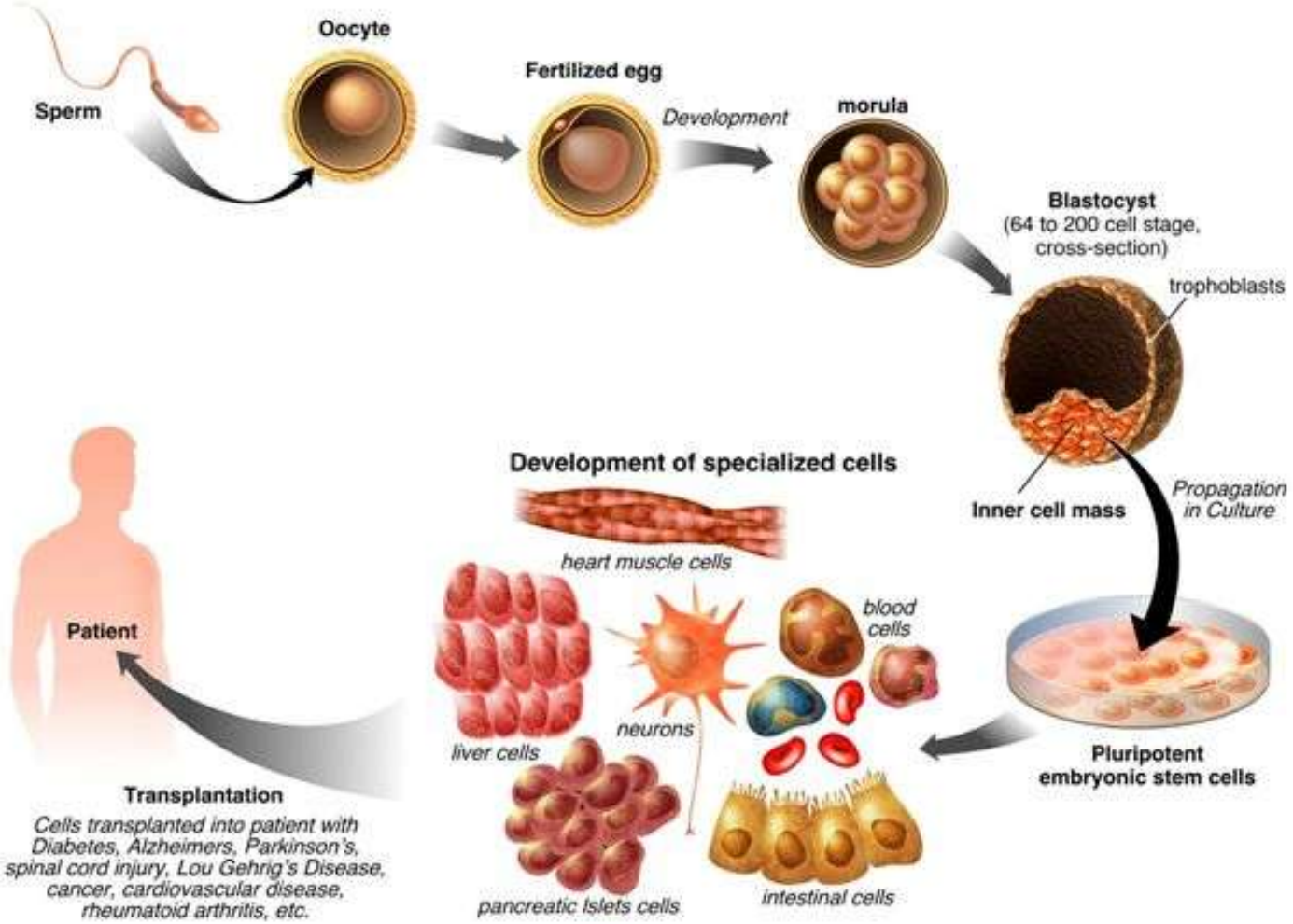
# Implementation of iPSC technology in producing patient-specific gametes - how close are we?

*Dusko Ilic, M.D., Ph.D.*

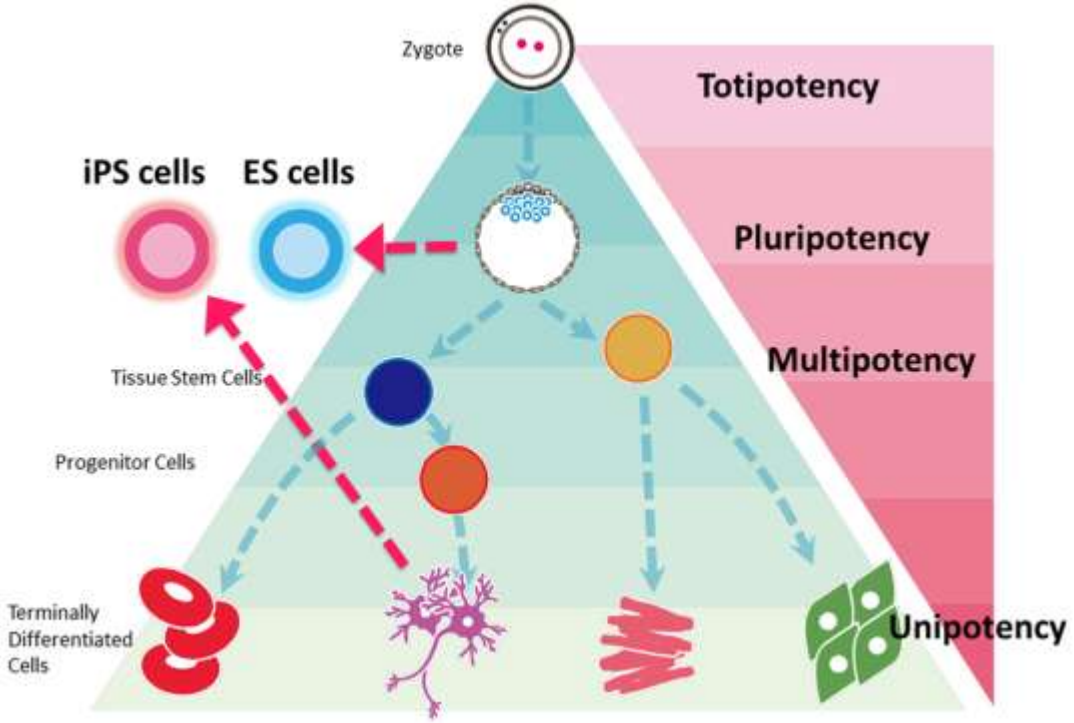
*Assisted Conception Unit, Guy's Hospital, London*


cleavage stages
compaction (morula)
blastocyst
ICM
day 1
day 2
day 3
day 4
day 5-7
Emma Stephenson (Assisted Conception Unit)


Oocyte
Sperm
Fertilized egg
Development
morula
Blastocyst
(64 to 200 cell stage, cross-section)
trophoblasts
Inner cell mass
Propagation in Culture
Development of specialized cells
heart muscle cells
blood cells
neurons
liver cells
pancreatic Islets cells
intestinal cells
Pluripotent embryonic stem cells
Patient
Transplantation
Cells transplanted into patient with Diabetes, Alzheimers, Parkinson's, spinal cord injury, Lou Gehrig's Disease, cancer, cardiovascular disease, rheumatoid arthritis, etc.

# iPS cells – induced pluripotent cells

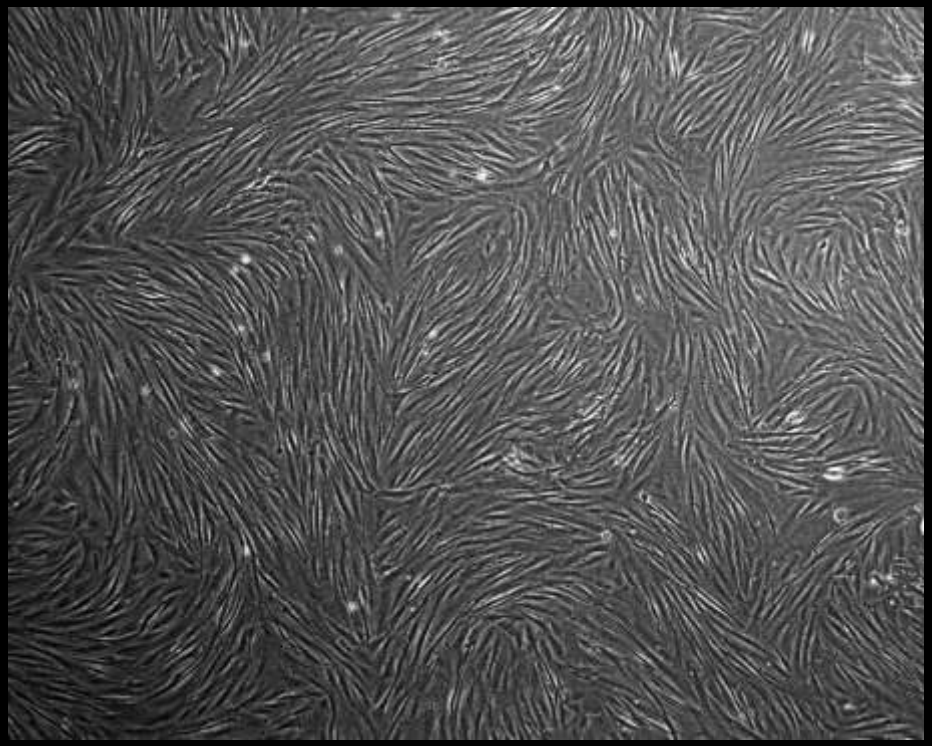
2006 Aug – the initial discovery by Shinya Yamanaka using mouse system;
2007 Nov – Yamanaka and Thomson independently demonstrated the approach in man;



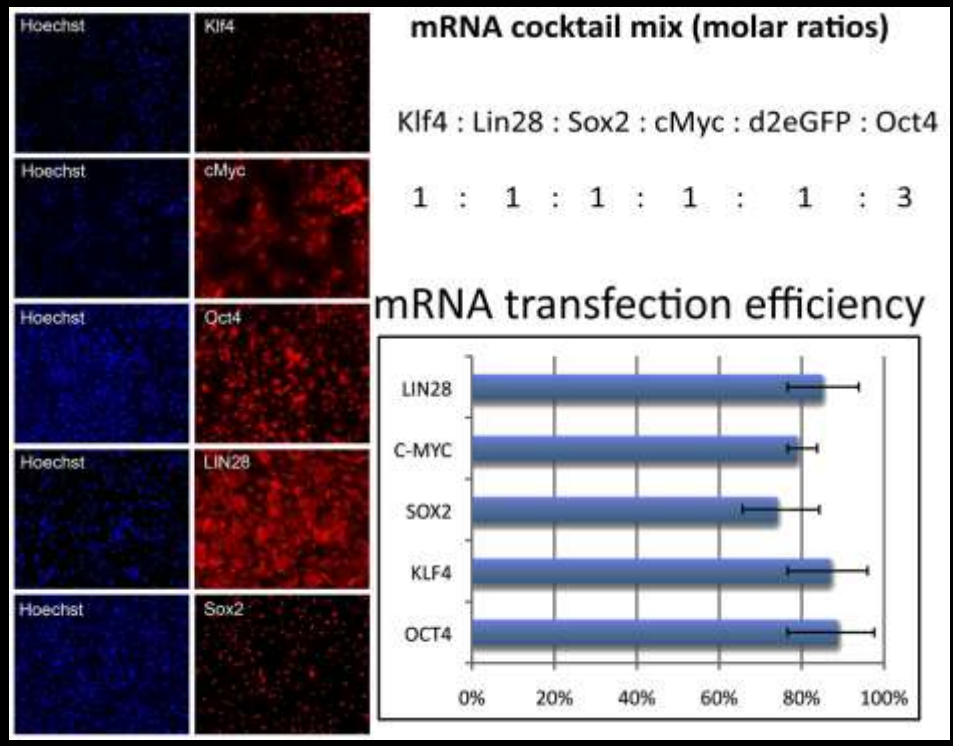

2012 Oct – Shinya Yamanaka and John Gurdon share Nobel Prize for Medicine or Physiology
2012/13 – Japanese government is investing about USD 320 million into his research

| Method of reprogramming | Delivery method |
| --- | --- |
| *Integrating* | |
| Viral‡ | Retrovirus |
| | Lentivirus |
| Non-viral | Transposon (excisable) |
| *Non-integrating* | |
| Viral‡ | Adenovirus |
| | Sendai virus |
| Non-viral | mRNA |
| | miRNA |
| | Small molecules |
| | Episomal vectors |
| | Protein |

