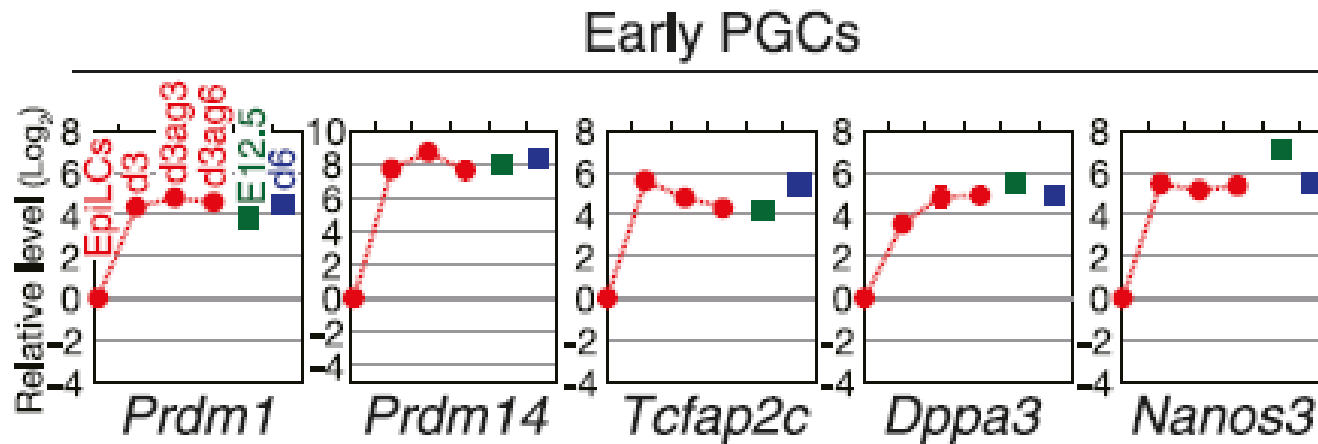
## 1) Produce PGCLC derived reconstituted ovaries in vitro:

- **Female** derived BVSC-ESCs induced to PGCLCs
- FACS sorted BV+ PGCLCs aggregate with embryonic female gonadal somatic cells (1000 PGCLCs + 10'000 gonadal cells)



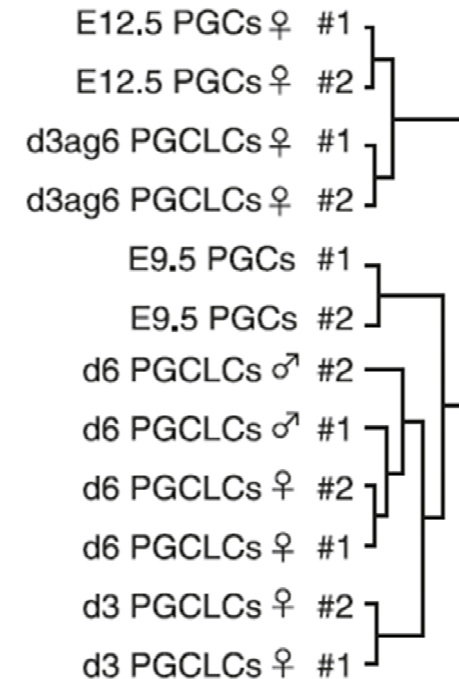
**1) d3ag3 and ag6 PGCLCs from reconstituted ovaries compared to natural E12.5 PGCs: Gene expression profiles**

