PRE-CONGRESS COURSE 13

Of stem cells and gametes: more similarities than differences?

Special Interest Group Stem Cells
Munich - Germany, 29 June 2014

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Of stem cells and gametes: more similarities than differences

Munich, Germany
29 June 2014

Organised by
The ESHRE Special Interest Group Stem Cells

Contents

Course coordinators

Karen Sermon (Belgium), Rita Vassena (Spain)

Course description

This is an advanced course on the latest developments in the differentiation of human pluripotent stem cells, both embryonic, adult and induced, into gametes, both oocytes and sperm. The course starts with a comparison of different shades of stem cells, and what their potential of differentiation towards gametes are. The differentiation into primordial germ cells starting from to the preimplantation embryo is drawn. Two speakers each then describe current knowledge on differentiation of hPSC into either male or female gametes. Alternative routes, such as from the adult ovary and testes are described next.

Target audience

Target audience are mainly scientists working in the field of stem cells, but also clinical embryologists with a general interest in fundamental embryology and clinicians interested in alternative ways to obtain donor gametes.

Scientific programme

Chairmen: Karen Sermon - Belgium and Cristina Eguizabal - Spain

09:00 - 09:30 Before the gamete, there was the primordial germ cell
Petra Hajkova - United Kingdom
09:30 - 09:45 Discussion
09:45 - 10:15 Dazlin' Germ Cells and Pluripotent Stem Cells
Niels Geijsen - U.S.A.
10:15 - 10:30 Discussion

10:30 - 11:00 Coffee break

Chairmen: Anna Veiga – Spain and Bjorn Heindryckx - Belgium

11:00 - 11:30 Current status of in vitro differentiation of HPSC into female gametes
Susana M. Chuva de Sousa Lopes - The Netherlands
11:30 - 11:45 Discussion
11:45 - 12:15 In vitro differentiation of hPSC into male gametes: current status and the road ahead
Cristina Eguizabal - Spain
12:15 - 12:30 Discussion

12:30 - 13:30 Lunch

Chairmen: Karen Sermon – Belgium and Rita Vassena - Spain

13:30 - 14:00 Functional characterization of adult ovary-derived oogonial stem cells in mice, monkeys and women
Jonathan L. Tilly - U.S.A.
14:00 - 14:15 Discussion
14:15 - 14:45 Do mitotically active female germline progenitors exist in postnatal mouse ovaries?
Kui Liu - Sweden
14:45 - 15:00 Discussion

15:00 - 15:30 Coffee break

Chairmen: Rita Vassena – Spain and Filippo Zambelli - Italy

15:30 - 16:00 Spermatogonia stem cells and future fertility
Ans van Pelt - The Netherlands
16:00 - 16:15 Discussion
16:15 - 16:45 Stem cell based approaches to restore spermatogenesis in monkeys
Stefan Schlatt - Germany
16:45 - 17:00 Discussion

Before the gamete, there was the primordial germ cell

Petra Hajkova
Reprogramming and Chromatin Group
MRC Clinical Sciences Centre
London UK

Imperial College London

MRC Clinical Sciences Centre

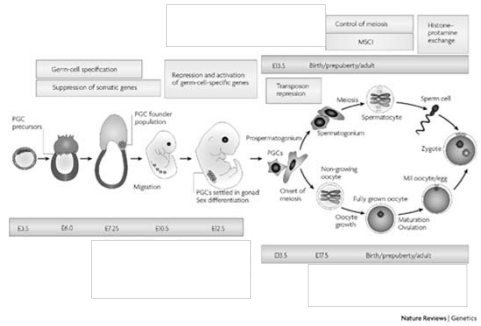
Conflict of interest:

Presenter declares no conflict of interest.

Learning objectives

- **Key aspects of germline development and epigenetic properties**
- **Concept and mechanistic outline of germline epigenetic reprogramming**
- **Derivation and properties of embryonic germ cells (EG cells)**
- **Relationship between germ line, pluripotency and stem cells**

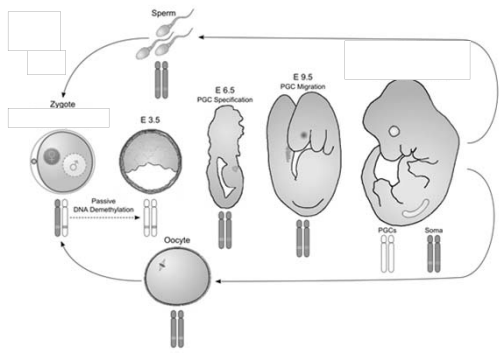
Mouse germline development



Hiroyuki Sasaki & Yasuhisa Matsui
Nature Reviews Genetics 9, 129-140 (February 2008)

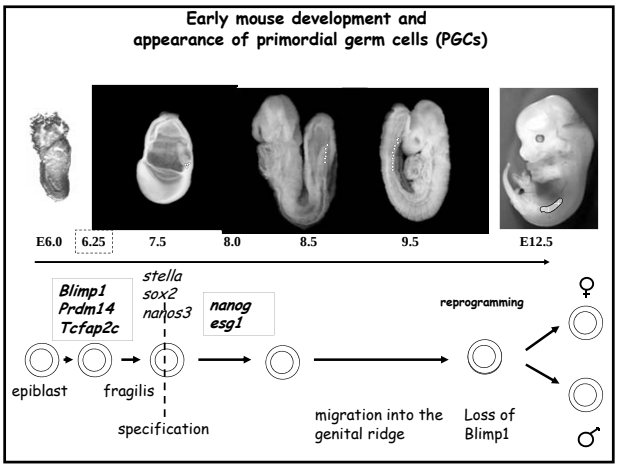
Specific epigenetic properties of germ line

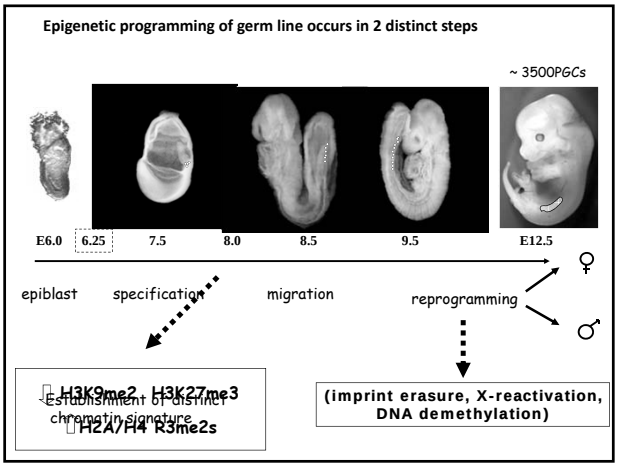
Mouse development & genomic imprinting

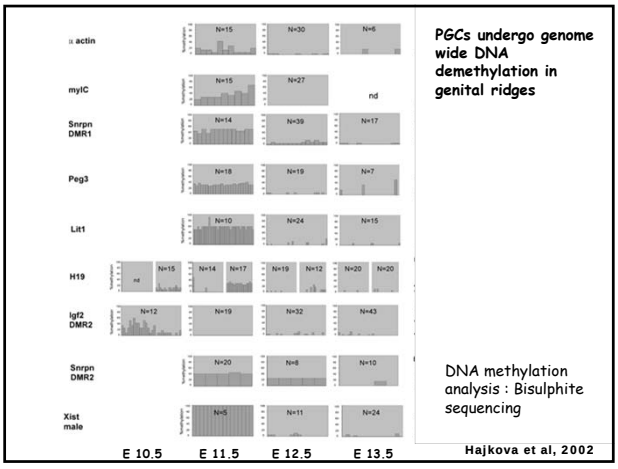


Surani and Barton, 1983, Barton, Surani, Norris 1984, McGrath and Solter, 1984

Hajkova et al, 2002; Lee et al, 2002

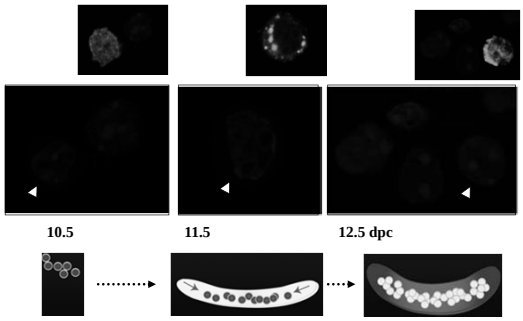

Early mouse development and
appearance of primordial germ cells (PGCs)
E6.0
6.25
7.5
8.0
8.5
9.5
E12.5
Blimp1
Prdm14
Tcfap2c
stella
sox2
nanos3
nanog
esg1
reprogramming
epiblast
fragilis
specification
migration into the
genital ridge
Loss of
Blimp1


Epigenetic programming of germ line occurs in 2 distinct steps
~ 3500PGCs
E6.0
6.25
7.5
8.0
8.5
9.5
E12.5
epiblast
specification
migration
reprogramming
H3K9me2, H3K27me3
H2A/H4 R3me2s
Establishment of distinct
chromatin signature
(imprint erasure, X-reactivation,
DNA demethylation)

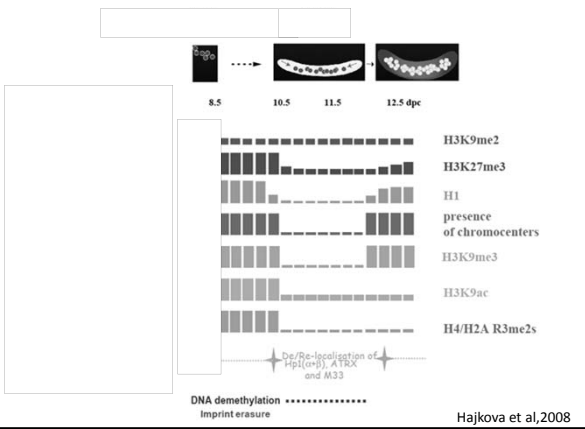

α actin
mylC
Snrpn DMR1
Peg3
Lit1
H19
Igf2 DMR2
Snrpn DMR2
Xist male
E 10.5
E 11.5
E 12.5
E 13.5
PGCs undergo genome wide DNA demethylation in genital ridges
DNA methylation analysis : Bisulphite sequencing
Hajkova et al, 2002

Bis seq or LC/MS data on germline demethylation

Impact on nuclear architecture



Chromatin changes in gonadal PGCs - overview



Hajkova et al,2008

Chromatin remodelling ? **DNA demethylation**

DNA demethylation → **Chromatin remodelling**

ssDNA breaks
(chromatin bound XRCC1)
PAR polymers
Activation of BER

→

Loss of H1

Loss of H2A.Z

Loss of many histone modifications

Translocation of histone chaperones

DNA demethylation (DNA repair)

Erasure of histone modifications

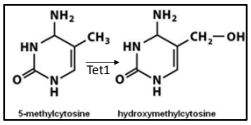
(histone replacement)

Hajkova et al,2008

Molecular mechanisms of epigenetic reprogramming in PGCs?

Many questions remaining…..

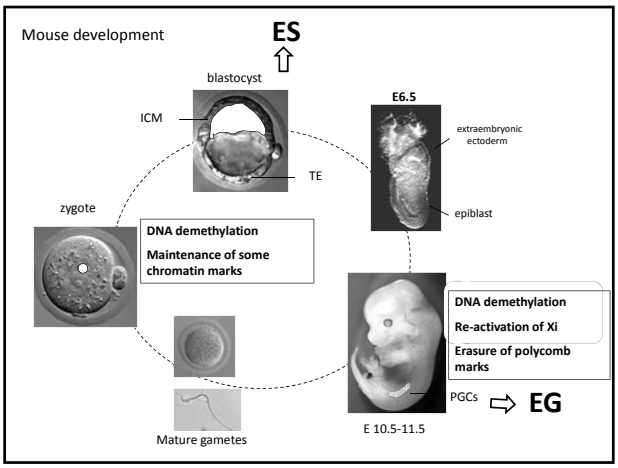
- Passive loss of DNA methylation
  (replication without maintenace of the DNA methylation pattern) ?
- Active DNA demethylation (Base excision repair pathway)?
- Conversion of 5mC (5methylcytosine) to 5hmC (5hydroxymethylcytosine) driven by Tet1 enzyme?



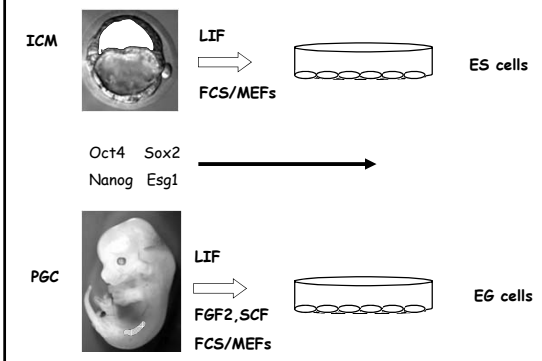
Germ line and pluripotency

Germ cells and pluripotency

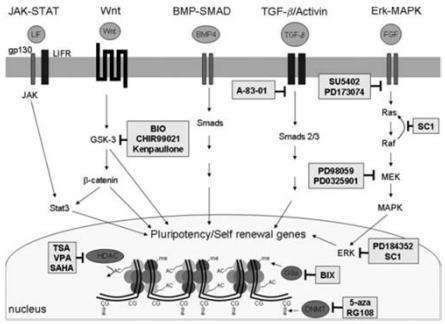
- **Primordial germ cells (PGCs) have the capacity to re-generate totipotency**
- **PGCs are not pluripotent** (ie do not contribute to chimaeras when injected into blastocyst (summarised in Leitch et al, 2013)
- PGCs express transcriptional network related to pluripotency (similar to mESCs)
- PGCs can give rise to pluripotent embryonic germ cells (EG cells) *in vitro*



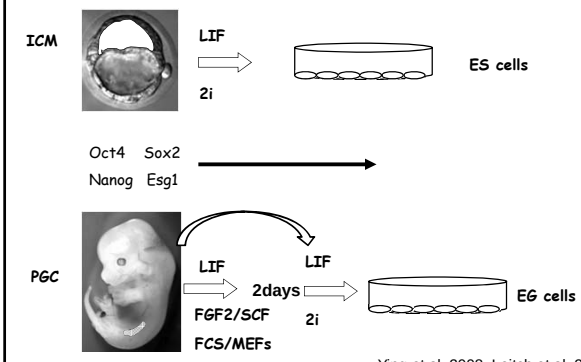
Stem cell derivation – „classic" protocol



Key signalling pathways regulating pluripotency (mESCs)



Stem cell derivation – „2i" protocol
(reaching the pluripotent ground state)

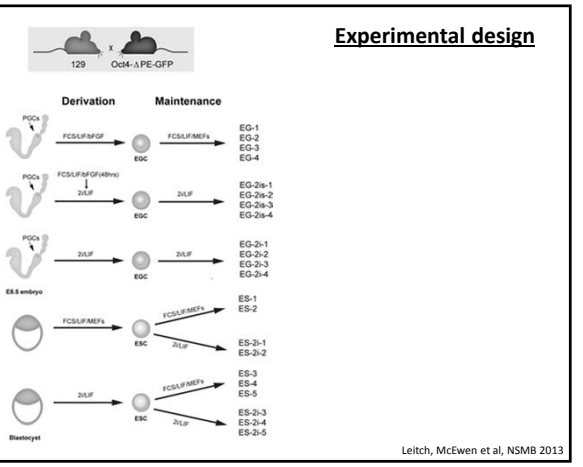


Ying et al, 2008, Leitch et al, 2010

In vitro models

- **EG vs ES comparison**
  - ES derived from ICM, EG derived from PGCs
  - lack of imprints in EG cells (x ES cells)
  - EG cells can erase imprints upon fusion with a somatic cell
  - due to their germline origin EG cells are believed to be globally (DNA) hypomethylated

Experimental design

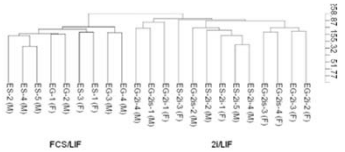


Leitch, McEwen et al, NSMB 2013

Gene expression analysis

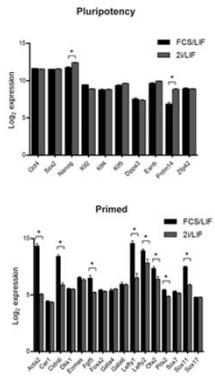
(Affymetrix gene array)

Unsupervised hierarchical clustering



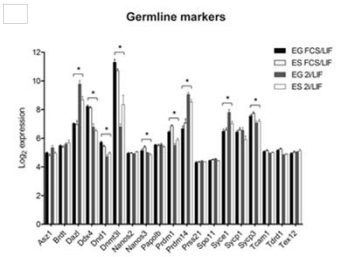
- The first principal component (i.e. the major difference in the sample set) separates cell lines maintained in 2i/Lif and FCS/LIF/MEFs (FLM) (>**2000 genes**, FDR<0.05, FC>1.5)
- The second and third principal components separate gender
- The third principal component also separates cell type (EG vs ES -) (**83 genes**)

2i vs "classic" culture conditions



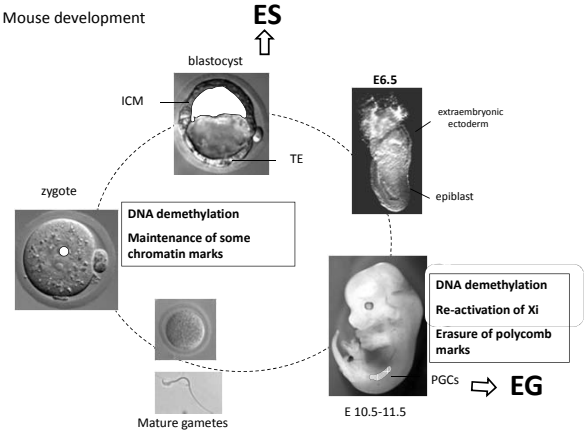
See also Marks et al, 2012

2i vs "classic" culture conditions

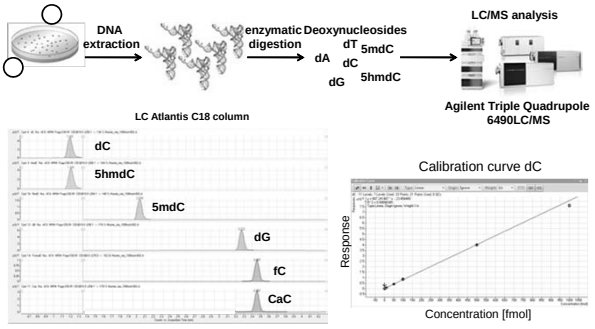


Leitch, McEwen et al, NSMB 2013

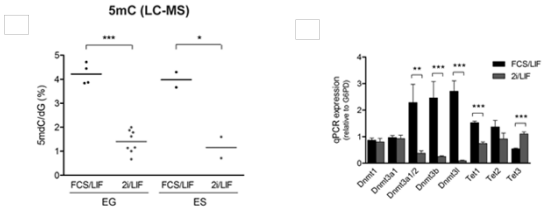
Mouse development



Measuring DNA methylation by LC-MS
- method -



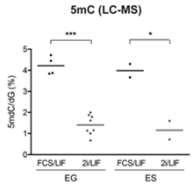
Global DNA hypomethylation in 2i



male

Leitch, McEwen et al, NSMB 2013

2i vs "classic" culture conditions
DNA methylation levels are reversible

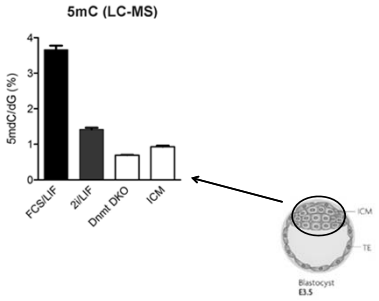


Leitch, McEwen et al, NSMB 2013

2i vs "classic" culture conditions
Regulation of DNA methylation - differentiation

Dnmt TKO ES cells die upon exit from pluripotency
Dnmt3a/3b KO ES cells fail to differentiate

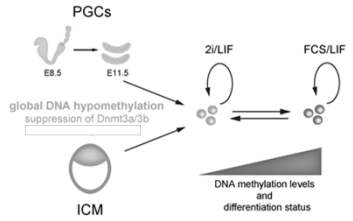
Leitch, McEwen et al, NSMB 2013



Leitch, McEwen et al, NSMB 2013

Summary I

- ES and EG cells are very similar at the transcriptional level , HOWEVER Major transcriptional differences found between pluripotent cells grown n FCS/LIF and in 2i conditions
- No difference in global levels of 5mC between ES and EG cells , but 2i induces genome wide DNA hypomethylation (downregulation of Dnmt3a, Dnmt3b and Dnmt3l, no change in Dnmt1 !)
- Global DNA methylation level is similar between mouse ICM and mouse pluripotent ESCs and EGCs grown in 2i medium

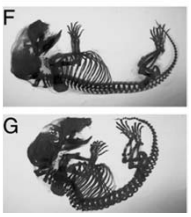


Leitch, McEwen et al, NSMB 2013

EGs , genomic imprints & chimaera formation

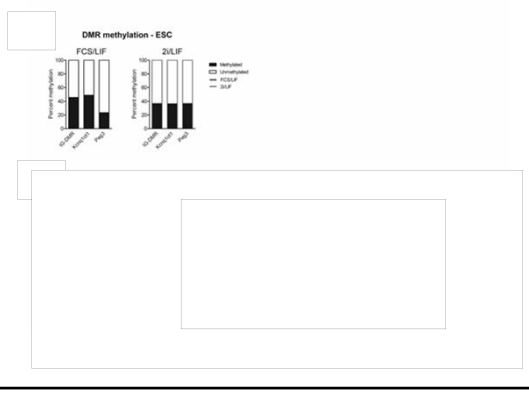
EGs , genomic imprints & chimaera formation

- EG cells lack genomic imprinting (x ES cells)
- Pluripotent, contribute to all germ layers in chimaeras
- Classic derivation protocol -> high contribution chimaeras lethal (lack of genomic imprinting)

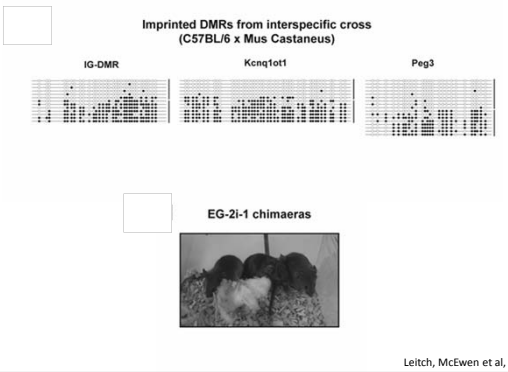


(Tada et al, 1998)

Maintenance of genomic imprints in 2i



Maintenance of genomic imprints in 2i and chimaera contribution



Leitch, McEwen et al, NSMB 2013

EGs , genomic imprints & chimaera formation

		DMR methylation status					
Cell line	Cell type	Kcnq1ot1	Peg3	IG-DMR	Blastocysts	Pups born	Chimaeras
ES-2i-4	ESC	✓	✓	✓	43	9	8
EG-1	EGC	x	x	✓	39	0	0
EG-4	EGC	nd	nd	nd	40	3	0
EG-2i-1	EGC	✓	✓	✓	39	15	14
EG-2i-4	EGC	x	x	x	27	2	?*

Leitch, McEwen et al, NSMB 2013

Summary II

- 2i allows for derivation of EG lines with intact imprints (human EG lines?)
- 2i derived EG lines can give rise to healthy high contribution chimaeras

Neither global hypomethylation nor lack of imprints are distinguishing features of EG cells

Acknowledgment

lab members

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Gopu Dharmalingam

CSC Mass spec facility

Vesela Encheva

CSC genomics laboratory

Laurence Game

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Harry Leitch
Billy Mansfield

Anne Ferguson-Smith (University of Cambridge)

Agilent Technologies
Epigenesys
MRC Medical Research Council
MARIE CURIE ACTIONS
PEOPLE
EMBO Young Investigator

Biochemical analysis of early Mammalian germ cell development

Niels Geijsen
Hubrecht Institute

No conflict of interest to report

Learning aims:

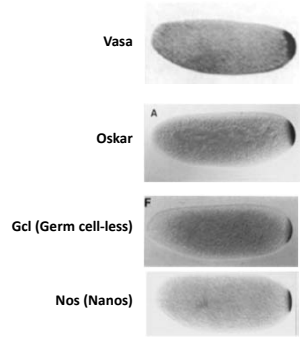
Differences between germ cell specification in lower animals and mammals

A method for the in vitro generation of primordial germ cells from pluripotent stem cells

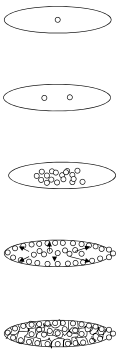
Dazl, a essential factor for germ cell specification, suppresses mRNA translation

Dazl targets mRNAs of pluripotency factors, differentiation mediators and pro-apoptotic factors.