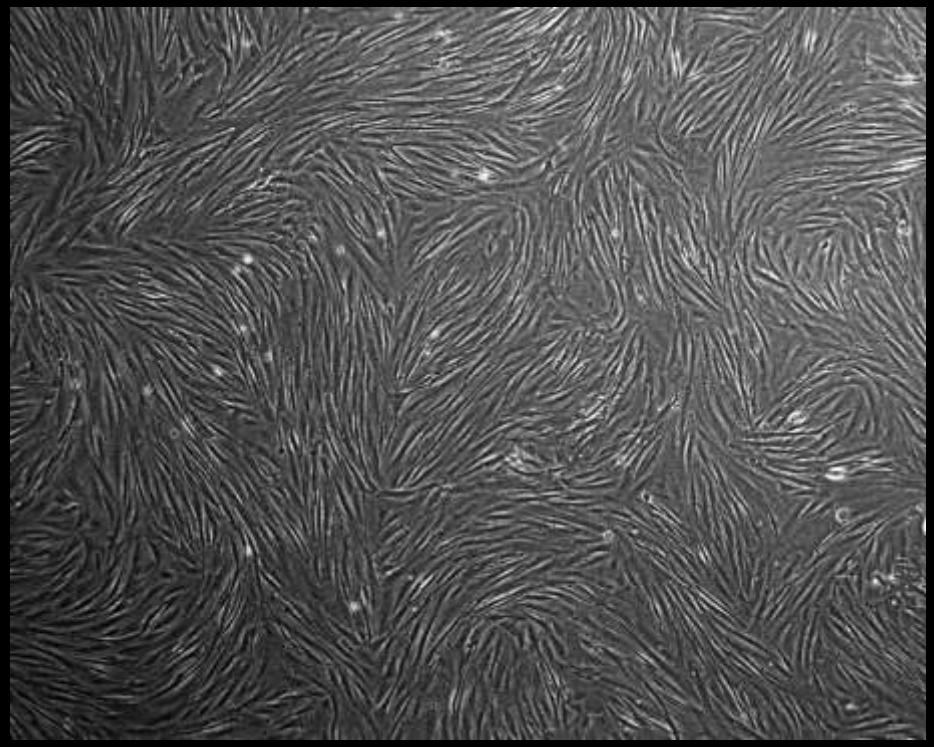
# Reprogramming of human foreskin fibroblasts with mRNA