d3ag6 PGCLCs reach pre-meiotic stage similar to **E12.5 PGCs**



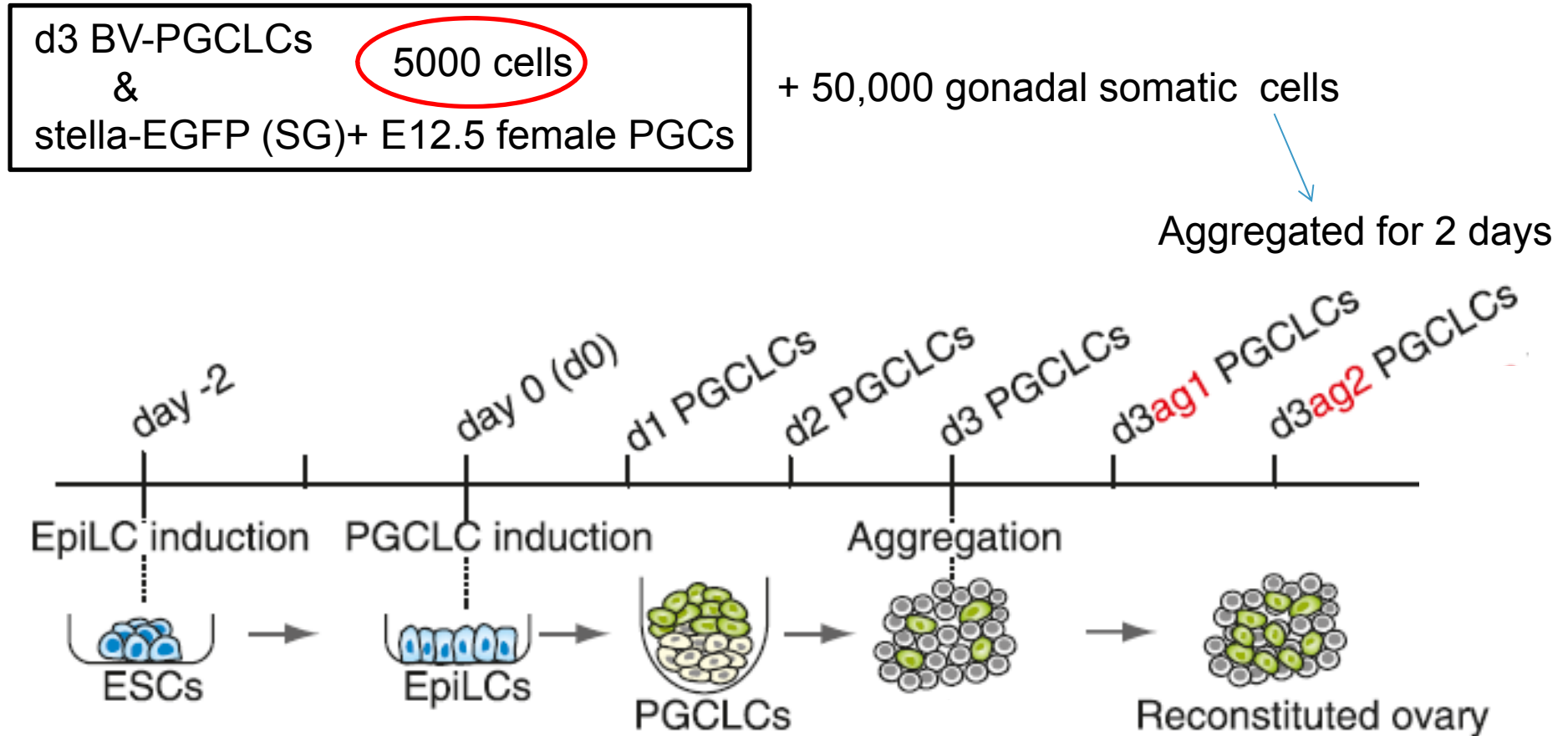
Blue d6 PGCLCs not aggregated with gonadal cells