Cell fate determination though germ-plasm localization



Early Drosophila embryogenesis



Egg

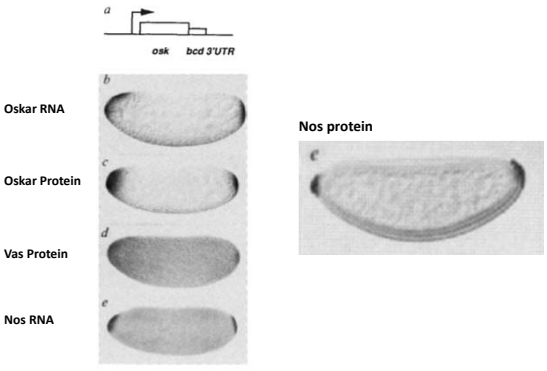
Nuclear division without cell cleavage

Syncytial Blastoderm (9 nuclear divisions)

Nuclear migration (4 nuclear divisions)

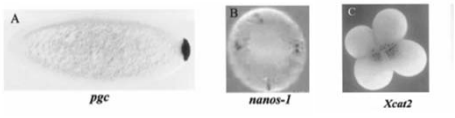
Cellular blastoderm (±6000 cells)

Mislocalization of germ plasm results in ectopic germ cell formation



Germ plasm is conserved across many species

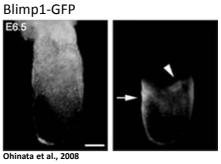
Drosophila Zebrafish Xenopus



Zhou and King 2004

Mammalian germ cell development

Blimp1-GFP

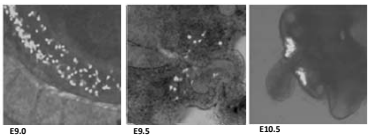


Ohinata et al., 2008

Stella/ Dppa3

MacLaren, 2003

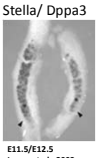
Oct4-GFP



E9.0 E9.5 E10.5

Molyneaux et al., 2001

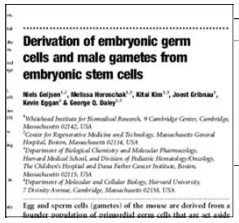
Stella/ Dppa3



E11.5/E12.5

Lange et al., 2003

In vitro generation of germ cells from embryonic stem cells



Derivation of embryonic germ cells and male gametes from embryonic stem cells

Niels Geijsen[1,2], Melissa Horoschak[1,3], Kitai Kim[1,4], Joost Gribnau[1], Kevin Eggan[1] & George Q. Daley[1,2]

Nature (2004) 427:148

RESEARCH ARTICLES

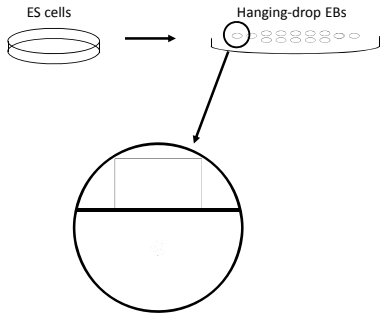
Derivation of Oocytes from Mouse Embryonic Stem Cells

Science (2003) 300:1251

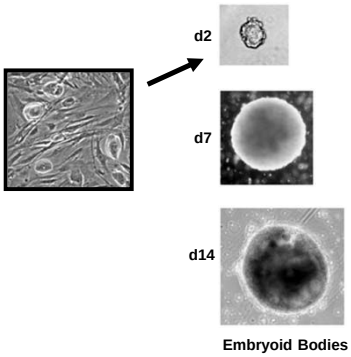
Embryonic stem cells can form germ cells *in vitro*

PNAS (2003) 100:11457

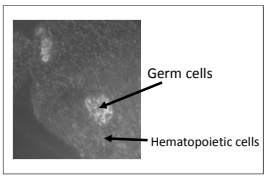
Modeling early embryonic development: Embryoid bodies

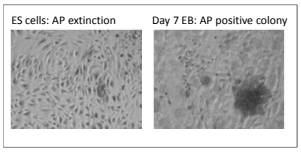


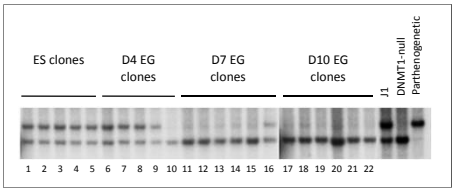
Modeling early embryonic development: Embryoid bodies



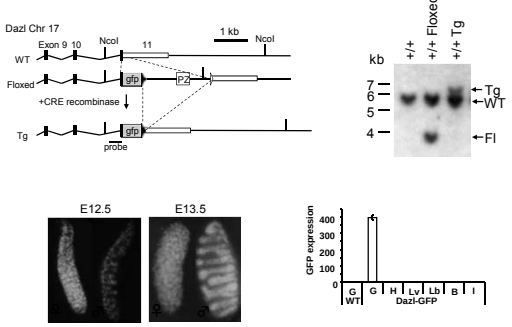
In vitro germ development of primordial germ cells



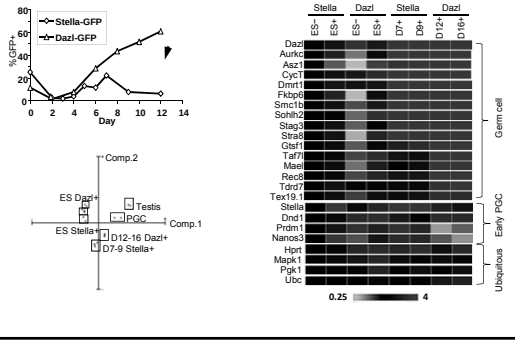




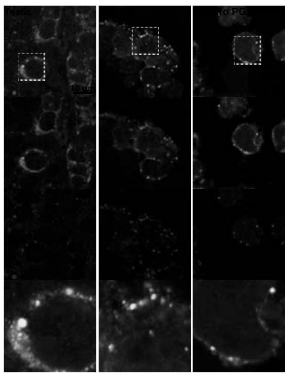
A primordial germ cell specific Dazl-GFP reporter



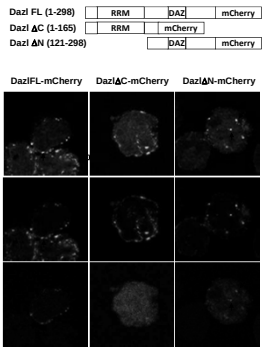
In vitro generation of Dazl-GFP germ cells



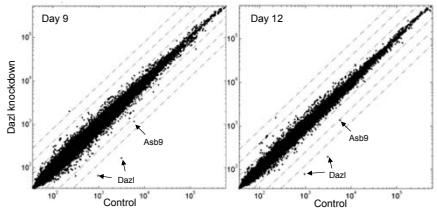
Dazl localizes in cytoplasmic granules (P-bodies)



The Daz-domain is essential for P-body localization



Loss of Dazl has minimal effect on PGG gene expression



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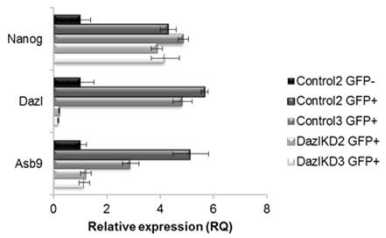
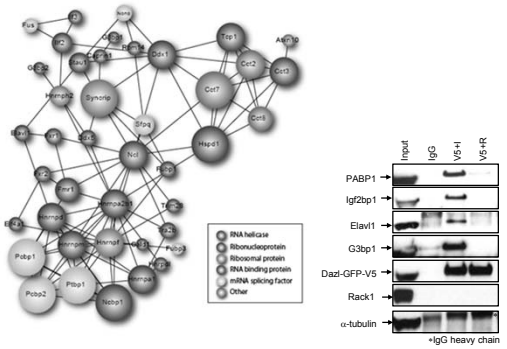
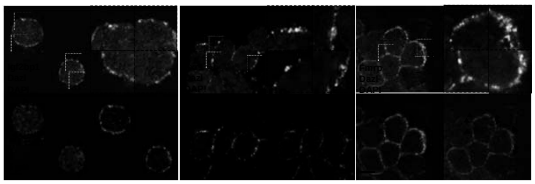


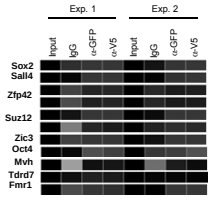
Dazl associates with a network of ubiquitously expressed RNA interacting proteins



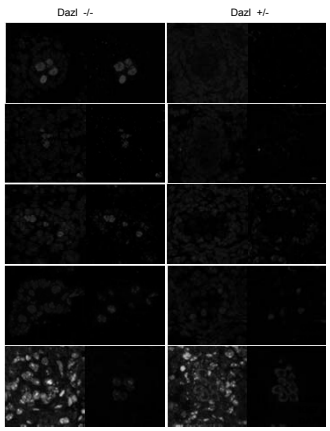
Dazl colocalization with Igfbp1 and Fragile-X proteins



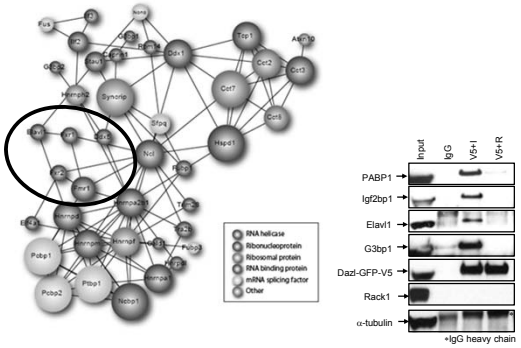
Dazl binds mRNA transcripts of key pluripotency genes



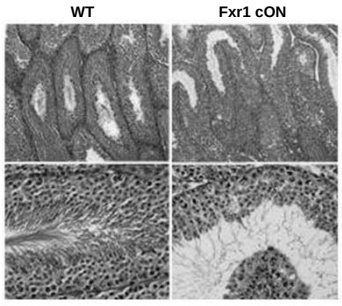
Loss of Dazl leads to aberrant expression of pluripotency genes



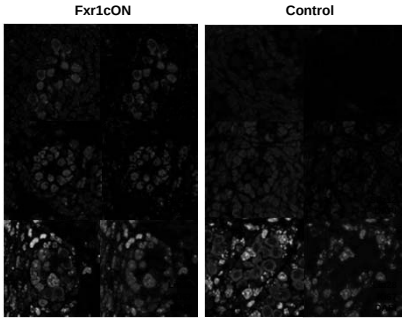
Dazl associates with a network of ubiquitously expressed RNA interacting proteins



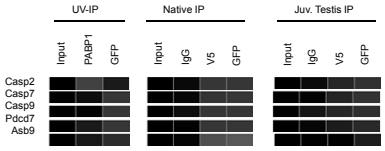
Fertility phenotype in Fragile-X mutant mice



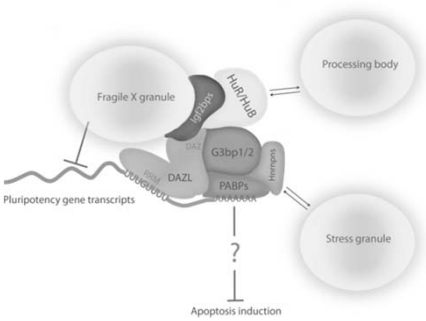
Fxr1 hypomorphic mice phenocopy Dazl-/- mutation



Dazl associates with transcripts of pro-apoptotic genes



Dazl recruitment of translational regulators to specific mRNA transcripts



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Pieter de Jong
Christine Jung

NIRM
NIH
NWO

Pre-congress course
PCC13: Of stem cells and gametes: more similarities than differences?

Current status of in vitro differentiation of HPSC into female gametes

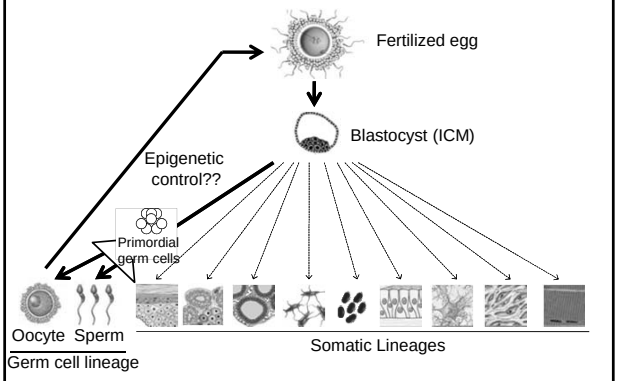
Susana M. Chuva de Sousa Lopes, PhD
Associate Professor
Dept. Anatomy and Embryology
Leiden University Medical Center
Leiden, The Netherlands

I have nothing to disclose

Learning objectives:

- What are HPSC (human pluripotent stem cells)?
- What are female gametes and why are they special?
- Why is there interest in generating female gametes from HPSC?
- What is the current state of in vitro differentiation to female gametes?
- What can you say in the clinic if you are asked about this topic?

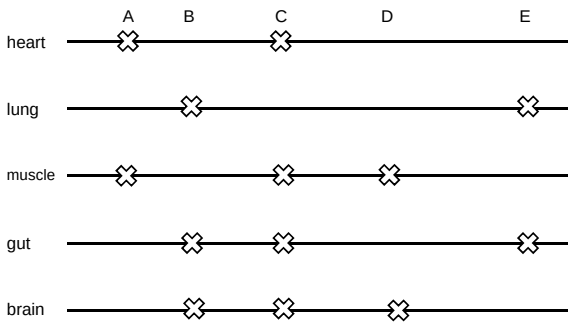
What are HPSC (human pluripotent stem cells)?



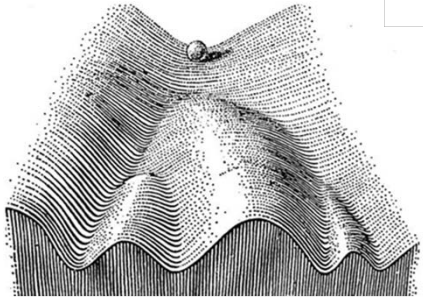
Courtesy of Dr. K. Hayashi

How?

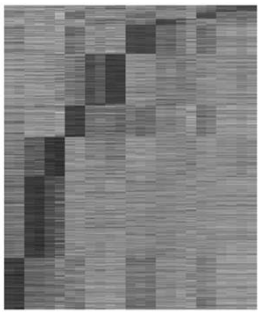
Epigenetic (re)programming as the possibility to be transcribed



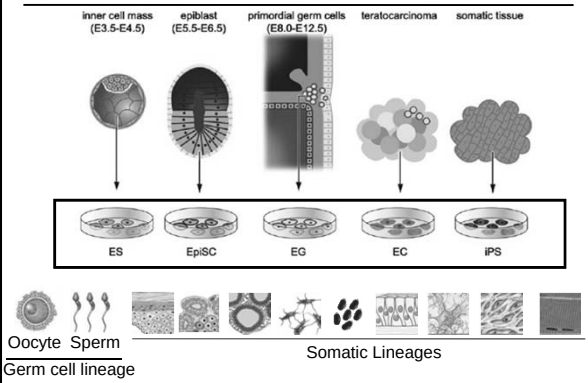
Waddington's epigenetic landscape



Tissue uniquely DNA hypomethylated regions: tissue barcode



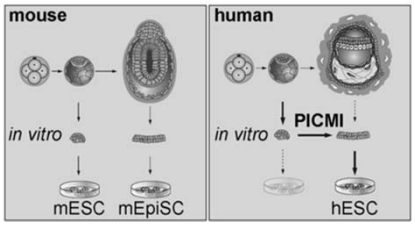
What are MPSC (mouse pluripotent stem cells)?



Chuva de Sousa Lopes and Roelen, 2010

What are HPSC (human pluripotent stem cells)?

hESCs:

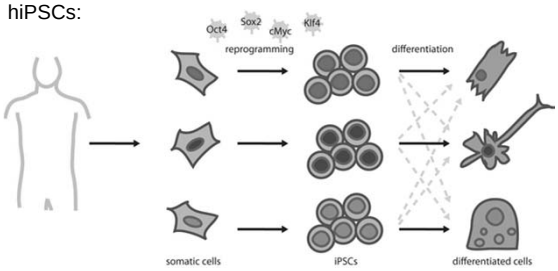


- EpiSC resemble human ESC
- Naïve, ground, primed,…..

O'Leary et al, 2012

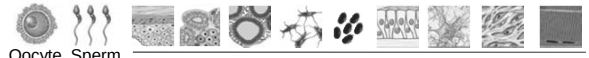
What are HPSC (human pluripotent stem cells)?

hiPSCs:



What are HPSC (human pluripotent stem cells)?

- In vitro differentiation (embryoid bodies, directed differentiation)
- Teratoma formation (tumor induction in mice)
- Chimera formation (integration of cells in embryo)
- Tetraploid complementation (generation of a complete embryo proper)

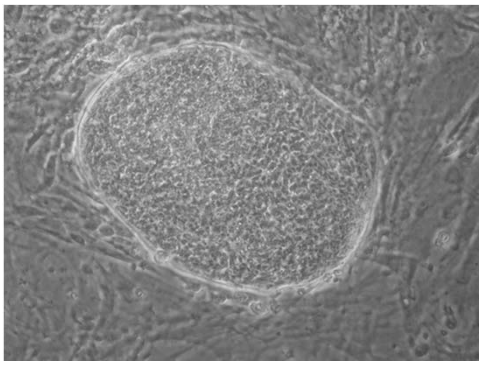


Oocyte Sperm

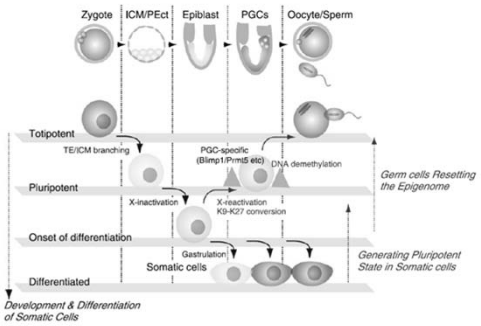
Germ cell lineage

Somatic Lineages

What are HPSC (human pluripotent stem cells)?

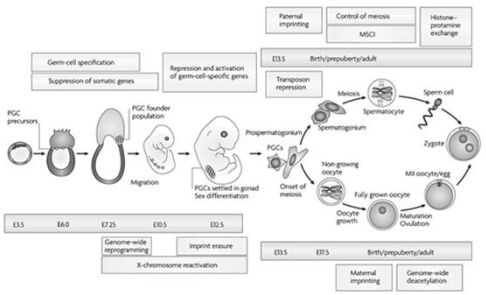


What are female gametes and why are they special?



Hayashi and Surani, 2009

What are female gametes and why are they special?



Sasaki and Matsui, 2008

What are female gametes and why are they special?

- Mature oocyte is totipotent (zygote, blastomeres)
- Very scarce….a few mature each time

- Know how to reprogram:
  - Spatial information to form an embryo
  - Formation of extraembryonic tissues (placenta, amnion)

- Can be used to make patient-specific embryonic stem cell lines:
  - Alternative to hiPSCs
  - Regenerative medicine
  - Drug testing (stem cells as the new patient)

- Used in assisted reproduction

Why is there interest in generating female gametes from HPSC?

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- Know how to reprogram:
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- Can be used to make patient-specific embryonic stem cell lines:
  - Alternative to hiPSCs
  - Regenerative medicine
  - Drug testing (stem cells as the new patient)

- Used in assisted reproduction:
  - Infertility can result in serious psychological problems
  - Unable to retrieve mature oocytes or gender issues
  - Problems associated with gamete donation (legislation)
  - Importance of the genetic link with the child

Current state of in vitro differentiation to female gametes?

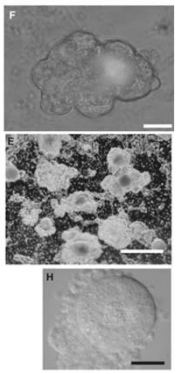
Mouse:

- **Hubner et al., Science, 2003, 300:1251**

Oct4GFP mESCs line
Differentiation in a monolayer
Expression of VASA after 12 days
Aggregates detached from colonies
Replated aggregates form follicle-like structures
Spontaneous activation of oocyte-like cells

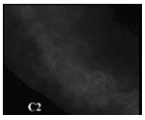
- **Novak et al., StemCells, 2007, 24:1931**

No evidence for meiosis !



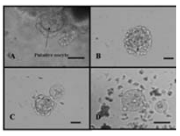
Current state of in vitro differentiation to female gametes?

Mouse:



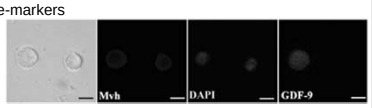
- **Lacham-Kaplan et al., StemCells, 2006, 24:266**

EBs cultured in mouse testis conditioned medium
After 6-7 days follicle-like structures
Oocyte-like cells 15-35µm
No zona pellucida
Expression of several oocyte-markers
No evidence for meiosis!



- **Qing et al., Differentiation, 2007, 75:902**

EBs cultured in DMEM+FCS
Germ cells observed in EBs after 4 days
Co-culture with granulosa cells from newborn mice
Expression of several oocyte-markers
No evidence for meiosis!



Current state of in vitro differentiation to female gametes?

Mouse:

Human Molecular Genetics, 2009, Vol. 18, No. 22 4376–4389
doi:10.1093/hmg/ddp393
Advance Access published on August 20, 2009

Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility

Cory R. Nicholas[1], Kelly M. Haston[1], Amarjeet K. Grewall[2], Teri A. Longacre[2] and Renee A. Reijo Pera[1,*]

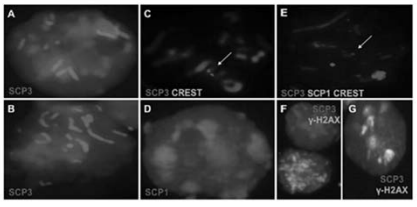
[1]Department of Obstetrics and Gynecology, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, CA 94304, USA and [2]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA

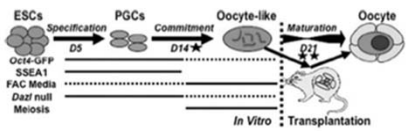
Received July 11, 2009; Revised and Accepted August 13, 2009

ous oocyte development *in vivo* by single-cell expression profiling and analysis of functional milestones including responsiveness to defined maturation media, shared genetic requirement of *Dazl*, and entry into meiosis. However, ESC-derived oocyte maturation ultimately fails *in vitro*; To overcome this obstacle, we transplant ESC-derived oocytes into an ovarian niche to direct their functional maturation and, thereby, present rigorous evidence of oocyte physiologic relevance and a potential therapeutic strategy for infertility.

Current state of in vitro differentiation to female gametes?

Mouse:

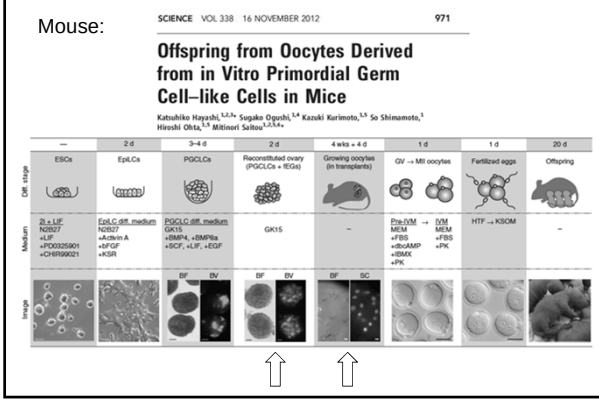




Nicholas et al., 2008

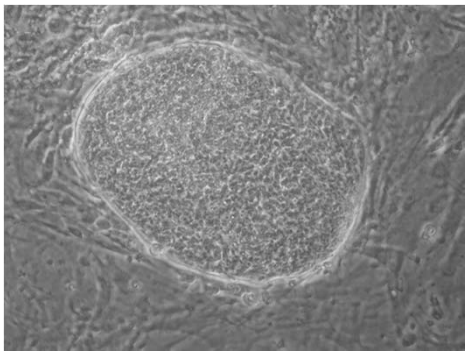
Current state of in vitro differentiation to female gametes?