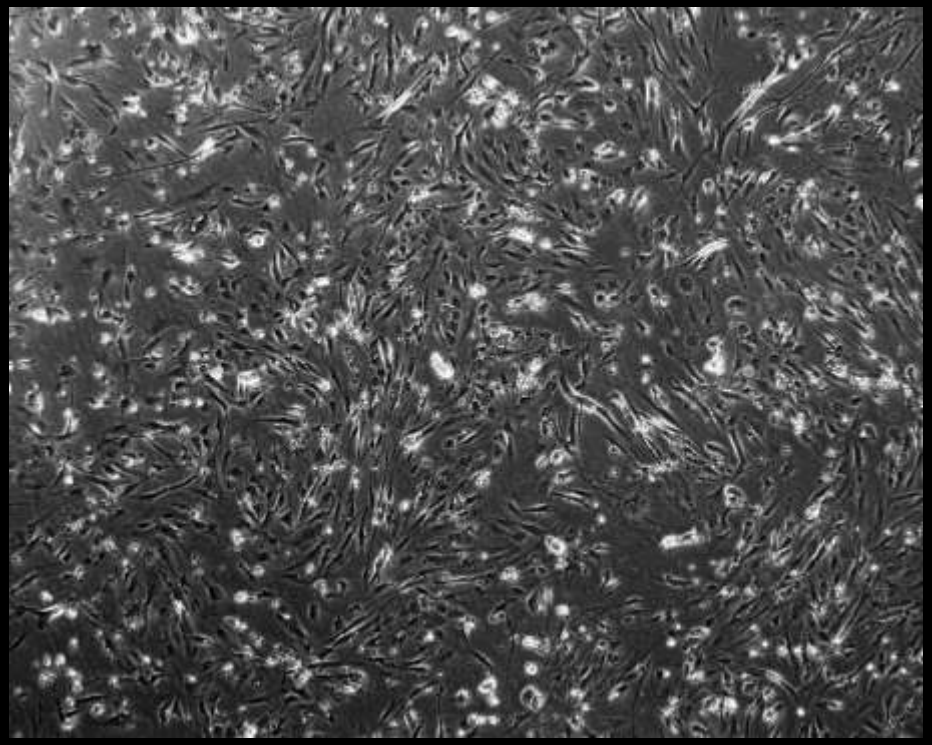
Day 0



# Reprogramming of human foreskin fibroblasts with mRNA

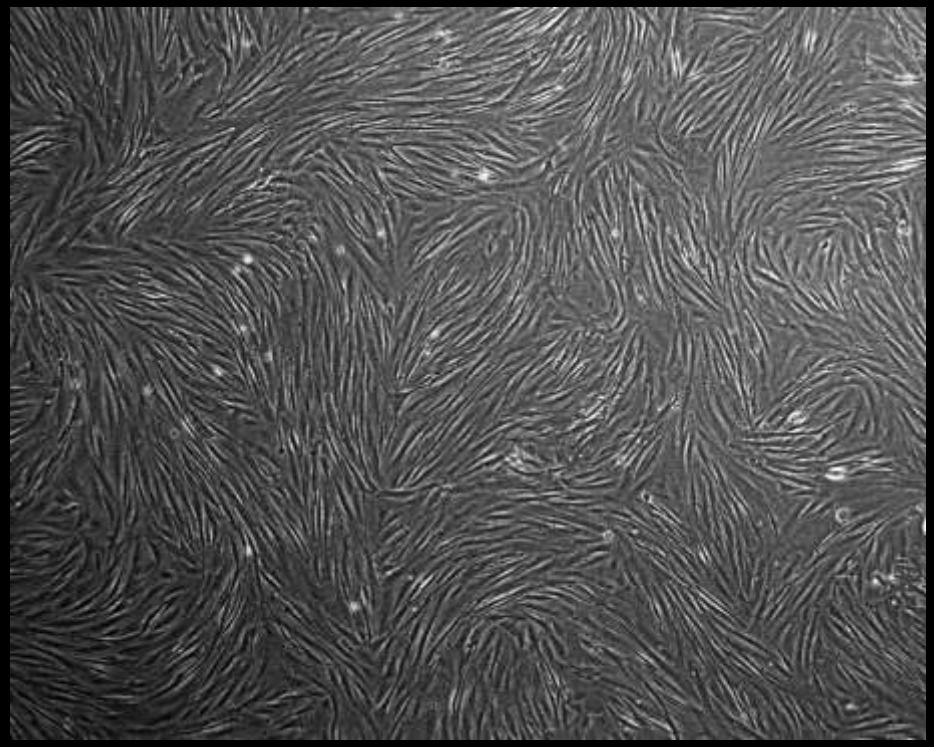


Day 0

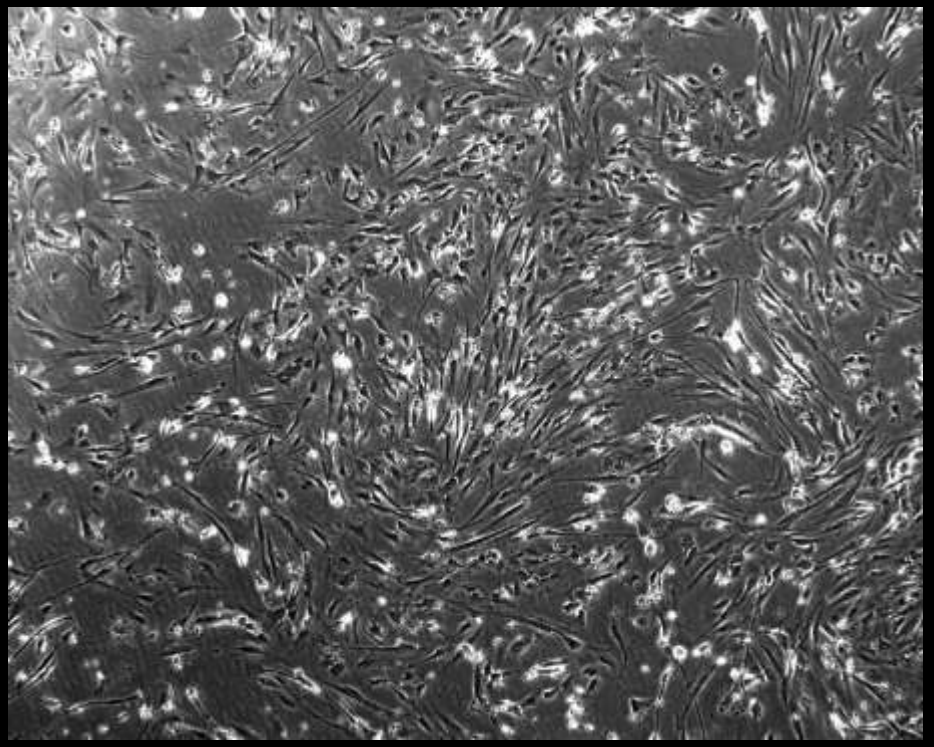


Day 8

# Reprogramming of human foreskin fibroblasts with mRNA

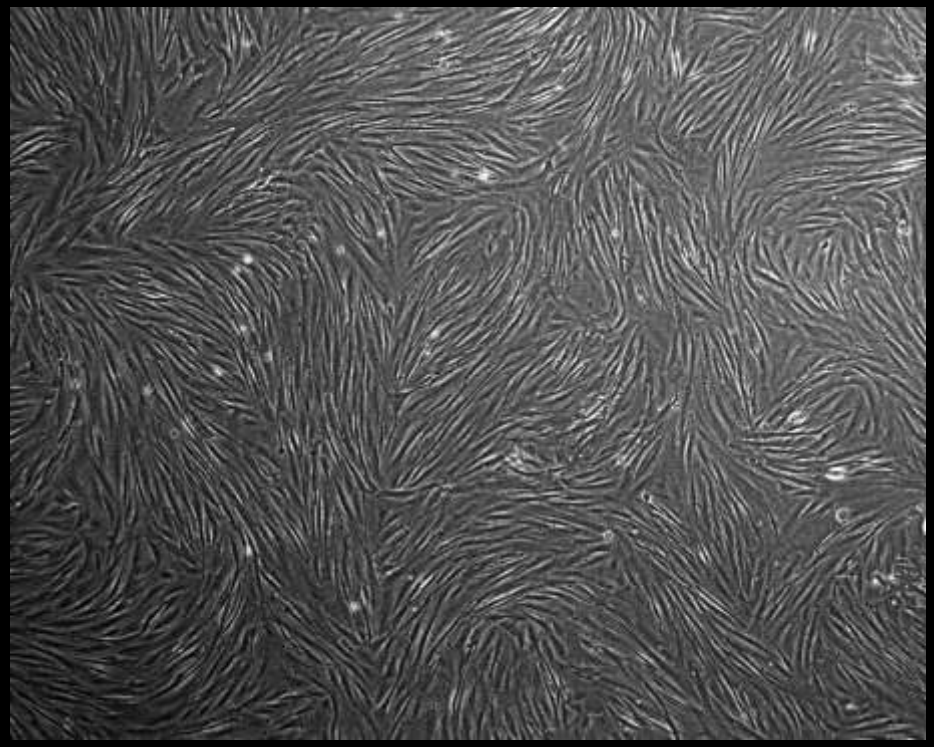


Day 0

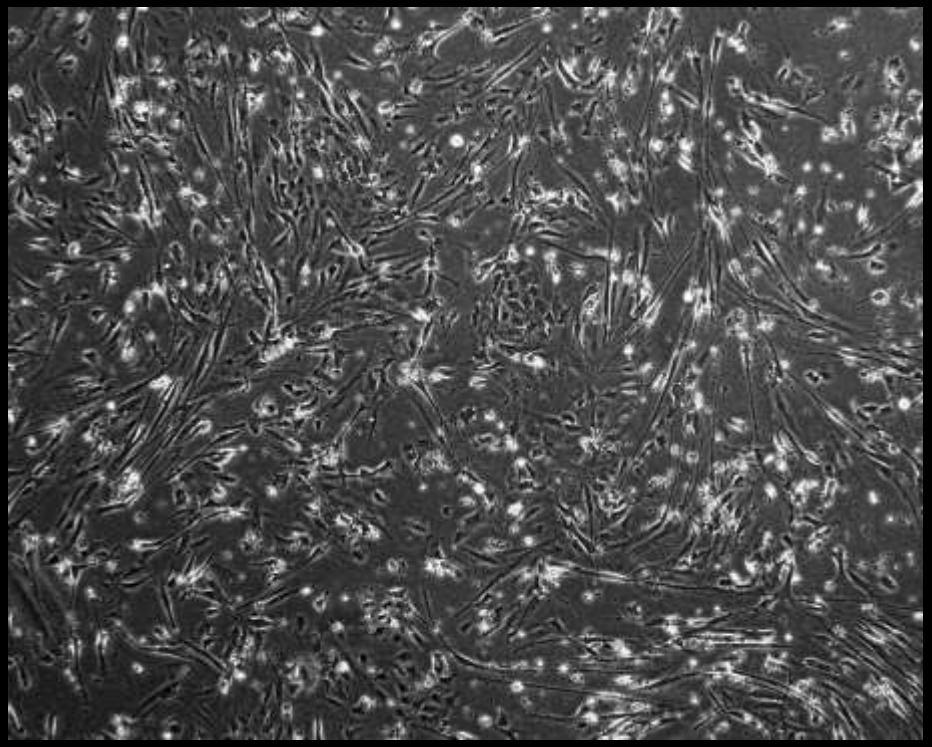


Day 10

# Reprogramming of human foreskin fibroblasts with mRNA

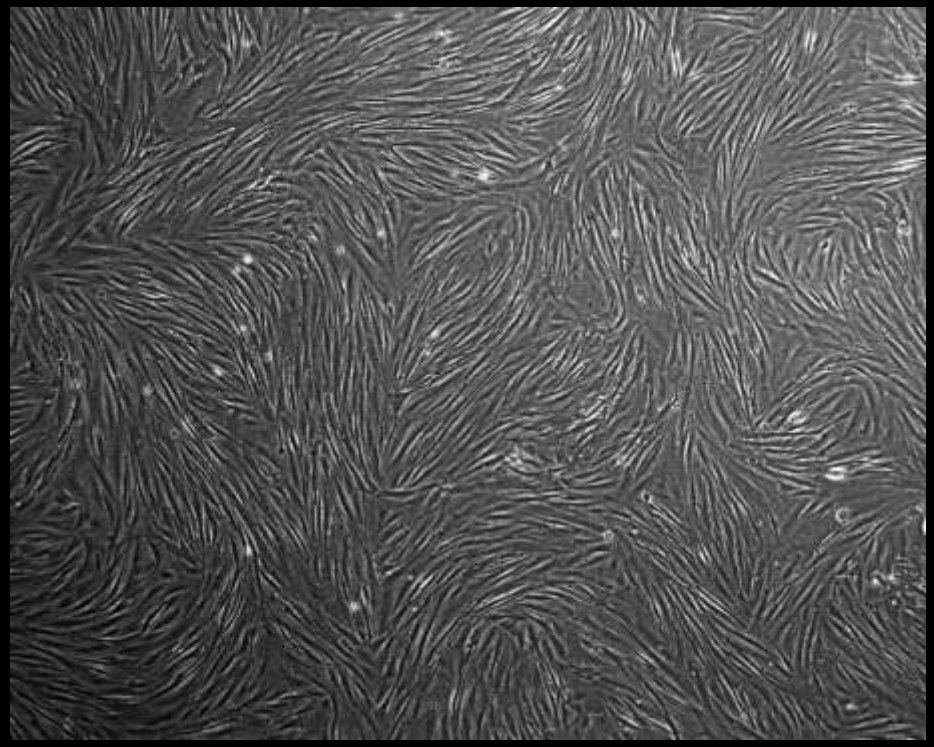


Day 0

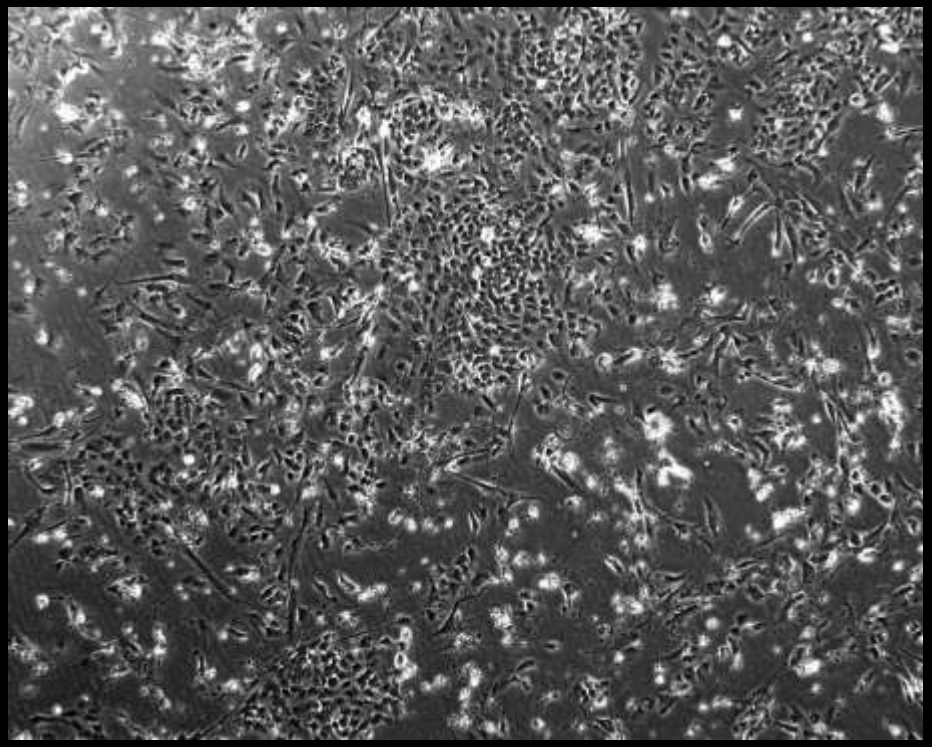


Day 12

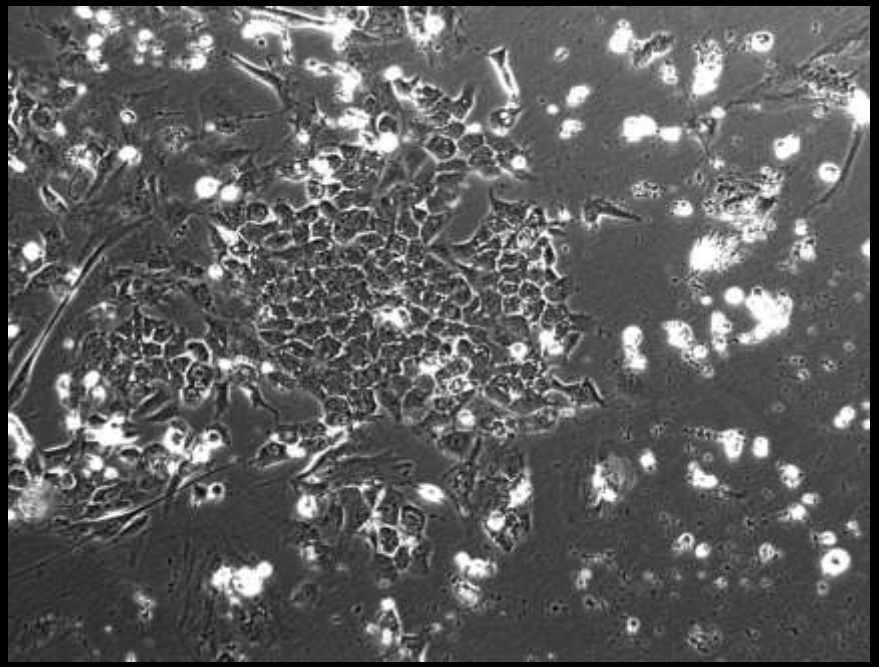
# Reprogramming of human foreskin fibroblasts with mRNA



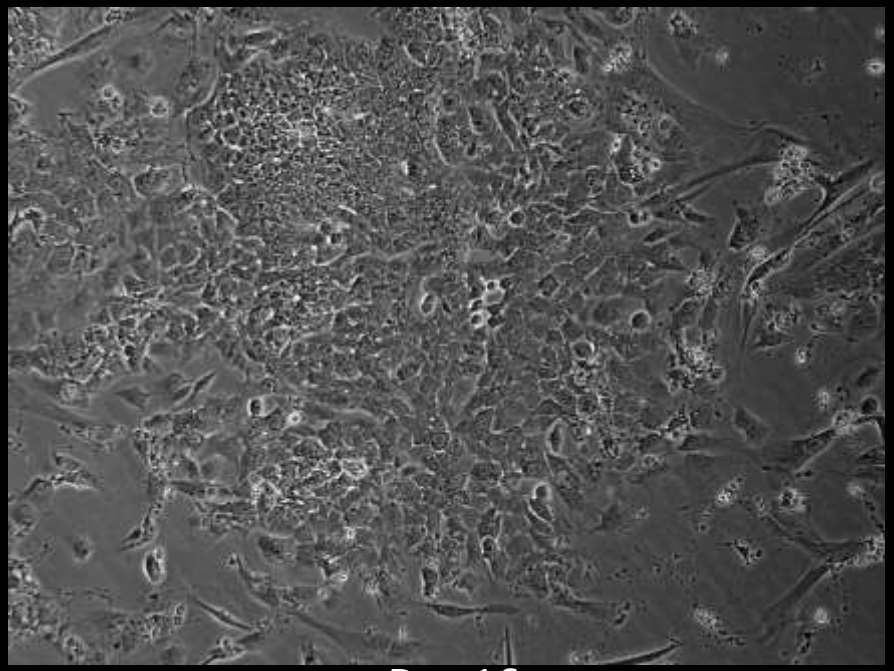
Day 0



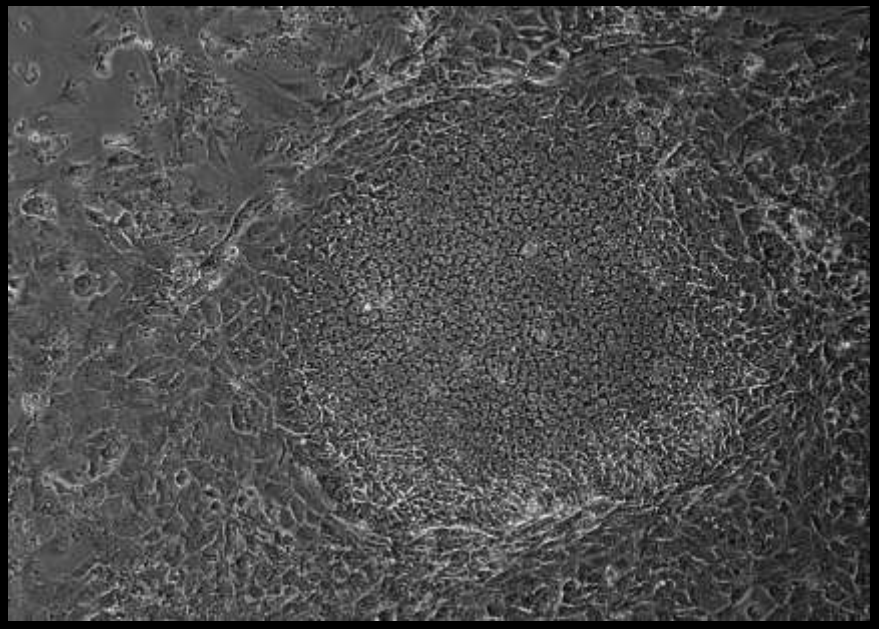
Day 14



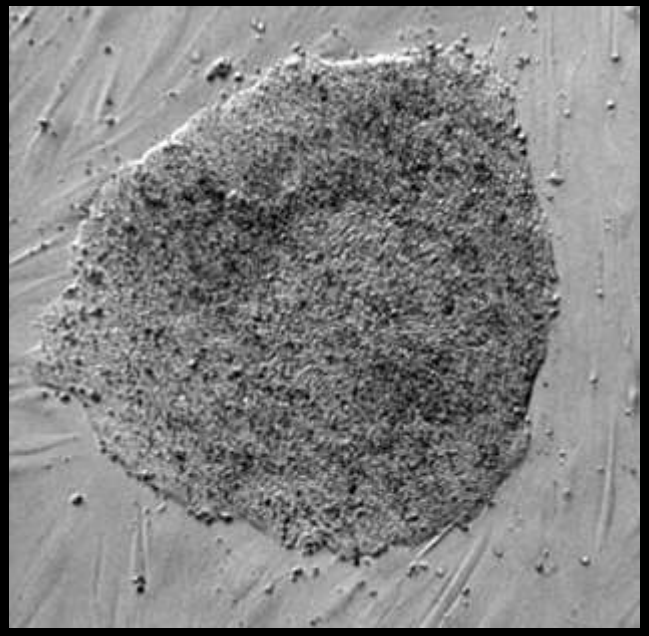
Day 14



Day 16

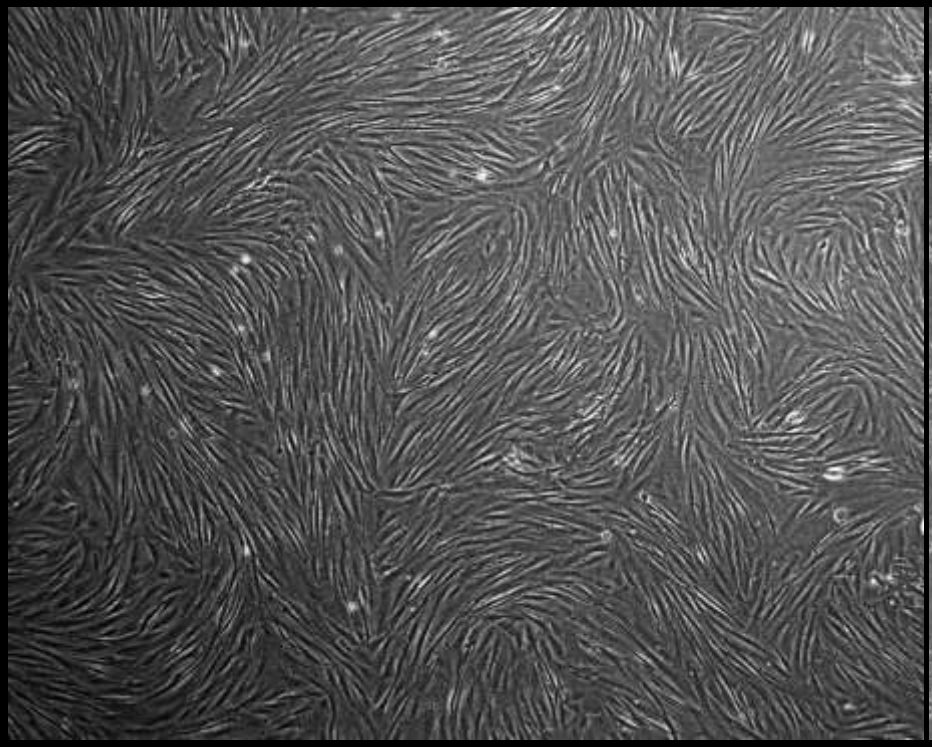


Day 18

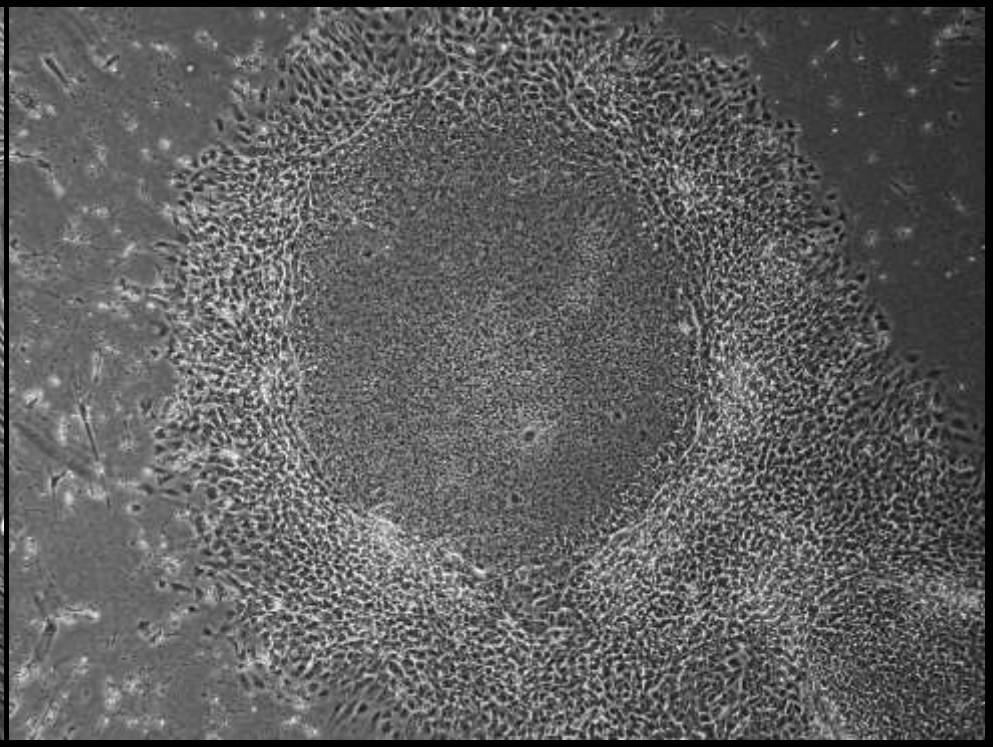


hES cell colony

# Reprogramming of human foreskin fibroblasts with mRNA

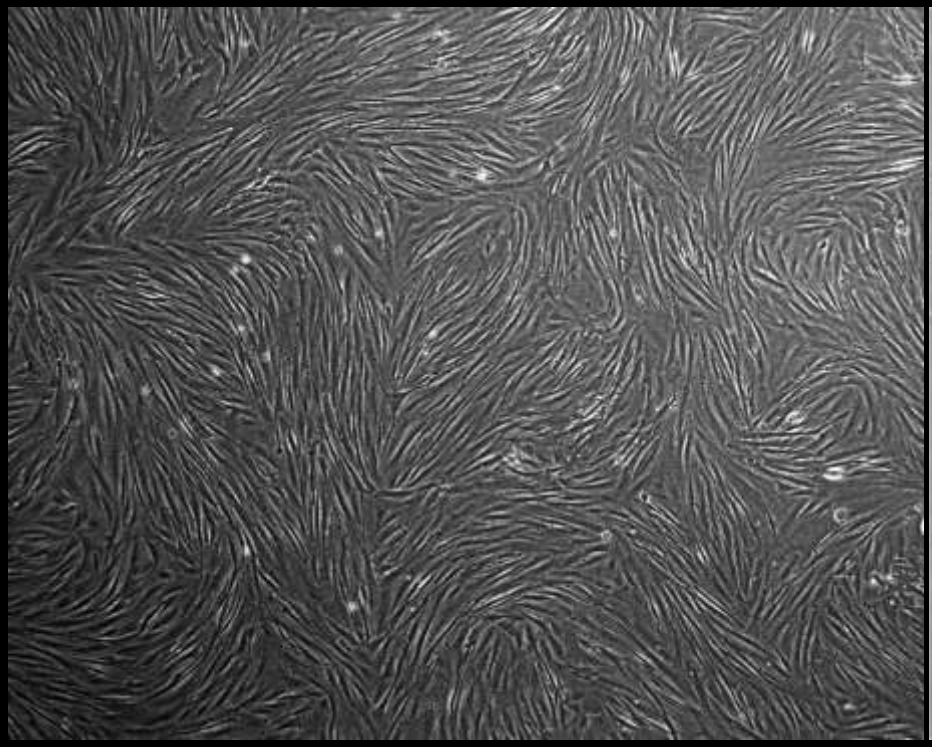


Day 0

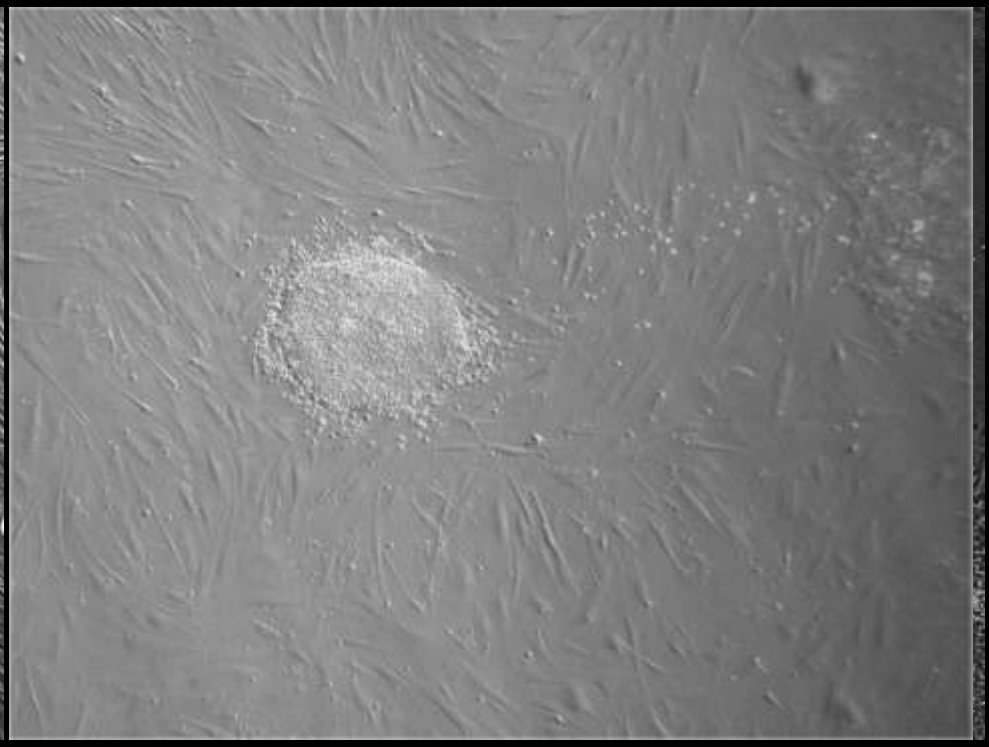


Day 18

# Reprogramming of human foreskin fibroblasts with mRNA



Day 0



Day 21 (passage 1)

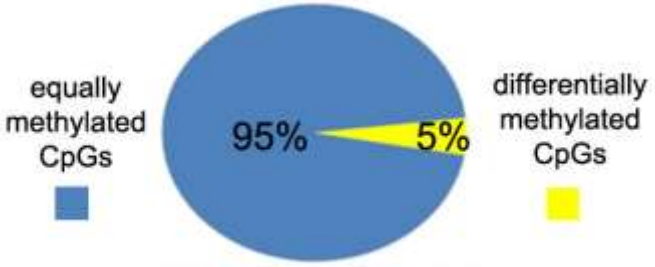
Negative side: accumulation of somatic mutation over lifespan

How we can be sure that our clinical grade iPS cells do not harbor dangerous mutations?

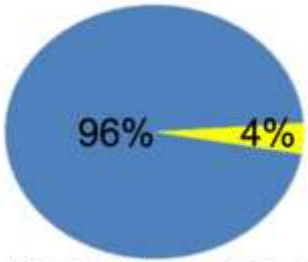
Negative side: accumulation of somatic mutation over lifespan

How we can be sure that our clinical grade iPS cells do not harbor dangerous mutations?

Exome sequencing



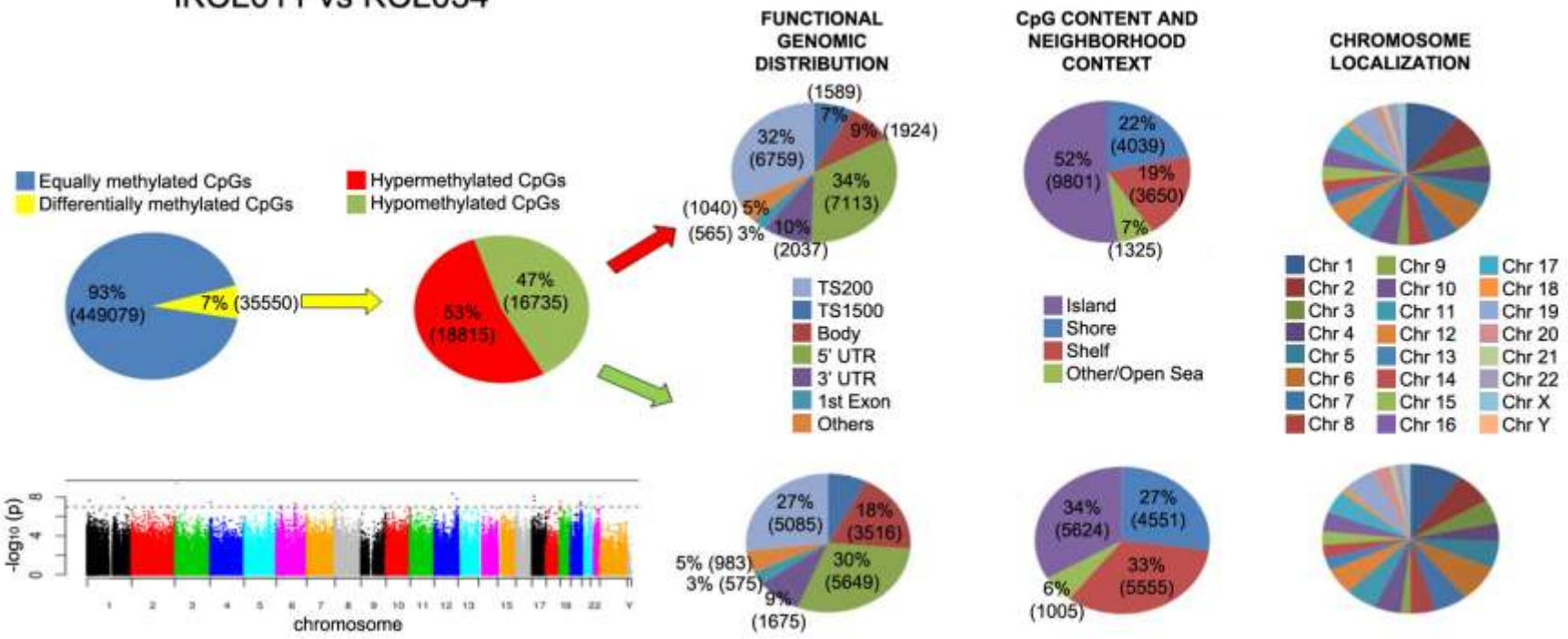

iKCL004 vs KCL034

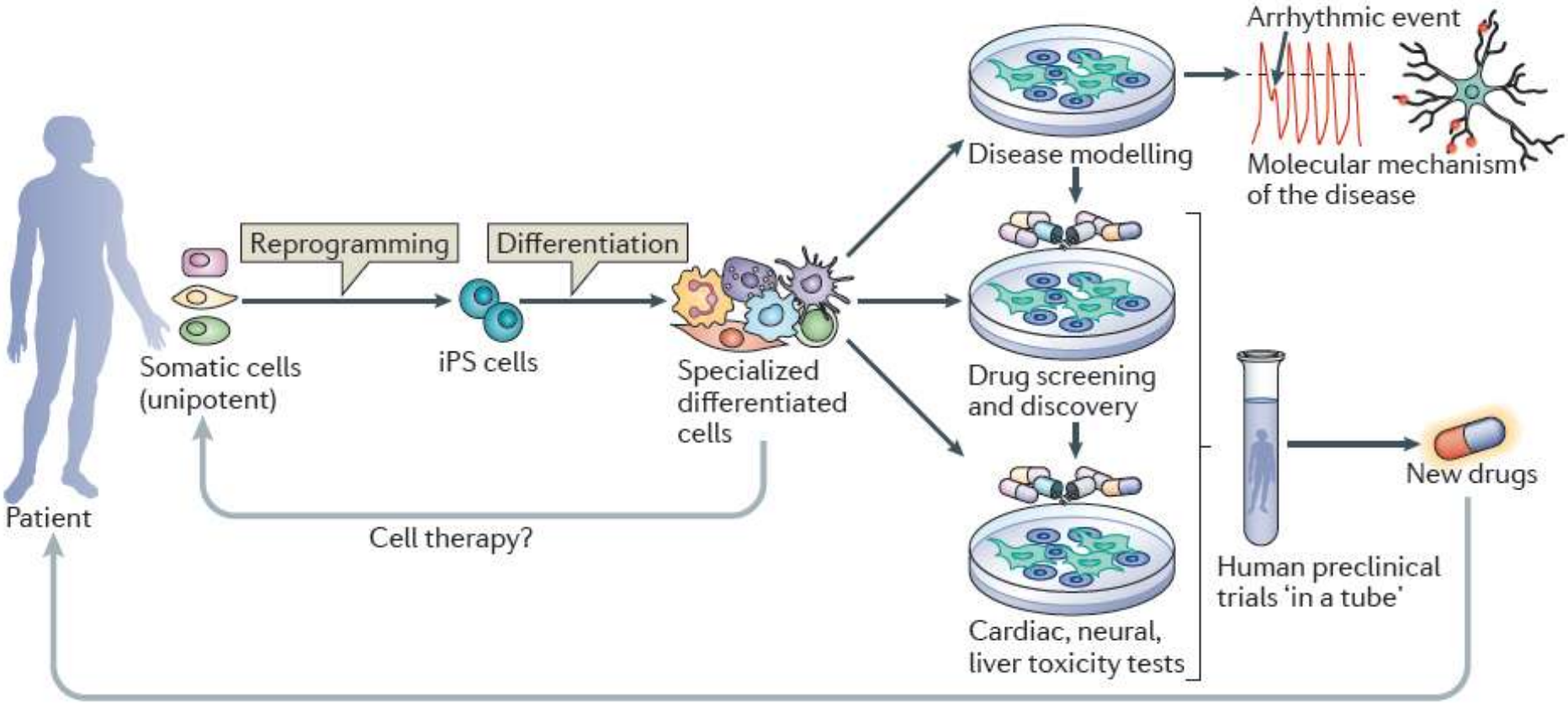



iKCL011 vs KCL034

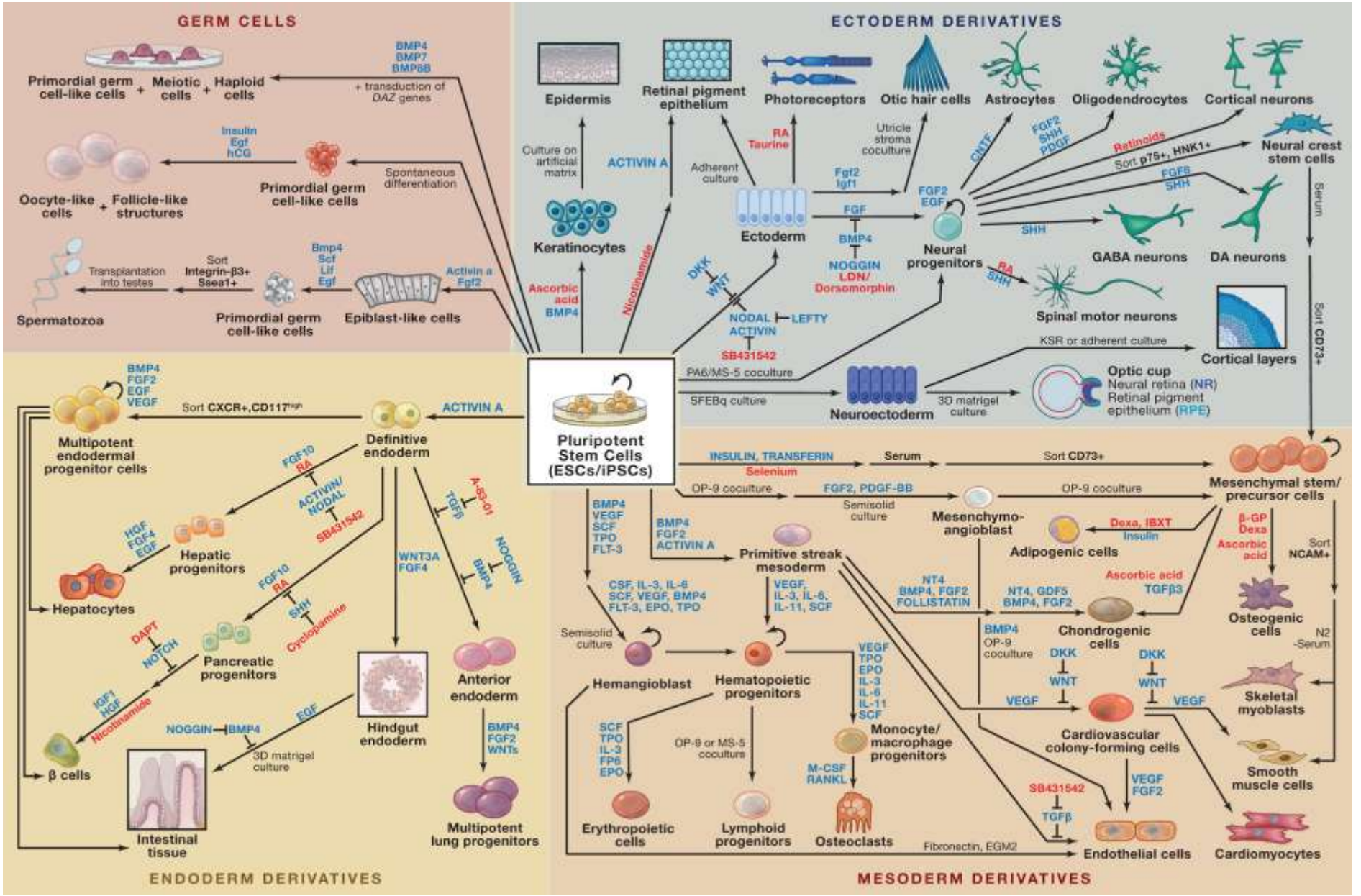
# DNA Methylation

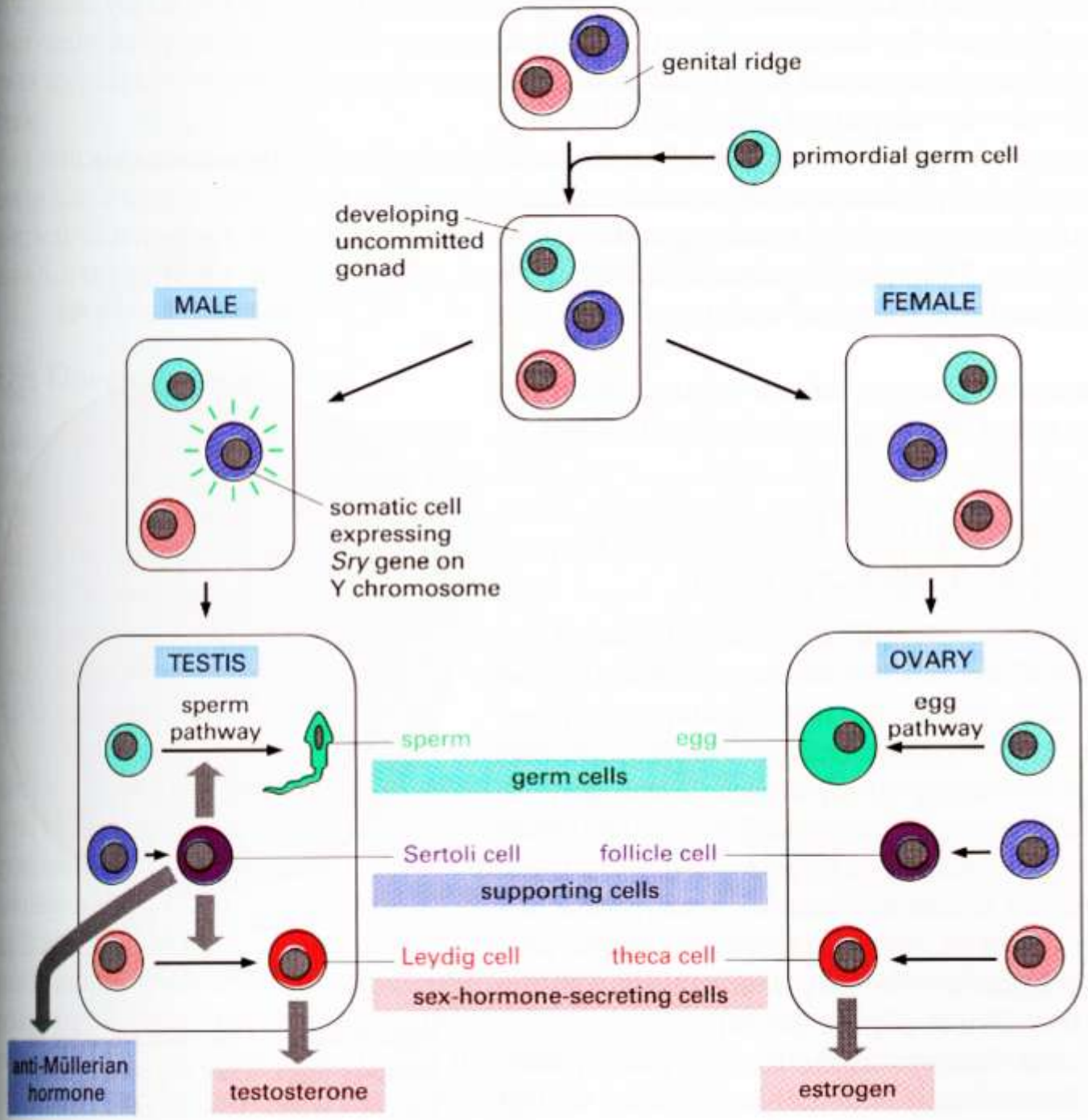
iKCL004 vs iKCL011

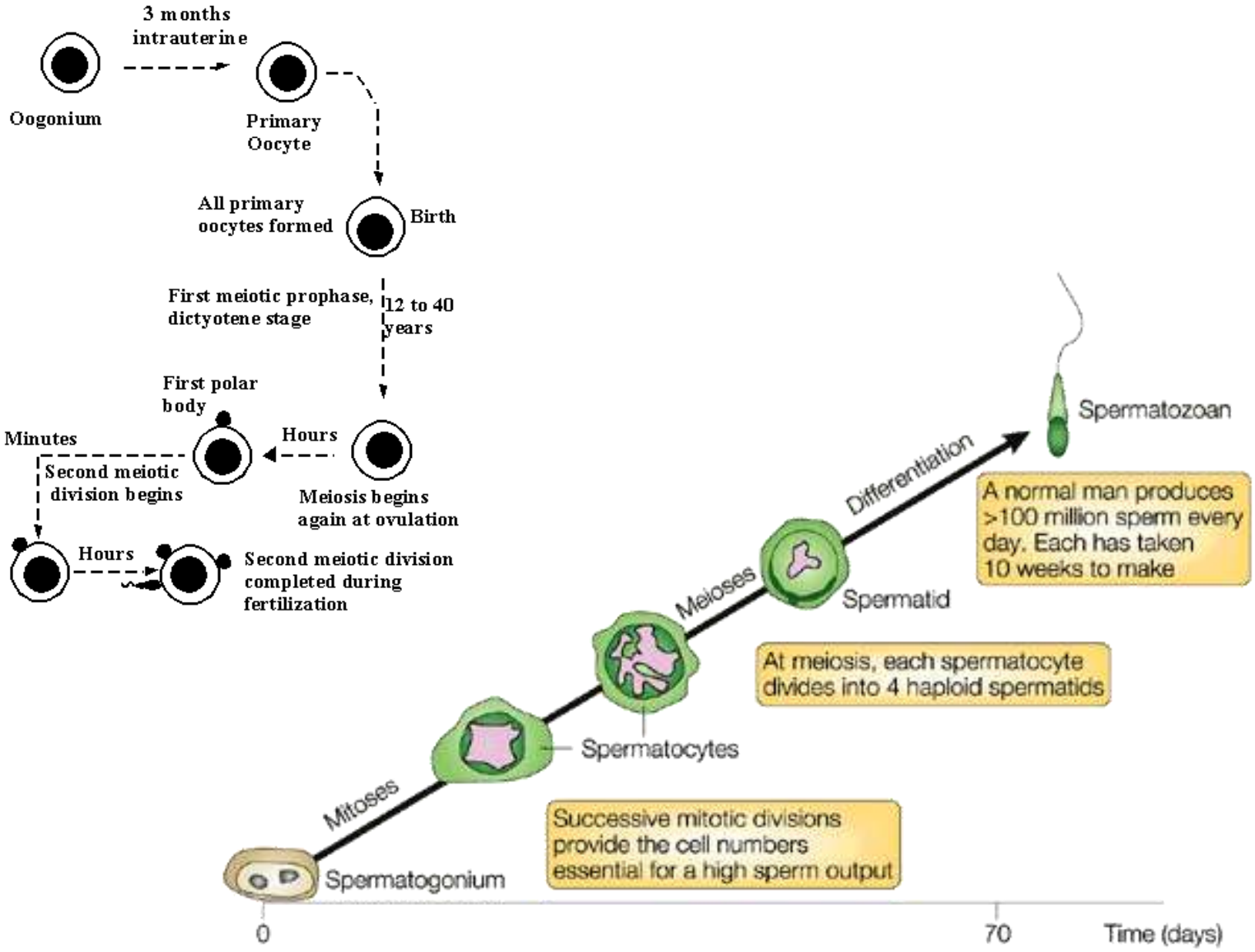


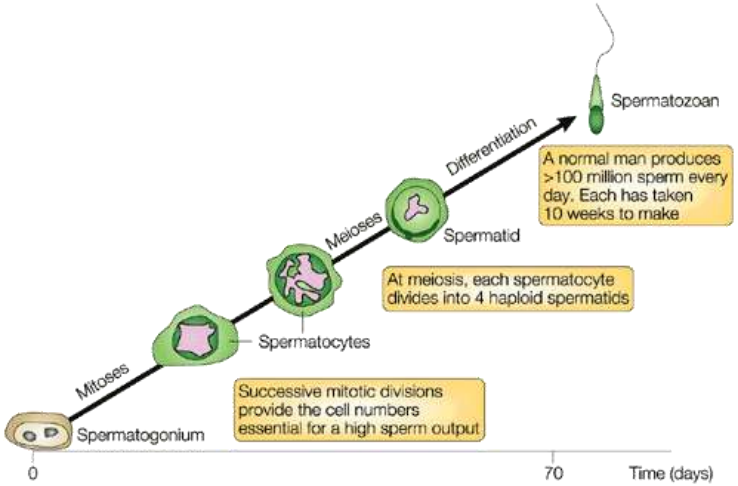


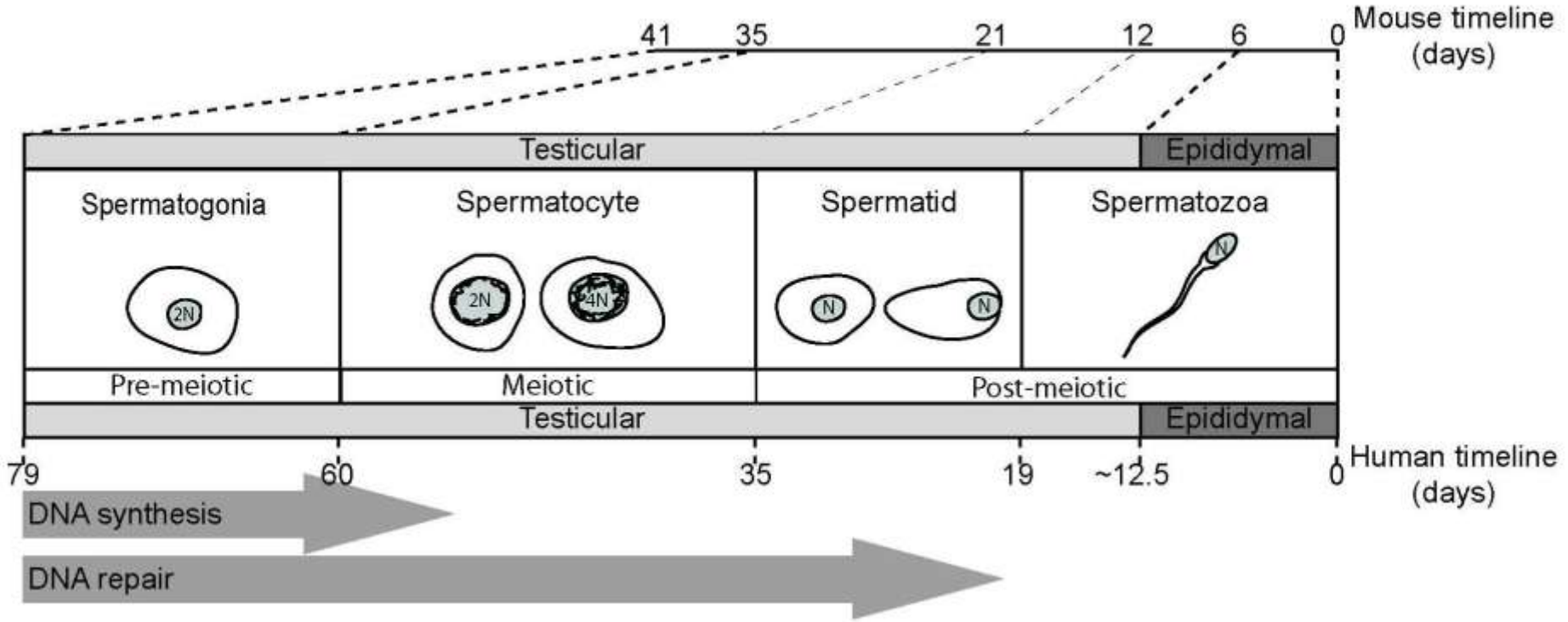

Bellin et al., Nat Rev Mol Cell Biol 2012;13:713-26.


GERM CELLS
Primordial germ cell-like cells
Meiotic cells
Haploid cells
BMP4
BMP7
BMP8B
+ transduction of DAZ genes
Insulin
Egf
hCG
Oocyte-like cells
Follicle-like structures
Primordial germ cell-like cells
Spontaneous differentiation
Transplantation into testes
Sort
Integrin-β3+
Ssea1+
Bmp4
Scf
Lif
Egf
Activin a
Fgf2
Spermatozoa
Primordial germ cell-like cells
Epiblast-like cells
ECTODERM DERIVATIVES
Epidermis
Retinal pigment epithelium
Photoreceptors
Otic hair cells
Astrocytes
Oligodendrocytes
Cortical neurons
Culture on artificial matrix
ACTIVIN A
Adherent culture
RA
Taurine
Utricle stroma coculture
CNTF
FGF2
SHH
PDGF
Retinoids
Sort p75+, HNK1+
Neural crest stem cells
Keratinocytes
Ectoderm
Fgf2
Igf1
FGF
BMP4
NOGGIN
LDN/
Dorsomorphin
FGF2
EGF
Neural progenitors
FGF8
SHH
SHH
GABA neurons
DA neurons
Serum
Ascorbic acid
BMP4
Nicotinamide
DKK
WNT
NODAL
LEFTY
ACTIVIN
SB431542
RA
SHH
Spinal motor neurons
Sort CD73+
KSR or adherent culture
Cortical layers
PA6/MS-5 coculture
SFEBq culture
Neuroectoderm
3D matrigel culture
Optic cup
Neural retina (NR)
Retinal pigment epithelium (RPE)
BMP4
FGF2
EGF
VEGF
Sort CXCR+,CD117high
ACTIVIN A
Multipotent endodermal progenitor cells
Definitive endoderm
Pluripotent Stem Cells (ESCs/iPSCs)
INSULIN, TRANSFERIN
Selenium
Serum
Sort CD73+
Mesenchymal stem/ precursor cells
OP-9 coculture
FGF2, PDGF-BB
Semisolid culture
Mesenchymo-angioblast
OP-9 coculture
β-GP
Dexa
Ascorbic acid
Sort NCAM+
FGF10
RA
ACTIVIN/ NODAL
SB431542
A-83-01
TGFβ
NOGGIN
BMP4
HGF
FGF4
EGF
Hepatic progenitors
Dexa, IBXT
Insulin
Adipogenic cells
BMP4
VEGF
SCF
TPO
FLT-3
BMP4
FGF2
ACTIVIN A
Primitive streak mesoderm
Ascorbic acid
TGFβ3
Osteogenic cells
N2
-Serum
Hepatocytes
FGF10
RA
SHH
Cyclopamine
WNT3A
FGF4
CSF, IL-3, IL-6
SCF, VEGF, BMP4
FLT-3, EPO, TPO
VEGF,
IL-3, IL-6,
IL-11, SCF
NT4
BMP4, FGF2
FOLLISTATIN
NT4, GDF5
BMP4, FGF2
Chondrogenic cells
DAPT
NOTCH
Pancreatic progenitors
IGF1
HGF
Nicotinamide
Semisolid culture
BMP4
OP-9 coculture
DKK
WNT
DKK
WNT
Skeletal myoblasts
Hemangioblast
Hematopoietic progenitors
VEGF
TPO
EPO
IL-3
IL-6
IL-11
SCF
VEGF
VEGF
Hindgut endoderm
Anterior endoderm
NOGGIN
BMP4
EGF
3D matrigel culture
β cells
BMP4
FGF2
WNTs
SCF
TPO
IL-3
FP6
EPO
OP-9 or MS-5 coculture
Monocyte/ macrophage progenitors
M-CSF
RANKL
Cardiovascular colony-forming cells
VEGF
FGF2
Smooth muscle cells
SB431542
TGFβ
Intestinal tissue
Multipotent lung progenitors
Erythropoietic cells
Lymphoid progenitors
Osteoclasts
Fibronectin, EGM2
Endothelial cells
Cardiomyocytes
ENDODERM DERIVATIVES
MESODERM DERIVATIVES


genital ridge
primordial germ cell
developing uncommitted gonad
MALE
FEMALE
somatic cell expressing *Sry* gene on Y chromosome
TESTIS
OVARY
sperm pathway
egg pathway
sperm
egg
germ cells
Sertoli cell
follicle cell
supporting cells
Leydig cell
theca cell
sex-hormone-secreting cells
anti-Müllerian hormone
testosterone
estrogen


3 months
intrauterine
Oogonium
Primary
Oocyte
All primary
oocytes formed
Birth
First meiotic prophase,
dictyotene stage
12 to 40
years
First polar
body
Minutes
Hours
Second meiotic
division begins
Meiosis begins
again at ovulation
Hours
Second meiotic division
completed during
fertilization
Spermatozoan
Differentiation
A normal man produces
>100 million sperm every
day. Each has taken
10 weeks to make
Meioses
Spermatid
At meiosis, each spermatocyte
divides into 4 haploid spermatids
Spermatocytes
Mitoses
Successive mitotic divisions
provide the cell numbers
essential for a high sperm output
Spermatogonium
0
70
Time (days)


Spermatozoan
Differentiation
A normal man produces >100 million sperm every day. Each has taken 10 weeks to make
Meioses
Spermatid
At meiosis, each spermatocyte divides into 4 haploid spermatids
Spermatocytes
Mitoses
Successive mitotic divisions provide the cell numbers essential for a high sperm output
Spermatogonium
0
70
Time (days)


41
35
21
12
6
0
Mouse timeline (days)
Testicular
Epididymal
Spermatogonia
Spermatocyte
Spermatid
Spermatozoa
2N
2N
4N
N
N
N
Pre-meiotic
Meiotic
Post-meiotic
Testicular
Epididymal
79
60
35
19
~12.5
0
Human timeline (days)
DNA synthesis
DNA repair

Cell

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

Katsuhiko Hayashi,[1,3] Hiroshi Ohta,[1,3] Kazuki Kurimoto,[1,3] Shinya Aramaki,[1] and Mitinori Saitou[1,2,3,*]
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[2]Institute for Integrated Cell-Material Sciences
Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
[3]JST, CREST, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
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DOI 10.1016/j.cell.2011.06.052

**SUMMARY**

The generation of properly functioning gametes in vitro requires reconstitution of the multistepped pathway of germ cell development. We demonstrate here the generation of primordial germ cell-like cells (PGCLCs) in mice with robust capacity for spermatogenesis. PGCLCs were generated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) through epiblast-like cells (EpiLCs), a cellular state highly similar to pregastrulating epiblasts but distinct from epiblast stem cells (EpiSCs). Reflecting epiblast development, EpiLC induction from ESCs/iPSCs is a progressive process, and EpiLCs highly competent for the PGC fate are a transient entity. The global transcription profiles, epigenetic reprogramming, and cellular dynamics during PGCLC induction from EpiLCs meticulously capture those associated with PGC specification from the epiblasts. Furthermore, we identify Integrin-β3 and SSEA1 as markers that allow the isolation of PGCLCs with spermatogenic capacity from tumorigenic undifferentiated cells. Our findings provide a paradigm for the first step of in vitro gametogenesis.

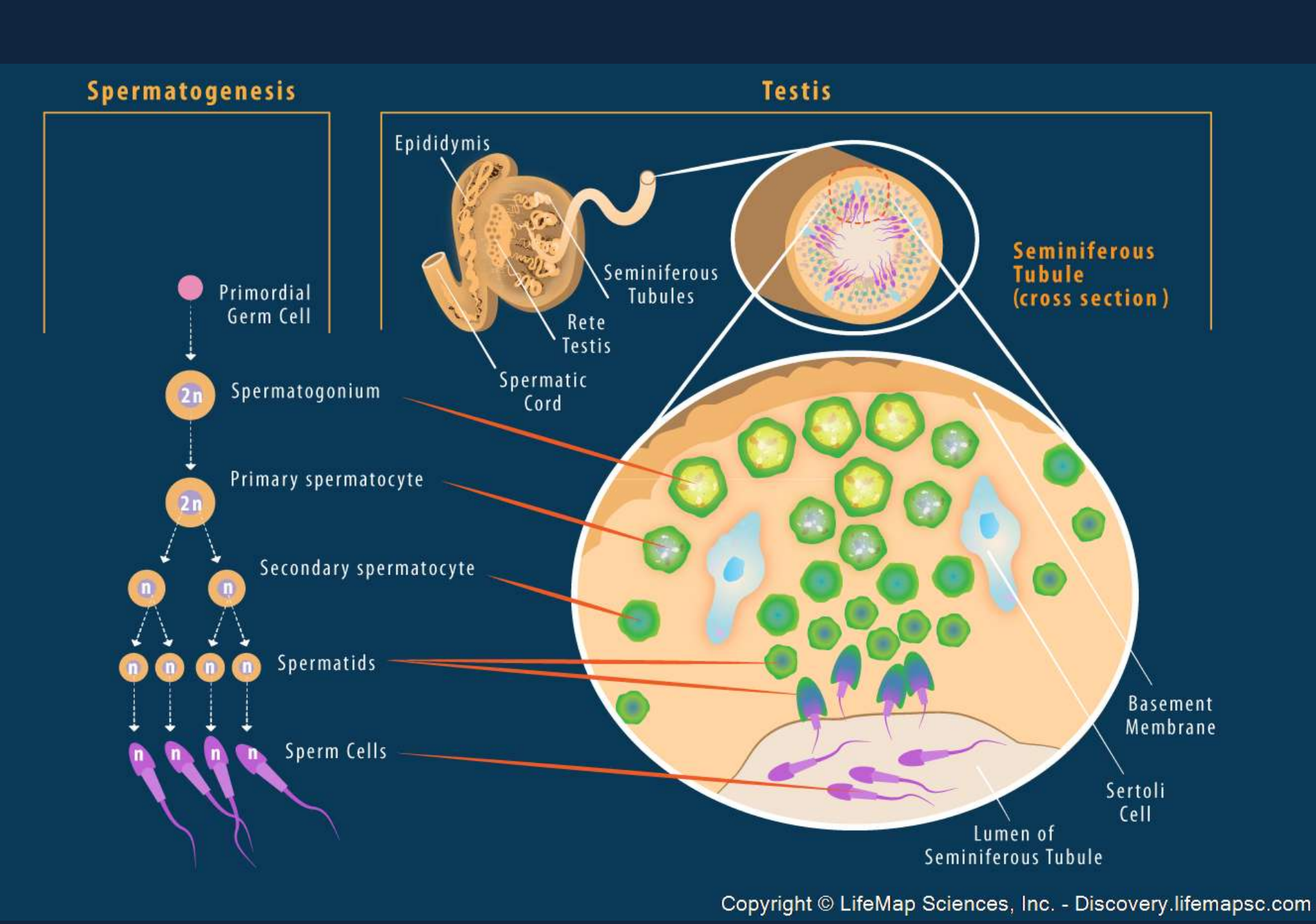
They differentiated iPSC into primordial germ cell-like cells (PGCLC), and then transplanted the passage 1 of PGCLC from the three lines into the seminiferous tubules of **W/Wv** mice and evaluated the recipients after 10 weeks.

No testes with cells from the 178B-5 or 492B-4 lines showed spermatogenesis, and two with the 178B-5 cells resulted in teratomas. Remarkably, 3 out of 18 testes with the cells from the 20D17 line exhibited proper spermatogenesis, and they observed no teratomas in the recipients of this line.

With ICSI followed by embryo transfer, the resultant sperm contributed to fertile offspring. Notably, some of the offspring died prematurely, apparently due to tumors around the neck region.

These findings demonstrate that, although iPSCs exhibit different induction properties depending on the lines, they can nonetheless form PGCLCs with proper function.

The offspring derived from spermatozoa from iPSCs.



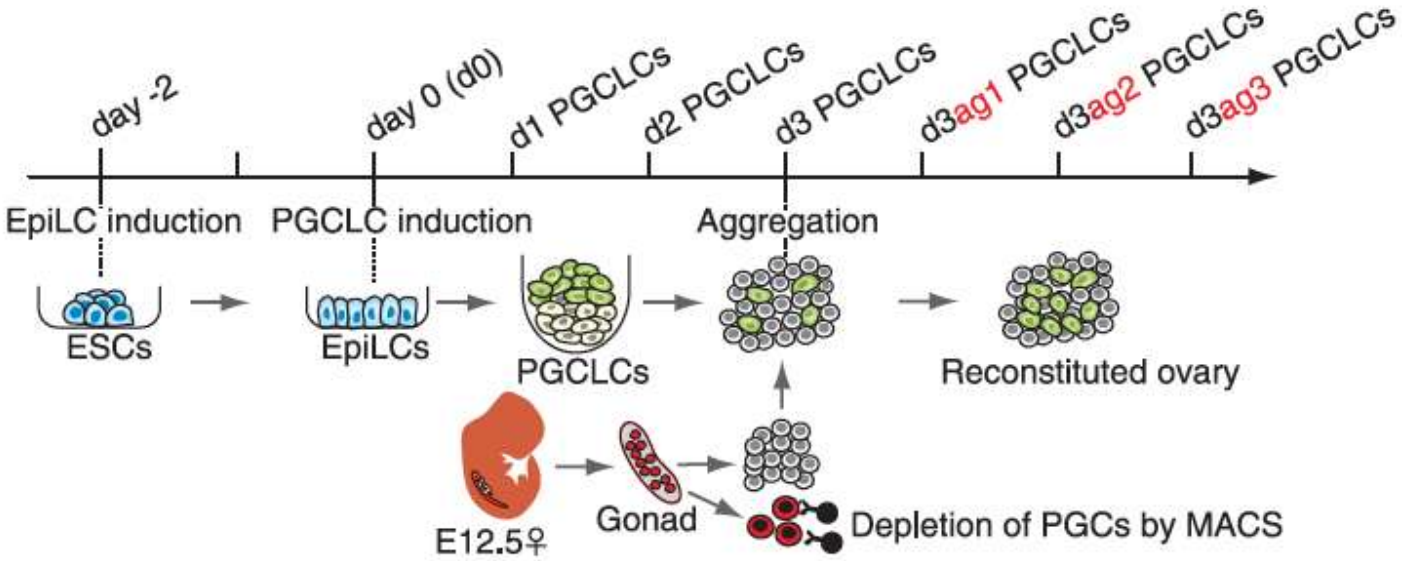
# Offspring from Oocytes Derived from in Vitro Primordial Germ Cell–like Cells in Mice

Katsuhiko Hayashi,[1,2,3]* Sugako Ogushi,[1,4] Kazuki Kurimoto,[1,5] So Shimamoto,[1] Hiroshi Ohta,[1,5] Mitinori Saitou[1,2,5,6]*

Reconstitution of female germ cell development in vitro is a key challenge in reproductive biology and medicine. We show here that female (XX) embryonic stem cells and induced pluripotent stem cells in mice are induced into primordial germ cell–like cells (PGCLCs), which, when aggregated with female gonadal somatic cells as reconstituted ovaries, undergo X-reactivation, imprint erasure, and cyst formation, and exhibit meiotic potential. Upon transplantation under mouse ovarian bursa, PGCLCs in the reconstituted ovaries mature into germinal vesicle-stage oocytes, which then contribute to fertile offspring after in vitro maturation and fertilization. Our culture system serves as a robust foundation for the investigation of key properties of female germ cells, including the acquisition of totipotency, and for the reconstitution of whole female germ cell development in vitro.

The germ cell lineage in mammals originates from pluripotent epiblasts as primordial germ cells (PGCs) and undergoes sexually dimorphic development, generating spermatozoa in males and oocytes in females. These cells fertilize to form zygotes with full developmental





*NATURE PROTOCOLS* | PROTOCOL

# Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells

**Katsuhiko Hayashi & Mitinori Saitou**

**Affiliations** | **Contributions** | **Corresponding authors**

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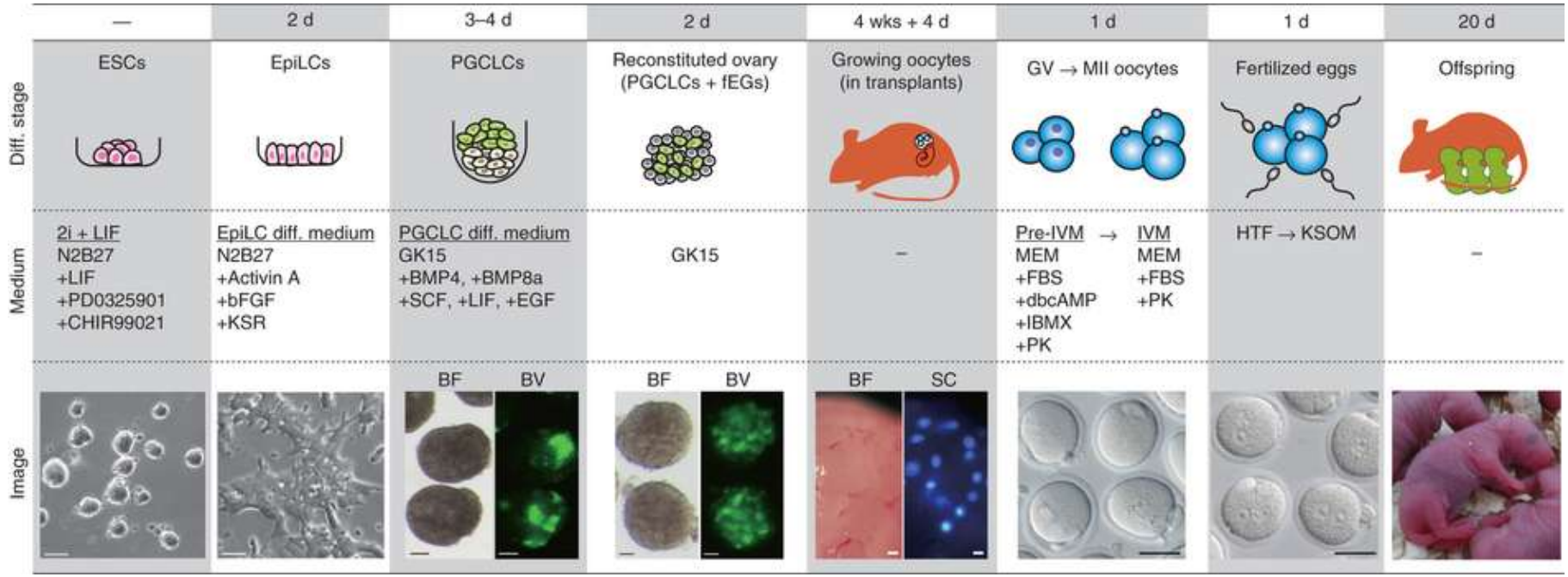

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Cell Reports
Article

# Fate of iPSCs Derived from Azoospermic and Fertile Men following Xenotransplantation to Murine Seminiferous Tubules

Cyril Ramathal,[1] Jens Durruthy-Durruthy,[1] Meena Sukhwani,[2] Joy E. Arakaki,[1] Paul J. Turek,[3] Kyle E. Orwig,[2] and Renee A. Reijo Pera[1,4,*]

[1]Institute for Stem Cell Biology & Regenerative Medicine, Departments of Genetics and Obstetrics and Gynecology, Stanford University, Stanford, CA 94305, USA

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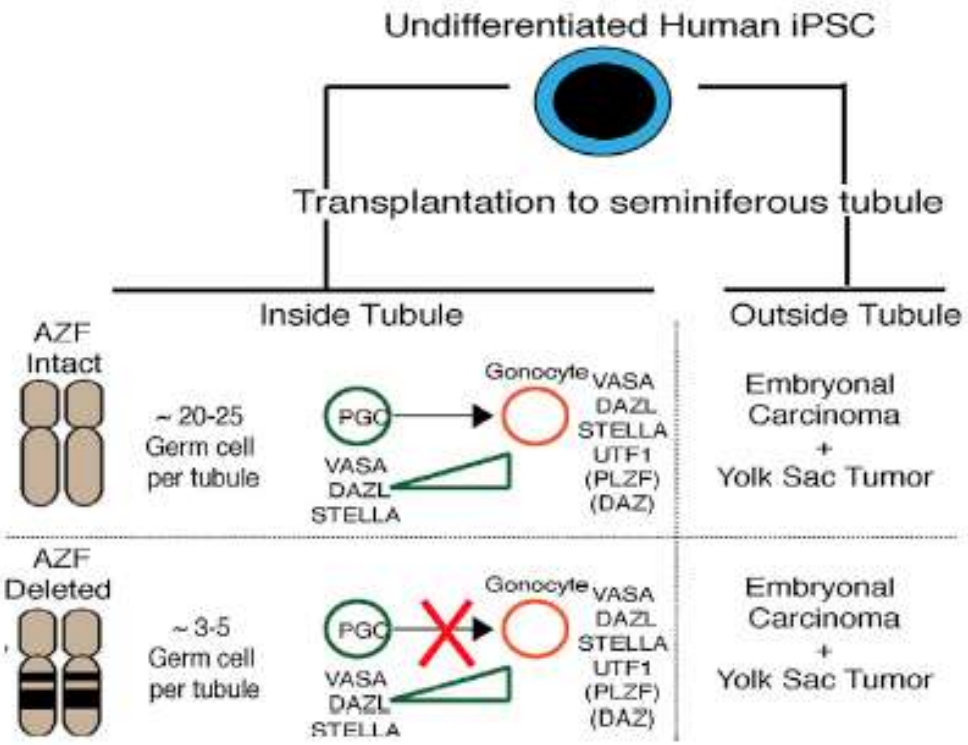
## SUMMARY

Historically, spontaneous deletions and insertions have provided means to probe germline developmental genetics in *Drosophila*, mouse and other species. Here, induced pluripotent stem cell (iPSC) lines were derived from infertile men with deletions that encompass three Y chromosome *azoospermia factor* (*AZF*) regions and are associated with production of few or no sperm but normal somatic development. *AZF*-deleted iPSC lines were compromised in germ cell development in vitro. Undifferentiated iPSCs transplanted directly into murine seminiferous tubules differentiated extensively to germ-cell-like cells (GCLCs) that localized near the basement membrane, demonstrated morphology indistinguishable from fetal germ cells, and expressed germ-cell-specific proteins diagnostic of primordial germ cells. Alternatively, all iPSCs that exited tubules formed primitive tumors. iPSCs with *AZF* deletions produced significantly fewer GCLCs in vivo with distinct defects in gene expression. Findings indicate that xenotransplantation of human iPSCs directs germ cell differentiation in a manner dependent on donor genetic status.




iPSCs were derived from patients with *azoosperimia factor* (AZF)-intact and AZF-deleted Y. When transplanted, undifferentiated male iPSCs specifically differentiate to PGC-like and gonocyte-like germ cells inside the mouse spermatogonial tubule environment, presumably in the Sertoli cell niche.

However, outside the tubule, all patient-derived iPSCs and hESCs remain undifferentiated as primitive tumors.

AZF-deleted iPSCs appear to have a lower potential to make germ cells in vivo as compared to AZF-intact iPSCs and appear to be restricted to forming PGCLCs.

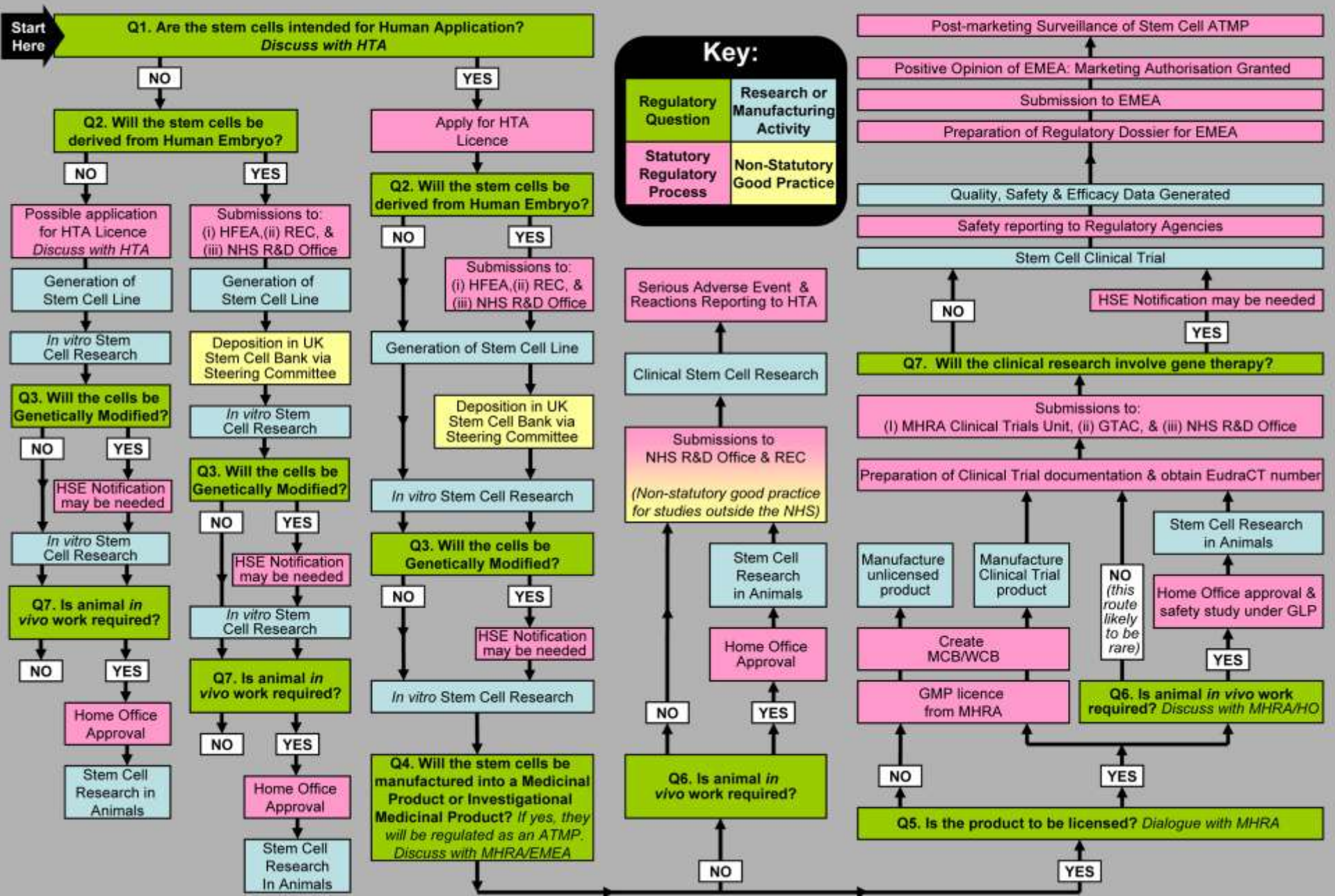
Let’s imagine that everything work and we found a way how to make gametes in the dish

What is the next?


How about quality control?

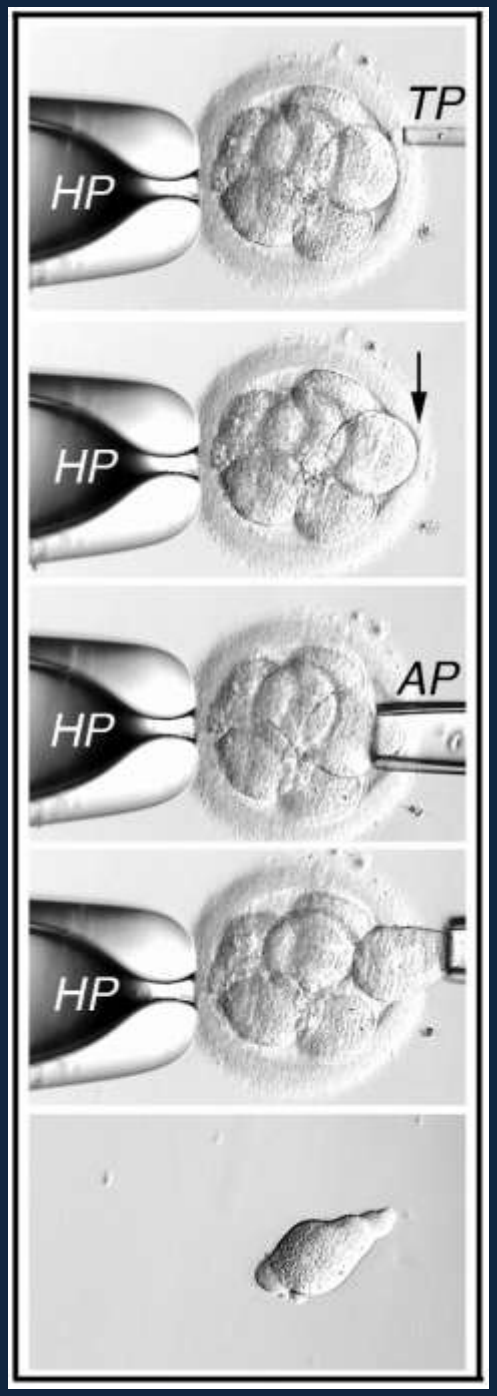
# Interim UK Regulatory Route Map for Stem Cell Research & Manufacture

**Version: 12.03.09**



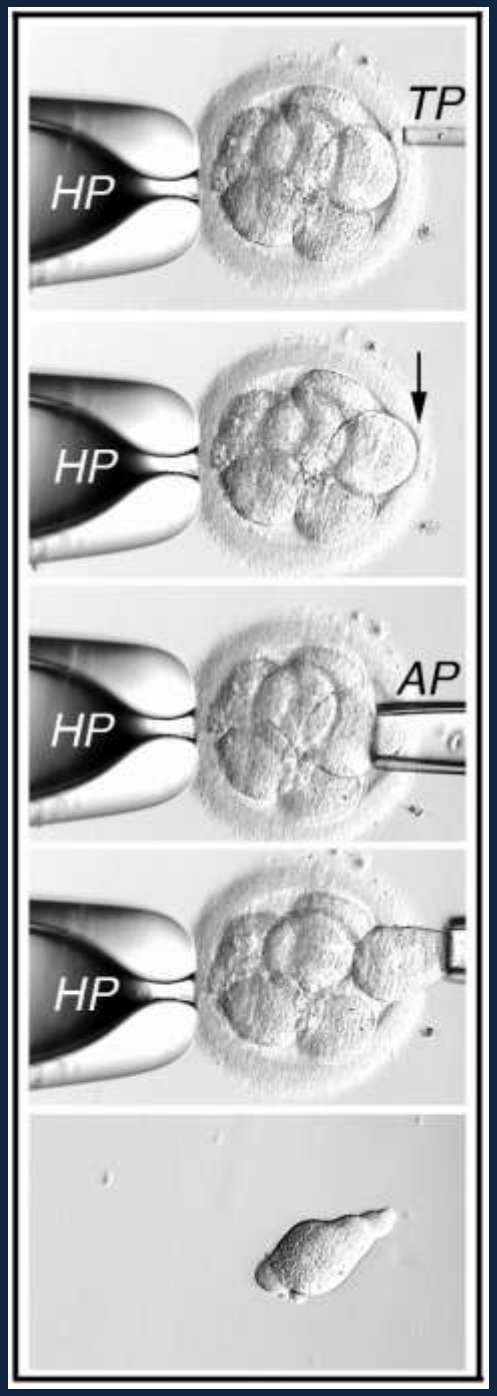
# MANUFACTURING hPSC FOR CELL THERAPY:
More legal than technical hurdle?

2009 Application to FDA for clinical trial (Geron)




Blastomere extraction
from the human generated from
iPSC-derived gametes

Cryopreservation of biopsied embryo
while single cell analyses were performed




Blastomere extraction
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The question is: which one?

- Molecular cytogenetics
- Transcriptome
- DNA Methylome

We have to build first database of normal embryo transcriptome, miRNAome and DNA methylome

Only then we will be able to compare the data from blastomeres of the embryos generated from iPSC-derived gametes

What are chances that iPSC-derived gametes will become a routine ART?

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