Hierarchical clustering of microarray data



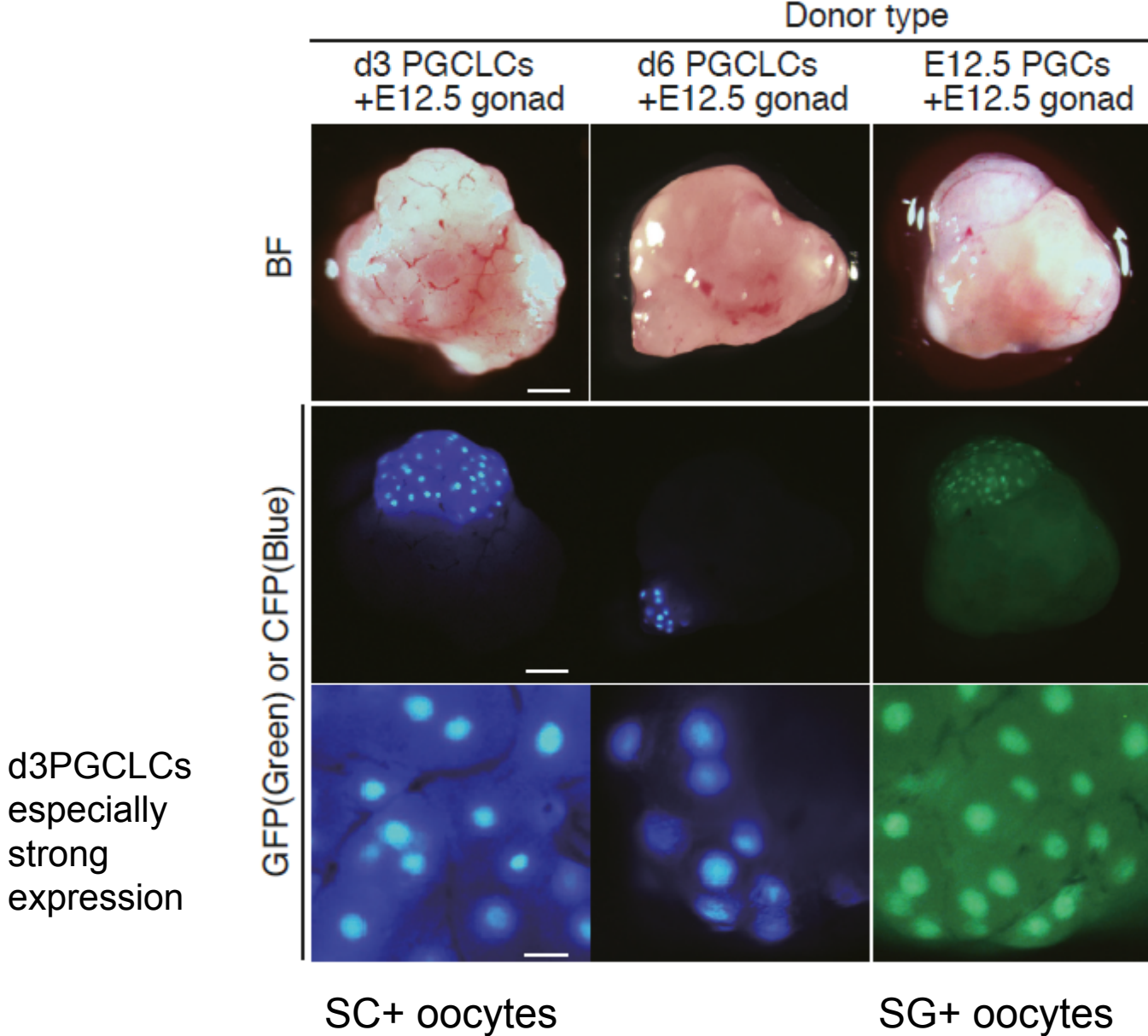
Potentially ready to produce oocytes

## 2.1) Transplantation of PGCLC derived reconstituted ovaries into nude mice:

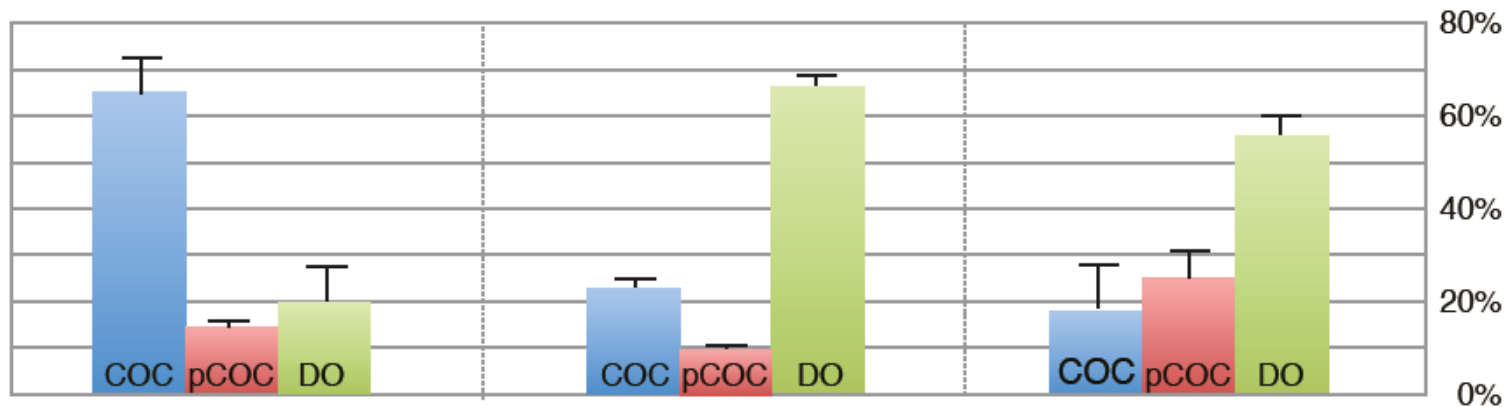