Mouse:



Current state of in vitro differentiation to female gametes?

Human:



Current state of in vitro differentiation to female gametes?

Human:

- **Clark et al., HumMolGen, 2004, 13:727**

EBs cultured in DMEM+FCS
Germ cells observed in EBs after 7-14 days
Expression of several oocyte-markers
No evidence for meiosis!

- **Chen et al., Hum Rep , 2006, 22:567**

EBs cultured in DMEM+FCS
Germ cells observed in EBs after 7-14 days
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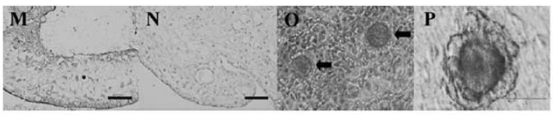
No evidence for meiosis!



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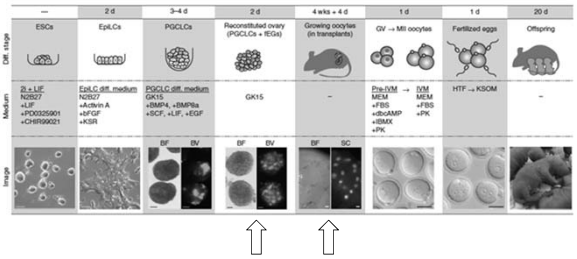
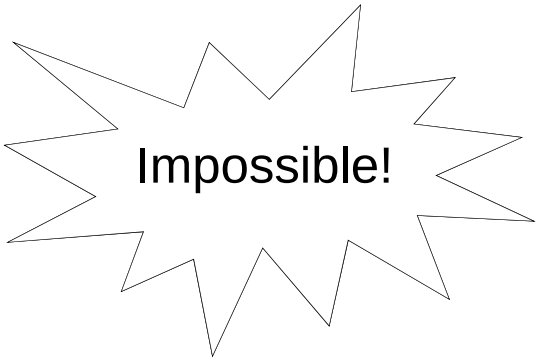


Duggal et al., 2014
Sun et al., 2014

What can you say in the clinic if you are asked about this topic?



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- Hayashi and Surani, 2009, Cell Stem Cell 4:493
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- Lacham-Kaplan et al., StemCells, 2006, 24:266
- Qing et al., Differentiation, 2007, 75:902
- Nicholas et al., 2008, Human Molecular Genetics 18:4376
- Hayashi et al., 2012, Science 338:971
- Clark et al., 2004, Human Mololecular Genetics 13:727
- Chen et al., 2006, Human Reproduction 22:567
- Duggal et al., 2014, Veterinary Quaterly PMID 24593843
- Sun et al., 2014, Journal Genetics Genomics, 41:87

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Jasin Taelman
Sharat Warrier

IAP-Phase VII

ESHRE Annual Meeting 2014

Pre-Congress Course 13

Munich, Germany

In vitro differentiation of PSC into male gametes: current status and the road ahead

Cristina Eguizabal, PhD

Stem Cell Therapy Unit

Basque Center for Blood Transfusion and Human Tissues

Disclosure

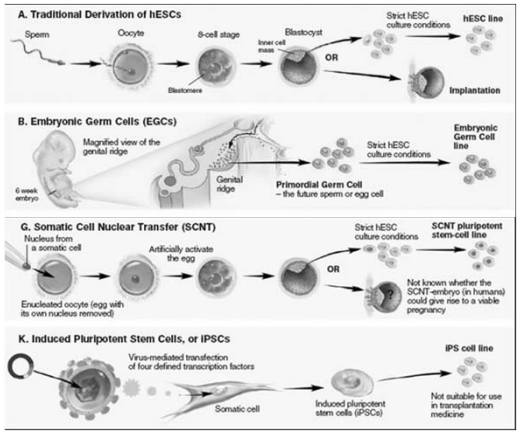
- Nothing to disclose
- I have no commercial or financial relationships with manufacturers of pharmaceuticals, laboratory supplies or medical devices

Learning objectives

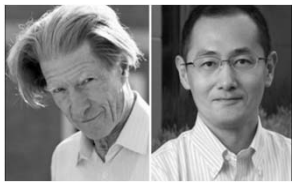
At the conclusion of this presentation, participants should be able to:

- Discuss pluripotent stem cells (PSC)
- Explain generation of pluripotent stem cells
- Why generation of gametes from PSC?
- Explain generation of male germ cells from mouse pluripotent stem cells
- Explain generation of male germ cells from human pluripotent stem cells
- Conclusions and Future remarks

Pluripotent Stem Cells **(PSC)** can be obtained from cells located in the inner cell mass of blastocysts **(ESC),** from primordial germ cells (EGC) and from nuclear reprogramming (SCNT and **iPS)**

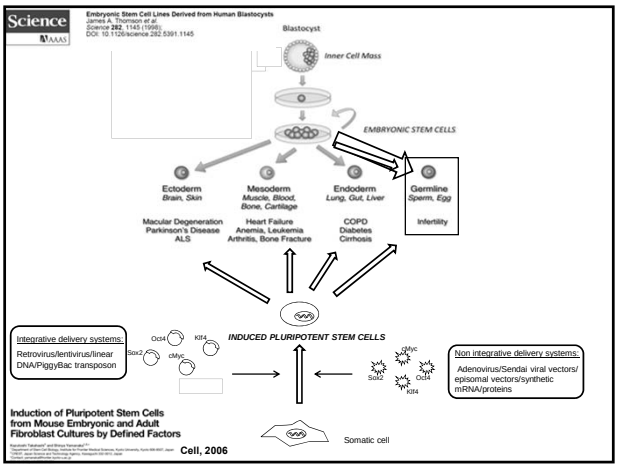


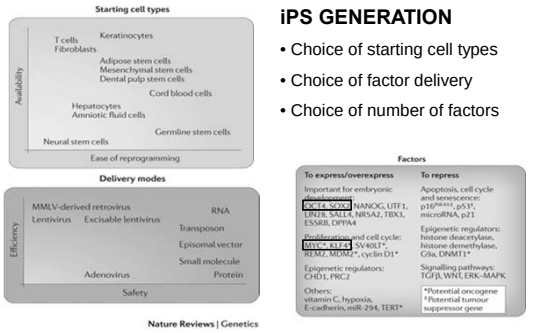
The Nobel Prize in Physiology or Medicine 2012 was awarded jointly to Sir John B. Gurdon and Shinya Yamanaka *"for the discovery that mature cells can be reprogrammed to become pluripotent"*



Sir John B. Gurdon → SCNT

Shinya Yamanaka → iPS





iPS GENERATION

- Choice of starting cell types
- Choice of factor delivery
- Choice of number of factors

González F. *et al.*, 2011

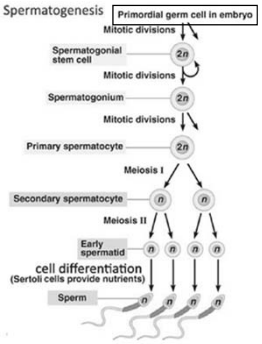
- Lentivirus/retrovirus mediated reprogramming methods are still major approaches for iPS generation

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

- To study gametogenesis *in vitro* for a better understanding of this process
- To study meiosis *in vitro*
- To check the capability of PSC (ES/iPS) to form germ cells *in vitro*
- PSC may constitute a future source of artificial gametes for clinical studies and potential therapeutic applications
- This system may provide a useful model for molecular genetic studies of human germline formation.

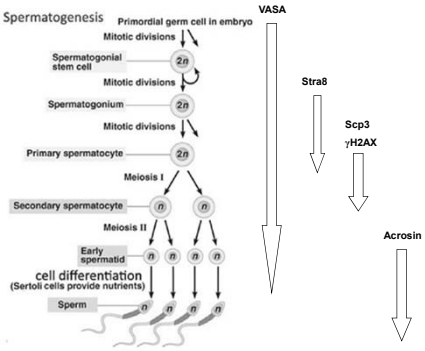
IMPORTANT POINTS FOR HAVING MALE FUNCTIONAL GAMETES DURING GERMLINE DIFFERENTIATION FROM PLURIPOTENT STEM CELLS:

The sequence of *in vivo* events: Spermatogenesis



IMPORTANT POINTS FOR HAVING MALE FUNCTIONAL GAMETES DURING GERMLINE DIFFERENTIATION FROM PLURIPOTENT STEM CELLS:

The sequence of *in vivo* events: Spermatogenesis



IMPORTANT POINTS FOR HAVING MALE FUNCTIONAL GAMETES DURING GERMLINE DIFFERENTIATION FROM PLURIPOTENT STEM CELLS:

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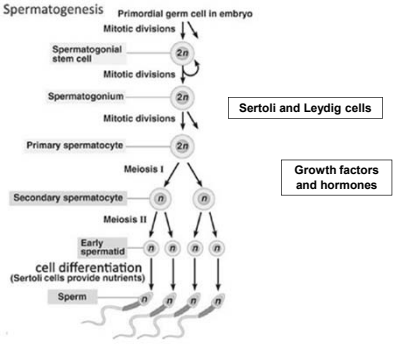
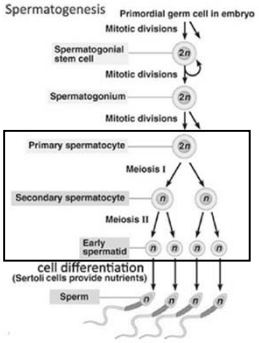


IMPORTANT POINTS FOR HAVING MALE FUNCTIONAL GAMETES DURING GERMLINE DIFFERENTIATION FROM PLURIPOTENT STEM CELLS:

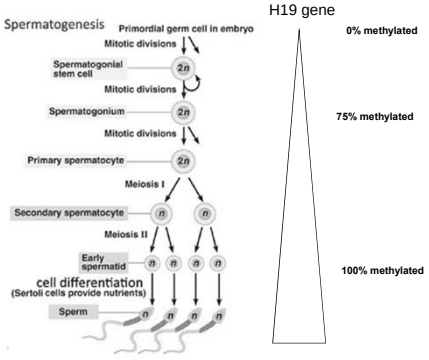
The sequence of *in vivo* events: Spermatogenesis



MEIOSIS

IMPORTANT POINTS FOR HAVING MALE FUNCTIONAL GAMETES DURING GERMLINE DIFFERENTIATION FROM PLURIPOTENT STEM CELLS:

The sequence of *in vivo* events: Spermatogenesis



MOUSE

- Toyooka *et al*. PNAS 2003
- Giejsen *et al*. Nature 2004
- Nayernia *et al.* Developmental Cell 2006
- Eguizabal *et al*. Differentiation 2009
- Hayashi *et al.* Cell 2011
- Peng *et al*. Biomed Res. Intl. 2013
- Cai *et al*, BBRC 2013
- Nakaki *et al*,. Nature 2013

Generation of male germ cells *in vitro* from **mouse** pluripotent stem cells

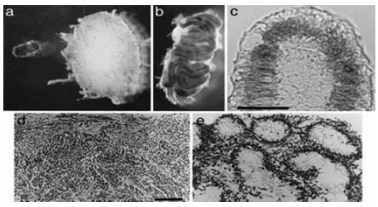
- **Pluripotent stem cells used**: ES, EG and iPS cells.
- **Sex of cell lines**: XY and XX.
- **Transgenic reporters / overexpression genes used**: MVH, Stra8, Prm1, Stella, Blimp1, Prmd14 and Tfap2c.
- **Differentiation method used**: EB formation and monolayer differentiation.
- **Culture conditions**: FBS, BMP4, N2B27, Activin A, bFGF, SCF, Retinoic Acid, Transferrin, Monothyoglicerol , Ascorbic Acid and Testosterone.
- **In vitro cells obtained**: Epiblast, PGCs, SSCs and male haploid-like cells.
- **Epigenetic status of imprinted genes**: correct
- **Functional assays**: Transplantation of SSCs into sterile testis or ICSI.

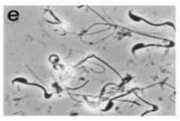
Embryonic stem cells can form germ cells *in vitro*

Yayoi Toyooka, Naoki Tsunekawa, Ryuko Akasu, and Toshiaki Noce*

Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya Machida-shi, Tokyo 194-8511, Japan

PNAS, 2003



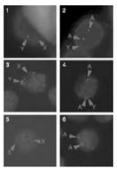


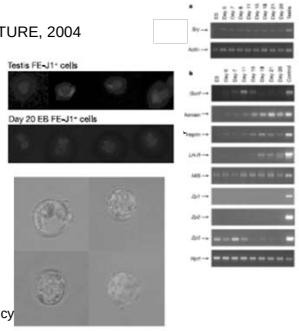
- EB formation and co-culture system in presence of BMP4.
- Transplantation of ESC aggregates to testicular capsule.
- ESC derived cells can participate in spermatogenesis and produce male germ cells *in vitro.*

Derivation of embryonic germ cells and male gametes from embryonic stem cells

Niels Geijsen[1,2], Melissa Horoschak[1,3], Kitai Kim[1,3], Joost Gribnau[1], Kevin Eggan[1] & George Q. Daley[1,3]

NATURE, 2004





- During embryoid body formation pluripotency markers expression decreased with some expression of male germ cell specific genes.
- EB microenvironment is permissive for male germ cell development and meiotic maturation, even though highly inefficient
- FISH: normal for sex chromosomes

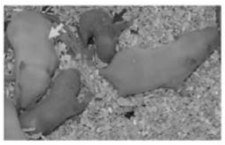
- ICSI with haploid cells into recipient oocytes
- 50% of the embryos cleaved to 2 cell stage
- 20% reach the blastocyst stage.

Developmental Cell 11, 125–132, July, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2006.05.010

In Vitro-Differentiated Embryonic Stem Cells Give Rise to Male Gametes that Can Generate Offspring Mice

Karim Nayernia,[1,7,*] Jessica Nolte,[1] Hans W. Michelmann,[2] Jae Ho Lee,[1] Kristina Rathsack,[1] Nadja Drusenheimer,[1] Arvind Dev,[3] Gerald Wulf,[3] Ingrid E. Ehrmann,[4] David J. Elliott,[4] Vera Okpanyi,[5] Ulrich Zechner,[5] Thomas Haaf,[5] Andreas Meinhardt,[6] and Wolfgang Engel[1]

Dev Cell, 2006



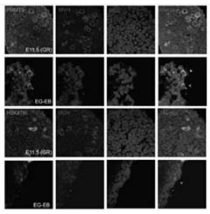
- Establishment of two cell lines (Spermatogonial stem cells- SSCs) with patterns of differentiation towards male germ cells .
- Derived from mESC (directed gene expression for premeiotic and haploid male germ cells)
- Formation of sperm with tail-like structures

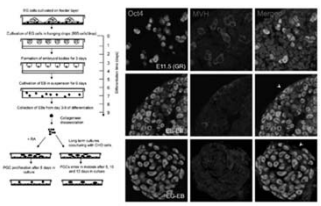
- ICSI of in vitro-generated cells (haploid cells into oocytes of wildtype females.
- Transfer of 65 embryos into the oviducts of pseudopregnant females
- Premature death (5 days to 5 months after birth).
- Abnormal methylation patterns and phenotypic abnormalities

Contents lists available at ScienceDirect
Differentiation 2009
journal homepage: www.elsevier.com/locate/diff

Generation of primordial germ cells from pluripotent stem cells

Cristina Eguizabal [*], Tanya C. Shovlin [1], Gabriela Durcova-Hills [2], Azim Surani, Anne McLaren [3]





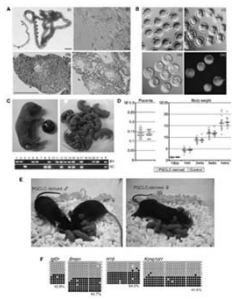
- Mouse EG and ES cells can form *in vitro* PGCs via embryoid body formation by adding RA
- The expression pattern of histone modification markers of late PGCs (Prmt5 and H3K27me3) is similar to *in vivo* embryonic day E 11.5
- When co-cultured with Chinese hamster ovary (CHO) cells , some of the PGC enter meiosis (SCP3 expression)

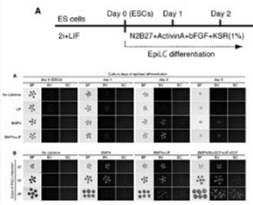
Cell

2011

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

Katsuhiko Hayashi,[1,2] Hiroshi Ohta,[1,2] Kazuki Kurimoto,[1,2] Shinya Aramaki,[1] and Mitinori Saitou[1,2,3,*]





- Generation of in vitro primordial germ cell-like cells from mES and miPS through epiblast-like cells having normal global transcription profiles, epigenetic reprogramming,and cellular dynamics.
- These PGCLCs express Integrin-b3 and SSEA1 markers of spermatogonias.
- Healthy offspring with normal methylation patterns of imprinted genes.

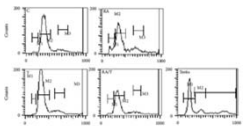
2013

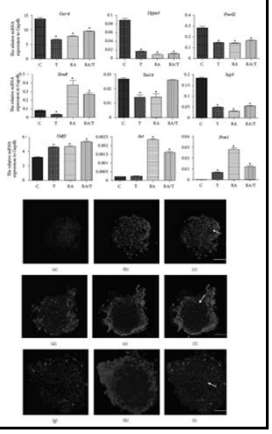
Research Article

Differentiation of Induced Pluripotent Stem Cells into Male Germ Cells *In Vitro* through Embryoid Body Formation and Retinoic Acid or Testosterone Induction

Peng Li,[1] Hongliang Hu,[1] Shi Yang,[1] Ruhui Tian,[1] Zhenzhen Zhang,[1] Wei Zhang,[1] Meng Ma,[1] Yong Zhu,[1] Xizhi Guo,[1] Yiran Huang,[1] Zuping He,[2,3,4] and Zheng Li[1]

- Generation of male *in vitro* germ cells by EB formation in presence of RA and Testosterone.
- iPSC derived cells can participate in spermatogenesis and produce male haploid-like cells *in vitro*.





2013

Contents lists available at SciVerse ScienceDirect

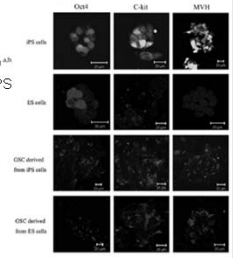
Biochemical and Biophysical Research Communications

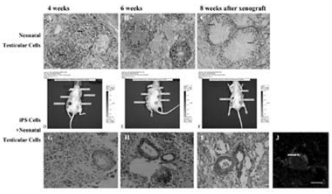
journal homepage: www.elsevier.com/locate/ybbrc

***In vitro* and *in vivo* differentiation of induced pluripotent stem cells into male germ cells**

Heng Cai [a,b,1], Xiaoyu Xia [a,b,1], Li Wang [a,b], Yue Liu [a,b], Zuping He [c], Qiangsu Guo [a,b], Chen Xu [a,b]

- Generation of *in vitro* primordial germ cell-like cells from miPS by EB formation in present of RA, FGF2 and SCF.
- These PGCLCs express Integrin-b3 and SSEA1 markers of spermatogonias.
- Generation of *in vivo* ectopic reconstitution of seminiferous tubules by transplantation of miPSCs with neonatal testicular cells.

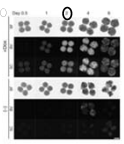




Induction of mouse germ-cell fate by transcription factors *in vitro*

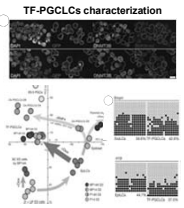
- Direct EpiLCs and mESC conversion into primordial germ cells –like cells (PGCLCs) by over-expressing 3 TFs : **BLIMP1 (B), PRDM14 (P14) and TFAP2C (A)**

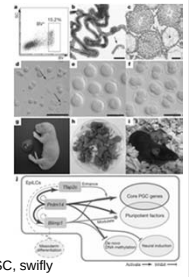
Transfection of BVSCR26rtTA ES cells with *piggyBac* transposon expressing the TFs



More than 80% of the cells expressed BV (Blimp1-mVenus) and 30% of the cells expressed SC (stella-eGFP).

TF-PGCLCs characterization





- Simultaneous overexpression of 3 TFs directs EpiLCs, but not ESC, swiftly and efficiently into a PGC state
- TFs showed faster kinetics of Blimp1 and Stella induction than the cytokines
- TFs directly activate a PGC program
- Normal offspring were obtained from TF-PGCLCs

Nakaki F. *et al.* Nature (2013)

HUMAN

- Bucay *et al*. Stem Cells 2008
- Tilgner *et al*. Stem Cells 2008; 2009
- Park *et al.* Stem Cells 2009
- Kee *et al*. Nature 2010
- Panula *et al.* HMG 2011
- Eguizabal *et al*. Stem Cells 2011
- Medrano *et al.* Stem Cells 2011
- Schatten *et al*. Cell Reports 2012
- Ramathal *et al*. Cell Reports 2014

PGCs

SSCs/haploid-like cells

Generation of germ cells *in vitro* from **human** pluripotent stem cells

• **Pluripotent stem cells used**: ES and iPS cells.

• **Sex of cell lines**: XY and XX.

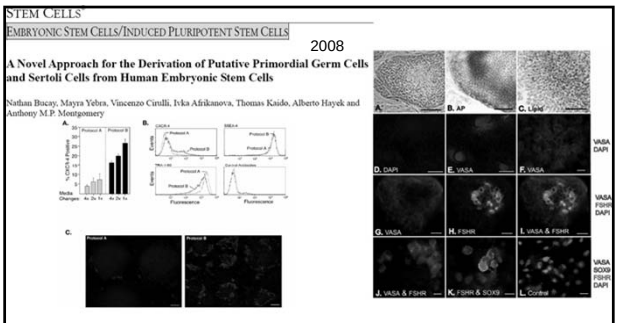
• **Transgenic reporters / overexpression genes used**: DAZ, DAZL, BOULE and VASA.

• **Differentiation method used**: EB formation and monolayer differentiation.

• **Culture conditions**: FBS, BMP4, -7, -8b, Activin A, bFGF, hLIF, Retinoic Acid, R115866, Nicotinamide, Transferrin, Insulin, Selenium, Monothyoglicerol and Ascorbic Acid.

• **In vitro cells obtained**: PGCs, Putative Sertoli, SSCs and male haploid-like cells

• **Epigenetic status of imprinted genes**: correct



• Generation a novel protocol based on the expression of CXCR4 receptor during the *in vitro* differentiation of PGCs from hESC.

• This differentiation process is accompanied by the development of putative Sertoli-like cells.

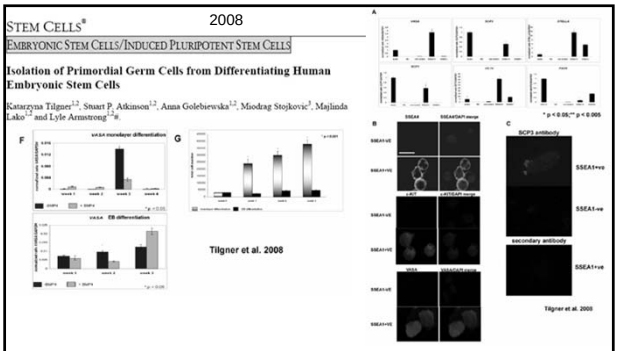
STEM CELLS®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

2008

Isolation of Primordial Germ Cells from Differentiating Human Embryonic Stem Cells

Katarzyna Tilgner[1,2], Stuart P. Atkinson[1,2], Anna Golebiewska[1,2], Miodrag Stojkovic[3], Majlinda Lako[1,2] and Lyle Armstrong[1,2]#.



• Generation a novel protocol (EBs + BMP4) for differentiating *in vitro* PGCs from hESC.

• These PGCs expressed specific germ cell markers and showed a removal of the parental imprints and chromating modification changes.

