GAMETE PRODUCTION,GENE EDITING AND OTHER NOVEL TECHNIQUES : READY FOR APPLICATION IN ART?

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No conflict of interest.

- Pluripotent stem cells (PSC)
- Generation of gametes from PSC
- iPS as a model for infertility
- Spermatogonial stem cells
- Gene editing
- Mitochondrial transfer

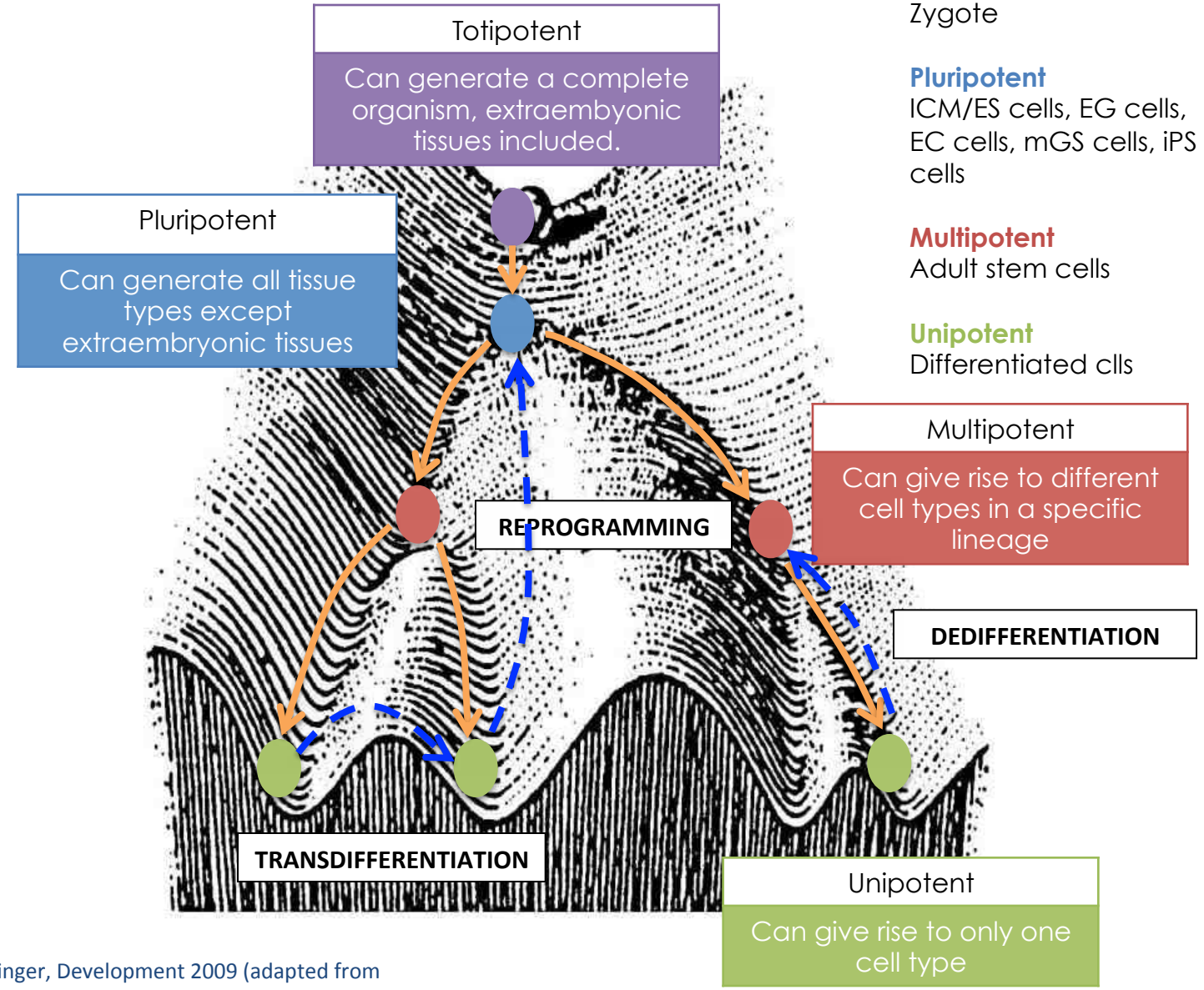
Developmental Potential

Totipotent
Zygote

Pluripotent
ICM/ES cells, EG cells, EC cells, mGS cells, iPS cells

Multipotent
Adult stem cells

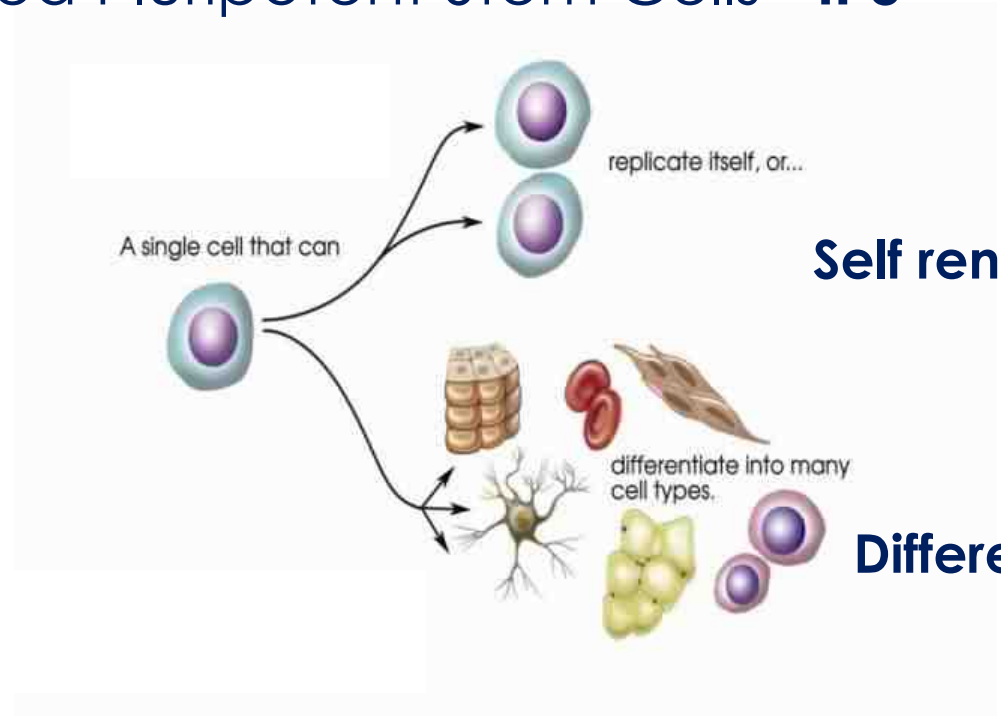
Unipotent
Differentiated clls



Adapted from Hochedlinger, Development 2009 (adapted from Waddington, 1957)