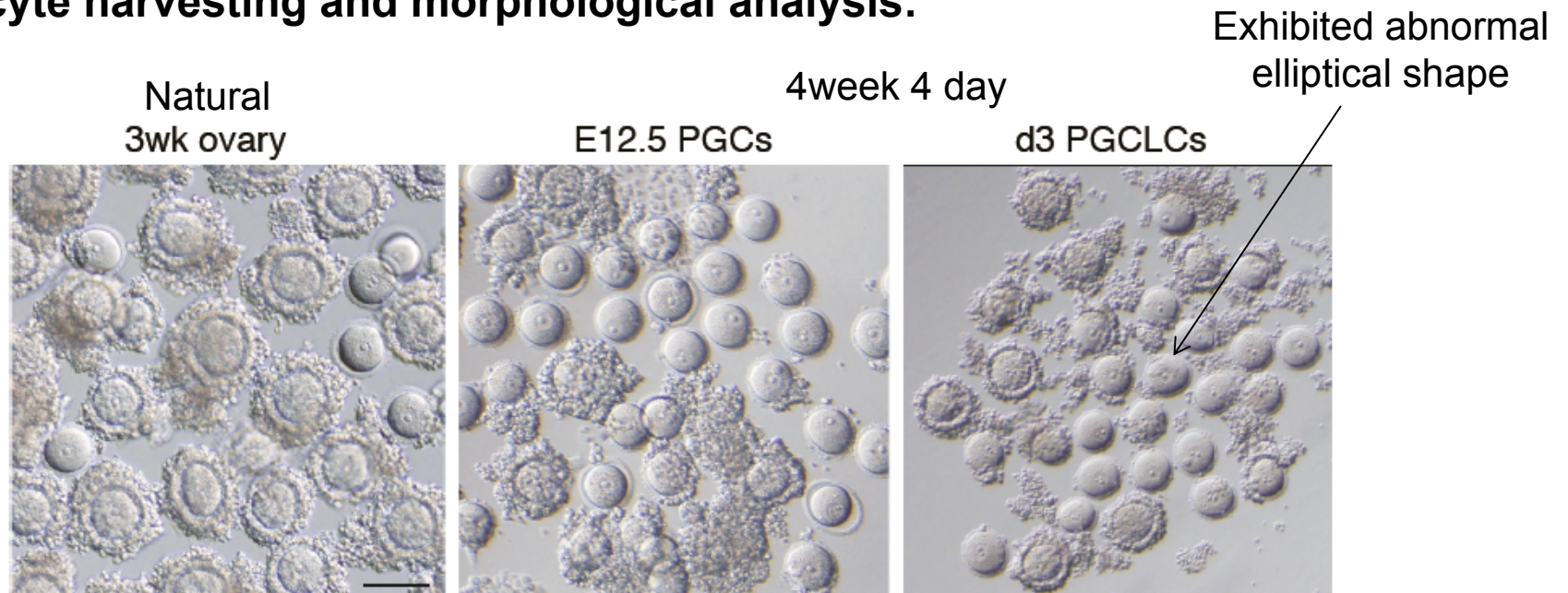


Transplanted under the ovarian bursa of nude mice  
(two reconstituted ovaries per recipient ovary).  
Reconstituted ovaries analysed 4 weeks and 4 days later