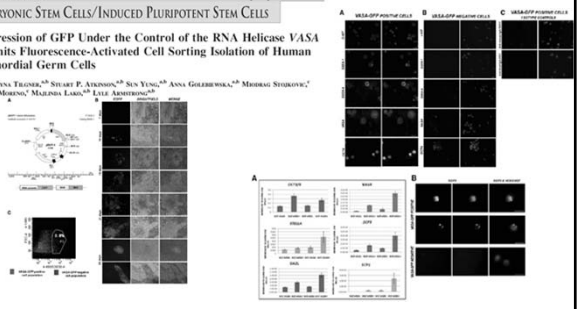
STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

2010

Expression of GFP Under the Control of the RNA Helicase *VASA* Permits Fluorescence-Activated Cell Sorting Isolation of Human Primordial Germ Cells

Katarzyna Tilgner,[a,b] Stuart P. Atkinson,[a,b] Sun Yung,[a,b] Anna Golebiewska,[a,b] Miodrag Stojkovic,[c] Ruben Moreno,[a] Majlinda Lako,[a,b] Lyle Armstrong[a,b]



• The generation and characterization of human embryonic stem cell lines stably carrying a VASA-pEGFP-1 reporter construct that expresses GFP in a population of differentiating human embryonic stem cells that show expression of characteristic markers of primordial germ cells.

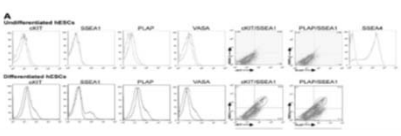
STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

2009

Derivation of Primordial Germ Cells from Human Embryonic and Induced Pluripotent Stem Cells Is Significantly Improved by Coculture with Human Fetal Gonadal Cells

Tae Sub Park,[a,b] Zoran Galic,[c] Anne E. Conway,[a,b] Anne Lindgren,[a,b] Benjamin J. van Handel,[a,b] Matteo Magnusson,[a,b] Laura Richter,[a,b] Michael A. Teitell,[d,e,f,g,h] Hanna K. A. Mikkola,[d,e,f,g,h] William E. Lowry,[a,b,f,g,h] Kathrin Plath,[a,f,g,h] Amander T. Clark[a,b,f,g,h]

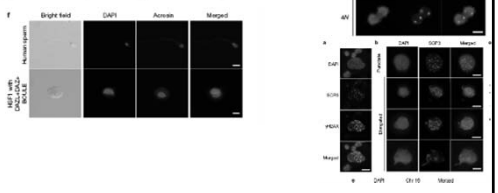


- Pluripotent stem cells (both hESC and iPS) give rise to *in vitro* PGC following 7 days of differentiation on hFGC
- They correspond to immature PGCs (developmental stage *in vivo* between specification and less than 9 week of gestation)
- Initiation of imprinting erasure is dependent on the epigenetic status of the pluripotent stem cell from which iPGCs are generated.

Nature, 2009

Human *DAZL, DAZ* and *BOULE* genes modulate primordial germ-cell and haploid gamete formation

Kehkooi Kee[1], Vanessa T. Angeles[1], Martha Flores[1], Ha Nam Nguyen[2] & Renee A. Reijo Pera[1]

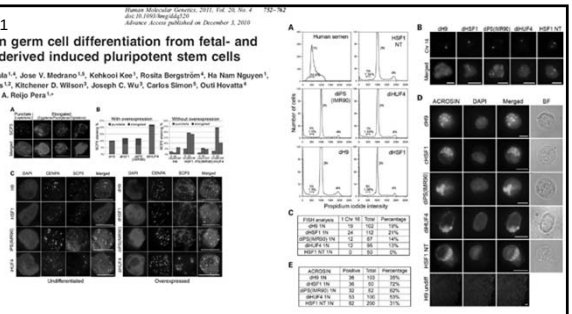


- Generation of *in vitro* PGC from male and female hESC
- DAZL, DAZ and BOULE promote PGC formation and germ-cell progression to meiosis and formation of haploid germ cells that ressemble round spermatids

2011

Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells

Sarita Panula[1,4], Jose V. Medrano[1,5], Kehkooi Kee[1], Rosita Bergström[4], Ha Nam Nguyen[1], Blake Byers[1,2], Kitchener D. Wilson[3], Joseph C. Wu[3], Carlos Simon[5], Outi Hovatta[4] and Renee A. Reijo Pera[1,*]



• iPSC can form *in vitro* meiotic and post-meiotic haploid cells over-expressing DAZ, DAZL and BOULE.

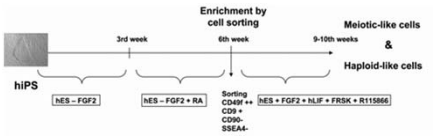
STEM CELLS

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

2011

Complete Meiosis from Human Induced Pluripotent Stem Cells

C. EGUIZABAL,[a] N. MONTSERRAT,[a] R. VASSENA,[a] M. BARRAGAN,[a] E. GARRETA,[a] L. GARCIA-QUEVEDO,[b] F. VIDAL,[b] A. GIORGETTI,[a] A. VEIGA,[a] J. C. IZPISUA BELMONTE[a,c]



• Generation of a novel differentiating 2 step-protocol without overexpressing any genes.

• iPS can form *in vitro* meiotic and haploid like-cells.

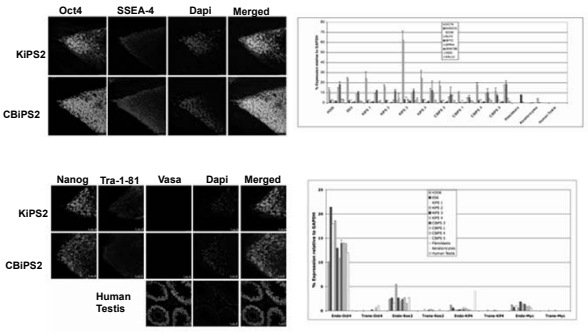
•The post-meiotic-like cells generated have features of human spermatids expressing Acrosin.

Table 1.List of human ES and iPS cell lines used in this study

Name of the cell line	Cell type	Culture conditions	Sex	Factors
HS306	hES	Feeders	F	
ES[6]	hES	Feeders	M	
KiPS1	KiPS	Feeders	M	4
KiPS2	KiPS	Feeders	M	4
KiPS3	KiPS	Feeders	M	3
KiPS4	KiPS	Feeders	F	4
CBiPS1	CBiPS	Feeders	M	4
CBiPS2	CBiPS	Feeders	M	2
CBiPS3	CBiPS	Feeders	F	3
CBiPS4	CBiPS	Feeders	M	2
CBiPS5	CBiPS	Feeders	F	2

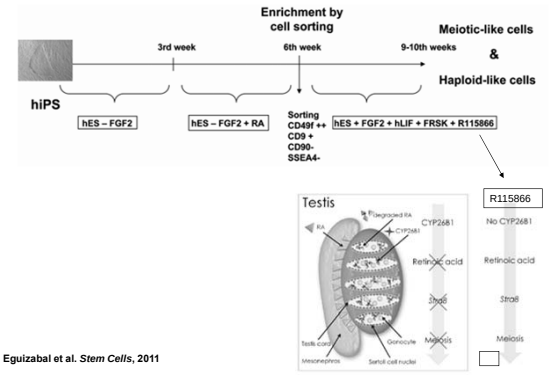
Eguizabal et al. *Stem Cells*, 2011

Characterization of hiPS cell lines



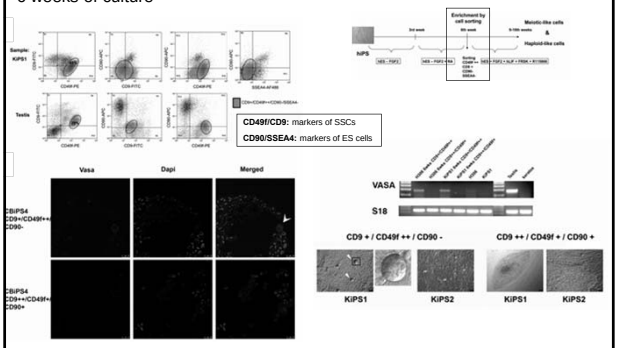
Eguizabal et al. *Stem Cells*, 2011

Timeline of *in vitro* differentiation of human iPS cells as a model for derivation of postmeiotic germ cells



Eguizabal et al. *Stem Cells*, 2011

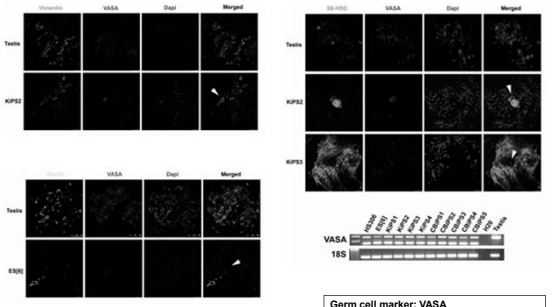
Characterization of human germ-like cells after enrichment by FACS sorting at 6 weeks of culture



CD49f/CD9: markers of SSCs
CD90/SSEA4: markers of ES cells

Eguizabal et al. *Stem Cells*, 2011

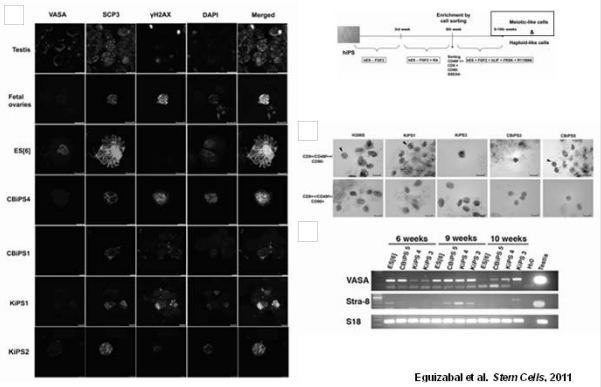
Differentiation towards human germ-like cells at 6 weeks of culture



Germ cell marker: VASA
Sertoli cell markers: Vimentin and Nestin
Leydig cell marker: 3β-HSD

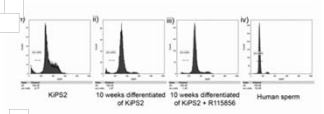
Eguizabal et al. *Stem Cells*, 2011

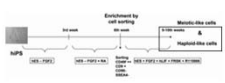
Meiotic progression of germ-like cells at 9 weeks of culture

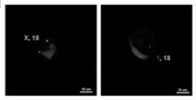


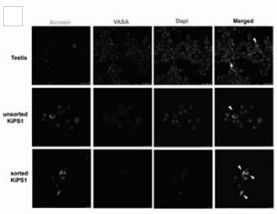
Eguizabal et al. *Stem Cells*, 2011

Haploid formation of germ-like cells at 10 weeks culture







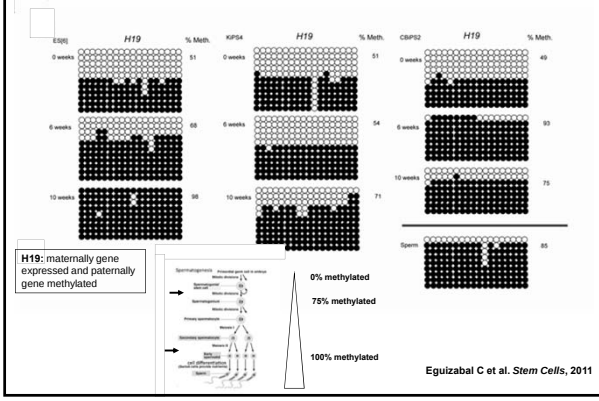


Cell line	+/- R115866	% haploid cells	% diploid cells	% tetraploid cells	% aneuploid cells
KIPS 2	-	0	73,11	1,05	25,84
KIPS 2	+	0,43±0,02	76,22±1,09	1,35±1,90	21,96±0,75
KIPS 4	-	0±0	78,51±3,23	1,49±1,49	20±1,73
KIPS 4	+	1,98±0,67	73,94±5,43	1,94±2	22,13±2,98
CBiPS 2	-	0,82	75,46	0,82	22,9
CBiPS 2	+	2,33	76,7	1,7	19,27
CBiPS 5	-	0	85,5	0	14,5
CBiPS 5	+	1,03±0,04	80,5±3,53	0,42±0,59	18,05±2,89
KIPS 1	+	0,5±0,19	78,64±2,20	0,45±0,63	20,41±11,10

0.43-2.33% haploid-like cells in presence of R115866

Eguizabal et al. *Stem Cells*, 2011

Methylation status of H19 promoter DMR were analyzed by bisulphite sequencing in undifferentiated hiPS and hES, and after 6 and 10 weeks of differentiation



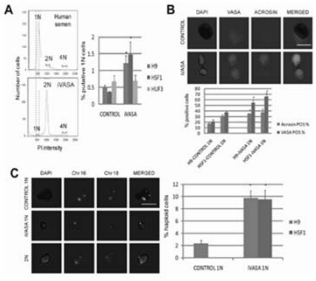
H19: maternally gene expressed and paternally gene methylated

Eguizabal C et al. *Stem Cells*, 2011

STEM CELLS®

Divergent RNA-binding Proteins, DAZL and VASA, Induce Meiotic Progression in Human Germ Cells Derived *in vitro* 2011

Jose V Medrano[1,2], Cyril Ramathal[1], Ha Nam Nguyen[1], Carlos Simon[2,*], & Renee A. Reijo-Pera[1]

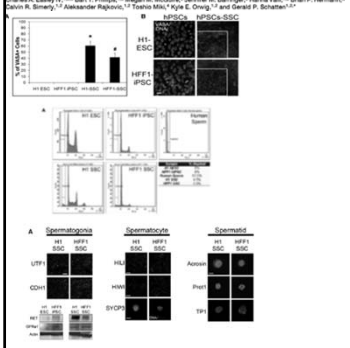


- Generation of *in vitro* PGCs from hESC and hiPS over-expressing VASA and/or DAZL.
- The hES/hiPS can form correctly *in vitro* meiotic and post-meiotic male haploid cells over-expressing VASA and/or DAZL in 14 days.
- The in vitro germ-like cells over-expressing only VASA present the typical pattern of methylation status for H19 as the normal germ cells.

Cell Reports
Report

2012

Direct Differentiation of Human Pluripotent Stem Cells into Haploid Spermatogenic Cells



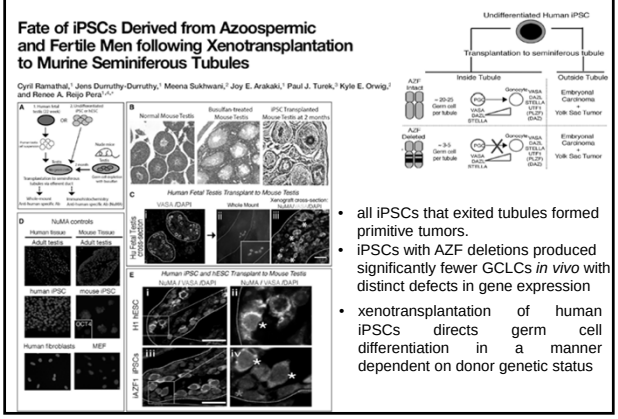
- hESCs and iPSCs cultured in SSC conditions differentiate directly into advanced male germ cell lineages including postmeiotic,spermatid-like cells *in vitro* without genetic manipulation (10 days)
- hPSCs differentiated in SSC culture conditions exhibit haploid features
- Differentiation of hPSCs in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

Cell Reports
Article

ACCESS

2014

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



- all iPSCs that exited tubules formed primitive tumors.
- iPSCs with AZF deletions produced significantly fewer GCLCs *in vivo* with distinct defects in gene expression
- xenotransplantation of human iPSCs directs germ cell differentiation in a manner dependent on donor genetic status

Conclusions and Future remarks

- Early germ cells and functional sperm have been obtained through mouse ESC and iPS using 2 step protocols (*in vitro* and *in vivo*).
- By using fully *in vitro* protocol for the generation of mouse male germ cells, the methylation pattern and offspring are abnormal.
- Normal healthy offspring with normal methylation patterns of imprinted genes if gametogenesis is resumed in *in vivo* conditions.
- Further studies will be necessary to develop efficient protocols to get *in vitro* germ cells.
- The use of such gametes in ART remains a “distant prospect“.
- Stem cell derived gametes can become a valuable resource for research: germ cell development, epigenetic reprogramming and germline gene modification.

Basque Center for Blood Transfusion and Human Tissues

cristina.eguizabalargaiz@osakidetza.net

THANKS FOR YOUR ATTENTION!!!

Northeastern

LAIR

Functional Characterization of Adult Ovary-derived Oogonial Stem Cells in Mice, Monkeys and Women

Jonathan L. Tilly, Ph.D.

Professor and Chair
Department of Biology
Laboratory of Aging and Infertility Research
Northeastern University
Boston, Massachusetts 02115, USA

j.tilly@neu.edu

Presented at the ESHRE Pre-congress Course 13 (SIG Stem Cells) , Munich, Germany – 29 June 2014

DISCLOSURES

Jonathan L. Tilly, Ph.D., declares the following:

interest in intellectual property described in U.S. Patents
7,195,775
7,850,984
7,955,846
8,642,329
8,647,869
8,652,840

interest in intellectual property described in U.S. Patent Applications
11/131,152
11/131,153
61/502,840
61/885,559
61/887,569
PCT US 2014/032010

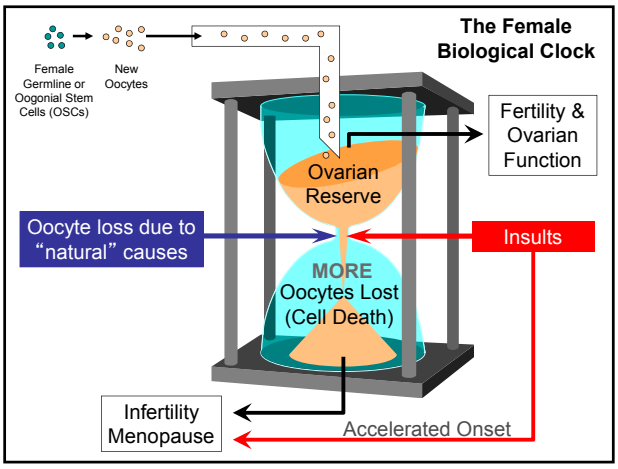
and,

interest as a scientific co-founder of OvaScience, Inc.
(Cambridge, MA; *www.ovascience.com*)

LEARNING OBJECTIVES

At the conclusion of this lecture, the participant:

1. Will be introduced to published studies regarding the identification, isolation and characterization of female germline or oogonial (oocyte-producing) stem cells (OSCs) in adult mammalian ovaries
2. Will be better informed of studies questioning the existence of OSCs, as well as the conceptual and experimental limitations of these studies which led to the conclusion drawn that OSCs do not exist
3. Will be able to integrate currently available evidence on the properties of OSCs in mice, monkeys and women into discussions of how regenerative medicine-based technologies involving OSCs could provide new tools to combat aging related infertility and menopause



NATURE | VOL 428 | 11 MARCH 2004 | articles

Germline stem cells and follicular renewal in the postnatal mammalian ovary

= OOCYTE RESERVE NOT FIXED AT BIRTH (OVARIES ≈ TESTES)

Joshua Johnson*, Jacqueline Canning*, Tomoko Kaneko, James K. Pru & Jonathan L. Tilly

Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, and Department of Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02114, USA

Independent Confirmation (mouse studies):

Zou *et al. Nature Cell Biology* 2009 12: 631–636
OSCs isolated from juvenile and adult ovaries
Transplanted OSCs produce eggs that fertilize and yield viable offspring

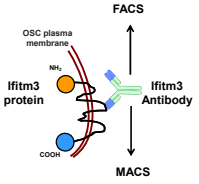
Pacchiarotti *et al. Differentiation* 2010 79: 159–170
OSCs isolated from neonatal and adult ovaries
Cultured OSCs generate oocytes that form follicles *in vitro*

Purification of OSCs based on cell surface expression of the germ cell-specific protein, Ddx4 (Vasa, Mvh)

- **In oocytes, Ddx4 is localized exclusively within the cytoplasm**
- **In OSCs, the C-terminus of Ddx4 is exposed on the outer plasma membrane**
- **Target this externalized epitope with a C-terminal Ddx4-specific antibody for live cell sorting**

AND,
if one still feels strongly against using Ddx4 antibodies to purify OSCs, antibodies against Ifitm3 (Fragilis), a well accepted transmembrane protein in germ cells, work equally well

Tilly lab, unpublished data



Stem Cell Dev 2011 20: 2197–2204

Why is this so important? Because at least one person has disputed publications from us and others based on his own lab's inability to viably sort OSCs using Ddx4 antibodies

NATURE | NEWS FEATURE

Reproductive biology: Fertile mind

Jonathan Tilly defied decades of dogma by suggesting that women can make new eggs throughout their lives. Now some of his critics are taking a second look.

Trisha Gura

14 November 2012

"Liu in Sweden says that he initially believed Wu's paper when it came out. But his group could not repeat the technique [of Ddx4/Vasa antibody-based sorting]. To bypass the cell-surface problem with vasa, Liu used an approach that tracks the protein inside the cells."

The Liu lab dissociates ovaries for analysis using trypsin!

PNAS

Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

12580–12585 | PNAS | July 31, 2012 | vol. 109 | no. 31

Hua Zhang[a,1], Wenjing Zheng[a,1], Yan Shen[a], Deepak Adhikari[a], Hiroo Ueno[b,2], and Kui Liu[a,2]

[a]Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30, Gothenburg, Sweden; and [b]Department of Stem Cell Pathology, Kansai Medical University, Moriguchi City, 570-8506, Osaka, Japan

Edited* by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved June 5, 2012 (received for review April 19, 2012)

PNAS

Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

"…our results show that no mitotically active Ddx4-expressing female germline progenitors exist in postnatal mouse ovaries."

Hua Zhang[a,1], Wenjing Zheng[a,1], Yan Shen[a], Deepak Adhikari[a], Hiroo Ueno[b,2], and Kui Liu[a,2]

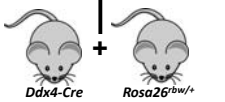
[a]Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30, Gothenburg, Sweden; and [b]Department of Stem Cell Pathology, Kansai Medical University, Moriguchi City, 570-8506, Osaka, Japan

12580–12585 | PNAS | July 31, 2012 | vol. 109 | no. 31

Collect ovaries at day 8, disperse, filter (40-μm pores) and culture cells

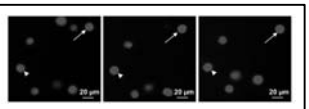
Ddx4-Cre;Rosa26$^{rbw/+}$

Ddx4 promoter activation = just those cells switch from GFP to RFP/YFP/CFP (Cre-based recombination)



Ddx4-Cre + ***Rosa26$^{rbw/+}$***

Ubiquitous GFP expression



Proliferation of RFP-positive (*Ddx4-Cre* recombined) cells in crudely dispersed ovarian cell preparations was not observed over short term culture (24, 48, 72 hours) = **OSCs do not exist**

Limitations and Caveats:

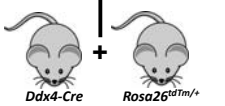
- No control experiments presented to document germ cell-specificity of Cre expression (no "leakiness")
- Even if specific for germ cells, *Ddx4-Cre* approach recombines **all germ cells equally**, and thus cells identified as "positive" by RFP expression could be oocytes, OSCs or any cell that has activated the *Ddx4* promoter fragment = **identity confirmation a must, but no effort was made to show that the cells studied are OSCs and not oocytes!**

2012 PNAS study from Liu and colleagues: a bold conclusion from inconclusive data … the major flaws

Collect ovaries

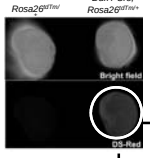
Ddx4-Cre;Rosa26$^{tdTm/+}$

Ddx4 promoter activation = just those cells activate *tomato red* expression (Cre-based recombination)



Ddx4-Cre + ***Rosa26$^{tdTm/+}$***

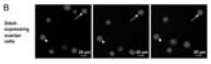
No reporter expression



MAJOR FLAW #1:

Ddx4 promoter-driven fluorescence pattern is widespread in ovaries, which is NOT consistent with germ cell-specific expression of the reporter = **"leaky" promoter!**

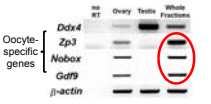
Ovaries dispersed **exactly** as described by Liu and colleagues (trypsin-based disruption, filtered through 40-μm cell strainer to obtain cells studied)



Simple PCR-based screen of this fraction (unclear why not done by Liu and colleagues)

MAJOR FLAW #2:

Crude cell fraction containing "Ddx4-expressing" cells studied by Liu and colleagues is **contaminated with Ddx4-expressing oocytes**, which are RFP-positive but **incapable of proliferation!**



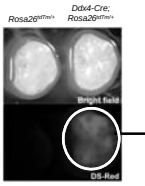
Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

If used the correct way, the *Ddx4-Cre* mouse provides clear experimental evidence that mitotically active female germline progenitors exist in postnatal mouse ovaries

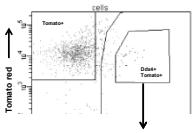
Collect ovaries

Ddx4-Cre;Rosa26$^{tdTm/+}$

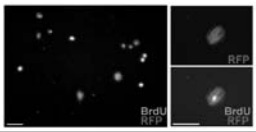
Ddx4 promoter activation = just those cells activate *tomato* red expression (Cre-based recombination)

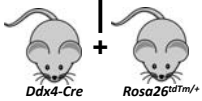


Ovaries dispersed and sorted for:
Tomato red
ecDdx4 (C-terminal antibody, APC)



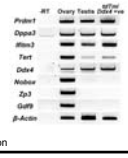
Collect ecDdx4/Tomato Red dual-positive cells and plate in culture: germ cell colony establishment and proliferation





Ddx4-Cre + ***Rosa26$^{tdTm/+}$***

No reporter expression



PNAS

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12580–12585 | PNAS | July 31, 2012 | vol. 109 | no. 31

Hua Zhang[a,1], Wenjing Zheng[a,1], Yan Shen[a], Deepak Adhikari[a], Hiroo Ueno[b,2], and Kui Liu[a,2]

[a]Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30 Gothenburg, Sweden; and [b]Department of Stem Cell Pathology, Kansai Medical University, Moriguchi City, 570-8506, Osaka, Japan

Edited* by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved June 5, 2012 (received for review April 19, 2012)

PNAS

Female mice lack adult germline stem cells but sustain oogenesis using stable primordial follicles

Lei Lei and Allan C. Spradling[1]

PNAS | May 21, 2013 | vol. 110 | no. 21 | 8585–8590

Howard Hughes Medical Institute Research Laboratories, Department of Embryology, Carnegie Institution for Science, Baltimore, MD 21218

Contributed by Allan C. Spradling, April 4, 2013 (sent for review April 15, 2013)

BOTH studies based on circumstantial and indirect 'negative' data

NEITHER study even attempted to reproduce what we and others have done by simply isolating the cells they claim do not exist using validated and detailed antibody-based sorting protocols – why not?

None of this should not be acceptable at this stage if one wishes to refute the findings of others and us, and claim that OSCs do not exist

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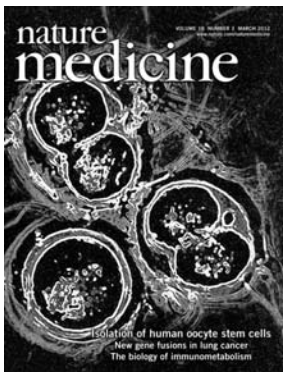
Evolutionary conservation of female germline stem cells



Annu Rev Genet 2005 39: 173–195
Cell Res 2007 17: 15–25

Science 2010 328: 1561–1563

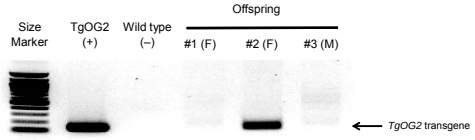
Mice
Rats
Giraffes
Cows
Baboons
Rhesus Macaques*

*NIH trial ongoing

Nature Medicine 2012 18: 413–421

Transplanted mouse OSCs carrying a traceable transgene generate functional oocytes *in vivo***

Genotype analysis of pups delivered by a wild type female mouse, transplanted at 2 months of age with OSCs isolated from ovaries of young adult TgOG2 (ΔPE*Oct4-Gfp*) transgenic female mice, confirms delivery of offspring carrying the transgene reporter (**independent confirmation of mouse OSC transplantation data reported by others: *Nature Cell Biology* 2009 11: 631–636 and *J Mol Cell Biol* 2011 3: 132–141)

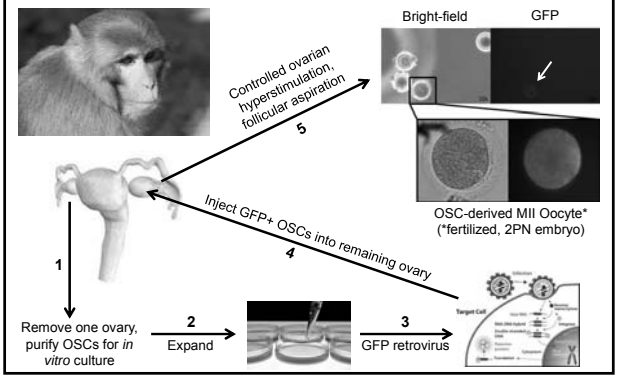


Wild type female 22919 (intraovarian transplant of TgOG2 OSCs) mated with wild type male:
2 pups (1 female, 1 male) negative for TgOG2 transgene (host oocytes)
1 female pup positive for TgOG2 transgene (TgOG2 OSC-derived oocyte)

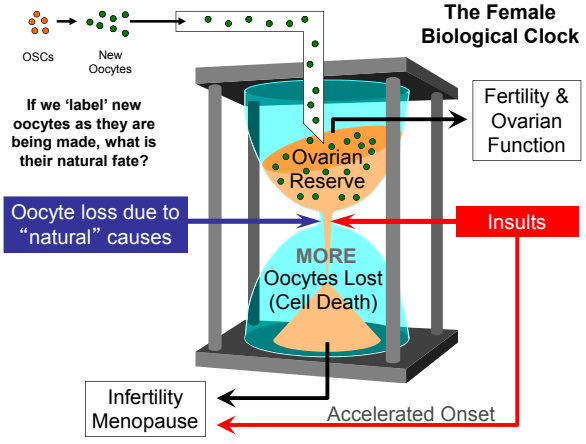
Four breeding pairs have produced 38 offspring, 6 of which carry the *TgOG2* transgene (15.8%)

In-vivo OSC function in adult female Rhesus macaques

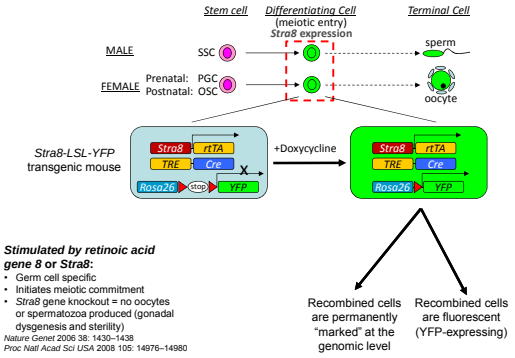
Wolff EF, Libfraind LL, Weitzel P, Woods DC, Feng Y, Tilly JL, DeCherney AH, Tisdale JF. Oogonial stem cells generate mature oocytes in an autologous Rhesus macaque transplantation model. *Reproductive Sciences* 2014; 21 (Supplement): 119A



The Female Biological Clock



Lineage tracing to map the *in-vivo* physiological fate of newly generated oocytes in adult ovaries

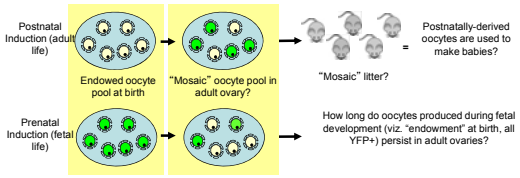


***Stimulated by retinoic acid gene 8* or *Stra8*:**

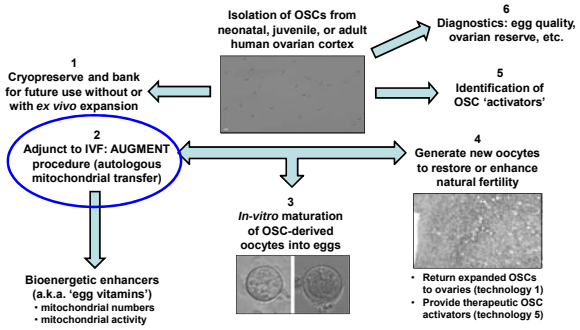
- Germ cell specific
- Initiates meiotic commitment
- *Stra8* gene knockout = no oocytes or spermatozoa produced (gonadal dysgenesis and sterility)

Nature Genet 2006 38: 1430–1438
Proc Natl Acad Sci USA 2008 105: 14976–14980

Lineage tracing to map the *in-vivo* physiological fate of newly generated oocytes in adult ovaries



What does the future hold for mammalian OSCs? Impact on women's reproductive health**



**Commercial isolation and use of OSCs for these and other purposes are protected by several issued patents and patent applications licensed exclusively to OvScience: U.S. Patents 7,955,846, 8,642,329, 8,647,869 and 8,652,840; U.S. Patent Applications 11/131,153, 11/131,152, 61/502,840, 61/885,559, 61/887,569 and PCT US 2014/032010)

Heterologous ooplasmic transfer: reinvigoration of energetically compromised eggs through donor mitochondria?

THE LANCET Vol 350 • July 19, 1997

Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs

Jacques Cohen, Richard Scott, Tim Schimmel, Jacob Levron, Steen Willadsen

Molecular Human Reproduction vol.4 no.3 pp. 269–280, 1998

Ooplasmic transfer in mature human oocytes

Jacques Cohen[1,4], Richard Scott[1], Mina Alikani[1], Tim Schimmel[1], Santiago Munné[1], Jacob Levron[2], Lizi Wu[1], Carol Brenner[1], Carol Warner[3] and Steen Willadsen[1]

FERTILITY AND STERILITY®
VOL. 74, NO. 3, SEPTEMBER 2000

Mitochondrial DNA heteroplasmy after human ooplasmic transplantation

Carol A. Brenner, Ph.D., Jason A. Barritt, Ph.D., Steen Willadsen, D.V.M., Ph.D., and Jacques Cohen, Ph.D.

Human Reproduction Vol.16, No.3 pp. 513-516, 2001

BRIEF COMMUNICATION

Mitochondria in human offspring derived from ooplasmic transplantation

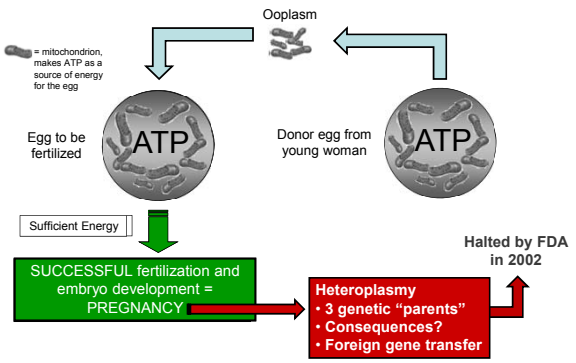
Jason A. Barritt, Carol A. Brenner, Henry E. Malter and Jacques Cohen

Heterologous ooplasmic transfer: reinvigoration of energetically compromised eggs through female germline mitochondrial transfer at ICSI?

Transfer of mitochondria from donor eggs	No. of Cycles	Pregnancies	Live Births	Success Rate
Cohen et al., 1997, 1998; Brenner et al., 2000; Barritt et al., 2000, 2001	30	13	16	43%
Dale et al., 2001	1	1	2	100%
Lanzendorf et al., 1999	4	1	2	25%
Huang et al., 1999	9	4	5	44%

44 attempts, 19 pregnancies achieved (25 babies): 43% success rates!

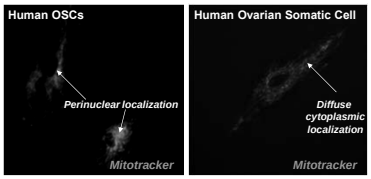
The promise and perils of heterologous (non-patient matched) ooplasmic transfer



Could we use autologous OSC mitochondria?

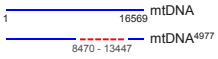
- **Perinuclear localization**

  Similar to other stem cell types

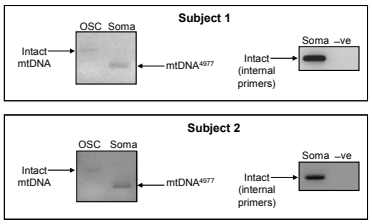


- **Mitochondrial DNA (mtDNA) mutations**

  Accumulate with age in somatic cells

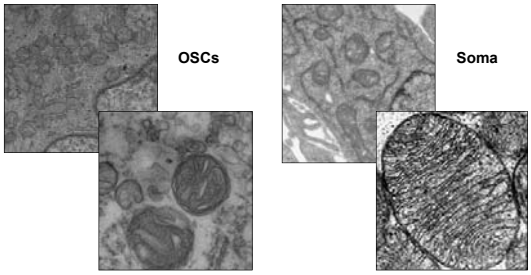


  OSCs free of 'common deletion' mutation

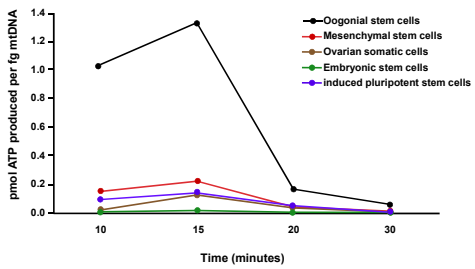


Electron microscopic evaluation of mitochondrial ultrastructure in oogonial stem cells

Very small, with electron dense matrices and few cristae, closely matching ultrastructural features of mitochondria in oocytes (very different from mitochondria in somatic cells)



Mitochondria present in human OSCs exhibit the highest energetic capacity compared with other human cell types



Improving IVF success with OSC-based technology

Poor *in-vitro* fertilization (IVF) success rates in women of advanced maternal age reflect unmet energy needs in the egg

= mitochondrion, makes ATP (energy for the egg)

Egg to be fertilized

ATP

Insufficient Energy

NO fertilization or POOR embryo development = FAILED pregnancy

AUGMENT**

(Autologous Germline Mitochondrial Energy Transfer)

Isolate Mitochondria

OSCs

From same patient

Inject mitochondria at time of ICSI

Egg to be fertilized

ATP

Sufficient Energy

SUCCESSFUL fertilization and embryo development = HEALTHY pregnancy

**AUGMENT is detailed in U.S. Patents 8,642,329 and 8,647,869

What does the future hold for mammalian OSCs? Impact on women's reproductive health**

Isolation of OSCs from neonatal, juvenile, or adult human ovarian cortex

1 Cryopreserve and bank for future use without or with *ex vivo* expansion

2 Adjunct to IVF: AUGMENT procedure (autologous mitochondrial transfer)

Bioenergetic enhancers (a.k.a. 'egg vitamins')
- mitochondrial numbers
- mitochondrial activity

3 *In-vitro* maturation of OSC-derived oocytes into eggs

4 Generate new oocytes to restore or enhance natural fertility

- Return expanded OSCs to ovaries (technology 1)
- Provide therapeutic OSC activators (technology 5)

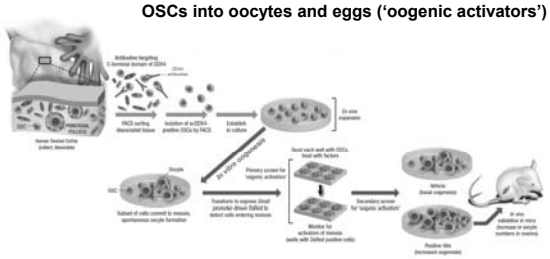
5 Identification of OSC 'activators'

6 Diagnostics: egg quality, ovarian reserve, etc.

**Commercial isolation and use of OSCs for these and other purposes are protected by several issued patents and patent applications licensed exclusively to OvScience: U.S. Patents 7,955,846, 8,642,329, 8,647,869 and 8,652,840; U.S. Patent Applications 11/131,153, 11/131,152, 61/502,840, 61/885,559, 61/887,569 and PCT US 2014/032010)

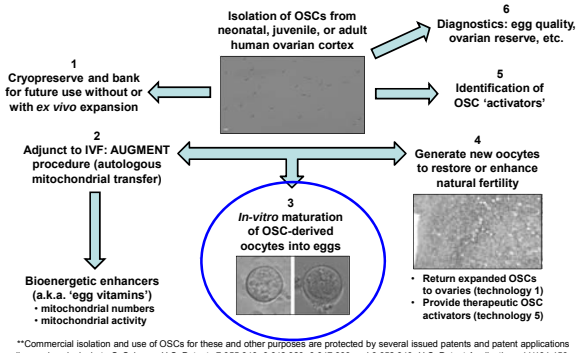
Woods DC, Tilly JL. Germline stem cells in adult mammalian ovaries. In: Ten Critical Topics in Reproductive Medicine, S. Sanders, editor; *Science*/AAAS, Washington, DC 2013, pp. 10–12

***In vitro* oogenesis: screening for factors that drive OSCs into oocytes and eggs ('oogenic activators')**



'Oogenic activators' identified thus far in OSCs:
- **Bone morphogenetic protein 4 (BMP4): see *Fertil Steril* 2013 100: 1468–1475**
- **Estrogen acting via its alpha-receptor isoform (blocked by progesterone)**
- **White adipocyte-derived growth factor (as-yet unidentified)**

What does the future hold for mammalian OSCs? Impact on women's reproductive health**



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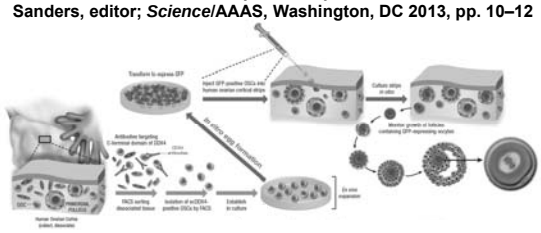
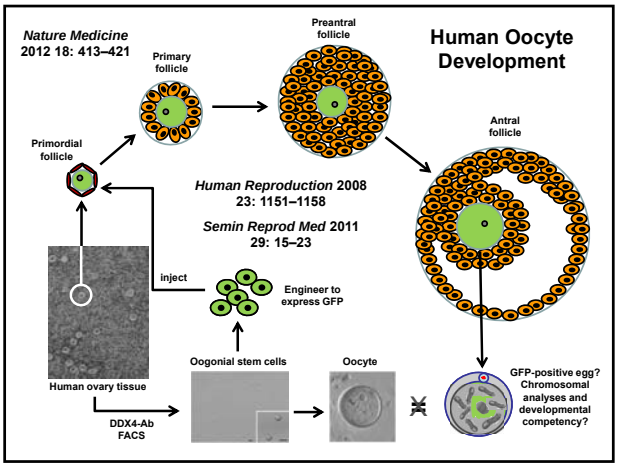
Woods DC, Tilly JL. Germline stem cells in adult mammalian ovaries. In: Ten Critical Topics in Reproductive Medicine, S. Sanders, editor; *Science*/AAAS, Washington, DC 2013, pp. 10–12

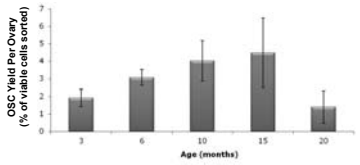


***In vitro* egg formation from purified OSCs: is it feasible and how do we do accomplish it?**



If OSC-based technologies can be developed and validated for clinical management of female fertility, are OSCs always present in ovaries to isolate and work with?

Mouse



Human

Yield of DDX4-positive cells from adult human ovarian cortical tissue across age:

Age (years)	% Yield
22	1.17
29	1.10
32	1.10
33	1.13
47	3.84
53	4.24
58	4.95

YES: OSCs are still present in ovaries after age-related ovarian failure

Can we 're-activate' OSCs in ovaries that have failed?

Can we derive fully functional eggs from OSCs *ex vivo*?

PNAS

Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

12580–12585 | PNAS | July 31, 2012 | vol. 109 | no. 31

Hua Zhang[a,1], Wenjing Zheng[a,1], Yan Shen[a], Deepak Adhikari[a], Hiroo Ueno[b,2], and Kui Liu[a,2]

[a]Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30, Gothenburg, Sweden; and [b]Department of Stem Cell Pathology, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, Japan

Edited* by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved June 5, 2012 (received for review April 19, 2012)

Thus, in contrast to the results published by Zou et al. (5) and White et al. (4), our results show that no mitotically active Ddx4-expressing female germline progenitors exist in postnatal mouse ovaries.

PNAS

Female mice lack adult germ-line stem cells but sustain oogenesis using stable primordial follicles

Lei Lei and Allan C. Spradling[1]

PNAS | May 21, 2013 | vol. 110 | no. 21 | 8585–8590

Howard Hughes Medical Institute Research Laboratories, Department of Embryology, Carnegie Institution for Science, Baltimore, MD 21218

Contributed by Allan C. Spradling, April 4, 2013 (sent for review January 15, 2013)

Thus, adult female mice neither require nor contain active germ-line stem cells or produce new oocytes in vivo.

The results presented to you today are even more compelling to consider if the outcomes observed by us and others over the past decade of work are actually due to the functional properties of cells that do not exist

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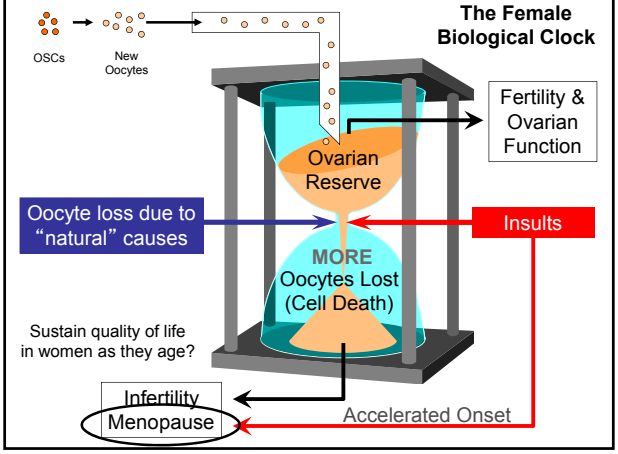


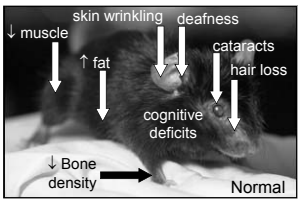
If we delay ovarian failure, females age better!!

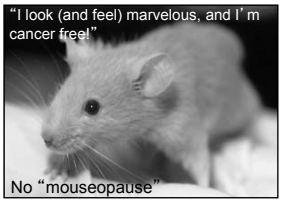
Nature Genetics 1999 21: 200–203

aged female mice

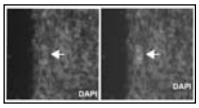
Normal

No "mouseopause"





"Ovarian replacement therapy" can dramatically improve the quality of life in aging females (*Proc Natl Acad Sci USA* 2007 104: 5229–5234)



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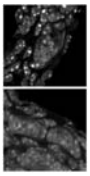

Collaborators

Northeastern University
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University of Edinburgh
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NICHD, NIH
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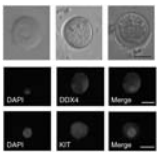
John Tisdale
Alan DeCherney



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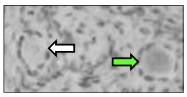


Do mitotically active female germline progenitors exist in postnatal mouse ovaries?

Kui Liu
Department of Chemistry and Molecular Biology
University of Gothenburg, Sweden

No conflict of interests

Learning objectives

To learn the current understandings of the ”female germ line stem cells”: if they exist, and if they are functinal.

Outline

- **Postnatal oogenesis: a topic under discussion.**
- **Female germline stem cells in adult ovary? The issues of isolated cells.**
- **Our own data: no mitotically active Ddx4 (Vasa)-positive female germline progenitors in postnatal ovaries.**

Challenging the classic principle of female reproduction: Follicular renewal in adult ovaries

Germline stem cells and follicular renewal in the postnatal mammalian ovary

Follicle counting
Mathematic model

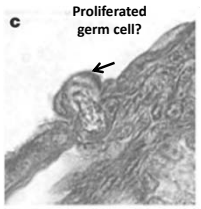
Johnson et al. and Tilly JL
Nature, 2004

Oocyte Generation in Adult Mammalian Ovaries by Putative Germ Cells in Bone Marrow and Peripheral Blood

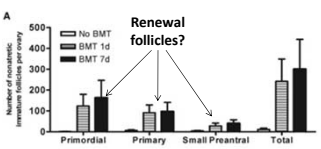
BM and Peripheral blood Transplantation

Johnson et al. and Tilly JL
Cell, 2005

Follicular renewal in adult ovaries: 77 follicles per day?



Johnson et al. and Tilly JL ,
Nature, 2004



Ovarian follicles are recovered 1 day after bone marrow transplantation

Johnson et al. and Tilly JL,
Cell, 2005

Similar experimental approaches showed no follicular renewal in adult ovaries

Fate of the initial follicle pool: Empirical and mathematical evidence supporting its sufficiency for adult fertility

Follicle counting
Mathematic model

Bristol-Gould et al., Dev Biol. 2005

The primordial follicle reserve is not renewed after chemical or γ-irradiation mediated depletion

Follicle counting

Kerr JB et al., Reproduction. 2012

Similar experimental approaches showed no follicular renewal in adult ovaries

Ovulated oocytes in adult mice derive from non-circulating germ cells

Transplantation and
Parabiotic mouse models

Eggan et al., Nature. 2005

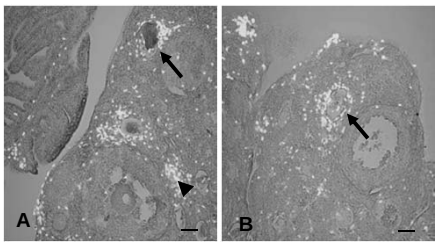
The oocyte population is not renewed in transplanted or irradiated adult ovaries

Transplantation

Begum S et al., Hum Reprod. 2008

Transplanting GFP-positive bone marrow into SCID mice never lead to any GFP oocytes.

Unpublished data, Kui Liu group



GFP-expressing bone marrow cells from Rainbow/+ females were transplanted into adult SCID females through tail vein injection. No fluorescent oocyte was observed in the ovary of recipients after 3 months of injection.

Challenging the classic principle of female reproduction: Female germline stem cells in postnatal ovaries

Production of offspring from a germline stem cell line derived from neonatal ovaries

Kang Zou[1], Zhe Yuan[1], Zhaojuan Yang[1], Huacheng Luo[1], Kejing Sun[1], Li Zhou[1], Jie Xiang[1], Lingjun Shi[1], Qingsheng Yu[1], Yong Zhang[1], Ruoyu Hou[1] & Ji Wu[1,2]

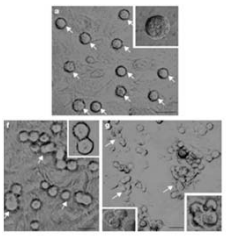
Zou et al. and Ji Wu, Nat Cell Biol, 2009

Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women

Yvonne A R White[1,2,4], Dori C Woods[1,2,4], Yasushi Takai[3], Osamu Ishihara[3], Hiroyuki Seki[3] & Jonathan L Tilly[1,2]

White et al. and Tilly JL, Nat Med, 2012

"Female germline stem cells" isolated from postnatal mouse ovaries by DDX4 (Vasa) antibody

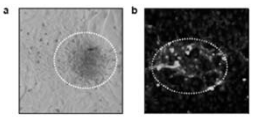


Mouse "female germline stem cells"

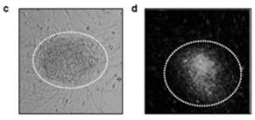
The cells were sorted by magnetic-activated cell sorting, by the use of the DDX4 (Mvh, or Vasa) antibody.

Zou et al. and Ji Wu, Nat Cell Biol, 2009

"Oogonial stem cells" isolated from postnatal mouse and human ovaries by DDX4 antibody



Mouse "oogonia stem cells"



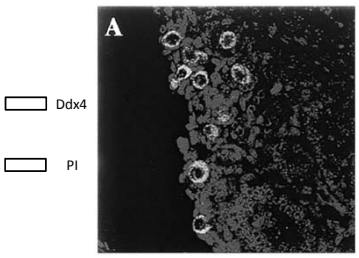
Human "oogonia stem cells"

The cells were sorted by Fluorescence-activated cell sorting which is also by the use of the DDX4 antibody.

White et al. and Tilly JL, Nat Med, 2012

The isolation methods

The Ddx4 proteins locate in cytoplasm of mouse germ cells



Ddx4 is a member of the DEAD box protein family which located in cytoplasm of germ cells.

Toyooka et al., Mech Dev, 2000

Transmembrane domain in Ddx4 (Mvh) protein?

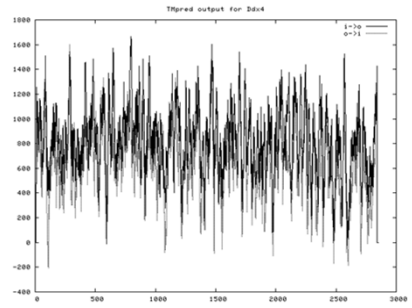
expressed in cytoplasm of germ cells[1, 2, 3]. Bioinformatics from website http://www.ch.embnet.org/cgi-bin/TMPRED_form-parser show that there are two strong transmembrane helices in MVH protein. Based upon the information above, we tried to

Zou et al. and Ji Wu, Nat Cell Biol, 2009

Our assessment of MVH using the TMpred program employed by Zou and colleagues (http://www.ch.embnet.org/software/TMPRED_form.html) confirmed the presence of these two consensus transmembrane domain sequences. Further, our orientation analysis is

Tilly JL and Telfer EE, Mol Hum Reprod, 2009

No transmembrane domain in Ddx4 protein after the TMpred prediction



Only scores above 2844 are considered significant.

Analysis by Kui Liu group.

http://www.ch.embnet.org/software/TMPRED_form.html

ch.EMBnet.org

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TMpred - Prediction of Transmembrane Regions and Orientation

The TMpred program makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring.

K. Hofmann & W. Stoffel (1993)
TMbase - A database of membrane spanning proteins segments
Biol. Chem. Hoppe-Seyler **374**,166

For further information see the TMbase and TMpredict documentation.

http://www.ch.embnet.org/software/TMPRED_form.html

Kay Hofmann [kay.hofmann@uni-koe... Actions

In response to the message from Hua Zhang, Wed 12/12

To: Hua Zhang

Wednesday, December 12, 2012 5:57 PM

- You forwarded this message on 12/12/2012 11:20 PM.

Dear Dr. Zhang,

First, don't use TMpred! it is never a good predictor in the first place and has not been maintained in 20 years.. I would use TMHMM or Phobius, or anything else.

I had a look at the protein in your link and agree with you - there are no signs for a TM region to be found, neither with TMpred nor with anything else.

Best Wishes,

Kay

> Mouse Ddx4NM_001145885.1:
> http://www.ncbi.nlm.nih.gov/nuccore/NM_001145885.1
> Best regards,
> **********
> ****Hua* Zhang*
> Ph.D
> Department of Chemistry and Molecular Biology

Did different groups independently repeat the isolation of female germline stem cells?

Aside from the fact that 4 different groups have now reported OSCs can be isolated and stably propagated long term,[1,2,5,8]

Woods DC, White YA, Tilly JL. Reprod Sci. 2013

Differentiation potential of germ line stem cells derived from the postnatal mouse ovary☆

No functional oocytes reported

Oct4 reporter cells J Pacchiarotti et al., Differentiation, 2010

GSK3 inhibitor-BIO regulates proliferation of female germline stem cells from the postnatal mouse ovary

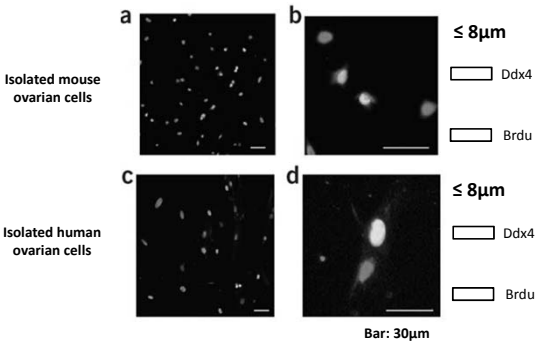
Not tested for functions

Cultured mixed ovarian cells Cao H et al., PLoS ONE, 2012

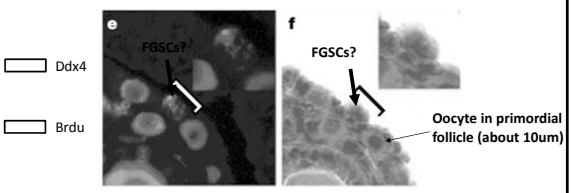
Differences between cells from Wu's group and Tilly's group

1. **Cell sizes.**
2. **Proliferation rate *in vitro.***
3. **Characteristics of these cells in culture.**
4. **Live pups obtained or not?**

White et al. and Tilly JL, Nature, 2012
The FACS isolated mouse ovarian cells are significantly smaller than natural PGCs



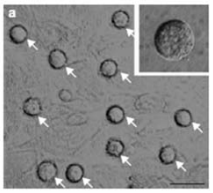
Johnson et al. and Tilly JL, Nature, ***2004***
The size of putative mouse female germline stem cells in ovary is similar with the size of oocyte in primordial follicle



No Bar in Figure

Proliferated Ddx4 positive cells in ovary: bigger than 10uM?

Zou et al. and Ji Wu, Nat Cell Biol, 2009
The size of putative mouse female germline stem cells in ovary is similar with the size of oocyte in primordial follicle



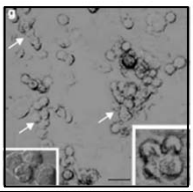
Bar: 25μm

isolated Ddx4 positive cells in mouse ovary by Ddx4 antibody: 12 to 20 μm

The isolated cells from Ji Wu and J Tilly labs are significantly different

Wu

MACS isolated cells



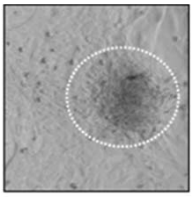
Cell size: **12-20 μm**

Proliferation: **Starting in 24h after seeding**

Colony formation in culture: **Never form colony in dish.**

Tilly

FACS isolated cells



Cell size: **≤ 8μm**

Proliferation rate: **Very slow in primary culture (no details reported)**

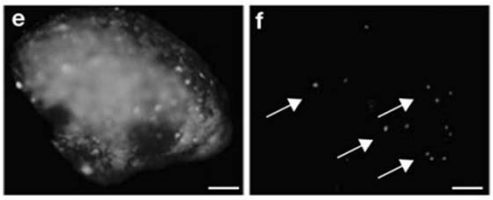
Colony formation in culture: **Colony formation after 4-5 weeks of culture**.

Functional identify of ”OSCs” from different group

Groups	Developmental Stages	References
Ji Wu’s group	Live pups	Zou et al., 2009, Nat Cell Biol.
J Tilly’s group	Blastocyst	White et al., 2012, Nat Med.
Izadyar’s group	Oocyte-like cells	Pacchiarotti et al., 2010, Differentiation.
JL Hua’s group	No functional study	Cao et al., 2012, PLoS One

Live pups are only obtained from the cells derived from Ji Wu’s group.

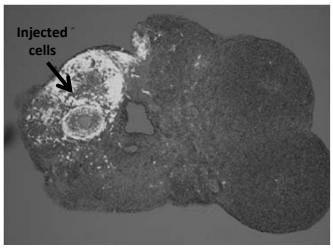
Do the transplanted GFP-positive “stem” cells migrate in donor ovaries?



The diffuse location of transplanted cells after injection.

Zou et al. and Ji Wu, Nat Cell Biol, 2009

The injected cells have no capability to migrate in whole ovary.

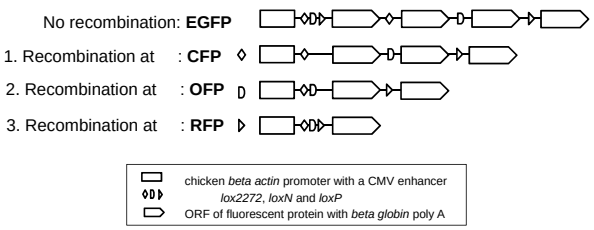


The injected GFP cells (arrow) only present around the site of injection.

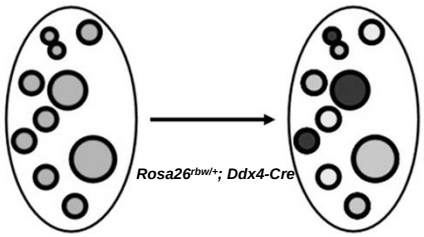
Unpublished data of Kui Liu group

A non-manipulated system: *Rosa26$^{rbw/+}$; Ddx4-Cre* system

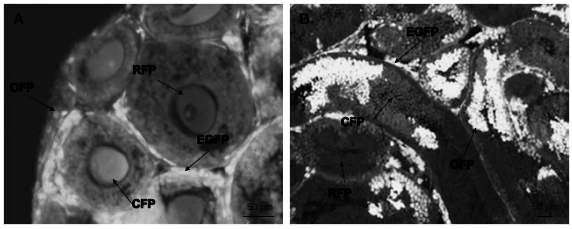
Multi-fluorescence reporter mouse strain: *Rosa26^{rbw}* (Rainbow)



Using an endogenous reporter to trace Ddx4 positive cells *in vitro.*



Multi-fluorescent germ cells in the gonads of postnatal *Rosa26$^{rbw/+}$;Ddx4-Cre* female and male mice.

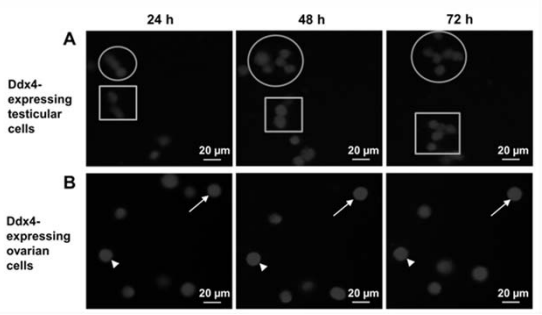


PD28 Rosa26$^{rbw/+}$;Ddx4-Cre ovary

PD28 Rosa26$^{rbw/+}$;Ddx4-Cre testis

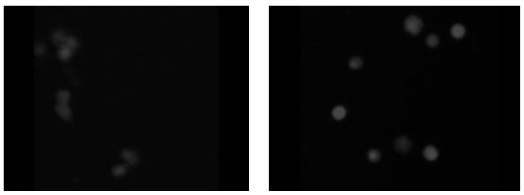
Hua Zhang et al., PNAS, 2012

The Ddx4-positive cells from postnatal mouse ovaries are mitotically inactive



Hua Zhang et al., PNAS, 2012

No Ddx4-expressing cells (0/1571) from postnatal mouse ovaries proliferated during 72 h *in vitro* culture.

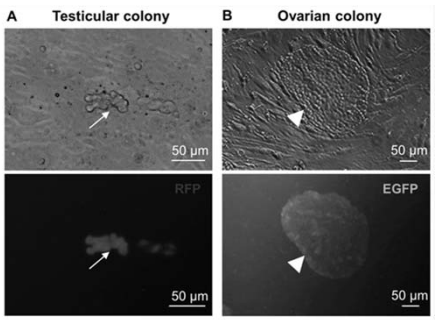


Ddx4-expressing cells from PD8 testes
Control group

Ddx4-expressing cells from PD8 ovaries
Experimental group

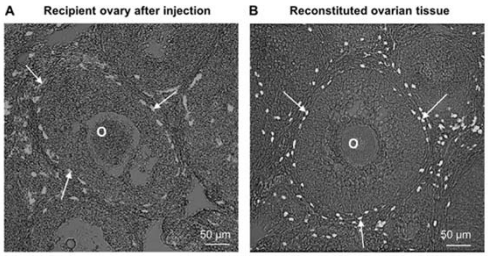
Hua Zhang et al., PNAS, 2012

No Ddx4-positive colony formed in long-term cultured ovarian cells



Hua Zhang et al., PNAS, 2012

The Ddx4-negative ovarian clonal cells from *Rosa26$^{rbw/+}$;Ddx4-Cre* females cannot differentiate into oocytes or granulosa cells *in vivo*.



The Ddx4-negative ovarian clonal cells in culture are not germline progenitors

Hua Zhang et al., PNAS, 2012

Conclusion:

•Ddx4 protein does not contain any transmembrane domain. Using the same Ddx4 antibody, isolated cells from Tilly's and Wu's labs turned out to be distinct.

•There is still a lack of evidence that neo-oogenesis occurs in the adult ovary. More research is needed.

Future Prospects:

1. **Does any follicular renewal occur in adult ovary under physiological or pathological conditions?**
2. **Are the isolated ovarian "stem cells" functional?**

References

Johnson, J., Canning, J., Kaneko, T., Pru, J.K., and Tilly, J.L. (2004). Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature *428*, 145-150.

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White, Y.A., Woods, D.C., Takai, Y., Ishihara, O., Seki, H., and Tilly, J.L. (2012). Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med.

Zou, K., Yuan, Z., Yang, Z., Luo, H., Sun, K., Zhou, L., Xiang, J., Shi, L., Yu, Q., Zhang, Y., et al. (2009). Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol *11*, 631-636.

Eggan, K., Jurga, S., Gosden, R., Min, I.M., and Wagers, A.J. (2006). Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature *441*, 1109-1114.

Bristol-Gould, S.K., Kreeger, P.K., Selkirk, C.G., Kilen, S.M., Mayo, K.E., Shea, L.D., and Woodruff, T.K. (2006). Fate of the initial follicle pool: empirical and mathematical evidence supporting its sufficiency for adult fertility. Dev Biol *298*, 149-154.

Zhang, H., Zheng, W., Shen, Y., Adhikari, D., Ueno, H., and Liu, K. (2012). Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries. Proc Natl Acad Sci U S A *109*, 12580-12585.

ESHRE Annual Meeting 2014
Pre-Congress Course 13
Munich, Germany

Spermatogonia stem cells and future fertility

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Academic Medical Center
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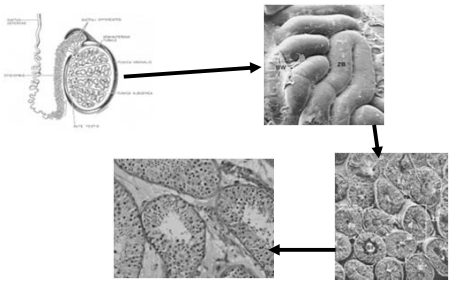
Disclosure

- I have nothing to disclose
- I have no commercial or financial relationships with manufacturers of pharmaceuticals, laboratory supplies or medical devices

Learning objectives

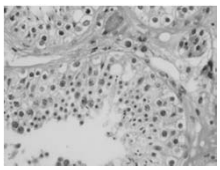
- Understand the function of spermatogonial stem cells (SSCs) in the testis
- Understand spermatogenesis
- Understand the germ cell depletion upon cancer treatment
- Understand the biological evidence for a possible fertility preservation using SSCs
- Learn about the translation of results on SSC culture and transplantation in animal studies to a future SSC based fertility preservation in men

Testis

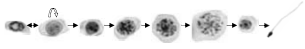


SSCs are among the spermatogonia on the basal membrane of the seminiferous epithelium

Sperm production



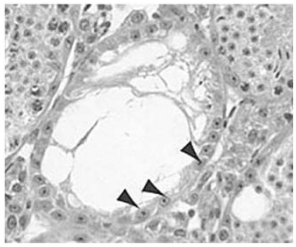
Spermatogonial stem cells (SSCs) form the basis of lifelong spermatogenesis with daily sperm production of ± 50-100 10^6 sperm. This requires a perfect balance between self renewal and differentiation to sperm.



Ad Ap B Spc Spt

Selfrenewal vs differentiation

PLZF-/- mouse

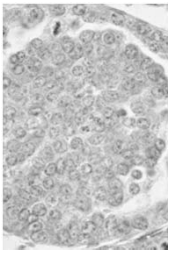


The balance has shifted to differentiation resulting in SSC depletion

Buaas et al., 2004

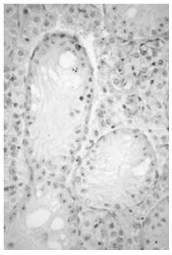
Selfrenewal vs differentiation

GDNF overexpressing mouse



Balance shifted to self renewal

GDNF +/- mouse

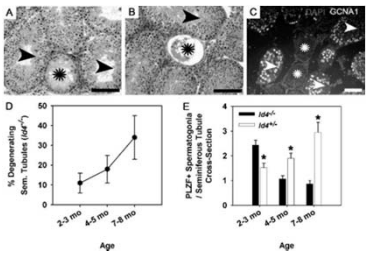


Balance shifted to differentiation

Meng et al., 2000

Selfrenewal vs differentiation

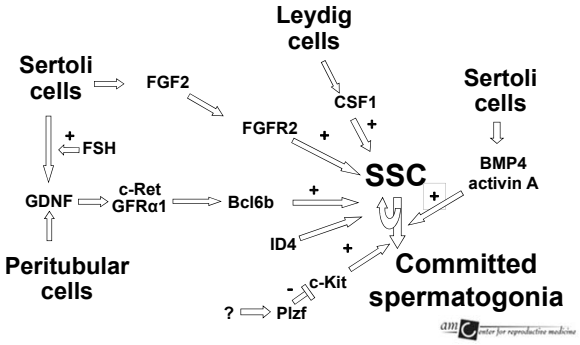
Id4-/- mouse



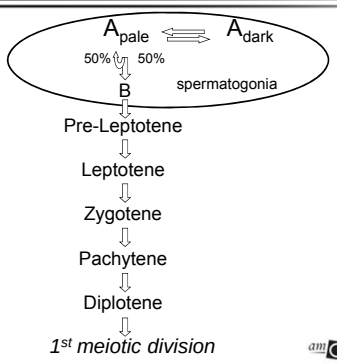
Balance has shifted to differentiation resulting in SSC depletion

Oatley et al., 2011

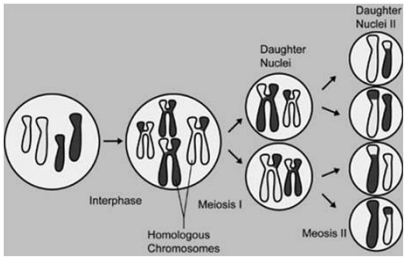
Regulation selfrenewal and differentiation



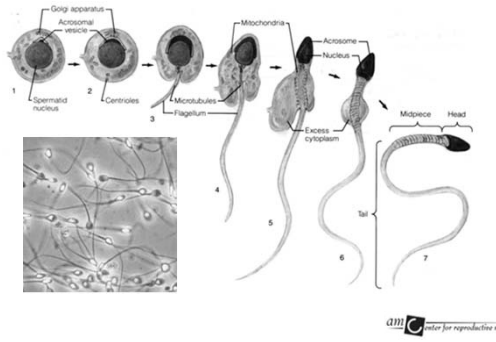
Spermatogenesis



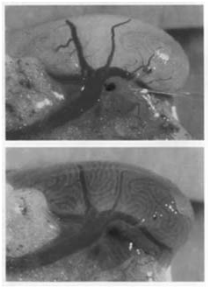
Meiosis

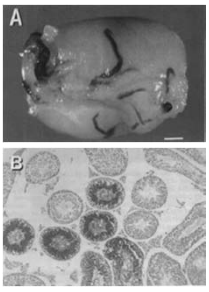


Spermiogenesis



Breakthrough I: mouse SSC transplantation

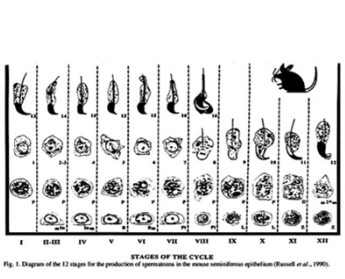


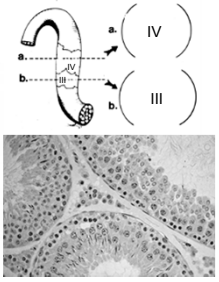


Brinster & Averbock, 1994

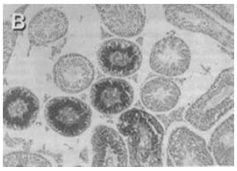
Brinster & Nagano, 1998

Germ cell associations in the mouse seminiferous epithelium



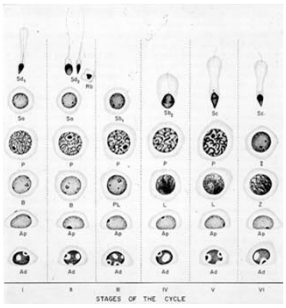


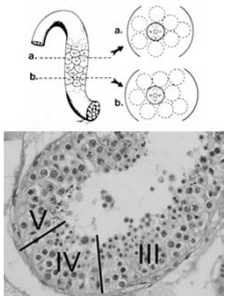
All associated germ cells stained blue



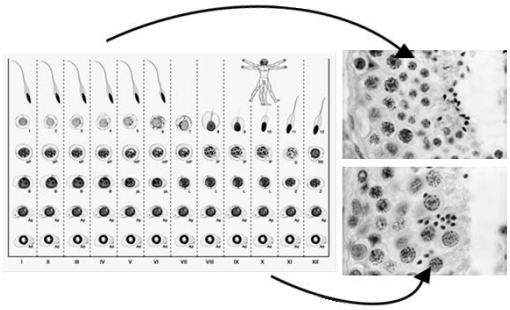
Brinster & Nagano, Semin Cell Dev Biol. 9, 401-409, 1998

Germ cell associations in human seminiferous epithelium





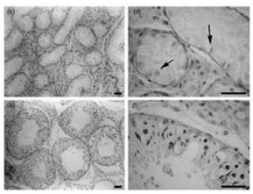
Germ cell associations in human seminiferous epithelium



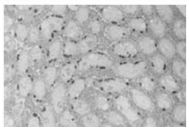
Muciaccia et al., 2013

SSC transplantation in various species

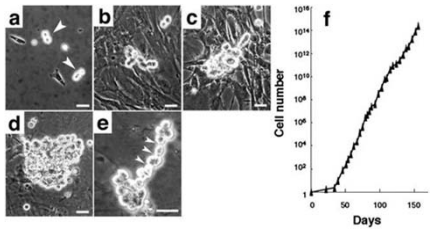
Autotransplantation:
Mouse to mouse
Bull to bull (Izadyar et al., Reproduction 2003)
Goat to goat (Honaramooz et al., Mol Reprod Dev 2003)
Rat to rat (Hamra et al., PNAS 2005)
Ram to ram (Ridriguez-sosa et al., Theriogenology 2006)
Dog to dog (Kim et al., Reproduction 2008)
Monkey to monkey (Herman et al., Cell Stem cells 2012)



Xenotransplantation:
Rat to mouse (Cloutier et al., Nature 1996)
Hamster to mouse (Ogawa et al., Biol Reprod 1999)
Rabbit/dog to mouse (Dobrinski et al., Biol Reprod 1999)
Baboon to mouse (Nagano et al., Biol Reprod 2001)
Bull to mouse (Izadyar et al., Reproduction 2002
Human to mouse (Nagano et al., Fert Steril 2002)

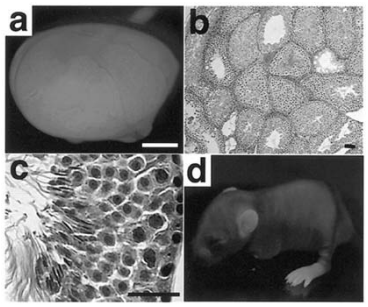


Breakthrough II: in vitro propagation of SSCs



Kanatsu-Shinohara et al., 2003

Spermatogenesis and offspring of transplanted cultured SSCs



Kanatsu-Shinohara et al, 2003

The human situation

Spermatogonial stem cells and future fertility

Clinical problem

Causes of male infertility

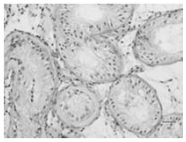
- Hyperprolactinemia
- Hypogonadotrophic hypogonadism
- Bilateral cryptorchidism
- Orchitis
- Genetic causes
  - Numerical and structural chromosome abnormalities
  - Y-chromosome deletions
- Previous chemo- or radiotherapy

Silber & Repping, 2002, Visser & Repping, 2010

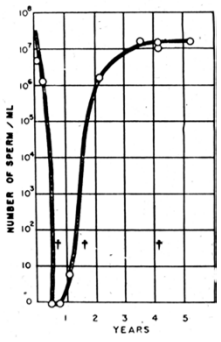
Clinical problem: loss of germ cells

Causes of male infertility

- Hyperprolactinemia
- Hypogonadotrophic hypogonadism
- Bilateral cryptorchidism
- Orchitis
- Genetic causes
  - Numerical and structural chromosome abnormalities
  - Y-chromosome deletions
- **Previous chemo- or radiotherapy**



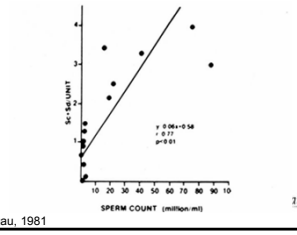
Fertility problems after irrradiation



Number of sperm in ejaculate after irradiation (human)

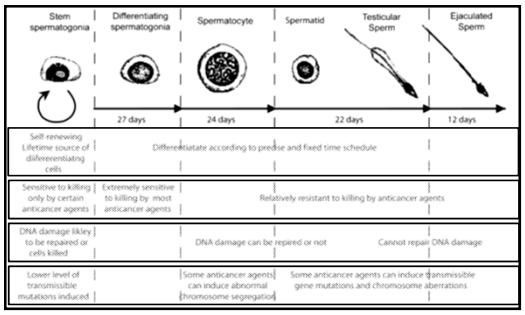
Recovery

- Difficult to predict
- May not occur at all
- May occur several years later
- May result in azoo- or oligozoospermia



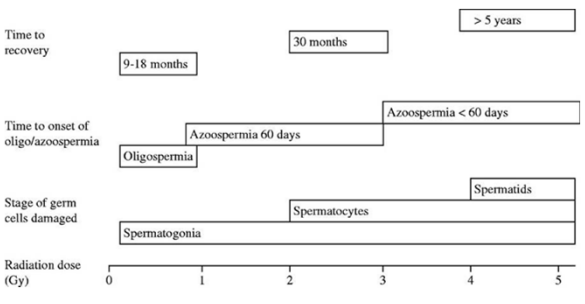
Silber & Rodriguez-Rigau, 1981

SSCs are sensitive for chemotherapy



Meistrich 2009

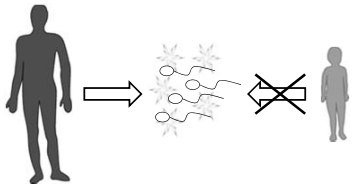
SSCs are sensitive for irradiation



Howell & Shalet, 2005

Fertility preservation

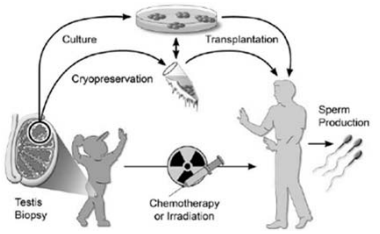
- Cryopreservatie of sperm before onset cancer treatment



For prepubertal boys with cancer there is no means to preserve fertility with sperm

Theoretical solution

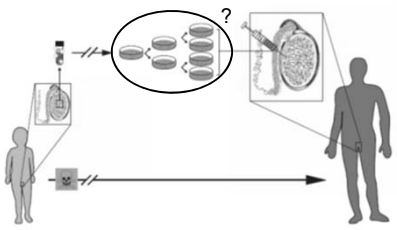
Cryopreservation of SSCs for later propagation and autotransplantation



Brinster, 2007

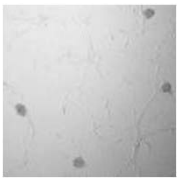
Translation to human

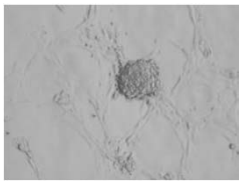
Propagation of human SSC



Culture of adult human testicular cells

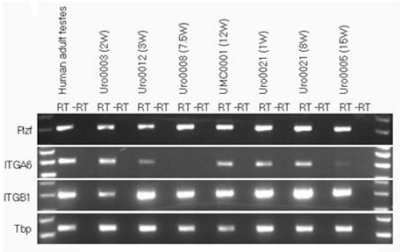
Establishment of human germ cell clusters in vitro





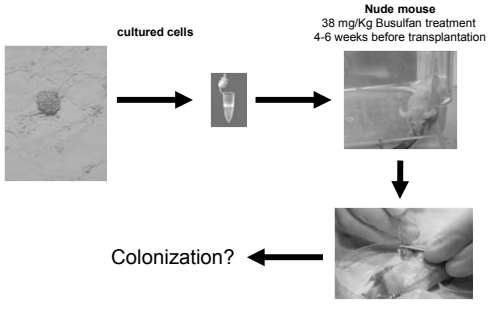
Sadri Ardekani et al., JAMA 302, 2127-2134, 2009

Expression spermatogonial markers

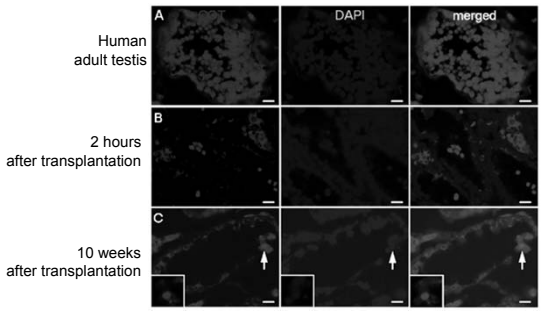


Sadri Ardekani et al., JAMA 302, 2127-2134, 2009

Xenotransplantation adult human SSCs



Human SSCs migrated after xenotransplantation



Human adult testis

2 hours after transplantation

10 weeks after transplantation

Sadri Ardekani et al., 2009

Xenotransplantation readout

Human Sample	Culture days	passage number	Number of injected cells(10^5)	Number of colonies /10^5 cells	Dilution factor	Human SSCs fold increase
Testicular cells culture						
UMC0001	63	4	1.3	0		
URO0003	14	1	3.5	0.7		
	14	1	0.2	12.5		
URO0005	14	1	2.7	0.9		
	42	3	0.3	0		
URO0008	28	3	0.7	3.6		
URO0012	21	1	0.6	0		
URO0021	28	3	0.1	0		
	56	7	0.4	0		
	28	2	2.55	2	↓ 133	53
	47	5	3.1	0.8		
GSCs subculture						
URO0005	91	6	2.5	0		
URO0021	77	7	2	1.25		
	84	8	0.5	5	↓ 8,870	18,450
	141	12	1.9	2.6		

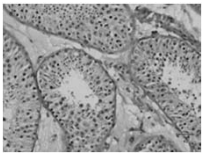
Successful propagation of adult human SSCs

Sadri Ardekani et al., 2009

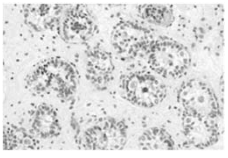
Adult vs prepubertal testis

Do prepubertal testicular cells also support SSC propagation in vitro?

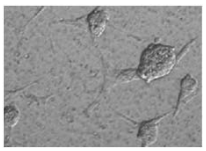
Adult

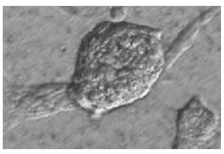


prepubertal



Prepubertal human testicular cell culture

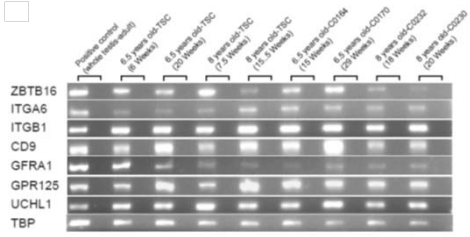




Sadri Ardekani et al., 2011

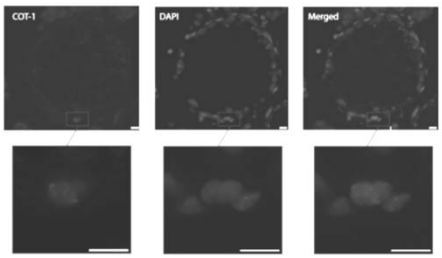
Long term culture of human spermatogonia

Expression of spermatogonial markers in cultured testicular cells



Sadri Ardekani et al., 2011

Xenotransplantation of prepubertal human SSCs



Successful prepubertal human SSCs migration after xenotransplantation

Sadri Ardekani et al., 2011

Xenotransplantation of prepubertal human SSCs

Patient I.D	Culture days (passage number)	Number of injected cells (10^5)	Number of colonies /10^5 cells	Dilution factor	Human SSCs fold increase
Testicular cells culture					
6.5 year old	70(5)	2.4	1		
	98(9)	3.6	0		
8 year old	46 (4)	2	0		
	63 (6)	5.1	0.5	↓ 1.2	9.6
	74 (7)	1.9	4		

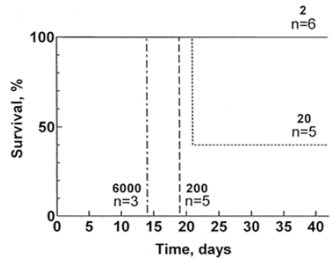
Successful propagation of prepubertal human SSCs

Sadri Ardekani et al., 2011

Leukemic patients

How to avoid risk of reintroduction of leukemic cells

Relapse intratubular transplantation of 20 leukemic cells



Jahnukainen K et al. 2001

Cancer cell elimination by FACS

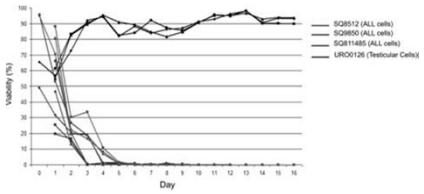
Quantitative assessment of tumor formation in recipient mouse testes

	Testis number (*n*)		Tumor formation [*n* (%)]	
Before sort[a]	**Intratubular**	**Interstitial**	**Intratubular**	**Interstitial**
Testis cells	29	n/a	0 (0%)	n/a
MOLT-4 cells	28	25	5 (18%)	18 (72%)
Testis cells + 10% MOLT-4 cells	32	26	13 (41%)	16 (62%)
After sort[a]				
EpCAM^{low}/CD49e^{+}/HLA-ABC^{-}	25	30	0 (0%)	0 (0%)
EpCAM^{+}/CD49e^{+}/HLA-ABC^{+}	22	29	5 (23%)	16 (55%)

[a]Unsorted (before sort) and sorted (after sort) cell fractions were transplanted into seminiferous tubules or interstitial space of recipient mouse testes. n/a, not applicable.

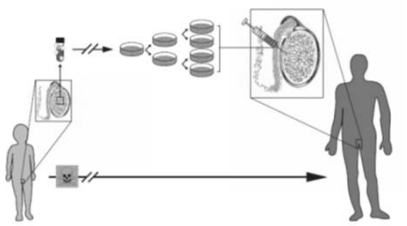
Dovey et al., 2013

Cancer cell contamination in culture



Sadri-Ardekani et al., 2014.

SSC autotransplantation: parents perspective



Retrospective opinion of the parents

- Van den Berg et al., Hum Rep 2007
  - Childrens Hospital Netherlands
  - 162 parents (median 7 years post-diagnosis)
  - 62% would have stored testicular biopsy
- Sadri-Ardekani, et al., Fert Steril 2013
  - Childhood Cancer Center Iran
  - 299 parents (children <12 year) (1 month to 19 years post diagnosis)
  - 54% would have stored a testicular biopsy

Retrospective opinion of the parents

- Decision depends on
  - Chance of infertility by chemotherapy
  - Chance that autotransplantation will be successful in the future
- What parents decide based on risks or chances of succes
  - Risk infertility ≥ 80% 65% would want a biopsy
  - Risk infertility ≥ 20% 35% would want a biopsy
  - Chance of success ≥ 80% 65% would want a biopsy
  - Chance of success ≥ 20% 26% would want a biopsy

Sadri Ardekani, et al., 2013

Summary SSC research

Year	Author	Highlighted findings	Species
1966	Clermont	Initial histological description of A_{pale} and A_{dark} spermatogonia	Human
1971	Huckins	Model for renewal and differentiation of spermatogonia and existence of 'spermatogonial stem cells' (SSCs)	Rat
1994	Brinster & Averbock	First successful transplantation of testis-derived cells from one mouse to another resulting in donor derived F1 progeny	Mouse
1998	Nagano *et al.*	*In vitro* maintenance of SSCs for 4 months on a somatic feeder layer	Mouse
1999	Schlatt *et al.*	Xenotransplantation of primate testis cell suspensions from one primate into the testes of another	Macaque
2002	Nagano *et al.*	First report on successful colonization of mouse testes after xenotransplanting human SSCs	Human
2003	Kanatsu-Shinohara *et al.*	Prolonged *in vitro* propagation of SSCs using GDNF, without immortalization of the cells in culture	Mouse
2005	Keros *et al.*	Proof of successful cryopreservation of testicular biopsies without decreasing structural integrity	Human
2005	Kanatsu-Shinohara *et al.*	Long-term propagation of SSCs under serum free and feeder free conditions	Mouse
2009	Sadri-Ardekani *et al.*	Long-term propagation of adult SSCs *in vitro* with retainment of functionality	Human
2011	Sadri-Ardekani *et al.*	Long-term propagation of prepubertal SSCs with retainment of functionality	Human
2012	Hermann *et al.*	Production of functional sperm by infertile prepubertal macaques after autotransplantation, capable of fertilizing oocytes	Macaque

Struijk et al., 2012

Conclusions

- Spermatogonia are extremely sensitive for killing by chemotherapy and irradiation.
- Cryopreservation of SSCs for later transplantation is the only option for prepubertal boys with cancer to preserve their fertility and parents are eager to preserve a testis biopsy from their son.
- SSCs (including those of human) can survive and proliferate in long term culture without losing their stem cell characteristics to migrate to their niche upon transplantation.
- ALL cells can be eliminated during culture of SSCs.

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- Hooman Sadri-Ardekani
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granted by: KiKa ZonMw

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ESHRE Annual Meeting 2014, Munich June 29-July 2, 2014
Pre-Congress Course 13
„Of stem cells and gametes: more similarities than differences"

Stem cell based approaches to restore spermatogenesis in monkeys

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Centre of Reproductive Medicine and Andrology
University Münster, Germany

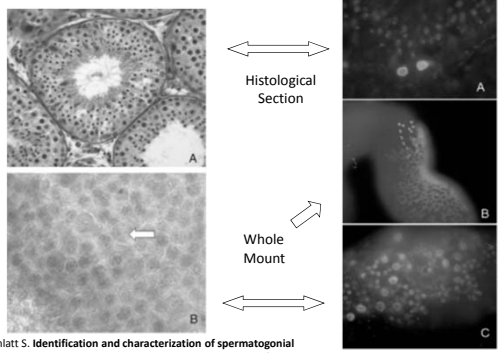
Disclosure

- Nothing to disclose
- I have no commercial or financial interests with manufacturers of pharmaceutical or laboratory supplies/medical devices

Learning Objectives

- Learning about primate specific features of spermatogonial stem cells (SSC)
- Recognize the central role of SSCs for male fertility
- Distinguish the potentials and risks of currently available and novel experimental strategies for male fertility protection
- Learn about perspectives of potential future cell based strategies for male germ cell development in vitro
- Provide clinical perspectives for establishing multidisciplinary programs on fertility preservation in boys

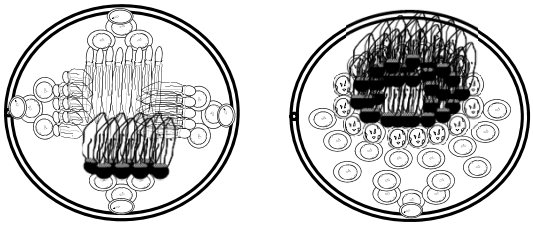
Observation of spermatogonia in primates



Ehmcke J, Schlatt S. **Identification and characterization of spermatogonial subtypes and their expansion in whole mounts and tissue sections from primate testes**. Methods Mol Biol 450:109-118 (2008)

Working Hypothesis

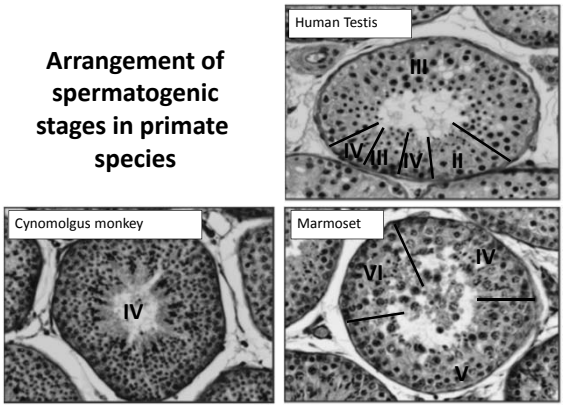
The extent of spermatogonial expansion determines the arrangement of spermatogenic stages



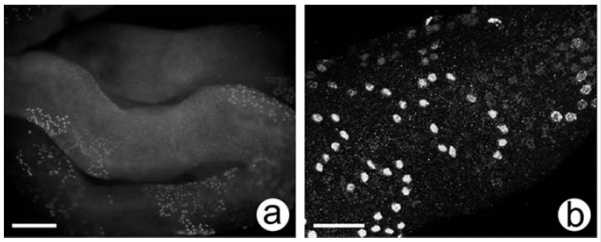
One mitotic division: Small clones
Man
Mixed stages

Four mitotic divisions: Large clones
Macaque
Longitudinal stages

Arrangement of spermatogenic stages in primate species



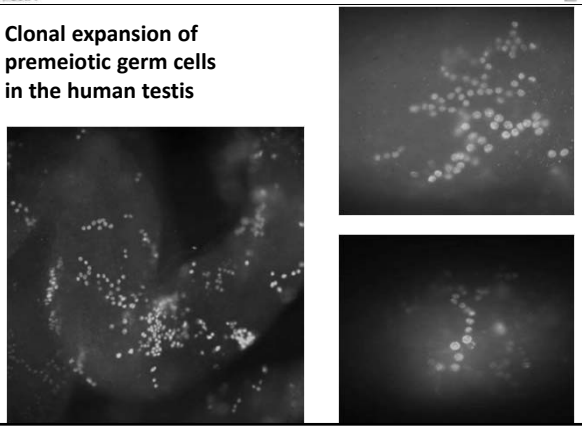
Confocal microscopy: clonal arrangement of spermatogonia



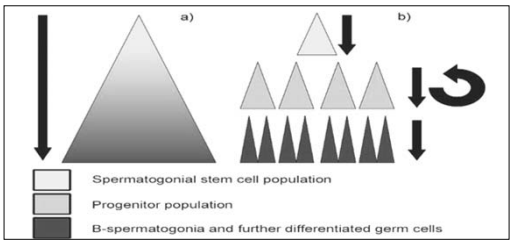
Immunohistochemistry:BrdU (green); acrosin (red)

Ehmcke J, Luetjens CM, Schlatt S**. Clonal organization of proliferating spermatogonial stem cells in adult males of two species of non-human primates, Macaca mulatta and Callithrix jacchus.** Biol Reprod 72: 293-300 (2005)

Clonal expansion of premeiotic germ cells in the human testis



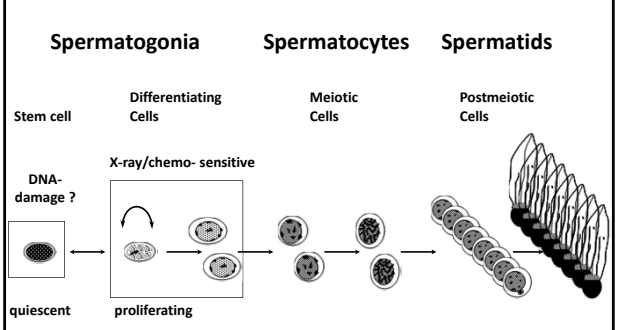
Species specific differences



- **Rodents**
  - **Very high mitotic expansion (one stem cell division generates several thousand sperm)**
  - **No self renewing progenitor cell under steady state conditions, small pool of mitotically active stem cells**
- **Primates**
  - **Low mitotic expansion (one stem cell division generates few sperm; human: 16, monkey: 256)**
  - **Self renewing progenitor cell and stem cell**

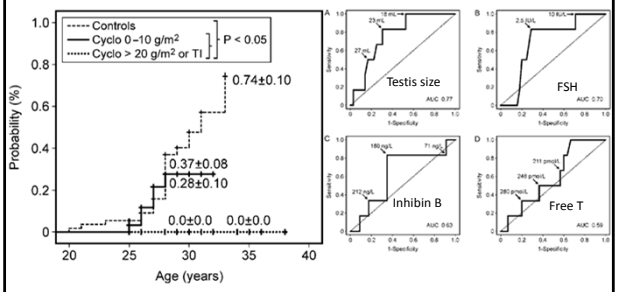
Ehmcke J, Schlatt S. A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132: 673-680 (2006)

Schematic Representation of Defects on Human Germ Cell Development After Oncological Therapy



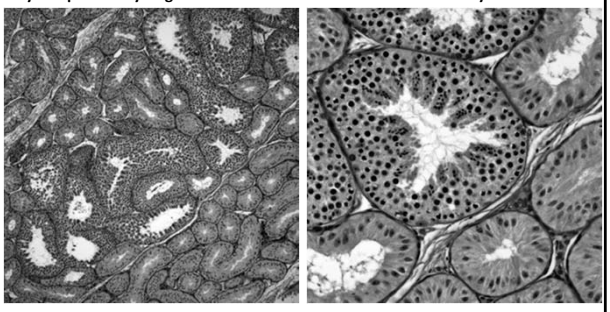
Recovery of fertility in boys after cancer therapy

(Data provided by Kirsi Jahnukainen, University of Helsinki)



Effects of testicular irradiation

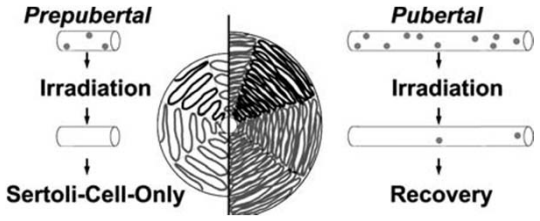
2 years post 10 Gy single irradiation in an immature rhesus monkey



Complete or focal absence of germ cells

Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates

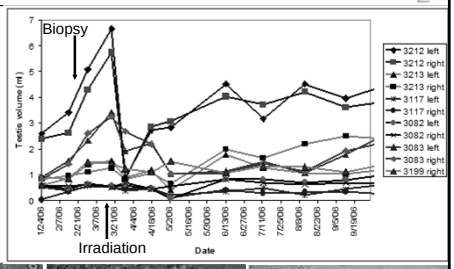
and can be enhanced by autologous germ cell transplantation.
Jahnukainen et al. Hum Reprod 26: 1945-1954 (2011)

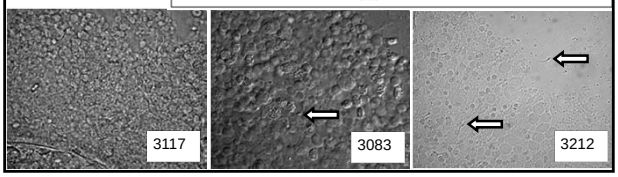


Testes from prepubertal monkeys were more radiosensitive than pubertal monkeys

Sperm in juvenile testes and radiation effects in prepubertal rhesus monkeys

single exposure, 10Gy



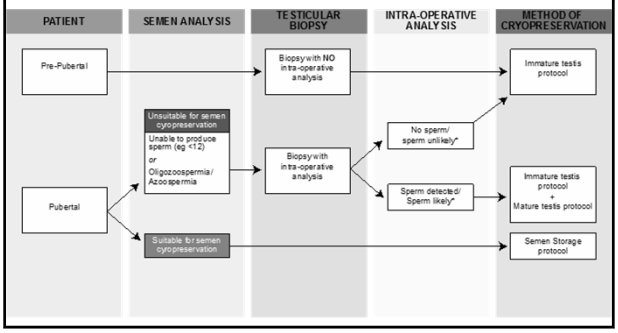


Prevention strategy:
Ex vivo maintenance of stem cells
Cryopreservation of immature testicular tissue

- Multidisciplinary team of clinicians (oncologist, paediatrician, urologist, andrologist, IVF group)
- Ethics Approval (Experimental procedure)
- Unilateral open testicular biopsies (several incisions)
- Dissection into small tissue fragments (<1 mm^3)
- No enzymatic digestion
- Cryopreservation using DMSO (1.4M) as protectant
- Slow Freezing Protocol

ESHRE Task Force for Fertility Preservation in Severe Diseases

Male Subgroup: A European Perspective On Testicular Tissue Cryopreservation For Fertility Preservation In Prepubertal And Adolescent Boys



Male Fertility Preservation
European Research Networks and Clinical Service Activities

Androprotect

FertiProtect (Germany)

PROSPERMA in CECOS
(Centre d`Etude et de Conservation des Oeufs et du Sperme humains)
GRECOT
(Group de recherche sur la conservation du tissue ovarien et testiculaire
(France)

Nordic Network for Fertility Preservation in Children
(Norway, Sweden, Denmark)

European Academic Medical Centres holding cryobanked immature human testes from oncological patients

ZU Brussels, Belgium
Catholic Univ, Brussels, Belgium
Rigshospitalet Kopenhagen, Denmark
Hopitaux de Rouen, France
CeRA, Germany
AMC, Amsterdam, Netherlands
Karolinska University Hospital, Sweden
University of Edinburgh, UK

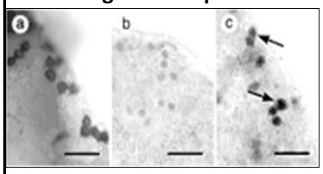
How to generate sperm from the cryopreserved tissue?

- Germ cell transplantation
- Testicular Grafting
- *In vitro* spermatogenesis

Development of Germ Cell Transplantation

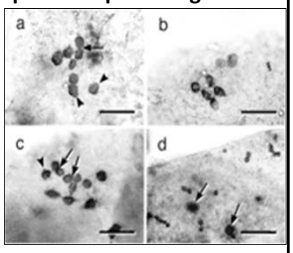
- **1994** **Spermatogenesis following male germ cell transplantation.** (Brinster and Zimmermann, PNAS 91: 11298)
- **1996** **Rat spermatogenesis in mouse testis**. (Clouthier et al., Nature 381: 418)
- **1996** **Reconstitution of** spermatogenesis **from frozen spermatogonial stem cells**. (Avarbock et al., Nat Med 2: 693)
- **1998** **Culture of mouse spermatogonial stem cells**. (Nagano et al., Tissue & Cell 30: 389)
- **1999** **Germ cell transfer into rat, bovine, monkey and human testes.** (Schlatt et al., Hum Reprod 14: 144)
- **2001** **Primate spermatogonial stem cells colonize mouse testes**. (Nagano et al., Biol Reprod 64: 1409)
- **2001** **Transgenic mice produced by retroviral transduction of male germline stem cells.** (Nagano et al. PNAS 98:13090)
- **2002** **Germ cell transplantation into X-irradiated monkey testes.** (Schlatt et al. Hum Reprod (17: 55)

Xenologous transplantation of primate spermatogonia



Baboon germ cell colonising mouse testes

Nagano et al., 2001 Primate spermatogonial stem cells colonise mouse testes. Biol Reprod 64: 1409-1416



Baboon germ cell colonising mouse testes after cryopreservation

Nagano et al., 2002 Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78: 1225-33

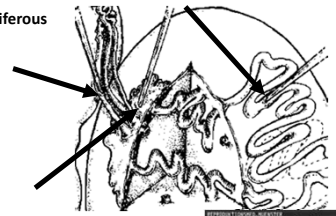
Colonisation of primate spermatogonia in mouse testes

Germ Cell Infusion into the Primate Testis

Microinjection of seminiferous tubules: difficult and inefficient

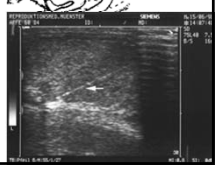
Injections into efferent ducts: surgically demanding and inefficient

Injections into the rete testis: Easy, efficient and reproducible

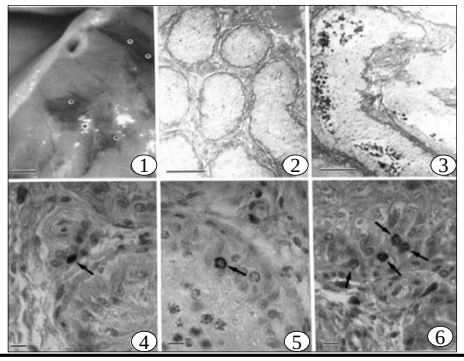


Involuted recipient testis

Ultrasound guidance

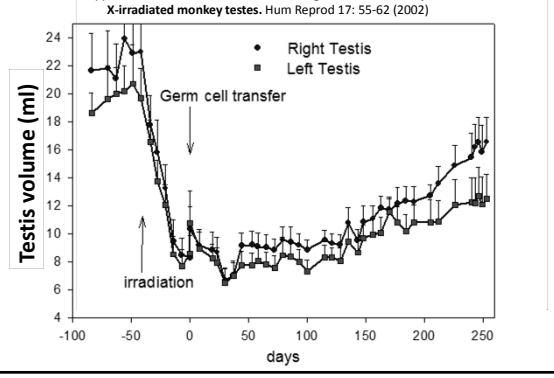


Germ Cell Transfer into Rat, Bovine, Monkey and Human Testes Schlatt et al., Hum Reprod 14: 144-150 (1999)



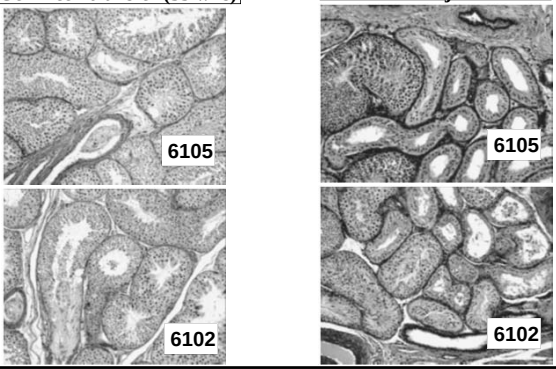
Testis Volume Following Irradiation and Germ Cell Transfer

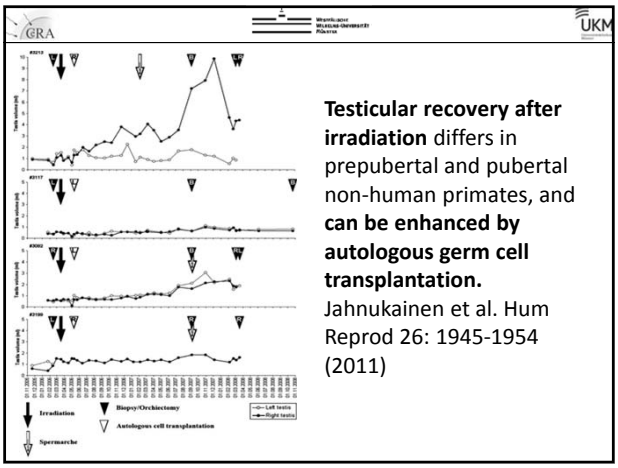
Schlatt S, Foppiani L, Rolf C, Weinbauer GF, Nieschlag E. **Germ cell transplantation into X-irradiated monkey testes.** Hum Reprod 17: 55-62 (2002)



Right testis
Germ cell transfer (35 wks)

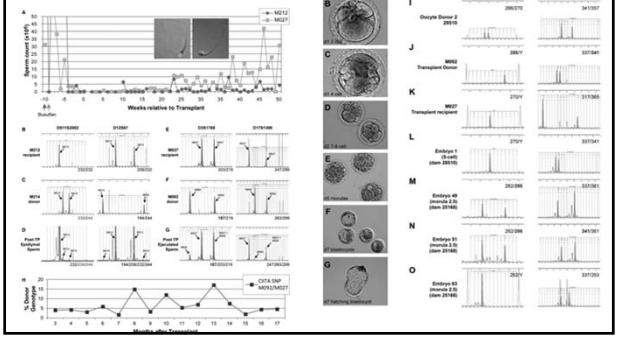
Left Testis
Saline Injection

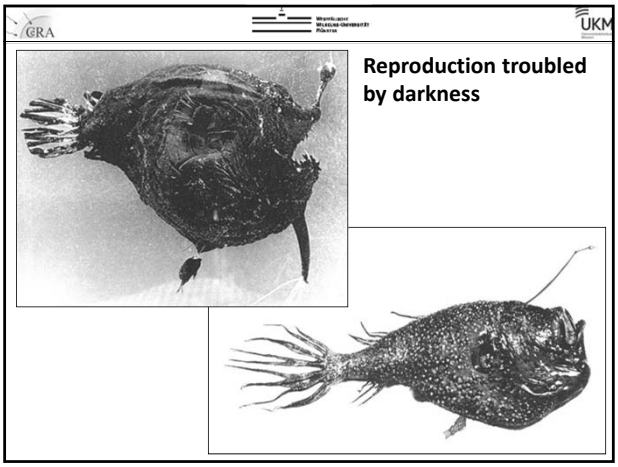




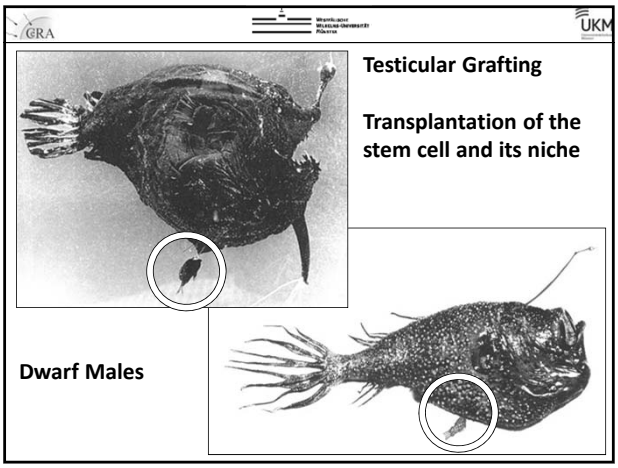
Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and **can be enhanced by autologous germ cell transplantation.**
Jahnukainen et al. Hum Reprod 26: 1945-1954 (2011)

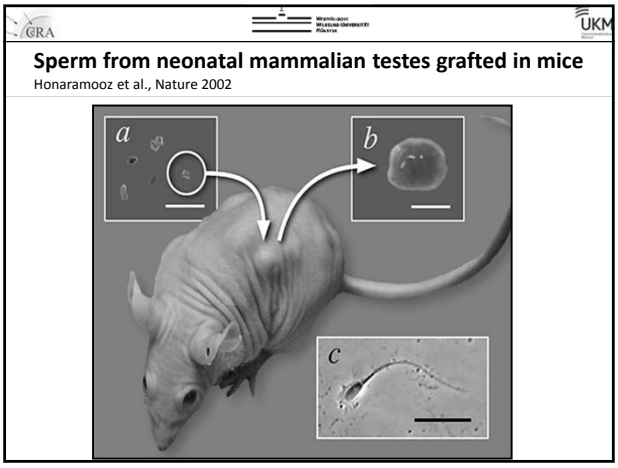
Spermatogonial stem cell transplantation into Rhesus testes regenerates spermatogenesis producing functional sperm.
Hermann et al. Cell Stem Cell 11: 715-726 (2012)

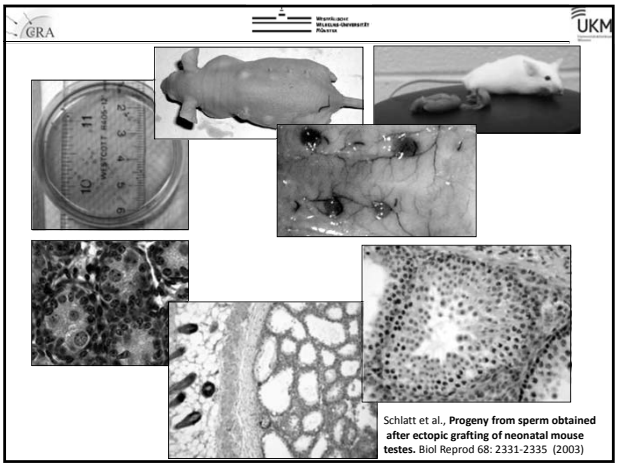




Reproduction troubled by darkness

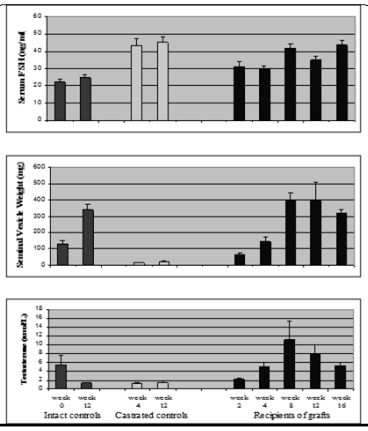

Testicular Grafting
Transplantation of the stem cell and its niche
Dwarf Males

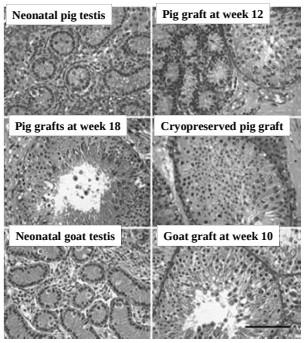

Sperm from neonatal mammalian testes grafted in mice
Honaramooz et al., Nature 2002
a
b
c


Schlatt et al., Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod 68: 2331-2335 (2003)

Endocrine response of castrated nude mice after grafting:

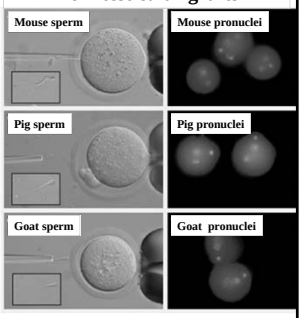
Substitution of androgens and suppression of FSH.





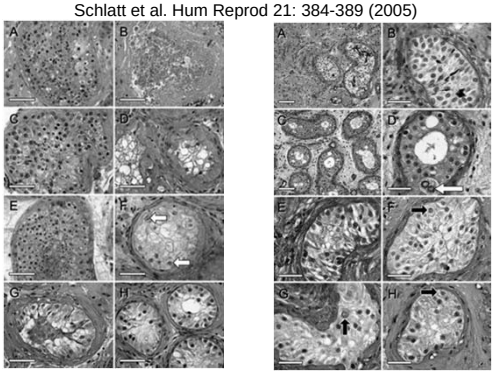
Histology of grafts from pig and goat testis

Sperm morphology and oocyte-activating competence of sperm from testicular grafts



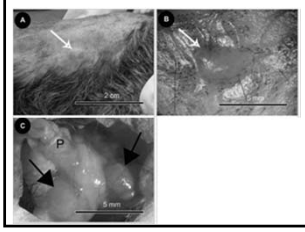
(All oocytes are from mice)

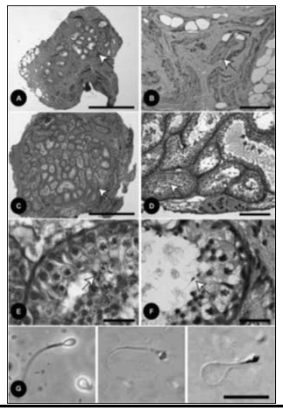
Limited survival of adult human testicular tissue as ectopic xenograft
Schlatt et al. Hum Reprod 21: 384-389 (2005)



See also: Geens et al. **Spermatogonial survival after grafting human testicular tissue to immunodeficient mice**. Hum Reprod 21: 389-395 (2005)

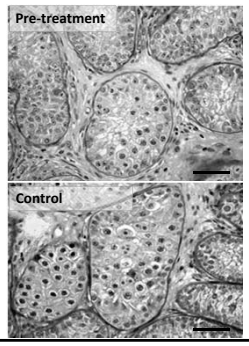
Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue Luetjens et al. Endocrinology 149: 1736-1747 (2008)

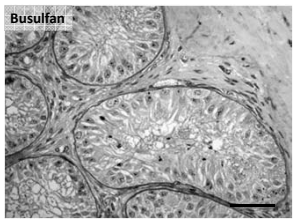




Testicular xenografts: A novel approach to study cytotoxic damage in juvenile primate testis

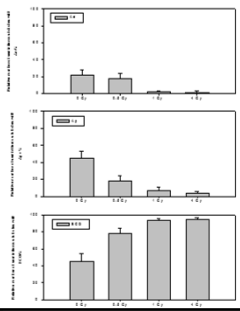
Jahnukainen et al., Cancer Research 66: 3813-3818 (2006)

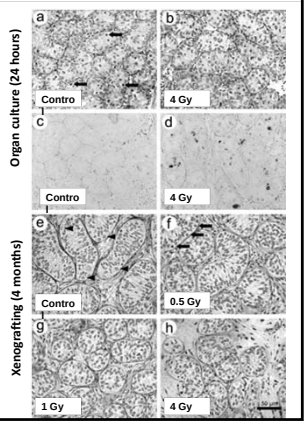




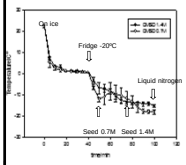
Conclusion: Xenografted monkey testis tissue shows the same changes to toxins as intact testes.

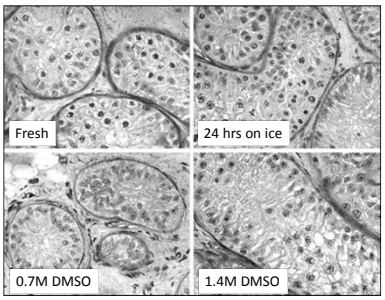
Irradiation causes acute and long-term spermatogonial depletion in cultured and xenotransplanted testicular tissue from juvenile non-human primates Jahnukainen et al., Endocrinology 2007; 148: 5541-5548





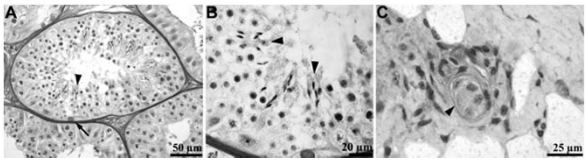
Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts.
Jahnukainen et al., Hum Reprod. 2007; 22:1060-7



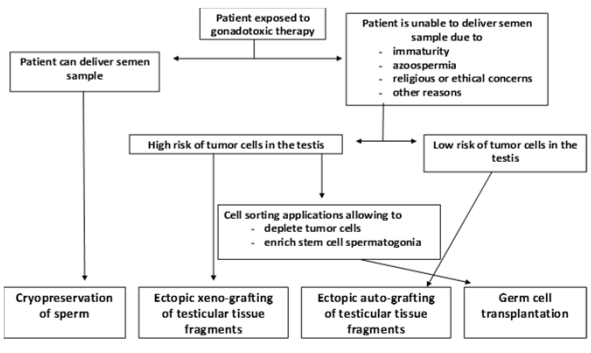


	Survival grafted/ recovered	Graft weight mg	Grafts with A-spg	Grafts with B-spg	Grafts with spct or sptd*
Ctrl	51% 33/64	5.4±0.5	100%	79%	12%
Fresh	52% 14/27	7.3±1.2	100%	100%	36%
DMSO	26% 6/23	4.5±0.9	83%	67%	0%
EG	7% 2/27	6.5±1.5	100%	100%	0%
Without	0% 0/23	-	-	-	-

Fertility preservation after sterilizing therapy through autologous ectopic grafting of cryopreserved testicular tissue in prepubertal monkeys
Jahnukainen et al., Cancer Research 72: 5174-78 (2012)



Autografting into scrotal area: sperm production (many grafts!)
Xenografting or ectopic grafting: no full spermatogenesis



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Eberhard Nieschlag

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UPCOMING ESHRE EVENTS

// ESHRE CAMPUS EVENTS

ESHRE's 30th Annual Meeting

www.eshre2014.eu

Munich, Germany
29 June - 2 July 2014

Epigenetics in reproduction

www.eshre.eu/lisbon

Lisbon, Portugal
26-27 September 2014

Endoscopy in reproductive medicine

www.eshre.eu/endoscopyoct

Leuven, Belgium
15-17 October 2014

Making OHSS a complication of the past: State-of-the-art use of GnRH agonist triggering

www.eshre.eu/thessaloniki

Thessaloniki, Greece
31 October-1 November 2014

From gametes to blastocysts – a continuous dialogue

www.eshre.eu/dundee

Dundee, United Kingdom
7-8 November 2014

Controversies in endometriosis and adenomyosis

www.eshre.eu/liege

Liège, Belgium
4-6 December 2014

Bringing evidence based early pregnancy care to your clinic

www.eshre.eu/copenhagen

Copenhagen, Denmark
11-12 December 2014

An update on preimplantation genetic screening (PGS)

www.eshre.eu/rome

Rome, Italy
12-13 March 2014

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or contact us at info@eshre.eu

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