Pluripotent stem cells

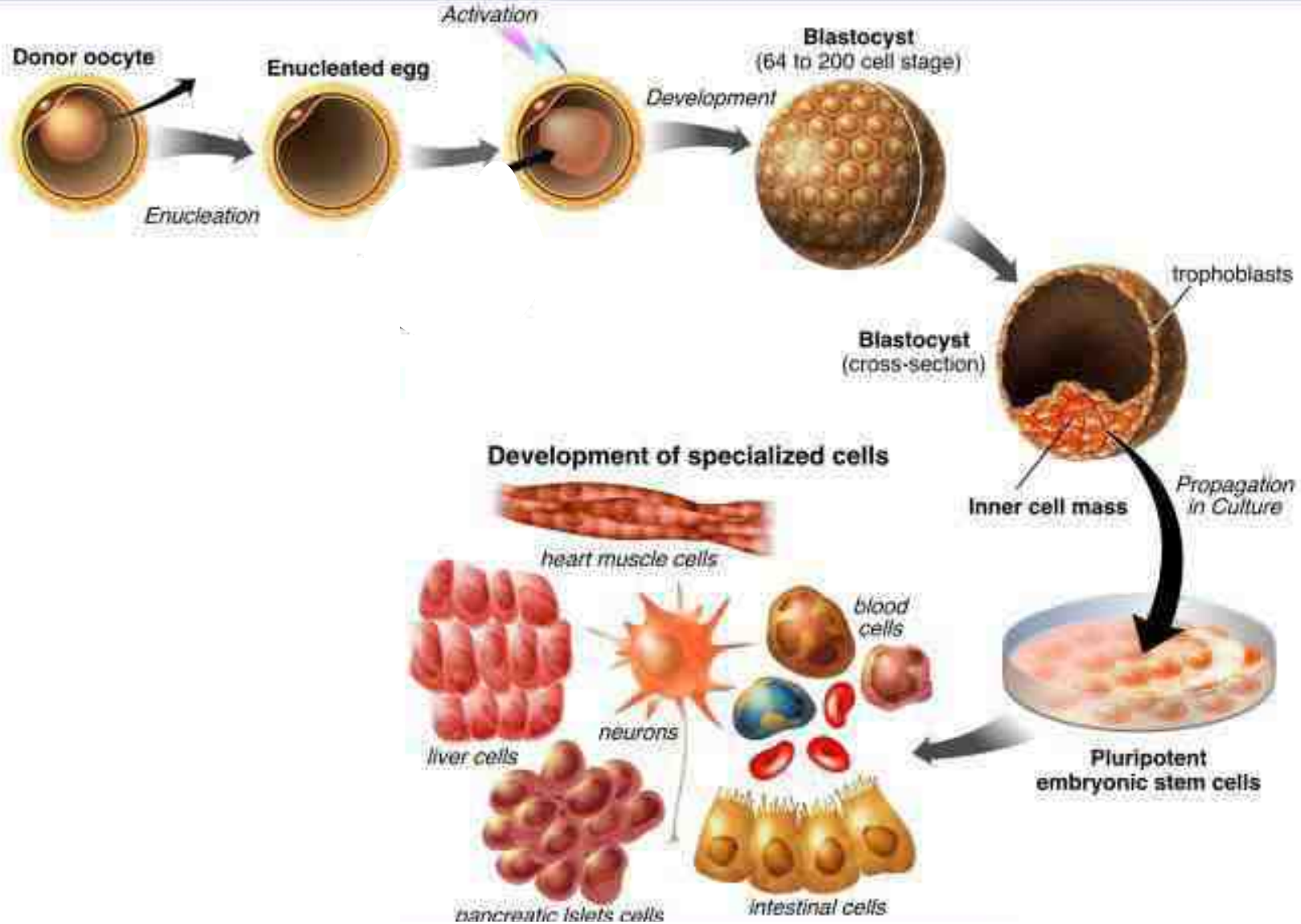
- Preimplantation embryos: **hESC**
- Nuclear reprogramming
  - Somatic Cell Nuclear Transfer – **SCNT**
  - induced Pluripotent Stem Cells - **iPS**



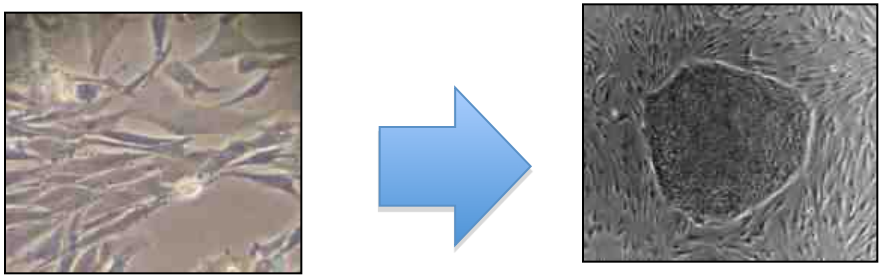
Self renewal

Differentiation

Embryonic stem cells



induced Pluripotent Stem cells (iPS)



- The first iPS cell line generated with 24 factors. (Takahashi K & Yamanaka S. Cell 2006)

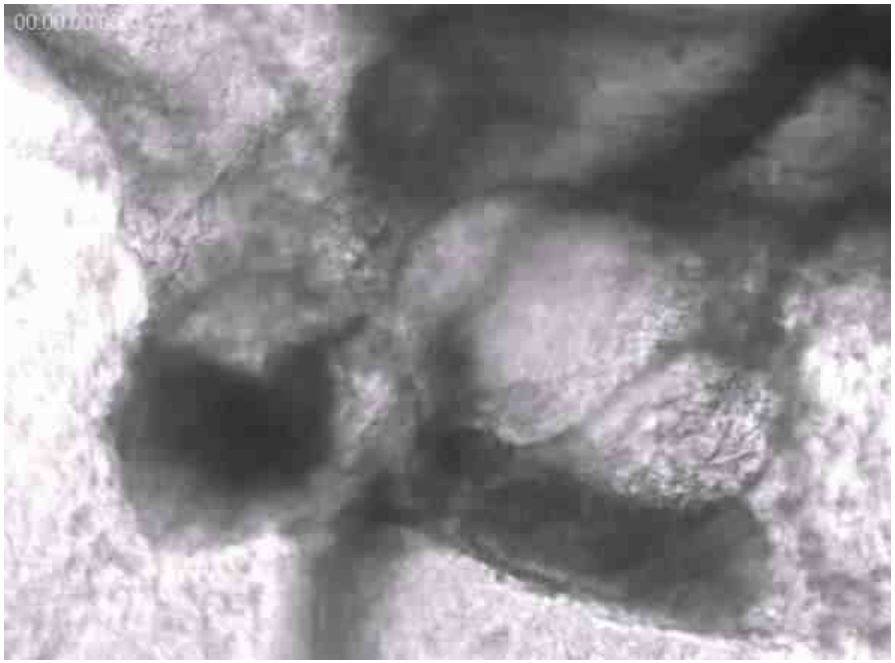
- The Classical 4 factors cocktail: **Oct4/3, Sox2, c- myc & KIF4 or Oct4/3, Sox2, Lin28 & Nanog** (Takahashi K & Yamanaka S. Cell 2006, Takahashi K et al, Cell 2007, Yu et al, Science NY, 2007Park et al, Nature 2008)

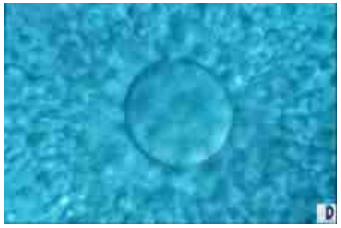
Stable Karyotype
Methylation of Nanog/Oct4 promoters
Transgene expression silencing
Expression of endogenous pluripotent associated markers
In vitro/In vivo differentiation
Chimera contribution*

Differentiation

Different cell types have been obtained

- Cardiomyocytes
- Neuronal cells
- Hematopoyetic cells
- Pancreatic cells
- Hepatocytes
- ……
- Gametes: oocytes and sperm

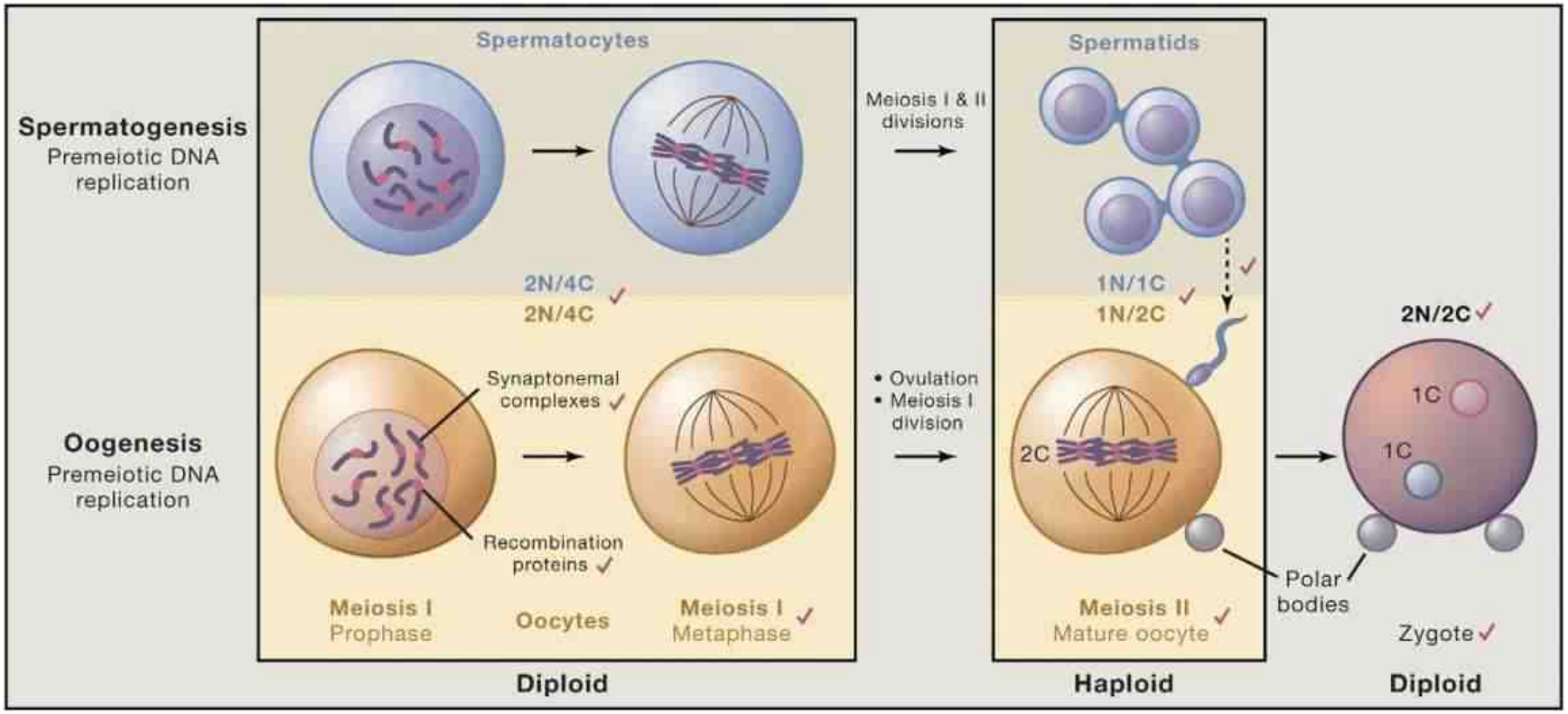




Why are we interested in the generation of germ cells *in vitro*?

- To study gametogenesis *in vitro*
- To study meiosis *in vitro*
- To check the capability of PSC to form germ cells *in vitro*

- PSC may constitute a future source of artificial gametes for research and potential future therapeutic applications
- This system may provide a useful model for molecular genetic studies of human germline formation.



Handel et al, 2014

Mammalian meiocyte development

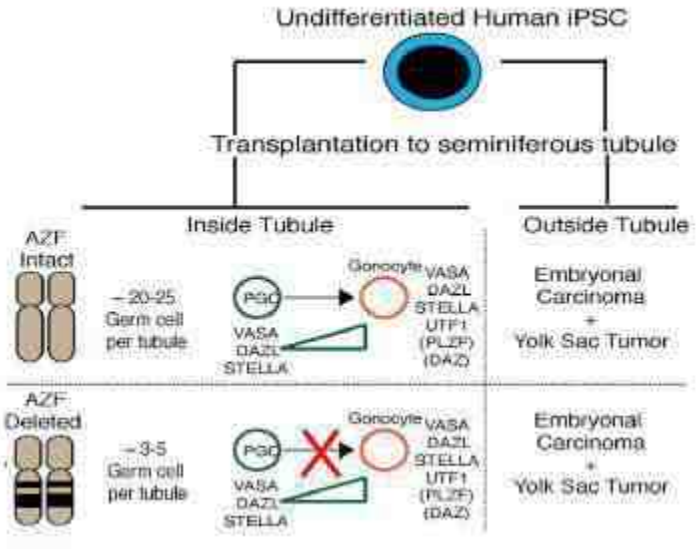
the final test is that any zygote obtained fom *in vitro* derived gametes should be able to form **viable progeny** following transfer to pseudopregnant female hosts

DISEASE MODEL

Fate of iPSCs derived from azoospermic and fertile men following xenotransplantation to murine seminiferous tubules

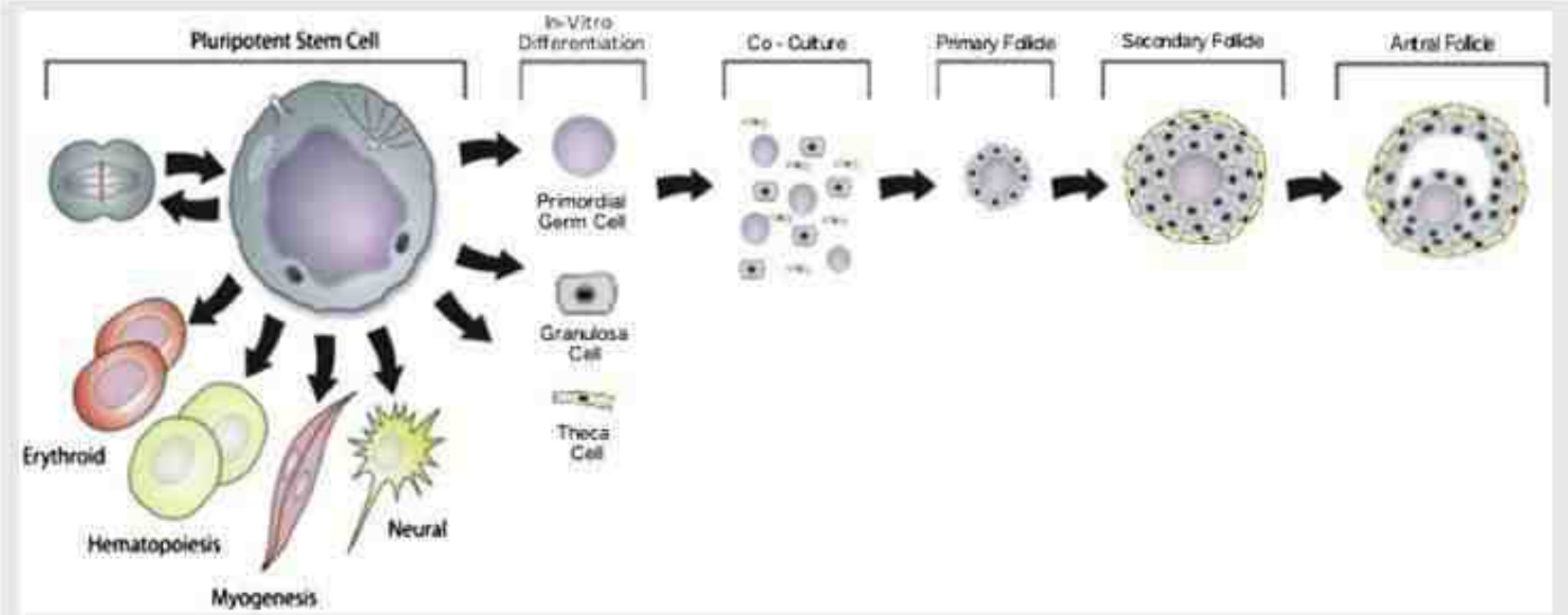
iPS derived from infertile men with AZF Y deletions

- AZF-deleted iPSC lines were **compromised in GC development *in vitro*.**
- AZF-deleted iPS produce **fewer GC-like cells *in vivo*** with defects in gene expression
- Undifferentiated iPSC transplanted in seminiferous tubules differentiated to GC-like cells.
- iPS that exited tubules produced primitive tumours.



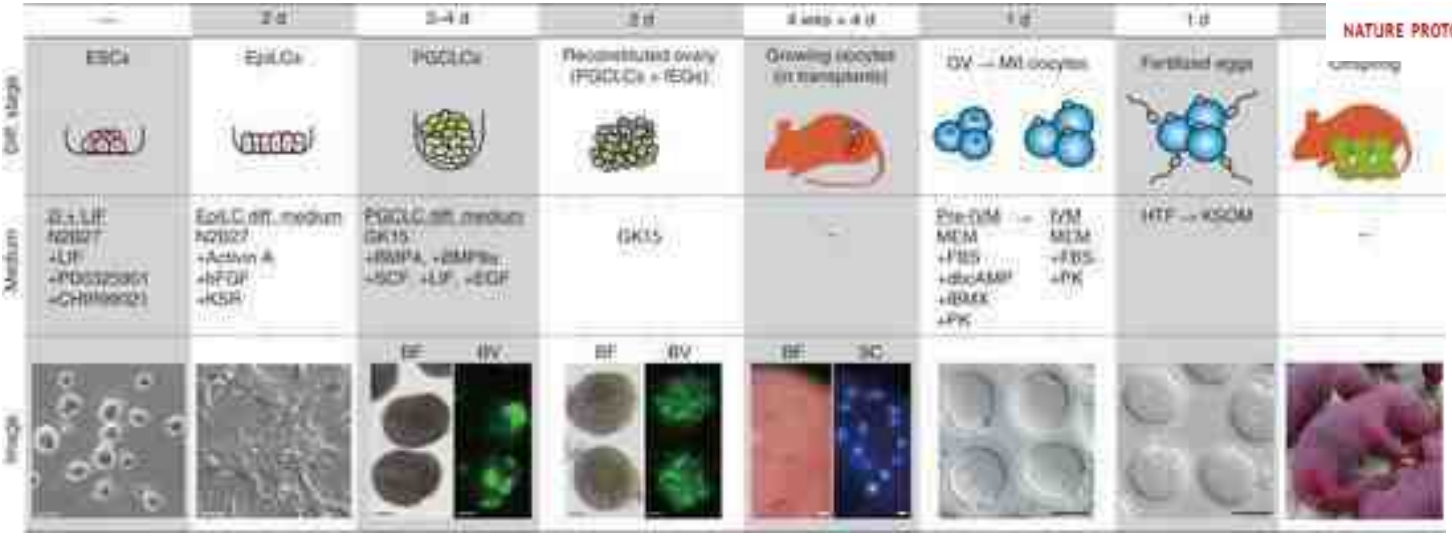
Ramathal et al, 2014

MOUSE OOCYTES



Easley. Adult somatic cells to the rescue. Fertil Steril 2014

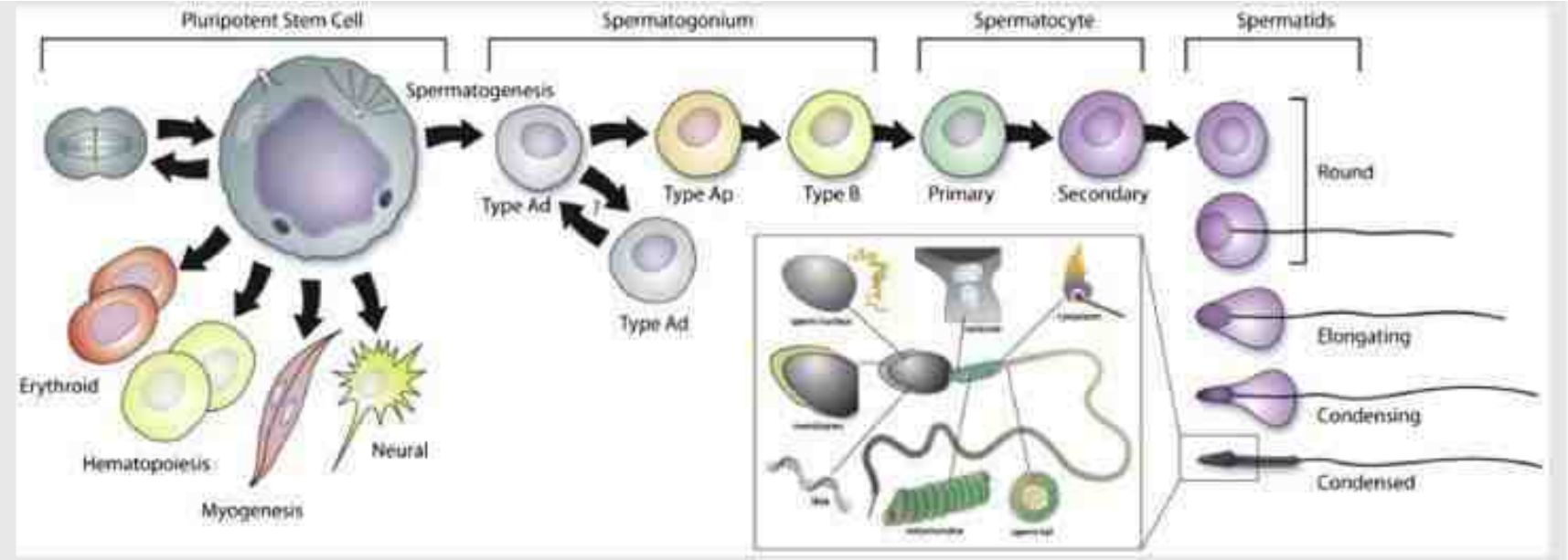
Mouse oocytes from iPS-derived PGCs



- Female ES cells and iPS are induced to **primordial germ cell like cells (PGCLCs**) that undergo X reactivation, imprint erasure, cyst formation and exhibit meiotic potential
- Upon transplantation, PGCLCs **mature into GV oocytes which contribute to fertile offspring** after in vitro maturation and fertilization.

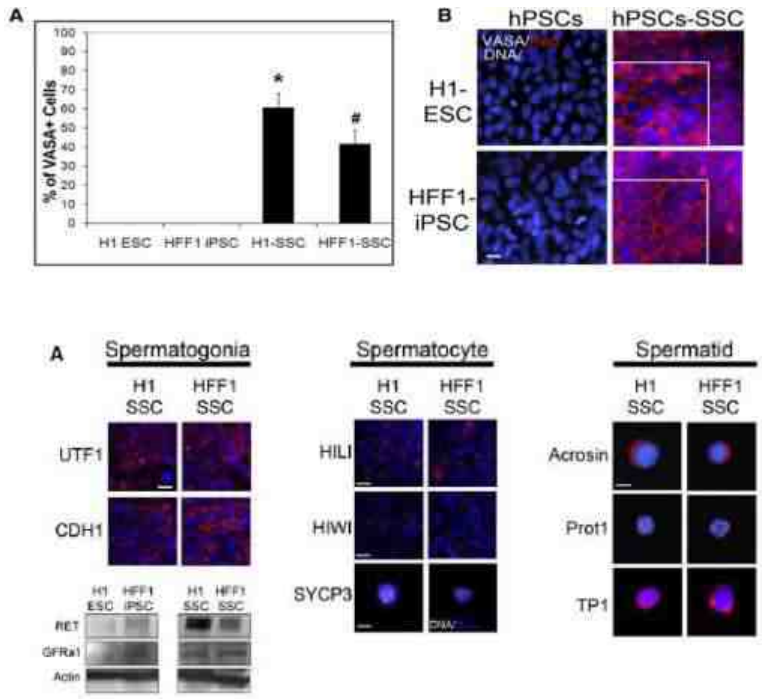
Hayashi, 2012, 2013

SPERM



Easley. Adult somatic cells to the rescue. Fertil Steril 2014

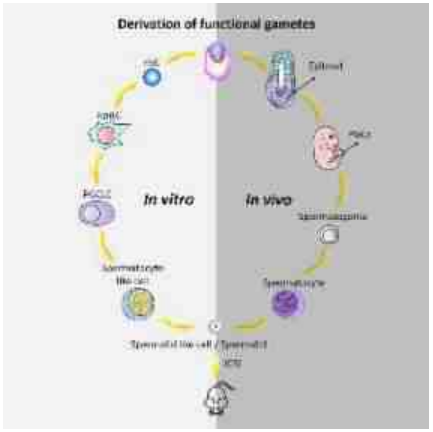
Direct differentiation of human pluripotent stem cells into haploid spermatogenic cells



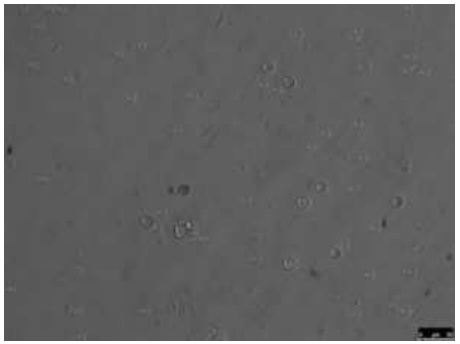
Easley et al, 2012

- hESCs and iPSCs cultured in **SSC conditions** differentiate directly into **advanced male germ cell lineages** including postmeiotic,spermatid-like cells in vitro without genetic manipulation (10 days)
- hPSCs differentiated in SSC culture conditions exhibit **haploid features**
- Differentiation of hPSCs in SSC culture yields cells that express **markers for spermatogonia, spermatocytes and spermatids**

Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro



- **Haploid spermatid-like cells** (SLCs) were derived by tepwise differentiation of ESCs
- This process completely **recapitulated meiosis in vitro**, meeting meiotic hallmarks
- Intracytoplasmic injection of SLCs produced **euploid and fertile offspring**



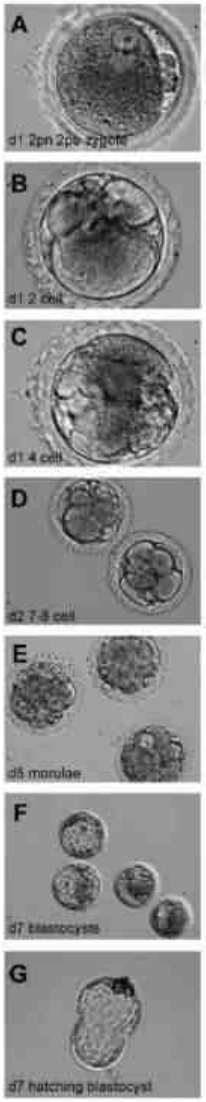
Zhou, Q , 2016

Spermatogonial Stem Cell (SSC) Transplantation into Rhesus Testes Regenerates Spermatogenesis Producing Functional Sperm

- Autologous and allogenic **SSC transplantation** into the testes of 18 adult anc 5 prepuberal infertile macaques
  - Autologous transplant: **donor genotype** from lentivirus marked SSC evident in the ejaculated sperm of 9/12 adult and 3/5 prepuberal recipeints
  - Allogenic transplantation: **Donor-recipient chimerism** in sperm in 2/6 adult recipients
- ICSI with Ejaculated sperm form allogenic transplantation:

✓ 81/85 fertilised oocytes

✓ 7/81 donor paternal origin



Hermann et al, 2012

Offspring production with sperm grown in vitro from cryopreserved testis tissues

- Testis tissues of neonatal mice cryopreserved either by **slow freezing or by vitrification**.
- After thawing, they were cultured on agarose gel and showed **spermatogenesis up to sperm formation**.
- Microinsemination was performed with round spermatids and sperm, leading to eight **healthy and fertile offspring**

Table 2 | Summary of microinsemination experiments.

Insemination exp. ID	Culture exp. ID	Cryoprotectant	Preservation period (days)	Microinsemination	No. of oocytes inseminated	No. of embryos transferred	No. of implantations	No. of offspring	Female/ male
1	15	CB	143	ICSI	8	5	2	0	0
				ROSI	17	16	7	4	3/1
2	67	SCK	85	ICSI (7)	15	7	5	0	—
				ROSI (8)					—
	71		72	ROSI	20	18	0	0	—
	81		28	ROSI	10	8	2	0	—
	82-1		28	ROSI	8	5	1	0	—
	82-2		28	ROSI	32	20	0	0	—
3	99	SCK	220	ICSI	46	30	15	2	2/0
				ROSI	52	30	12	0	—
	100		219	ICSI	22	12	3	2	0/2
				ROSI	6	5	2	0	—

CB, Cell Banker 1; ROSI, round spermatid injection; SCK, Stem Cell Keep.

Yokonishi et al, 2014

human reproduction ESHRE PAGES

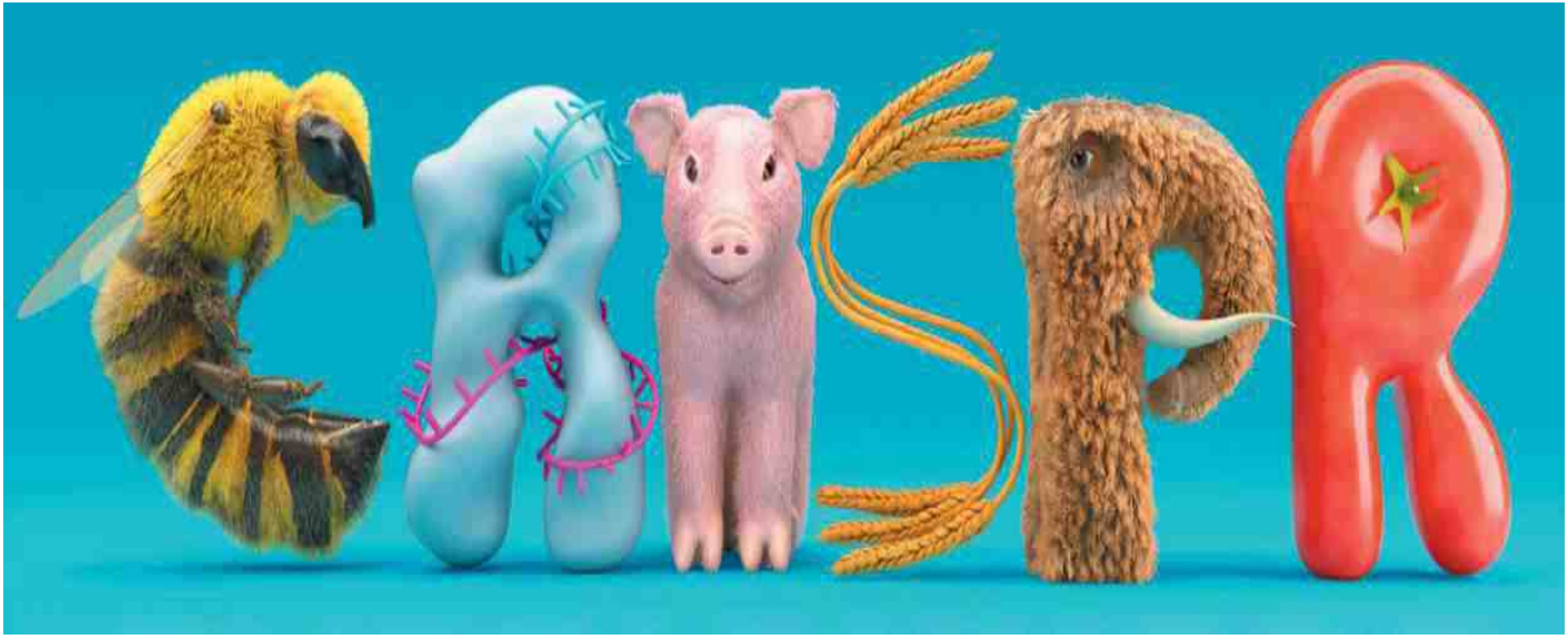
2015

Stem cells in reproductive medicine: ready for the patient?

R. Vassena[1,*], C. Eguizabal[2], B. Heindryckx[3], K. Sermon[4], C. Simon[5,6], A.M.M. van Pelt[7], A. Veiga[8,9], and F. Zambelli[4,10] on behalf of the ESHRE special interest group Stem Cells[†]

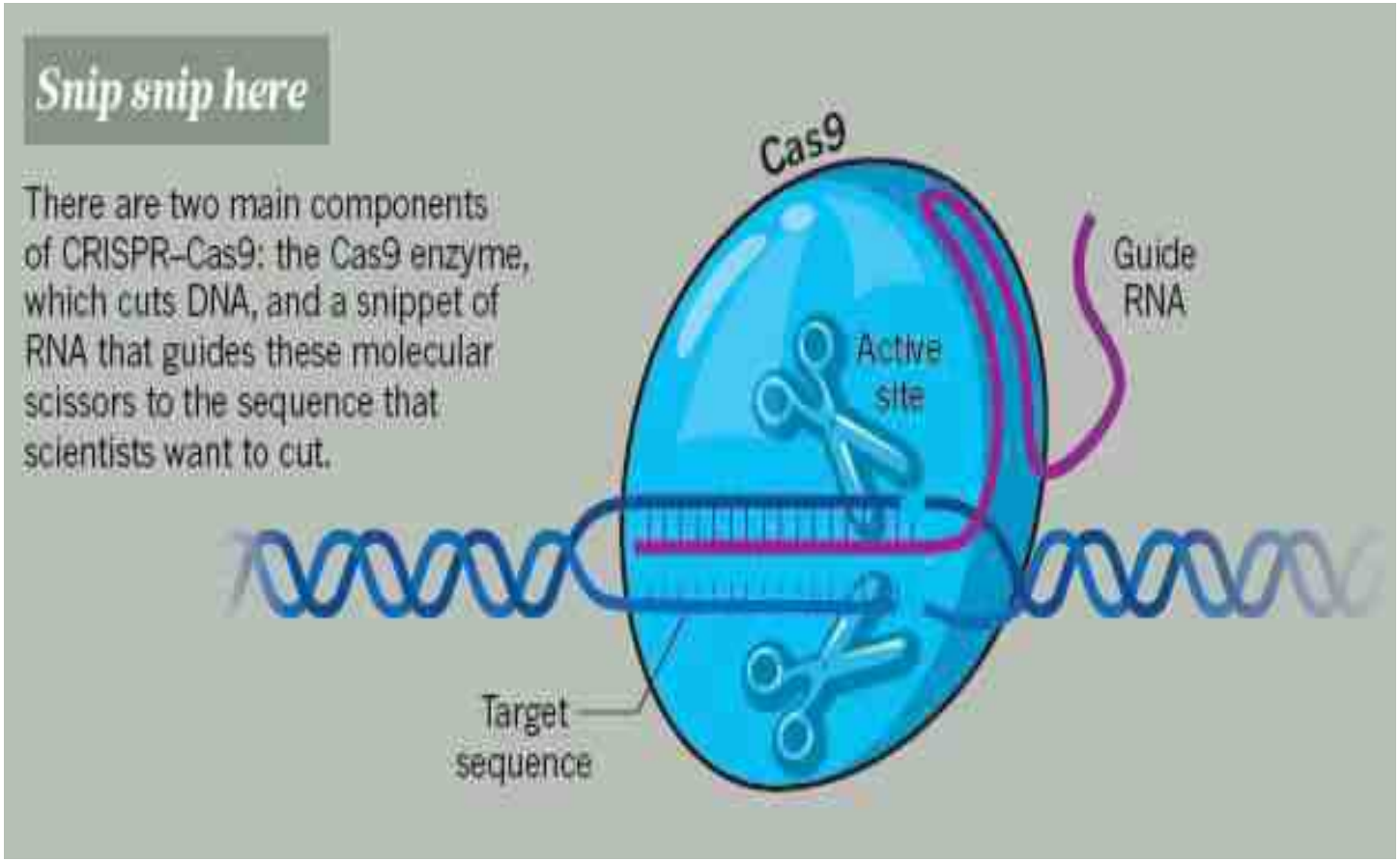
- No proven stem cell based means to:
  - improve reproductive function, either by producing functional gametes in vitro
  - stimulating the resident stem cell population (were it confirmed as being present in our species) in the ovary to elicit de novo oocyte production.
- Development of therapies from adult stem cells in the treatment of reproductive tract alterations, such as erectile dysfunction or damaged endometrial lining (animal models, clinical results still preliminary)
- Patients and physicians should be wary of unfounded claims of improvement of existing medical conditions
- Actually, stem cell treatment for reproductive diseases and alteration is not feasible.

Gene Editing
CRISPR CAS9 System

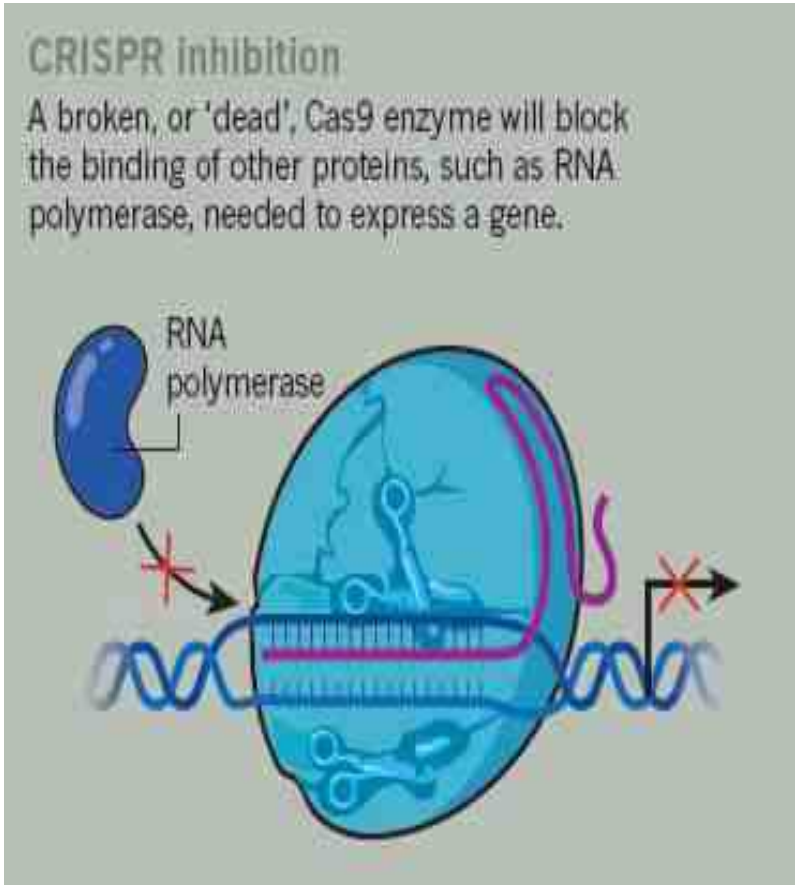


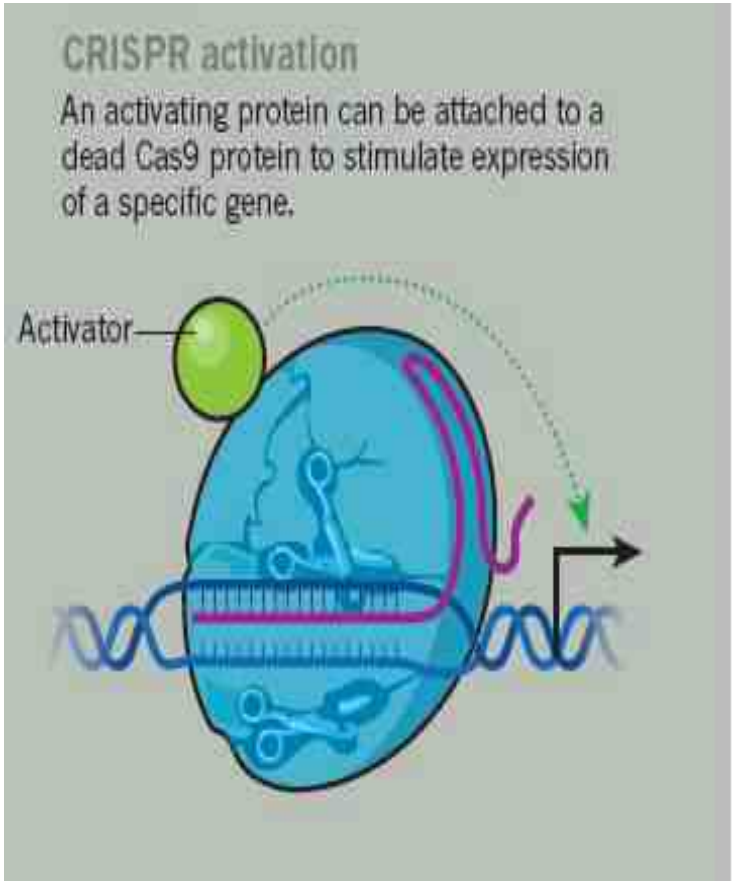
CRISPR-CAS 9 SYSTEM

- 2005 - 2009 (Mojica,2005) microbial genetic studies revealed that bacteria have a programmable mechanism that directs nucleases, such as Cas9, to bind and cut invading DNA that matches "guide RNAs" encoded in specific bacterial genome regions containing **clustered regularly interspaced short palindromic repeats (CRISPR).**
- 2010 and 2011: Moineau and Charpentier defined the critical components of the **CRISPR-Cas9 system**
- Biochemical studies in 2012, by Charpentier and Doudna and by Siksnys, confirmed these results in vitro.
- 2013, Zhang and Church described how to repurpose the **CRISPR-Cas9 system to work in mammalian cells**, creating a general-purpose tool for editing the genome in living human cells.



Riding the CRISPR wave. Nature, March 2016





Riding the CRISPR wave. Nature, March 2016

- Editing a patient's immune cells to delete the *CCR5* gene to treat **human immunodeficiency virus (HIV)** infection, physicians might, conferring the resistance to HIV

- Inactivating the mutant allele in retinal cells to treat progressive blindness caused by dominant forms of **retinitis pigmentosa**

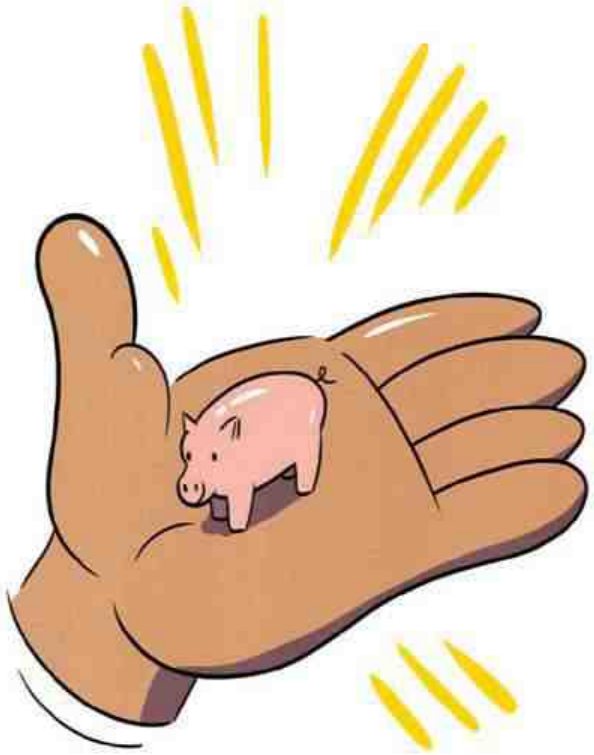
- Editing liver cells to restore a functional copy of the gene encoding low-density lipoprotein receptors to prevent myocardial infarctions (**homozygous familial hypercholesterolemia**)

- Editing blood stem cells for **sickle cell anemia and hemophilia**

THE CRISPR ZOO

DISEASE CONTROL:
"hygienic" bees less likely to succumb to mites, fungi and other pathogenes. Edit genes associated with this behaviour in honeybees might stop their dramatjc loss.

DE-EXTINCTION:
gene editing might be used to transform endangered Indian elephants into whooly mammoths or at least cold resistant elephants



IMPROVING PETS:
micropigs for research or as pets . Modify adverse patterns of behaviour.

Reardon, 2016

human germline editing

Indications: **preventing monogenic diseases**, such as Huntington's disease.

- Genome editing would require making IVF embryos, using preimplantation genetic diagnosis (PGD) to identify those that would have the disease, repairing the gene, and implanting the embryo.

- Easier and safer simply to use PGD to identify and implant the embryos that aren't at risk (a parent heterozygous for a dominant disease (50%) or two parents who are carriers for a recessive disease (75%))

human germline editing

- **Another potential application** is reducing the risk of common diseases, such as heart disease, cancer, diabetes, and multiple sclerosis. The heritable influence on disease risk is polygenic, shaped by variants in dozens to hundreds of genes.

- A more distant frontier would be to reshape nonmedical traits.

human germline editing.

- **Genome editing** cannot actually be performed with sufficient precision to permit scientists to responsibly contemplate creating genetically modified babies (**inaccurate editing, and off-target mutations**).

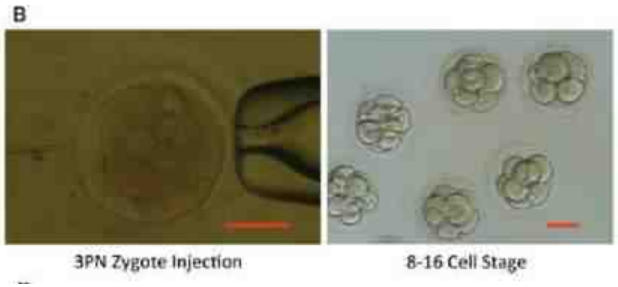
CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

- CRISPR/Cas9 can cleave the endogenous β -globin gene (HBB)
- The efficiency of homologous recombination directed repair (HDR) of HBB is **low and the edited embryos were mosaic.**
- **Off-target cleavage** was also apparent in these 3PN zygotes.
- Repair of the HBB locus occurs preferentially through the non-crossover HDR pathway.
- Need to further improve the fidelity and specificity of the CRISPR/Cas9 platform, a prerequisite for any clinical applications of CRSIPR/Cas9-mediated editing.

Liang,2016

Introducing precise genetic modifications into human 3PN embryos by CRISPR/Cas 9-mediated genome editing

- Results By co-injecting Cas9mRNA, gRNAs, and donorDNA, successfull introduction of the naturally occurring CCR5 Δ 32 allele into early human 3PN embryos.
- In the embryos containing the engineered CCR5 Δ 32 allele, the other alleles at the same locus could not be fully controlled because they either remained wild type or contained indel mutations.

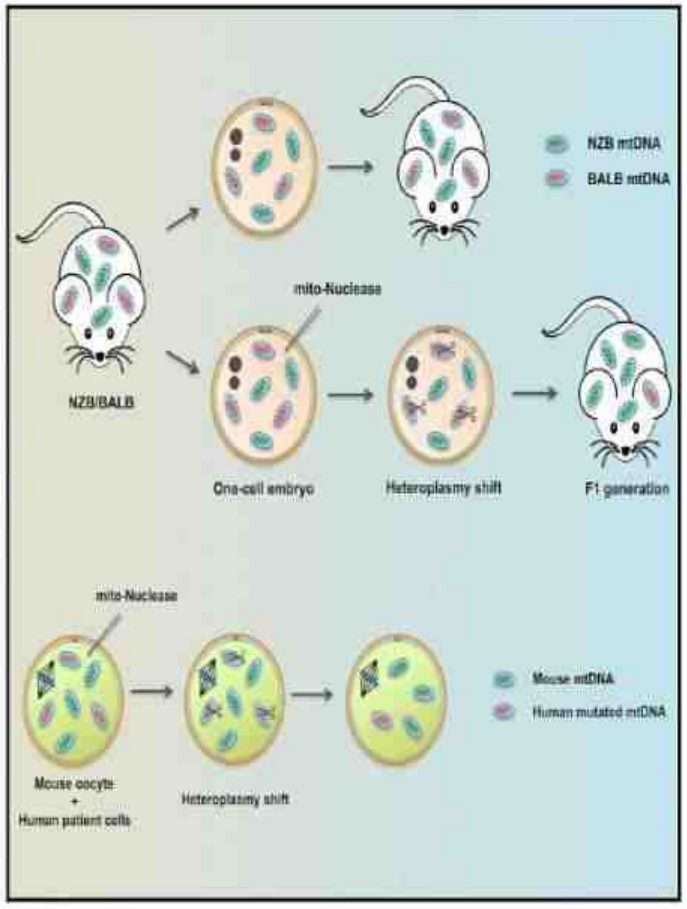


Groups*	Injected 3PN zygotes	Development (%)		Genetic modification	
		Cleavage	8–16 cell	NHEJ mutations (percent)	Δ32 allele (%)
Control water	18	15 (83)	13 (72)	0	0
Cas9 + gRNA1	11	9 (82)	7 (64)	4 (57)	0
Cas9 + gRNA2	13	10 (77)	8 (62)	5 (63)	0
Cas9 + gRNA1 + ssODN1 (PN injection)	23	14 (61)	11 (48)	4 (36)	0
Cas9 + gRNA1 + ssODN1	32	28 (88)	20 (63)	10 (50)	1 (5)
Cas9 + gRNA2 + ssODN2	46	39 (85)	27 (59)	13 (48)	0
Cas9 + gRNA1 + 1 kb dsDonor	25	21 (84)	15 (60)	7 (47)	1 (7)
Cas9 + gRNA1 + gRNA2	45	37 (82)	26 (58)	13 (50)	4 (15)

*CRISPR/Cas system was delivered to 3PN zygotes by cytoplasmic injection in all groups, except the group labeled with PN (pronuclear) injection

Kang, 2016

Selective Elimination of Mitochondrial Mutations in the Germline by Genome Editing



- Mitochondria-targeted nucleases selectively **reduce mtDNA haplotypes in germline**
- Germline heteroplasmy shift prevents transmission on of mt DNA haplotypes to offspring
- **Human mutated mtDNA can be reduced in oocytes** by mitochondria-targeted nucleases

Reddy, 2015

human reproduction update

2016

Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells

R. Vassena[1,*], B. Heindryckx[2], R. Peco[3], G. Pennings[4], A. Raya[3,5,6], K. Sermon[7], and A. Veiga[3,8]

- Review of critical technical and ethical issues that should deter from employing CRISPR/Cas9 based technologies in human reproduction until clarified.

Cytoplasmic transfer

- To counteract the decrease in **developmental competence** of oocytes collected from aged oocyte donors or those with repeated fertilisation failure by transferring ooplasm (mRNAs, proteins, mitochondria,..)from unfertilized oocytes donated from young donors with proven fertility. (Malter and Cohen, 2002; Levy et al., 2004)
- >30 babies born (1990's)
- **Benefit never demonstrated and safety questioned** (heteroplasmy). 2/17 45 XO + 1/17 sex-linked autism-related disorder.
- **Banned by FDA in 2001**

The AUGMENT℠ Treatment: Physician Reported Outcomes of the Initial Global Patient Experience

- **Egg precursor cells** can be readily isolated from the protective outer lining of the ovarian cortex.
- The AUGMENT treatment was used in a population of **difficult-to-treat patients** with a poor prognosis.
- Marked improvements in **pregnancy rates** above the historic IVF success rate.
- Morphogenetic embryo selection and transfer from the AUGMENT treatment group was significantly higher, suggesting **that improved embryo quality** may have resulted in the **improved pregnancy rates** observed

Fakih, 2015

The use of mitochondrial transfer to improve ART outcome
ESHRE SIG stem cells

- Wrong citations (Van Blerkhom, Cohen)
- Wrong control group
- No blinded morphology assessment
- Methodology
  - Mitoch. Isolation and purification
  - Number of mtDNA copies needed
  - mtDNA heteroplasmy
  - Epigenetic changes
  - No animal models

Heindryckx 2015

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