**2.1) Transplanted ovaries showing SC+ growing/grown oocytes:**



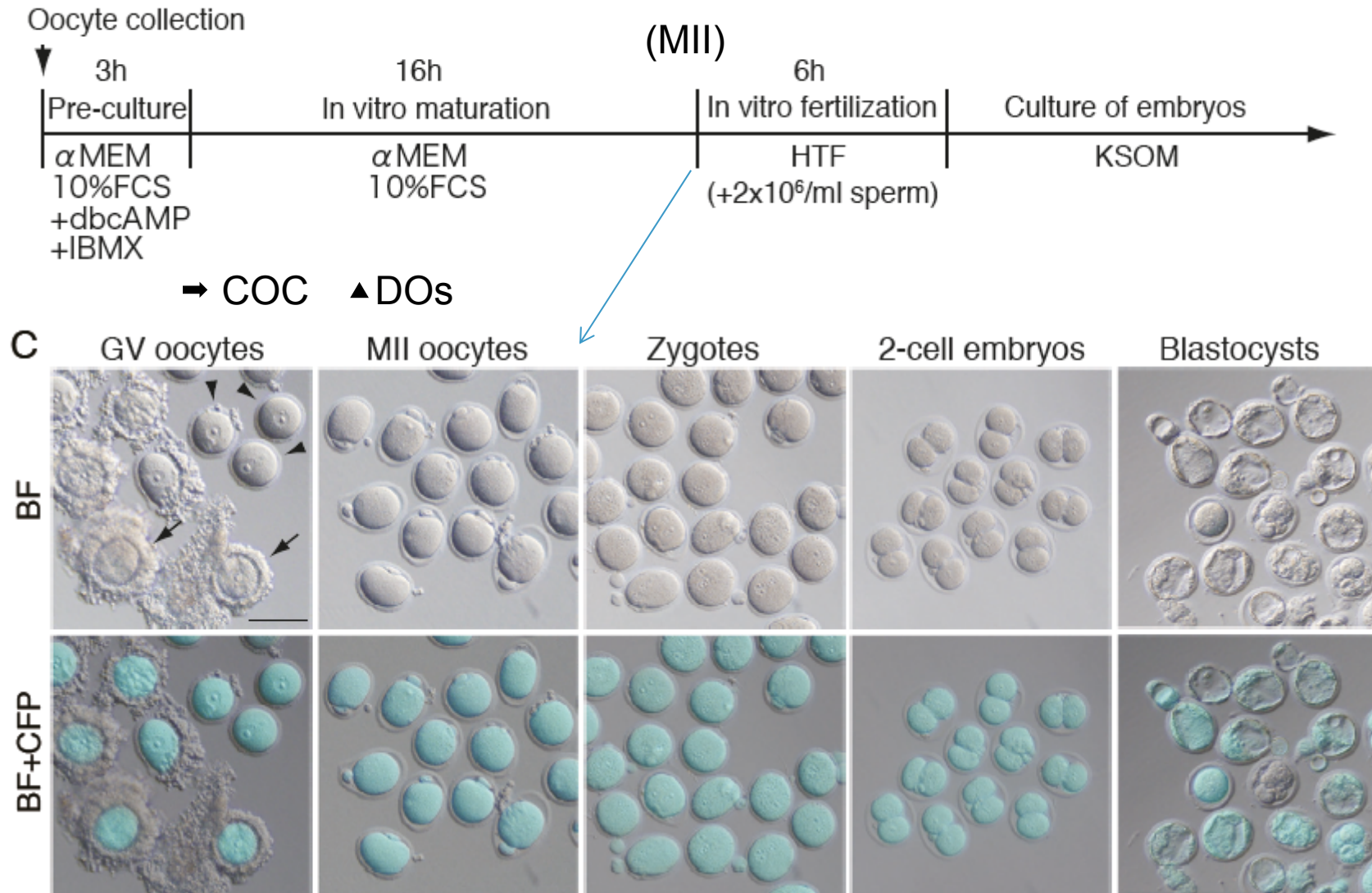
## 2.2) Oocyte harvesting and morphological analysis:



Cumulus cell-oocyte complex (COC)  
 Partial COC (pCOC)  
 Denuded oocytes (DOs)

Oocytes from PGCLCs may exhibit some cytoskeletal immaturity and/or fragility with a certain frequency & instability in COC formation

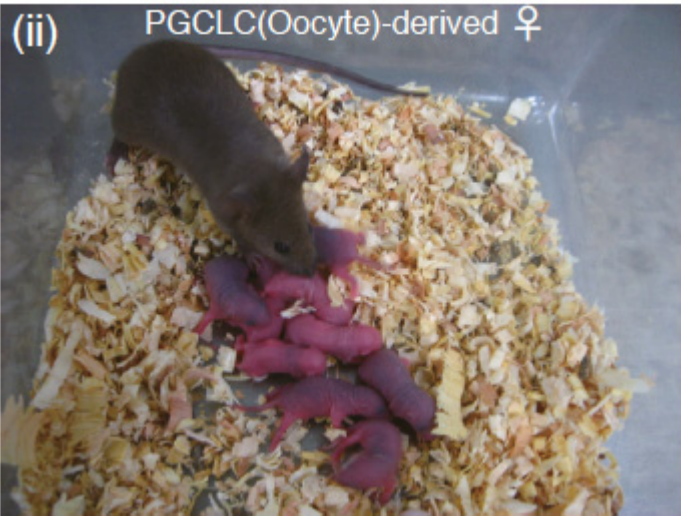
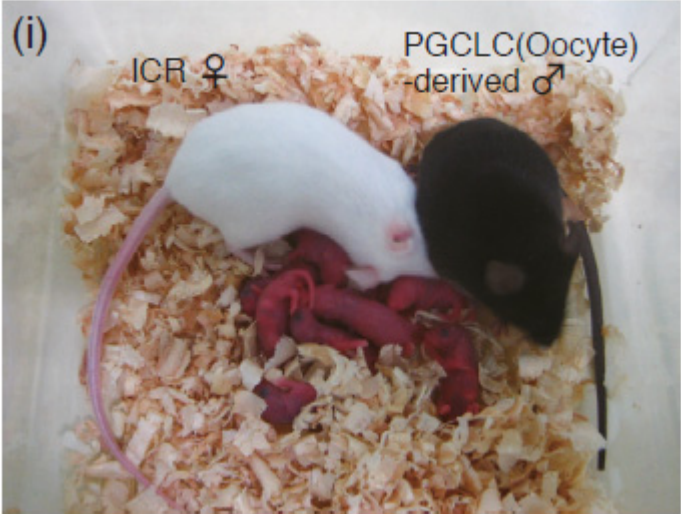
## 2.2) in vitro maturation (IVM) & in vitro fertilization (IVF) of the harvested oocytes:



SC+ expression Despite differences in COC stability and shape, the PGCLC-derived oocytes reached metaphase II (MII), were fertilized, and developed into two-cell embryos. (19/46) ~39% developed into blastocysts



### 2.3) Two-cell embryos transferred to foster mothers: Fertile offspring



Efficiency of obtaining pups:

PGCLCs = 3.9%

E12.5 PGCs = 17.3%

WT = 12.7%

PGCLC-derived offspring:

+ BVSC transgenes

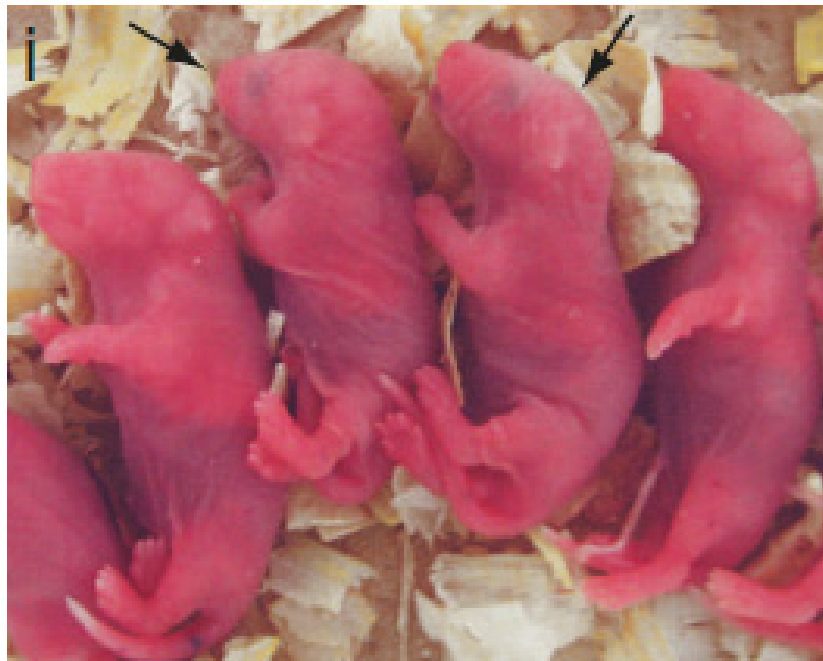
Normal imprinting

Full fertility

Post IVF some zygotes retained 3 pro nuclei = triploid chromosomes  
 Failure to extrude polar bodies after fertilization

## Induction of iPSCs into PGCLCs and repeated the same protocol to obtain oocytes.

SSEA1+ Integrin b3+ PGCLCs were selected → reconstituted ovaries → oocyte harvesting  
→ IVM and IVF → 2 cell embryos transferred to foster mothers → offspring



# Significance of these studies:

- Still requires a mouse to host the developing eggs, the big prize: Deriving egg cells entirely in vitro.
- But it does demonstrate that ES cells can give rise to fertile oocytes & spermatozoa
- Further characterisation of male and female germ line development
- Can open new leads to treating human infertility
- Better understand some kinds of infertility

Thank you for  
your attention!!

# Epiblasts & pluripotency in mice:

