PRE-CONGRESS COURSE 10

Pluripotent stem cells, cancer and fertility preservation: science fact or science fiction?

Special Interest Group Stem Cells and Task Force Fertility Preservation in Severe Diseases
London - UK, 7 July 2013

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Pluripotent stem cells, cancer and fertility preservation: science fact or science fiction?

London, United Kingdom
7 July 2013

Organised by
The ESHRE Special Interest Group Stem Cells and the Task Force Fertility Preservation in Severe Diseases

Contents

Course coordinators

Rita Vassena (Spain), Anis Feki (Switzerland), Helen Picton (UK) and Karen Sermon (Belgium)

Course description

This advanced course will try to unveil the link between pluripotent stem cells and cancer, and how this has repercussions on fertility preservation. The course will cover the current knowledge on stem cells in gonads, how these stem cells are related to cancer of reproductive organs and how this affects cancer treatment as well as infertility treatment

Target audience

Stem cell biologists, fertility specialists with an interest in fertility preservation after cancer treatment

Scientific programme

Chairman: Anis Feki - Switzerland
Chairman: Helen M. Picton - United Kingdom

09:00 - 09:30	Stem cells in ovarian tissue- the case for and against *Evelyn E. Telfer - United Kingdom*
09:30 - 09:45	Discussion
09:45 - 10:15	Female fertility preservation and reproductive outcome *Claus Yding Andersen - Denmark*
10:15 - 10:30	Discussion
10:30 - 11:00	Coffee break

Chairman: Rita Vassena - Spain
Chairman: Karen Sermon - Belgium

11:00 - 11:30	Stem cells in testis and their role in fertility preservation *Ans van Pelt - The Netherlands*
11:30 - 11:45	Discussion
11:45 - 12:15	Advances in male germ cells preservation and transplantation *Christine Wyns - Belgium*
12:15 - 12:30	Discussion
12:30 - 13:45	Lunch

Chairman: Anis Feki - Switzerland
Chairman: Rita Vassena - Spain

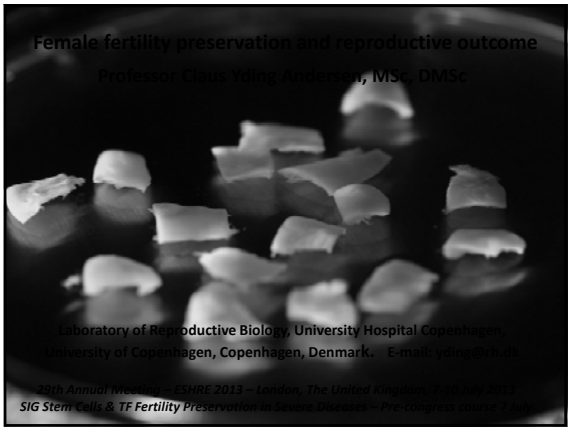
13:45 - 14:20	Epigenetics of pluripotent cells *John Huntriss - United Kingdom*
14:20 - 14:40	Discussion
15:00 - 15:30	Coffee break

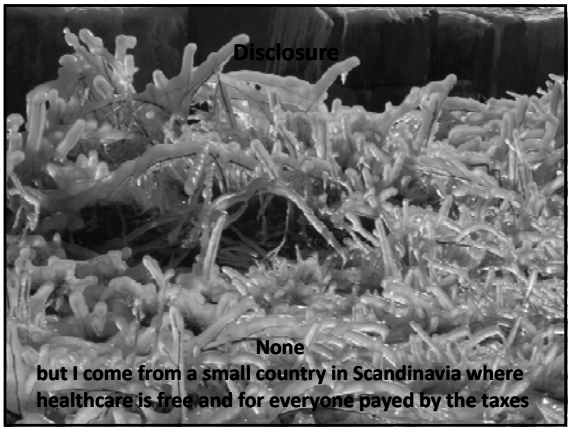
Chairman: Helen M. Picton - United Kingdom
Chairman: Karen Sermon - Belgium

15:30 - 16:00	Stimulation protocols in cancer patients *Juan Garcia Velasco - Spain*
16:00 - 16:15	Discussion
16:15 - 16:45	Cancer stem cells and their role in male germline cancers *James Korkola - U.S.A.*
16:45 - 17:00	Discussion
17:00 - 18:00	Business meeting of the SIG Stem Cells

Stem cells in ovarian tissue- the case for and against – **Evelyn E. Telfer (United Kingdom)**

Contribution not submitted by the speaker





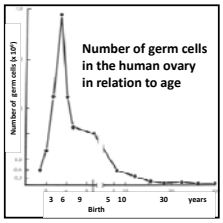
Outline

- Why focus of fertility preservation
- Fertility preservation options available
- Freezing of MII oocytes from cancer patients
- Freezing ovarian tissue including transport of tissue
- Experience with transplantation of frozen/thawed ovarian tissue
- Safety of transplanting ovarian tissue

29th Annual Meeting – ESHRE 2013 – London, The United Kingdom, 7-10 July 2013
SIG Stem Cells & TF Fertility Preservation in Severe Diseases – Pre-congress course 7 July

Why focus on fertility presevation

- Survival rates among young cancerpatients have increased significantly during recent years and is usually around 80%
- Modern treatment regimes bears a high risk of gonadotoxic effects
- The cancer patients want it
- Technical developments have made fertility preservation a realistic option



Patient with breast cancer asking for advise

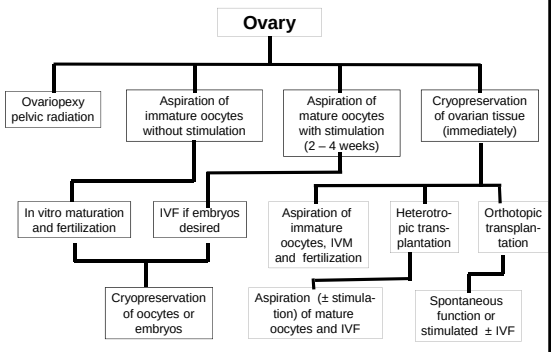
March 15th 2010 – letter from a Serbian woman

"Prior to exposure to chemotherapy and radiology therapy I wish to save my genetic material in order to use the same when restored to health. This is my one and only treasure and presently the main, if not the only, reason for fighting this illness"

Danish patient having tissue transplanted (June 2012)

"Having back my menstrual cycles and being a woman again was as good as having my hair back after having completed chemotherapy"

Fertility preservation options in female cancer patients



Cryopreservation of mature oocytes for fertility preservation

Ovarian response to stimulation for fertility preservation in women with malignant disease: a systematic review and meta-analysis

TABLE 1

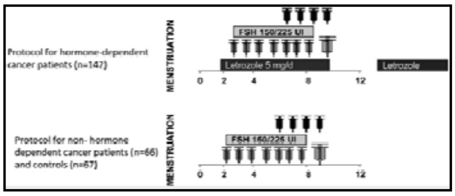
Patients' age and malignancies, and indication for in vitro fertilization in the controls.

Study	No. of patients Study (N = 216)	No. of patients Control (N = 153)	Age (mean ± SD) Study	Age (mean ± SD) Control	Breast cancer	Lymphoma	Leukemia	Gynecologic cancer Ovarian	Gynecologic cancer Uterine	Gynecologic cancer Cervix	Other malignancies[a]	Indication for IVF/ICSI in controls Male factor	Indication for IVF/ICSI in controls Tubal
Pal et al., 1998 (24)	5	12	31 ± 2	32 ± 1						3	2		12
Oktay et al., 2006 (28)	47	56	36 ± 3.6	36 ± 3.9	47								56
Knopman et al., 2009 (34)	28	135	34 ± 5.1	32 ± 5	10	4	1	4	4	1	4	135	
Klock et al., 2010 (35)	28	57	31 ± 5.3	31 ± 4.6	11	4	3	1			9	57	[illegible]
Quintero et al., 2010 (36)	50	50	32 ± 5	32 ± 5	28	11	8				11	50	
Michaan et al., 2010 (37)	22	22	32 ± 8	34 ± 4.2	12		2				8		22
Robertson et al., 2011 (33)	38	921	34 ± 5	35 ± 4	16	9	9	3	1	1	11	921	

- ❖ The lenght of stimulation was 10.9 days for both groups
- ❖ The number of MII oocytes was 9.0 (1.8 reduced) in the cancergroup
- ❖ The incidence of low response were 8% (no definition)
- ❖ 20 patients received ET from frozen/thawed oocytes:
  10 deliveries, two ongoing, one ectopic and one biochemical

Friedler S. et al., *Fertil Steril*. 2012;

Ovarian response to COH in cancer patients is diminished even before oncological treatment



Patient demographics according to the type of stimulation.

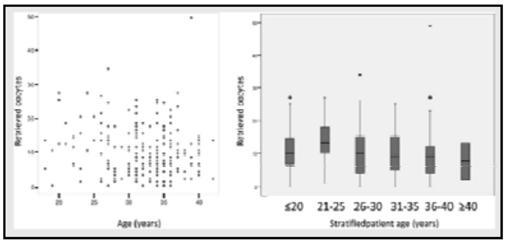
	Non-HD, antagonist FSH (n = 66)	HD, letrozole FSH (n = 142)	Control (n = 97)
Age	30.6 ± 5.7	33.2 ± 4.3	31.9 ± 5.3
Days of stimulation	8.7 ± 1.7*	9.6 ± 2.4	9.9 ± 1.6
Total FSH, IU	1,803 ± 889	1,755 ± 1,114	1,947 ± 808
Peak serum E_2, pg/mL	1,744 ± 1,242	381 ± 191*	2,109 ± 1,260
Retrieved oocytes	12.2 ± 6.5	9.8 ± 7.1*	12.4 ± 5.4
% MII oocytes	75.3 ± 18.5	74.4 ± 22.1	74.2 ± 17.7

* P< .05.

Domingo. Fertility preservation in cancer patients. Fertil Steril 2012.

Domingo J. *et al., Fertil Steril* 2012;**97**:930

Outcome of controlled ovarian stimulation in cancer patients



- ❖ On average a total of 7.8 MII oocytes were obtained in cancer patients
- ❖ 21% had fewer than 5 oocytes and 40% had fewer than 8 oocytes
- ❖ Cancellation rate was 2.5%
- ❖ The mean time to start stimulation was 9.5 days from the first consultation
- ❖ The mean lenght of stimulation was 9.1 days

Domingo J. *et al., Fertil Steril* 2012;**97**:930

Cryopreservation of ovarian tissue

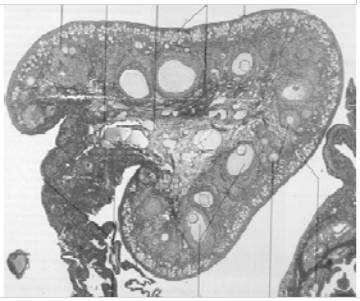
Advantages:

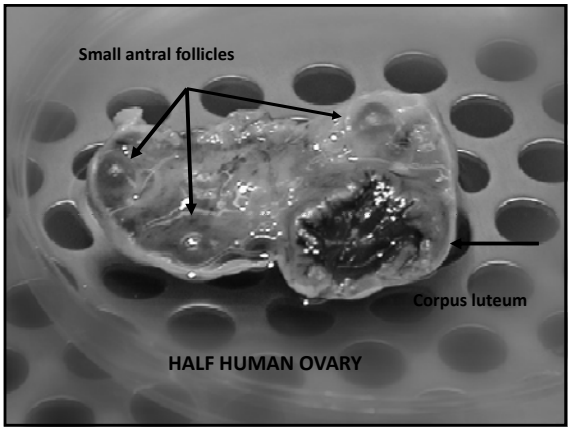
- Available on a short notice
- Preserves the functional unit of the ovary – the follicle
- Preserves potentially a large number of follicles
- Only option available for prepubertal girls

Limitations:

- Experimental and the efficacy is unknown
- Risk of transplanting the original disease
- Functional duration of the transplants

Only the ovarian cortex is cryopreserved





Preparation of human ovarian tissue for cryopreservation

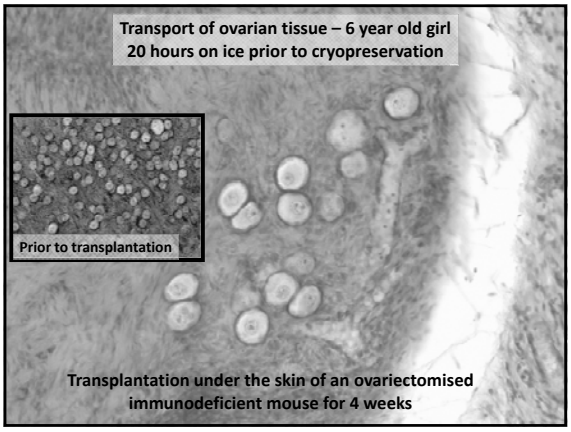



Centralised Service in Denmark

Transport Ovarian Cryopreservation

- The woman receives gonadotoxic treatment at the local hospital
- Ovarian tissue is removed at the local hospital and transported to a central laboratory where cryopreservation and storage is performed
- Cryostored ovarian tissue is transported to the local hospital for transplantation

Transport of ovaries for cryopreservation within Denmark for 4 – 5 hours



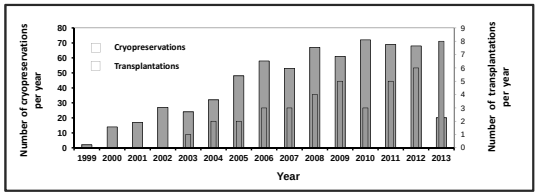
Diagnosis for cryopreservation of ovarian tissue in Denmark: cummulative (January 2013)

Diagnosis	No.	Diagnosis	No.
Mammary cancer	170	Emdometriosis	1
Mb. Hodgkin	96	Turner syndrome	4
Non-Hodgkin	17	BRAC-gen	2
Leukaemia (AML, ALL, CML)	52	Aplastic Anaemia	11
Ewing & Synovial sarcoma	56	Autoimmune (SLE)	7
Ovarian & cervical cancer	31	Thallasaemi	4
Lymphoma	17		
Other malignant diseases	49	Others diseases	55

Age distribution of girls/women having ovarian tissue cryopreserved in Denmark

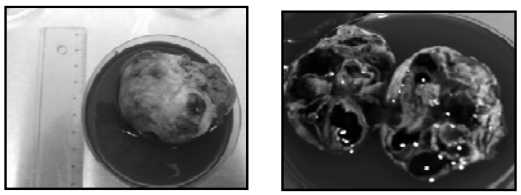
Age (years)	0–5	5–10	10–15	15–20	20–25	25–30	30–35	35-40
No. pt.	22	28	39	75	87	143	131	43
Mean no. of cortex	9	12	18	23	23	24	26	25
Range	4-18	3-22	1-37	11-47	6-43	2-69	3-56	10-42
Mean Ovarian volume (ml)	1,1	1,5	3,2	6,6	6,7	7,0	8,6	7,2

Annual activity of cryopreservation and transplantation of ovarian tissue in Denmark



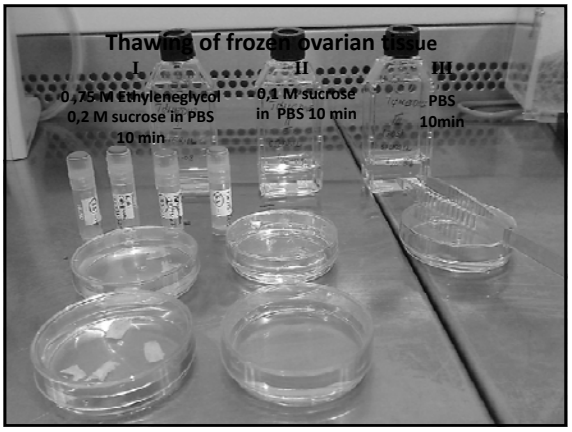
Around 14 cases per million inhabitants receive freezing per year

Difficult to perform ovarian stimulation and cryopreservation simultaneously

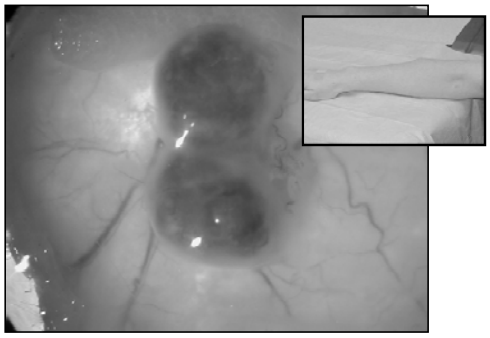


Enlarged ovary two days after oocyte retrieval

Focus the light on transplantation of ovarian tissue

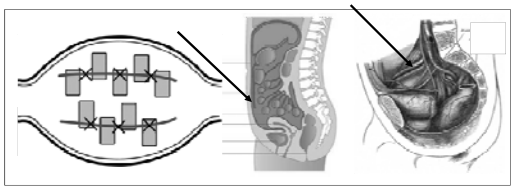


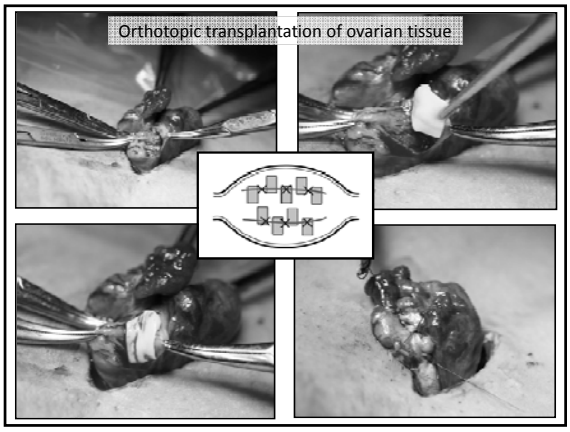
Frozen thawed human ovarian cortex implanted under the skin of ovariectomized SCID mice for 4 weeks

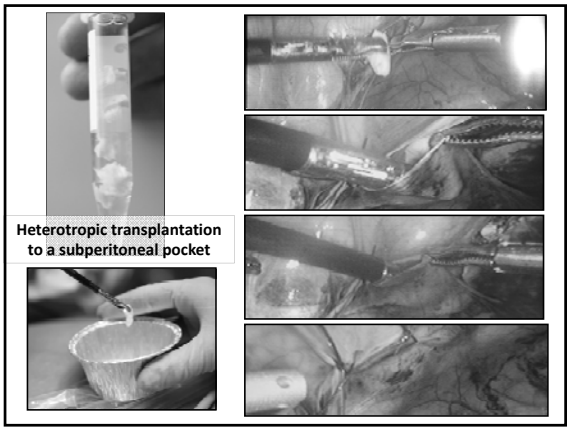


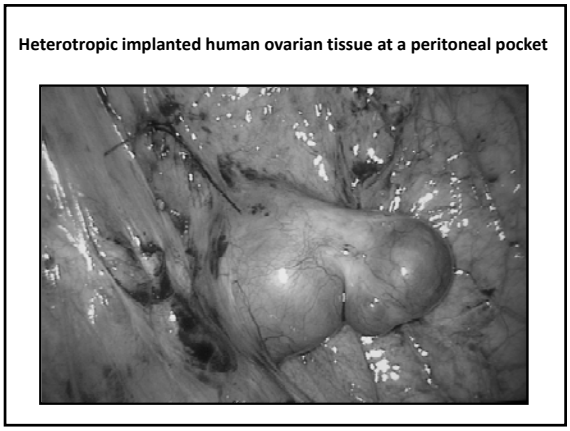
Clinical results - transplantation

- Laparoscopy / mini-laparotomy
- Orthotopic (ovary)
- Heterotopic (sub-peritoneal on anterior abdominal wall and lateral pelvic wall)




Orthotopic transplantation of ovarian tissue


Heterotropic transplantation
to a subperitoneal pocket


Heterotropic implanted human ovarian tissue at a peritoneal pocket

Transplantation of frozen/thawed tissue in Denmark (March 2013)

- **In Denmark: 29 women/girls have been transplanted with frozen/thawed ovarian tissue a total of 39 times**
- **Transport ovarian cryopreservation: 18 women/girls have been transplanted a total of 28 times**

No relapse due to the ovarian graft
The tissue have started to work in each individual case

Results of transplanting frozen/thawed ovarian tissue in Denmark

Transport:

☆ **Four children born (two women)**
- **One ongoing pregnancies**
- **Two legal abortions (natural)**
- **Three clinical pregnancies (abortion)**

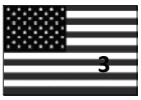
Immediately:
- **One ongoing pregnancy**
- **One clinical pregnancy (IVF)**
- **Two biochemical pregnancies (heterotropic transplants)**

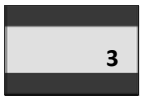
Diagnosis for transplantation of frozen/thawed ovarian tissue in Denmark

Diagnosis	Number
Hogdkin's lymphoma	7
Breast cancer	9
Non-Hodgkin's lymphoma	2
Ewing's sarcoma	2
Aplastic anaemia	1
Cervical cancer	1
Various others	6

Children born from transplantation of frozen/thawed ovarian tissue

All slow freezing
12 girls ; 8 boys
Spontaneous (n=13) vs IVF (n=7)
Normal weight and duration
Orthotopic >> heterotopic





Two ongoing pregnancies: one in South Africa and one in Australia

AMH levels in women transplanted with frozen/thawed ovarian tissue

	Age at cryo (years)	Months since transplantation	AMH (ng/ml)	Menstrual cycles	Overall highest AMH (ng/ml)
1	32	53	0.1	Yes	0.1
2	28	5	0.2	Yes	0.2
3	26	12	< Detection	Yes	0.6
4	27	57	< Detection	Yes	
5	27	15	0.1	Yes	0.1
6	19	42	< Detection	Yes	0.82
7	25	17	< Detection	Yes	0.06
8	26	24	< Detection	Yes	0.39
9	9	28	< Detection	Yes/no	
10	23	26	< Detection	Yes	
11	34	11	< Detection	Yes	
12	30	7	< Detection	Yes	

Greve T *et al., Fertil. Steril.*, 2012:**97**:1394

Longevity of frozen/thawed ovarian tissue transplanted to Danish women (July 2013)

Age at cryo	First Transplant			Second transplant			Remai-ning
	Pieces	Month	Still work	Pieces	Month	Still work	Pieces
32 (1)	6	56	No	12	25	No	15
28 (2)	12	54	No	8	53	Yes	7
25 (3)	12	26	No	6	45	No	0
26 (4)	10	15	Yes	12	85	Yes	10
31 (7)	8	25	Yes	8	21	Yes	4
25 (11)	7	58	Yes				10
19 (10)	12	63	Yes				24
27 (8)	6	91	Yes				7

The amount of tissue transplanted is depended on:

1. **Fertility restoration**
2. **Endogenous hormone production**
3. **Upper age limit?**

Instead of transplanting around 1/3 of an ovary we are now graft at least one half: two out of four became pregnant shortly after the tissue had regained function

We suggest to freeze one whole ovary in case of fertility restoration

Danish patient having tissue transplanted (June 2012)

"Having back my menstrual cycles and being a woman again was as good as having my hair back after having completed chemotherapy"

"Having my tissue transplanted made me feel like a whole woman again"

Follow-up study of women having one ovary removed for fertility preservation

- **143 women unilateral oophorectomy (>18 years; >24 months from excision; 78% participation)**
- **Mean follow-up time 58 months (24-129);**
- **80% confirmed they wanted to use the tissue if necessary**
- **31/143 (22%) were parous prior to freezing**
- **57 women had attempted to become pregnant – 41 (72% succeeded); 5 additional unwanted pregnancies**
- **84 had not yet a pregnancy wish (23% still on medication or advised against it)**

We do no harm and a number of these women may utilise their tissue to enter menopause at a normal age

Schmidt KT *et al., RBMOnline* (in press)

We do no harm and provide reassurance

A number of these women have not yet entered menopause, but their ovarian reserve is dimished and they may need their tissue in order to avoid entering menopause too early

The actual utilisation rate requires long term follow-up studies, which we in Denmark – due to our personal number system – is well suited to undertake.

Factors affecting reproductive outcome and efficacy

- **Young women have tissue transplanted to obtain menstrual cycles**
- **Women may devorce their partner after transplantation**
- **Women may have a relapse after transplantation**
- **Their life situation may change – looking into the future**
- **Some women have too little tissue stored to provide a good of fertility**

The true efficacy of transplanting ovarian tissue is currently not known, it is not high and will certainly be improved

This is still early days

CONCLUSIONS

- **Ovarian cryopreservation, including transportation, is now a clinical option**
- **Transplanted frozen/thawed tissue restores ovarian function with high efficacy and maintain function for periods of time a lot longer than expected**
- **This procedure is important to women and we don't do harm by taking out ovarian tissue**
- **Transplanted tissue restore fertility but the efficacy is probably not high, but perhaps refinements are slowly being developed**
- **Results are encouraging for a continued effort**

Collaborators

University Hospital of Copenhagen:
- Anders Nyboe Andersen
- Anne Loft
- The Fertility Clinic
- Christian Ottesen
- Department of Gynaecology

University Hospital of Aarhus:
- Erik Ernst
- Margit Dueholm
- Ditte Trolle
- Sara Markholt
- The Fertility Clinic
- Department of Gynaecology

Odense University Hospital
- Per Emil Rasmussen
- The Fertility Clinic
- Department of Gynaecology

Laboratory of Reproductive Biology:
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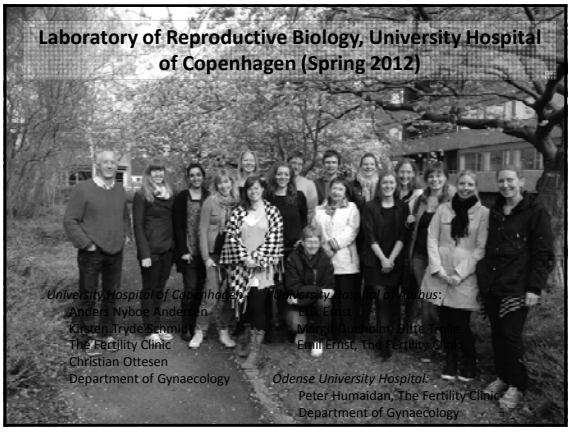
- Mikkel Rosendahl
- Stine Gry Kristensen
- Inga Husum
- Tiny Roed
- Mario Westphal
- Tine Greve
- Annette Rasmussen
- Anne Grete Byskov
- Claus Yding Andersen

The Danish Medical Research Council

The Danish Cancer Foundation

Laboratory of Reproductive Biology, University Hospital of Copenhagen (Spring 2012)



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- The Fertility Clinic
- Christian Ottesen
- Department of Gynaecology

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- Erik Ernst
- Margit Dueholm, Ditte Trolle
- Emil Ernst, The Fertility Clinic

Odense University Hospital:
- Peter Humaidan, The Fertility Clinic
- Department of Gynaecology

ESHRE Annual Meeting 2013
Pro-Congress Course 10
London, UK

Stem cells in testis and their role in fertility preservation

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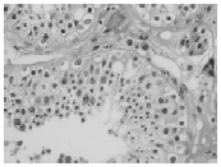
Disclosure

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

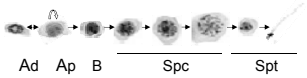
Learning objectives

- Understand the function of spermatogonial stem cells (SSCs) in the testis
- Understand the limitation to recognize SSCs
- 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 fertilty preservation in men

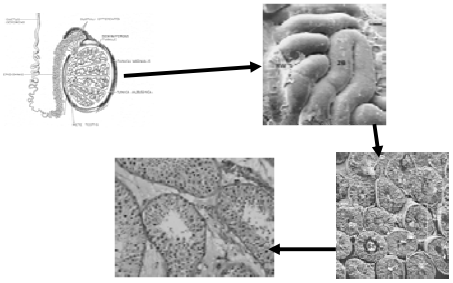
Sperm production



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.



Testis



In testis sections, SSCs cannot morphological be distinguished from their differentiating daughter cells

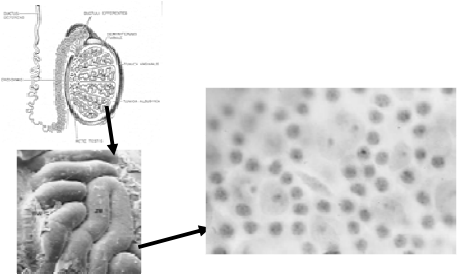
Molecular Characteristics of spermatogonia

A_s and A_{pr}	GFRalpha1
A_s, A_{pr} and A_{al}	PLZF, OCT4, NGN3, NOTCH-1, SOX3, c-RET
A spermatogonia	RBM
Spermatogonia	EP-CAM
Pre-meiotic germ cells	STRA8, EE2
Cells on basal membrane and interstitium	CD9
Spermatogonia, spermatocytes and round spermatids	GCNA1, Hsp90α
Spermatogonia and spermatids	TAF4B

A specific molecular marker for SSCs does not exist

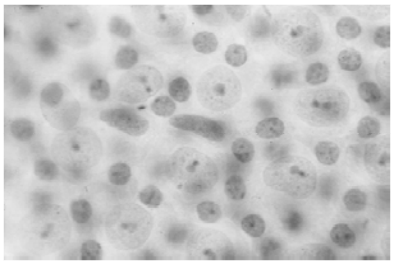
Aponte et al., APMIS 113, 727-742, 2005

Spermatogonial stem cells in the testis



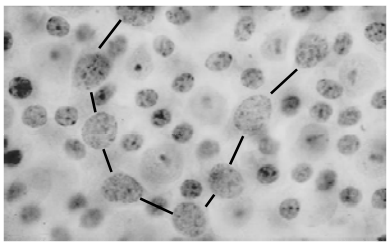
SSCs are single cells on the basal membrane of the seminiferous tubules

A_{pr} – first differentiation step



Upon differentiation SSCs form pairs of cells connected by intercellular bridges

Chain of 8 A-al spermatogonia in prophase



Upon further division, differentiation spermatogonia form chains of cells connected by intercellular bridges

Relative low numbers of SSCs in testis

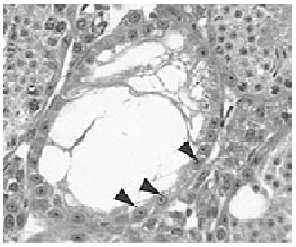
- 0.03 % of all germ cells
- 1.3 % of all spermatogonia
- 3.3 % of all A spermatogonia
- 10.6 % of all A_s, A_{pr} and A_{al} spermatogonia

35.000 stem cells per mouse testis

Tegelenbosch & de Rooij, Mut Res 290, 193-200,1993

Selfrenewal vs differentiation

PLZF-/- mouse

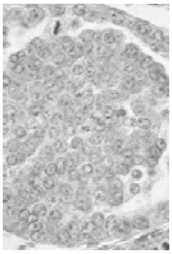


The balance has shifted to differentiation resulting in SSC depletion

Buaas et al., Nat Genet 36, 647-652, 2004

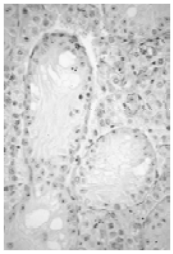
Selfrenewal vs differentiation

GDNF overexpressing mouse



Balance shifted to self renewal

GDNF +/- mouse

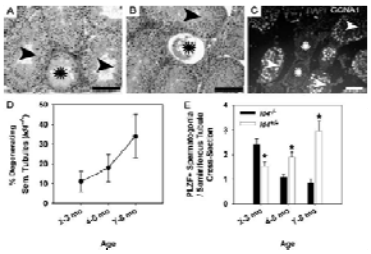


Balance shifted to differentiation

Meng et al., Science 2000

Selfrenewal vs differentiation

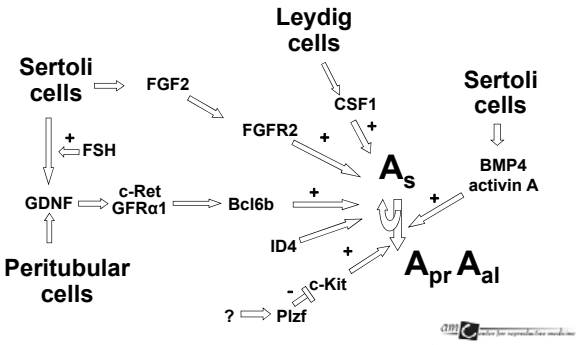
Id4-/- mouse



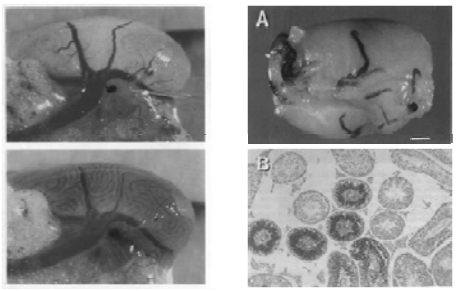
Balance has shifted to differentiation resulting in SSC depletion

Oatley et al., Biol Reprod 85, 347-356, 2011

Regulation selfrenewal and differentiation



First SSC transplantation in mouse

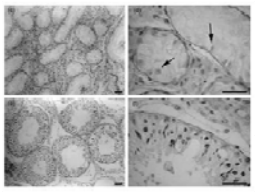


Brinster & Averbock, PNAS 91, 11303, 1994

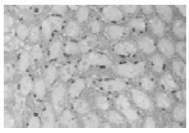
Nagano et al., Int J Dev Biol 41, 111-122, 1997

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)

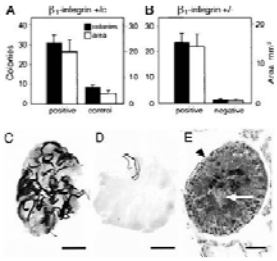


Xenotransplantation:
Rat to mouse (Cloutier et al., Nature 1996)
Hamster to mouse (Ogawa et al., Biol Reprod 1999)
Rabbit/dog to mouse (Dobrinski et al., Biol Reprod 1999)
Baboon to mouse (Nagano et al., Biol Reprod 2001)
Bull to mouse (Izadyar et al., Reproduction 2002
Human to mouse (Nagano et al.,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



Transplantation as readout

Enrichment of SSCs with antibodies against integrins



Shinohara et al. PNAS 96, 5504-5509, 1999

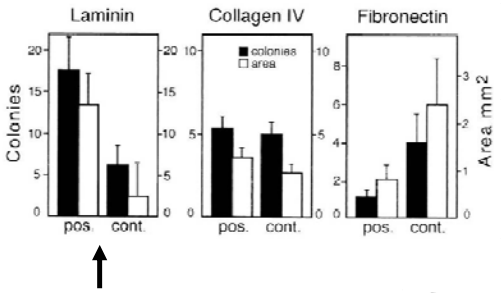
Membrane markers to enrich for SSCs

Markers used for positive selection	CD9, integrinα6, integrinβ1, integrinαV, THY-1, CD24
Markers used for negative selection	c-kit, MHC1, LY6A, CD34

Aponte et al., APMIS 113, 727-742, 2005

Transplantation as readout

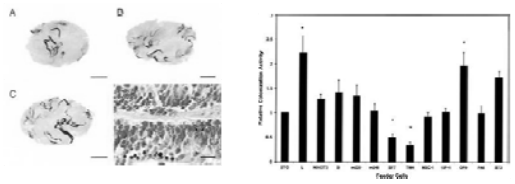
Selection of matrix components supporting maintanance of SSCs in vitro



Shinohara et al. PNAS 96, 5504-5509, 1999

Transplantation as readout

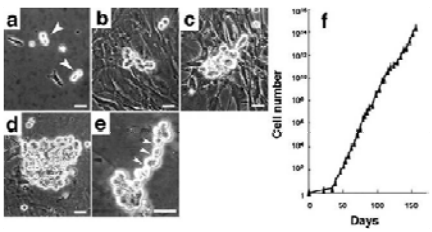
Effect of feeder cells on maintanance of SSCs in vitro



Fibroblast support spermatogonia better then Sertoli cell lines

Nagano, M. et al. Biol Reprod 68:2207-2214, 2003

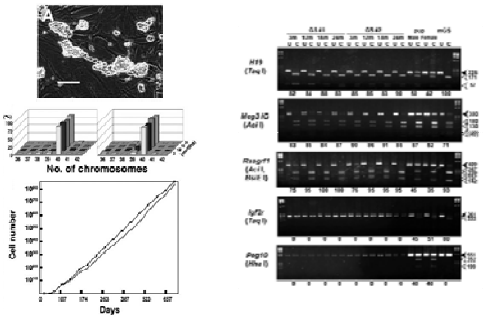
First in vitro propagation of SSCs



Propagation of mouse SSCs in vitro was succesful when using MEF feeder cells and GDNF as growthfactor

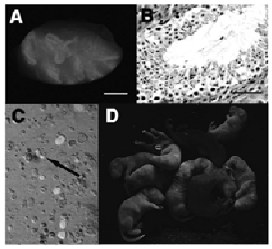
Kanatsu-Shinohara, Biol Reprod 69, 612-616, 2003

Stablity of cultured mouse SSCs



Kanatsu-Shinohara, M. et al. Development 2005;132:4155-4163

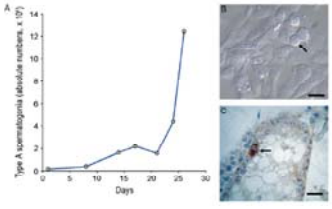
Spermatogenesis and offspring of transplanted cultured SSCs



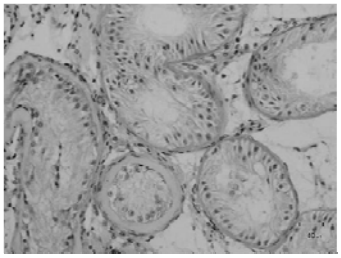
Kanatsu-Shinohara, M. et al. Development 2005;132:4155-4163

Successful long term culture of SSCs of various species

- Mouse
- Rat (Hamra et al., PNAS 2005)
- Hamster (Kanatsu-Shinohara et al., Biol Reprod 2008)
- Bull (Aponte et al., Reproduction 2008)

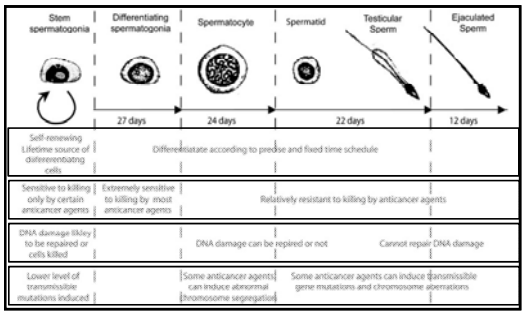


Clinical problem: loss of germ cells



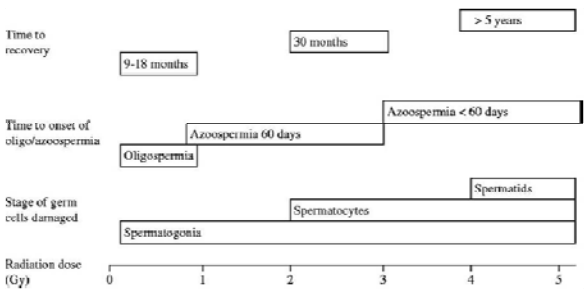
High frequency of azoospermia after chemotherapy or irradiation

SSCs are sensitive for chemotherapy



Meistrich Pediatr Blood Cancer 2009

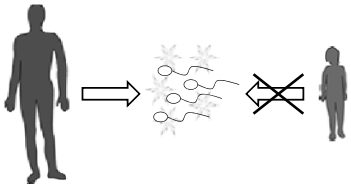
SSCs are sensitive for irradiation



Howell & Shalet, J Natl Cancer Inst Monogr 2005

Fertility preservation

- Cryopreservatie of sperm before onset cancer treatment

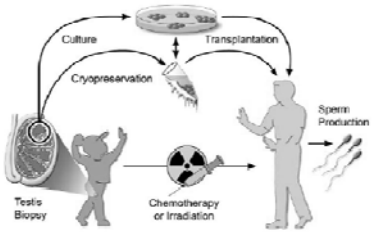


For prepubertal boys with cancer there is no means to preserve fertility with sperm

Blatt, et al., Med Pediatr Oncol. (1999), Wallace, et al., Lancet (2005)

Theoretical solution

Cryopreservation of SSCs for later propagation and autotransplantation



Brinster, Science 316, 404-405, 2007

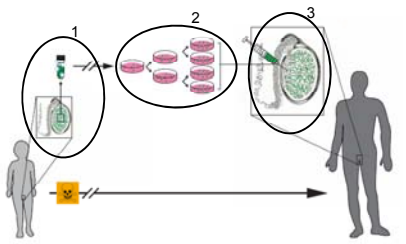
Parents desire

Survey among parents

- Van den Berg et al., Hum Rep 2007
  - Retrospective 162 parents (median 7 years post-diagnosis)
  - 62% would have stored testicular biopsy
- Ginsberg, et al., Hum Rep 2009
  - Prospective 21 parents
  - 76% stored testicular biopsy
- Sadri-Ardekani, et al., Fert Steril 2012
  - Retrospective 299 parents (children <12 year) (1 month to 19 years post diagnosis)
  - 54% would have stored a testicular biopsy
  - Risk perception differs between parents
    - Risk infertility ≥ 80% — 65% would cryopreserve a biopsy
    - Risk infertility ≥ 20% — 35% would cryopreserve a biopsy
    - Chance of success ≥ 80% — 65% would cryopreserve a biopsy
    - Chance of success ≥ 20% — 26% would cryopreserve a biopsy

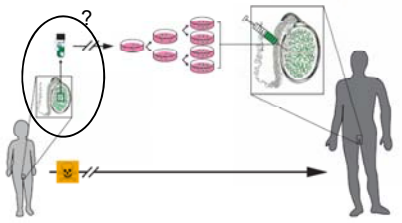
Translation to human: How far are we?

1. Cryopreservation of testis biopsies
2. In vitro propagation of human SSC
3. Transplantation human testis

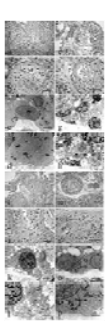


Translation to human

1. Cryopreservation of testis biopsies



Morphology after freezing testis biopsy

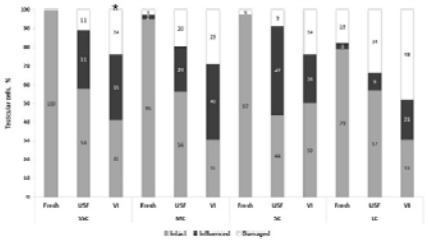


Cryoprotectant

- Control
- Glycerol based: 88% tubules damaged
- Propanediol based: 70% tubules damaged
  41 and 24% undamaged spermatogonia
- DMSO based: 30% tubules damaged
  73 and 57% undamaged spermatogonia

Keros et al. Hum. Reprod. 20:1676-1687, 2005

Morphology after freezing testis biopsy



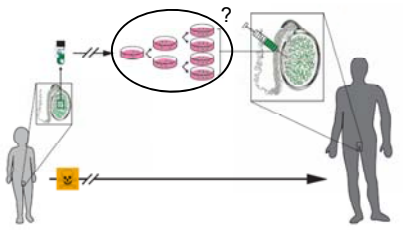
Freezing method

USF: uncontrolled slow freezing
VI: Solid surface vitrification

Baert et al. Fert Steril. 97, 1152-1157, 2012

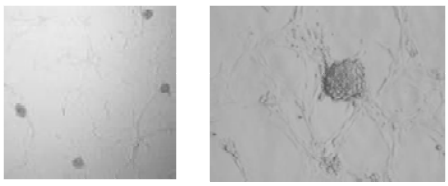
Translation to human

2. Propagation of human SSC



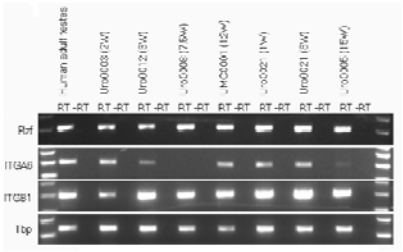
Culture of adult human testicular cells

Establishment of human germ cell clusters in vitro



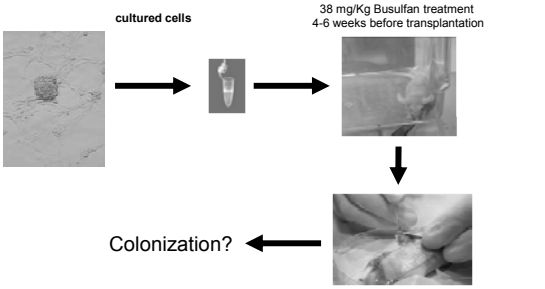
Sadri Ardekani et al., JAMA 302, 2127-2134, 2009

Expression spermatogonial markers

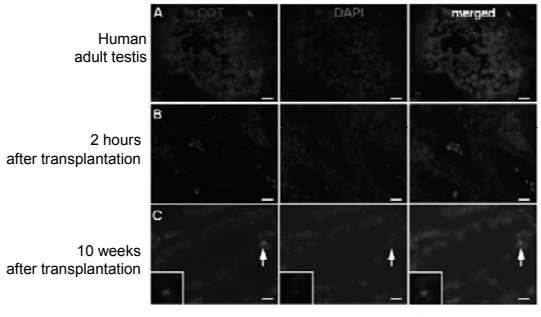


Sadri Ardekani et al., JAMA 302, 2127-2134, 2009

Xenotransplantation adult human SSCs



Human SSCs migrated after xenotransplantation



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

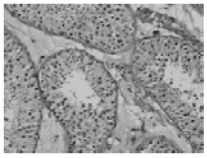
Successful propagation of adult human SSCs

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

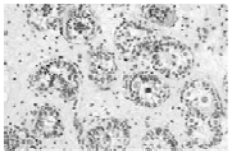
Adult vs prepubertal testis

Do prepubertal testicular cells also support SSC propagation in vitro?

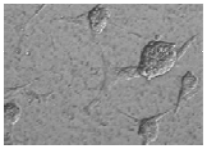
Adult

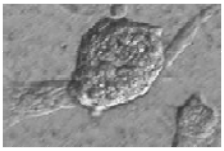


prepubertal



Prepubertal human testicular cell culture

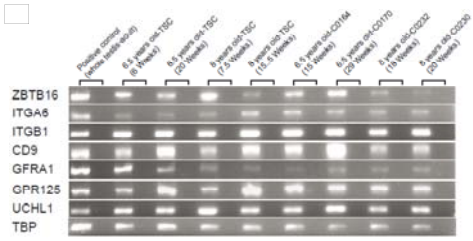




Sadri Ardekani et al., JAMA 305, 2416-2418, 2011

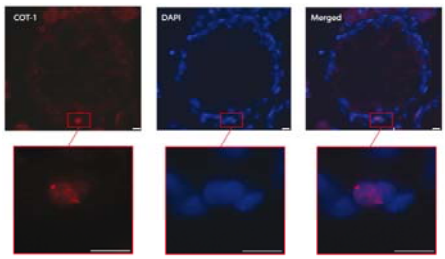
Long term culture of human spermatogonia

Expression of spermatogonial markers in cultured testicular cells



Sadri Ardekani et al., JAMA 305, 2416-2418, 2011

Xenotransplantation of prepubertal human SSCs



Successful prepubertal human SSCs migration after xenotransplantation

Sadri Ardekani et al., JAMA 305, 2416-2418, 2011

Xenotransplantation of prepubertal human SSCs

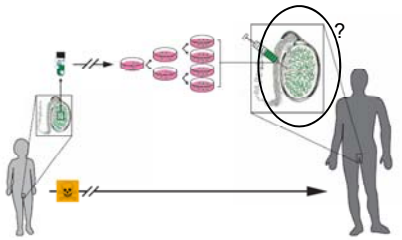
Patient I.D	Culture days (passage number)	Number of injected cells (10^5)	Number of colonies /10^4 cells	Dilution factor	Human SSCs fold increase
Testicular cells culture					
6.5 year old	70(5)	2.4	1		
	98(9)	3.6	0		
8 year old	46 (4)	2	0		
	63 (6)	5.1	0.5	↓ 1.2	9.6
	74 (7)	1.9	4		

Successful propagation of prepubertal human SSCs

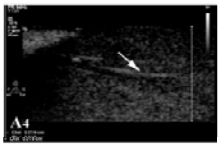
Sadri Ardekani et al., JAMA 305, 2416-2418, 2011

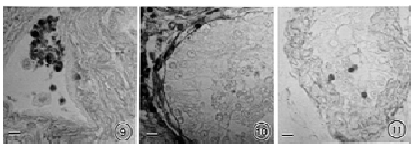
Translation to human

3. Transplantation human testis



Transplantation to human testis

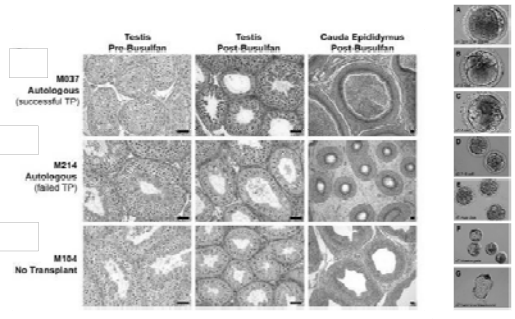




Infusion of contrast liquid into the rete testis guided by ultrasonography is the best transplantation option in human testis

Schlatt et al., Hum Rep 14, 144-150, 1999; Ning et al., Fert Steril, 98, 1443-1448, 2012

First SSC autotransplantation in primates



Hermann et al., Cell Stem Cell 11, 715-726, 2012

Summary milestones 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., BioMed Res Int, 2012 http://dx.doi.org/10.1155/2013/903142

Conclusions

For prepubertal boys with cancer or other disease that need to undergo chemotherapy or irradiation as part of a treatment, SSCs are a good target for fertility preservation.

Prepubertal boys diagnosed with cancer or other disease that need gonadotoxic treatement, should now be offered the possibility to cryopreserve a testis biopsy for future SSC autotransplantation.

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London – United Kingdom
7 to 10 July 2013
Pre-congress course 10

Advances in male germ cells preservation and transplantation

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Catholic university of Louvain
Belgium

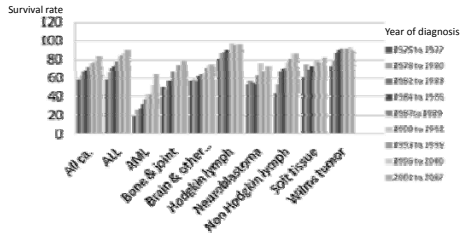
- Nothing to disclose
- No conflict of interest

Learning objectives

- Provide current knowledge about male germ cell preservation
  - Why preserving male germ cells?
  - Which germ cells should be preserved?
  - Who can benefit from the technique?
  - How can the germ cells be preserved?
- Provide current knowledge about transplantation of male germ cells as a fertility restoration strategy
  - Who can benefit from the technique?
  - What is the progress towards clinical application?

Why preserving germ cells?

- Well known germ cell toxicity of cancer therapies
- Growing success of oncologic treatments

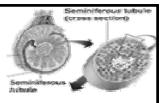


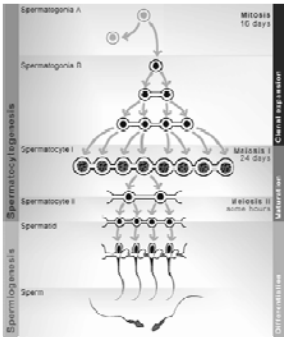
Source: Ca Cancer J Clin 2012; 62:10-29

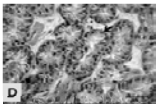
Why preserving germ cells?

- Growing population of patients undergoing fertility threatening therapies:
  - increase in cancer incidence
  - extension of gonadotoxic therapies to benign diseases
- Quality of life after cancer
  - Loss of fertility in adult life is a major and psychologically traumatic consequence of fertility-threatening therapies. In a quality-of-life analysis of former oncological patients (Schover et al., 1999) :
    - about 80% viewed themselves as potential parents
    - the vast majority of younger cancer survivors saw their cancer experience as pivotal in preparing them to be better parents

Which germ cell?

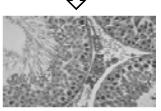






Immature Tissue SSCs

Spermatogenesis starts at puberty



Mature Tissue Routinely applied

Who can benefit from SSC preservation?

Malignant	Non-Malignant
• Leukemia • Hodgkin's disease • Non-Hodgkin's lymphoma • Myelodysplastic syndromes • Solid tumors • Soft tissue sarcoma	(1) HSCT in case of: • hematological disorders: thalassemia major, sickle cell disease, aplastic anemia, Fancori anemia • primary immunodeficiencies • severe autoimmune diseases unresponsive to immunosuppressive therapy: juvenile idiopathic arthritis, juvenile systemic lupus erythematosus, systemic sclerosis, immune cytopenias • osteopetrosis • enzyme deficiency disease: Hurler's syndrome (2) Risk of testicular degeneration • Klinefelter syndrome

HSCT, hematopoietic stem cell transplantation

Wyns et al., HR Update, 2010

How can the germ cells be preserved?

Cryobanking of

- isolated immature testicular cell suspensions
- immature testicular tissue pieces
- whole testes

Cryopreservation of testicular cell suspensions

1. Collagenase/trypsin-EDTA digestion

   → cell viablity (human): 66% (identical for all morphological cellular types)

2. Cryopreservation: no significant influence of CPA on cell viability

Cryoprotective agents (1.5M)	Mean % viability (range)[a]
Glycerol	54 (52–57)
DMSO	54 (51–57)
1,2-propanediol	58 (55–59)
Ethylene glycol	52 (51–53)

Brook et al., 2001

Cryopreservation of testicular cell suspensions

3. Innovative techniques:
   Open pulled straw vitrification of human diploïd germ cell suspensions
   Higher cell viability than slow freezing
   Sa et al., 2012

Cryopreservation of testicular pieces

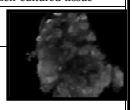
Why cryopreserving pieces of testicular tissue instead of cell suspensions?

- Maintains an intact functional stem cell niche for subsequent maturation of spermatogonia (Ogawa, 2005)
  Disruption of the stem cell niche may influence epigenetic patterns of germ cells (Goossens et al., 2011)
- Preserves the interstitial compartment (hormone substitution)
- Avoids germ cell loss due to tissue digestion
- Does not exclude alternative clinical uses in the future

BUT cell heterogeneity in tissue pieces renders tissue freezing more challenging

Freezing of prepubertal testicular tissue in humans: literature overview

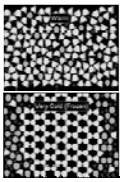
Reference	CPA	Freezing protocol	Type of evaluation	Outcome (germ cells)
Kvist et al, 2006	EG1.5 M Sucrose 0.1 M	Slow controlled	Culture 2 weeks	Well preserved STs Presence of intact SG (c-kit+)
Keros et al, 2007	DMSO 0.7 M	Slow controlled	Culture 24 h	70±7% ISTs (vs 77±4% in fresh-cultured tissue) 94±1% intact SG (vs 83±1% in fresh-cultured tissue)
		Rapid controlled		20±14% ISTs in frozen-cultured tissue 50±43% intact SG in frozen-cultured tissue
Wyns et al, 2007	DMSO 0.7 M Sucrose 0.1 M	Slow controlled	Xenografting 3 weeks	82.19±16.46% ISTs 14.5% SG recovery
Wyns et al, 2008	DMSO 0.7 M Sucrose 0.1 M	Slow controlled	Xenografting 6 months	55±42% ISTs 3.7±5.5% SG recovery 21% proliferating SG Differentiation up to pachytene stage; abnormal spermatids



Cryopreservation: Slow-freezing

- Liquid→**Crystalline** phase
- Gradual freeze
- Long process (±3h)
- Low [Cryoprotectant]
- **Ice crystal formation**

Molecular



Tissular

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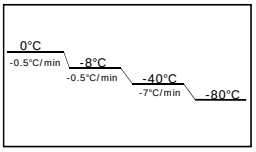
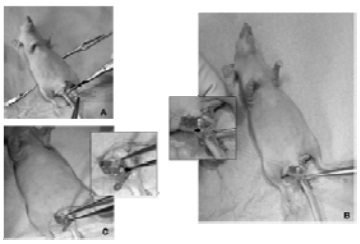
Slow-freezing protocol for immature testicular tissue pieces

- Biopsy transferred to Falcon tubes containing HBSS at 4°C, placed on ice
- Remaining piece cryopreserved within 10 minutes of recovery
- DMSO 0.7 mol/l + 0.1 mol/l sucrose + HSA 10mg/ml
- Slow-freezing protocol:
  - 0° for 9 min
  - cooling at a rate of 0.5°C/min to -8°C
  - holding for 5 min
  - manual seeding at -8°C
  - holding for 15 min at -8°C
  - cooling rate of 0.5°C/min from -8°C to -40°
  - final dehydration for 10 min at -40°C
  - cooling at 7°C/min to -80°C

→ Liquid nitrogen



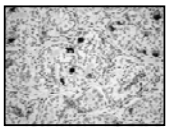
Tissue evaluation after cryopreservation: development of an orthotopic xenografting model in nude mice



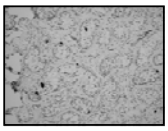
Avascular xenografting procedure: castration of the mouse through a scrotal incision (A); the peritoneal bursa (right arrow) is held open (B) and the testicular tissue fragment (left arrow) is placed without suture in the bursa (C)

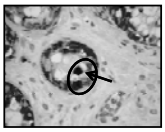
Slow-freezing
xenografting for 3 weeks of Human ITT

Fresh



Frozen/thawed/grafted





MAGE-A4 *(marker of SG)*
0.55 ±0.52 SG/ST

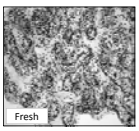
0.08 ±0.13 SG/ST

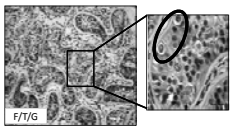
Ki67 and vimentin
(Sertoli cell marker)

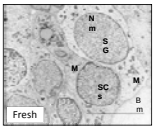
Maintenance of spermatogonia: 14.5%
Preservation of spermatogonia able to proliferate

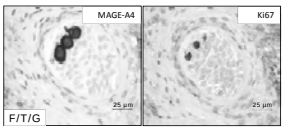
(Wyns et al., 2007)

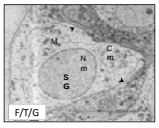
Slow-freezing : In vivo evaluation
xenografting for 6 months of human ITT











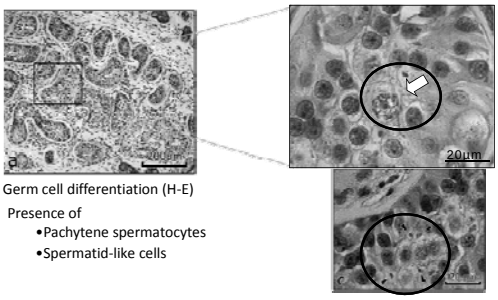
Testicular tissue from a 12-year-old boy

Wyns et al., 2008

Maintenance of spermatogonia: 3.74%

Preservation of spermatogonia able to proliferate

Slow-freezing : In vivo evaluation
xenografting for 6 months of human ITT



Germ cell differentiation (H-E)

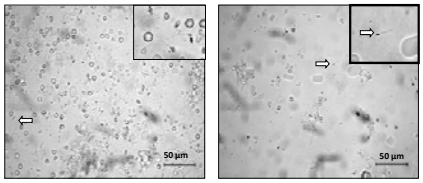
Presence of
- Pachytene spermatocytes
- Spermatid-like cells

Wyns et al., 2008

Slow-freezing : In vivo evaluation xenografting for 6 months of human ITT

TESE

Fresh mature testicular tissue (control)	Frozen/thawed/grafted tissue (14-year-old boy)



⇨ Sperm

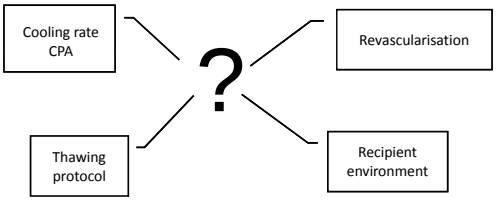
No characteristic sperm found after freezing, thawing and xenografting
Spermatozoon-like cells

Wyns et al., 2008

Why spermatogonial loss ? Why abnormal differentiation ?

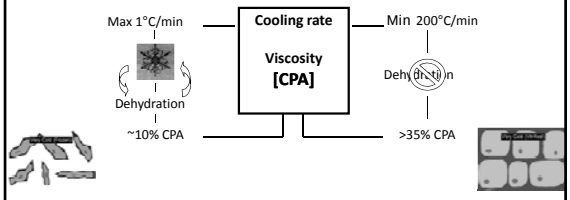
Freezing procedure

Grafting procedure



CRYOPRESERVATION METHODS

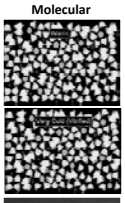
- **Slow-freezing**
  - Liquid phase
  - → Cristalline phase (stable)
- **Vitrification**
  - Liquid phase
  - → Vitreous phase (metastable)



Cryopreservation: Vitrification

- Liquid → **Vitreous** phase
- High cooling rate
- Short process (±20 minutes)
- High [Cryoprotectant]
- **No ice crystal formation**

Molecular



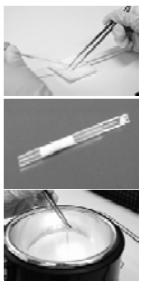
Tissular

Immature testicular tissue vitrification in animals: literature overview

Authors	Species	Vitrification solution	Evaluation	Outcome
Abrishami et al., 2009	Piglet	DMSO 15% vs glycerol 7% +EG 15% +FBS 20% +Sucrose 0.5M	Xenografting	Better cell viability with DMSO Complete maturation only with DMSO
Zeng et al., 2009	Piglet	EG +Raffinose O.5M	Xenografting	Similar germ cell viability Lower germ cell recovery Reduced germ cell differentiation compared to controls
Curaba et al., 2010	Mouse	EG 15% +DMSO 15%	Organotypic culture (3 days)	Good tissue and cell integrity Similar outcome to slow-freezing on a qualitative basis

Vitrification/warming protocol for human ITT

- Pre-treatment with equilibration solution:
  - 7.5% DMSO
  - 7.5% EG
  - 0.25M sucrose + 25mg/ml HSA
  - 10' at 4°C
- Vitrification solution:
  - 15% DMSO
  - 15% EG
  - 0.5M sucrose + 25mg/ml HSA
  - 5' at 4°C
- Remove excess vitrification medium on sterile gauze
- Put in 0.5 ml straw and plunge directly into LN_2



- 20'' in air
- L15 + sucrose + 25mg/ml HSA
  - 0.5M → 0.25M → 0 M sucrose
- 5' at 35°C/bath

Abrishami et al 2010, slightly modified

Sterilization of LN_2

- Sterilization of 500 ml of LN_2 by UVC (λ=254nm) with UV G25T8 at 15 cm of LN_2 for 15 min(Parmegiani et al; Fertil Steril 2009)

UVC are used for sterilization of medical materials, water, surfaces FDA,(Guidelines for Drinking Quality Water, vol, 1, World Health Organization, Geneva, Switzerland, 1993, p. 135)

Sterilization of liquid nitrogen with ultraviolet irradiation for safe vitrification of human oocytes or embryos

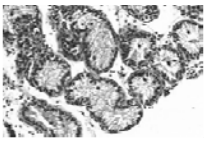
Ledirlee Parmegiani, B.Sc.,[a] Antonio Accorsi, B.Sc.,[a] Graciela Enela Cognigni, M.D.,[a] Silvia Bernardi, B.Sc.,[a] Enzo Troilo, B.Sc.,[a] and Marco Filicori, M.D.[a]

[a] Reproductive Medicine Unit, CyroBio Medical Centers, Bologna, Italy; and [b] Department of Occupational Medicine, University of Bologna, Bologna, Italy

crobial contamina-
LN_2 demonstrates
ue and in particular
, the fact that LN_2
can be quickly and safely sterilized could encourage the wider appli-
cation of human oocyte and embryo vitrification, especially with
"open carriers."

Immature testicular vitrification in non-human primates: in vivo evaluation

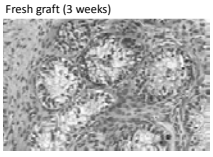
Fresh



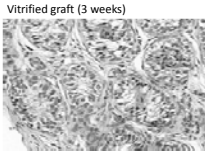
Rhesus monkey (4 years old)

Tissue integrity based on histological characteristics

Fresh graft (3 weeks)



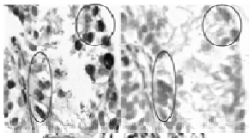
Vitrified graft (3 weeks)



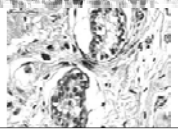
Poels et al., 2013

Immature testicular vitrification in non-human primates: in vivo evaluation

MAGE-A4/ Ki67



3β-HSD



→Survival of proliferating spermatogonia
→Leydig cells preservation

Poels et al., 2013

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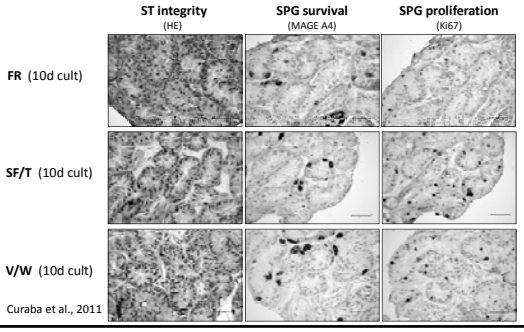
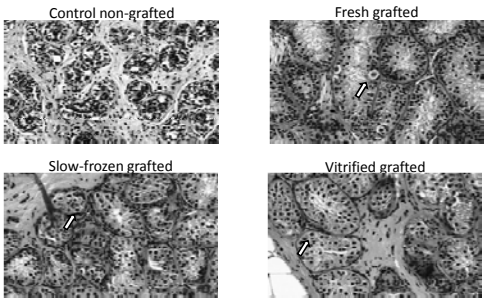
Immature testicular tissue vitrification in humans: in vitro evaluation



Curaba et al., 2011

Immature testicular tissue vitrification in humans: in vivo evaluation (8-year-old boy)



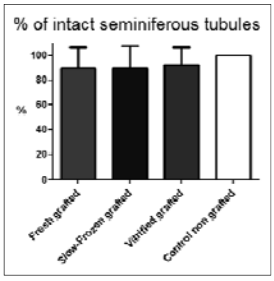
Poels et al., 2013

Histological evaluation

One section every 50 µm was stained with HE and evaluated by LM

Intact seminiferous tubules show:

- ✓ No sclerosis
- ✓ Good adhesion of cells to the basal membrane
- ✓ Good cell cohesion



→ **Good integrity of seminiferous tubules**

Poels et al., 2013

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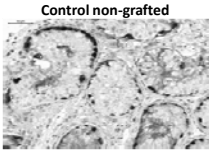
Immunohistochemical evaluation
Spermatogonial survival after 6 months (MAGE-A4)

Control non-grafted



Fresh grafted

Slow-frozen grafted

Vitrified grafted

Poels et al., 2013 → **Survival of SG after vitrification and grafting**

Spermatogonial recovery

	Grafted tissue 6 months			Non grafted tissue
	Fresh	**Slow-Frozen**	**Vitrified**	**Control**
Mage A4 positive cells/ST	0.23±0.27[a]	0.28±0.52[a]	0.49±1.14[a]	6.71±7.02[b]
%Recovery	**3.4%**	**4.1%**	**7.3%**	**100%**

a and b differ significantly (P<0.001)

→ **Significant loss of spermatogonial cells**
→ **No significant difference between grafting groups**
→ **Similar results to those obtained in previous study**

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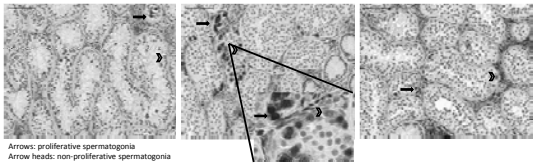
Proliferating spermatogonia

Double IHC (MAGE-A4 (red) and Ki67 (brown))

Fresh grafted **Slow-frozen grafted** **Vitrified grafted**

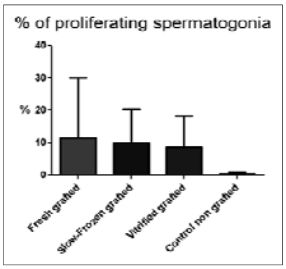


Arrows: proliferative spermatogonia
Arrow heads: non-proliferative spermatogonia

→ **Preservation of proliferating SG after vitrification and grafting**

Proliferating spermatogonia

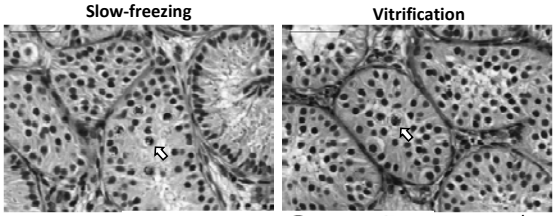
% of MAGE-A4 (spermatogonia) and Ki67 (proliferation) positive cells



→Proliferative activity of spermatogonia is higher after grafting
No difference between F, SF and V grafts

Spermatogonial differentiation

Slow-freezing **Vitrification**



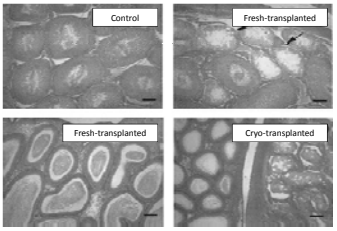
n=1/10 **n=2/10**

→ Spermatogonial differentiation to pachytene stage

35

Cryopreservation of whole testes

Isotransplantation of rat testes with vascular anastomosis
(Yin et al., 2003)

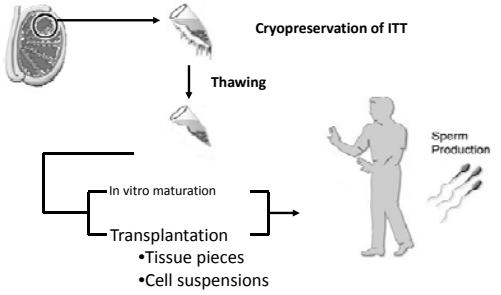


Active spermatogenesis in 42% of fresh isotransplants

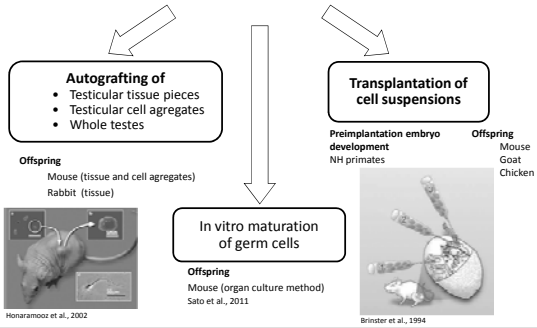
Organ perfusion with ice-cold M2 medium containing 0.1M fructose + gradient of DMSO (0 to 1.5M) 30 min
SF (0 to -7°C at 2°C/min; seeding; 0.3°C/min to -40°C and 10°C/min to -85°C→LN2

Transplantation of intact cryopreserved testes: none found to be functional

Male germ cell preservation for prepubertal boys: preserving the spermatogonial stem cell (SSC) with a view to later fertility restoration strategies



Fertility restoration options: where are we now?



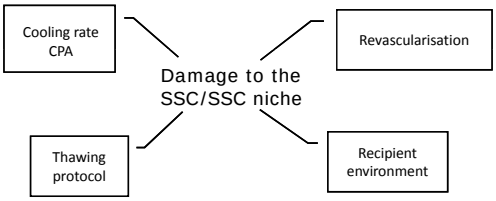
Xenografting of human immature testicular tissue: literature overview

Reference	Fresh/ frozen	Mature/ immature	Source of the tissue	Grafting period	Grafting site in mice	Outcome
Wyns et al., 2007	**Frozen**	**Immature**	Prepubertal boys undergoing orchidopexy for cryptorchidism	3 weeks	Scrotal bursa	Well preserved tissue integrity (histo-IHC) 82.19±16.46% ISTs 14.5% remaining SG
Goossens et al., 2008	Fresh	**Immature**	Prepubertal boys undergoing BMT	4 & 9 months	Back skin	Mainly Sertoli-cell only tubules Some surviving SG
Wyns et al., 2008	**Frozen**	**Immature**	Prepubertal boys with cancer or benign pathology requiring BMT	6 months	Scrotal bursa	Well preserved tissue integrity 3.7 % remaining SG Differentiation up to pachytene spermatocytes
Wyns et al., 2013	**Fresh/ Frozen/ Vitrified**	**Immature**	Prepubertal boys with cancer or benign pathology requiring BMT	6 months	Scrotal bursa	Well preserved tissue integrity 4.1 to 7.3 % remaining SG Differentiation up to pachytene spermatocytes

Transplantation of tissue pieces: the cryopreservation procedure is only partially responsible for spermatogonial stem cell loss and/or impairment

Cryopreservation procedure

Grafting procedure

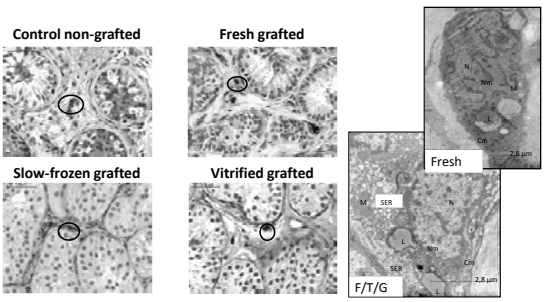


→ Need for studies on the transplantation technique

Challenges for tissue transplantation

- Competent testicular environment of the recipient:
  - Xenografting: species differences (preclinical evaluation: inadequacy of current xenografting models)
  - Autografting: exposure of the SSC niche to chemo- or radiotherapy, hormonal support of the graft (clinical application)
- Oxygen supply in grafts: ischemic stress affecting spermatogonial cells and their niche
- Safety issues

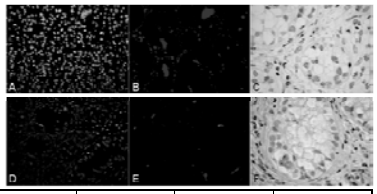
Transplantation procedure:
sufficient hormonal support of the graft?



Tissue transplantation : ischemic stress before revascularization or inadequate recipient environment responsible for increased apoptosis?

3 days

3 weeks

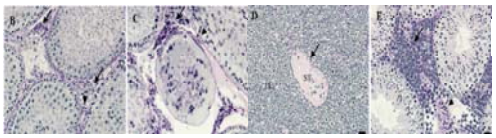


	Fresh *n=3*	Grafts (3 days) *n=3;* G=5	Fresh *n=9*	Grafts (3 weeks) *n=9;* G=9
CASPASE-3	0.1±0.163	0.063±0.056	0.096±0.135	0.014 ±0.022
TUNEL	0.004±0.007	+++	0.001* ±0.003	0.032 * ±0.040

*p=0.044

Cancer cell contamination of the stored testicular tissue

As few as 20 leukemic cells injected into a testis can induce disease relapse (Jahnukainen et al., 2001)



Hou et al., 2007

Tumor growth without potential to differentiate germ cells into gametes

Testicular cell aggregate transplantation: literature overview

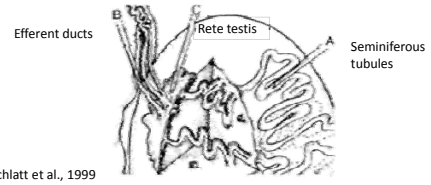
References	Donor species	Recipient species	Graft localization	Tubule reconstitution	Sperm differentiation	Steroidogenesis
Gassei et al., 2006	Rat (after culture)	Nude mouse	Back skin	Yes	Few putative spermatogonia No further differentiation	IHC identification of Leydig cells Production of bioactive testosterone
Kita et al., 2007	Mouse Rat	Nude/SCID mouse	Back skin	Yes	Round spermatids **Offspring from mouse-testis-cell transplants**	Not assessed
	Pig			Yes	Not assessed	
Honaramooz et al., 2007	Pig	Nude/SCID mouse	Back skin	Yes	Complete spermatogenesis	IHC identification of Leydig cells Production of bioactive testosterone
Arregui et al., 2008	Sheep	Nude mouse	Back skin	Yes	Complete spermatogenesis	Production of bioactive testosterone
Zhang et al., 2008	Bovine (after culture 3-7 days)	Nude mouse	Back skin	Yes	No germ cells	Not assessed
			Testis	Yes	No germ cells	
Watanabe et al., 2009	Neonatal pig	Nude/SCID /NOG mouse	Back skin	Yes	Complete spermatogenesis	Not assessed

Challenges for testicular cell suspension transplantation

- Competent testicular environment of the recipient:
  - Xenotransplantation: species differences (preclinical evaluation: inadequacy of models due to phylogenetic distance)
    Nagano et al., 2002: colonization of SSC niches, long term survival but no differentiation of human SSCs in mice
  - Autografting: exposure of the SSC niche to chemo-or radiotherapy, hormonal support of the transplanted cells (clinical application)
- Safety issues

Testicular cell transplantation: progress towards clinical application

Preclinical studies : injection techniques



• Schlatt et al., 1999
Rete testis: 70% of tubules filled with cell suspension in ~ 30 min
• Brook et al., 2001
Intratubular injection: 50 to 70% of tubules filled with cell suspension

Testicular cell transplantation: progress towards clinical application

Clinical study

Radford, 2003
Manchester (UK) in 1999: germ cell transplantation in cancer patients

• No information on the fertility of these patients

• Impossible to distinguish between endogenous spermatogenesis and spermatogenesis issuing from transplanted cells

Cancer cell contamination: SSC cell transplantation after cell sorting

Table III Studies on isolation of germ cells with detection of cancer cell contamination

Reference	Species	Cell-sorting technique	Markers	Evaluation after cell sorting	Outcome (% of residual contamination/number of contaminated samples or mice)
Fujita et al., 2005	Mouse	FACS	H-2Kb/H2Db$^-$ (MCH cl I) CD45$^-$	Cell transplantation Histology: testis, bone marrow, peritoneal exudate of recipient mice	No contamination of recipient mice
Fujita et al., 2006	Human	FACS	MCH cl I CD45$^-$	RT-PCR for germ cell markers (DAZL, HIWI, vASA, NANOG, STELLAR, OCT4)	(<0% K562 cells (CML), 0% K562 cells after Fr_3 (for reduction of MCH cl I)
Geens et al., 2007	Mouse	MACS + FACS	H2Kb$^-$ (MCH cl I) CD49f$^+$ (α6 integrin)	FACS In vitro culture Cell transplantation	0.29% H2Kb$^+$ cells 3.1% (1/32) contaminated cultures 1/20 contaminated testis
	Human	FACS	H2Kb$^-$ (MCH cl I)	FACS; in vitro culture; PCR for B cell receptor	0.58% SD$^-$ cells; 1/1 contaminated samples

MCH cl: major histocompatibility complex class I marker of somatic cells; α6 integrin: marker of SSCs; CD45: surface marker of leukemic cells; Fr_3: interferon-γ; CML: Chronic myelogenous leukemia.

Wyns et al., 2010

Post-sorting purity checks are required to confirm elimination of malignant cells (Hermann et al.,2011)

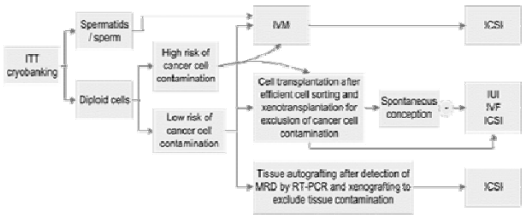
Advances in male germ cell preservation and transplantation: general conclusion

- Crucial to inform patients and parents of the potential consequences of their therapy on future fertility
- The inability to father one's own genetic children might have a huge impact on the psychological well-being of patients in adulthood (Schover et al , 2005; van den Berg et al., 2007)
- Methods to cryostore immature germ cells are available
- Preservation of testicular tissue from today's prepubertal patients will allow them to consider various fertility restoration options emerging in the next 20–30 years, giving them hope of fathering children with their own genetic heritage.

BUT we face:

- absence of proven reproductive potential of cryopreserved ITT in humans
- unsolved questions regarding restoration techniques from cryostored ITT
- safety issues after transplantation: risk of chromosomal abnormalities, abnormal imprinting, risk of cancer recurrence

Fertility restoration strategy after gonadotoxic therapies in prepubertal boys



Storage of patient blood and/or tumor samples before therapy assessment for later detection of malignant cells among normal cells (Jolkowska et al., 2007).

References

ITT cryopreservation: clinical experience (2005-2010)

human reproduction

ORIGINAL ARTICLE *Andrology*

Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain

C. Wyns[1], M. Curaba[1], S. Petit[1], B. Vanabelle[1], P. Laurent[1], J.-F.X. Wese[2], and J. Donnez[1,*]

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Human Reproduction Update Advance Access published January 4, 2010

human reproduction update

Options for fertility preservation in prepubertal boys

Christine Wyns, Mara Curaba, Bernard Vanabelle, Anne Van Langendonckt, and Jacques Donnez[1]

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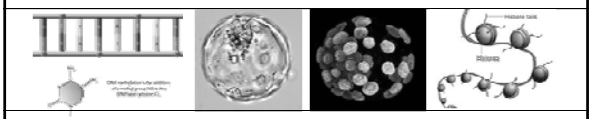
Reproduction and Genetics

Epigenetics of Pluripotent Cells



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Learning Objectives

To cover the salient facts regarding the epigenetic control of pluripotent cells including:

1) Cellular potency
2) Unique Properties of Stem Cells
3) Pluripotent Cell Types (ESCs, iPSCs)
4) Epigenetics
5) Control of the Pluripotent 'State'
6) Unique Epigenetic Features in Pluripotent Cells
7) Epigenetic Problems with Stem Cells
8) Conclusions

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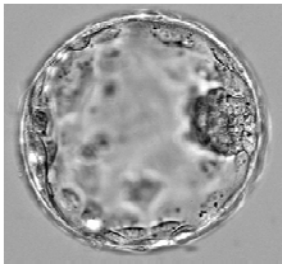
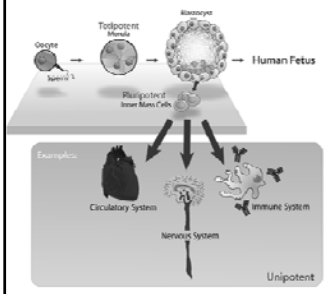
Cellular Potency



Cellular 'Potency'



- Oocyte & cleavage stage embryos are **totipotent** (potential for forming any cell-type).
- 4 days after fertilization, cells begin to differentiate and become specialized
- In blastocyst, ICM and TE cells are distinct cell types:-
- TE cells (**differentiated**) form the placenta and related tissues
- ICM cells are **pluripotent** potential to develop into any of the three germ layers

Unique Properties of Stem Cells

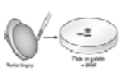
http://stemcells.nih.gov/info/2001report/appendixC.asp

- 3 general properties of stem cells:
  - Capable of dividing and renewing themselves for long periods
  - They are unspecialized cells
  - They can give rise to specialized cell types by **Differentiation**

Pluripotent Cell Types

- The Inner Cell Mass (ICM)
- Embryonic Stem Cells (ESCs)
- Induced Pluripotent Stem Cells (iPSCs)
- Germline Stem Cells



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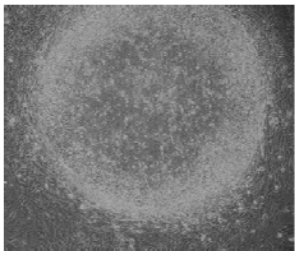
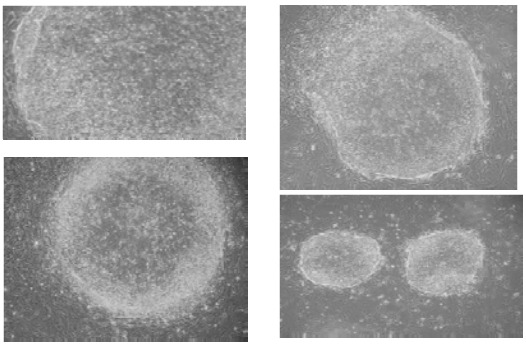
Embryonic Stem Cells
(ESCs)



Embryonic Stem Cells

- Derived from preimplantation or peri-implantation embryos
- Capable of prolonged undifferentiated proliferation
- Maintain potential to form all three embryonic germ layers even after prolonged culture
- Upon differentiation, ES-cells form cell aggregates termed embryoid bodies containing a wide variety of cell types
- These relatively uncommitted cells contain exhibit a broad pattern of gene expression
- Form teratomas when injected SC into mice

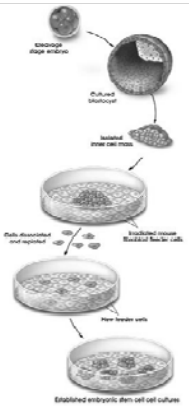
H1 Human ES Cell Colonies (Passage 44)



Generation of Embryonic Stem Cells

- ICM is isolated by immunodissection and plated onto mitotically inactivated murine embryonic fibroblast (MEF) feeders in culture
- With serum, ICM cell outgrowths are propagated, and colonies with undifferentiated morphology are selected for expansion
- Mouse ESCs need only LIF for undifferentiated proliferation
- Human ES cells require feeder layers, + serum (or alternatively bFGF addition for serum-free medium)
- Human ES cells express telomerase enzyme, which adds repeats to chromosome ends
- The enzyme is highly correlated with immortality in human cell lines

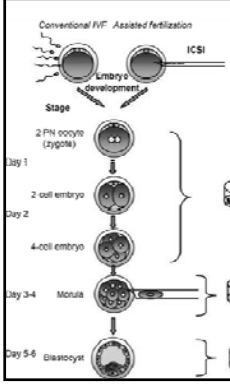
Generation of Human Embryonic Stem Cells



Blastocyst
↓
ICM immunodissection
↓
Plate onto mitotically inactivated MEFs, add serum or bFGF
↓
Passage
↓
Differentiate to Embryoid Body
↓
Futher differentiation to desired cell type

http://stemcells.nih.gov/info/2001report/appendixC.asp

Embryonic Stem Cells Can be Obtained from Several Stages of Preimplantation Development *Lei et al., Cell Research* (2007) **17**:682–688.



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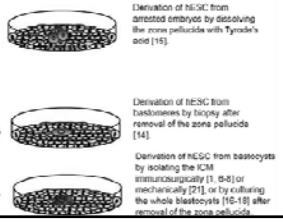

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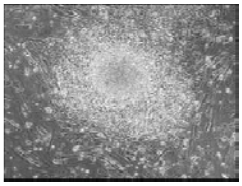
Induced Pluripotent Stem Cells (iPSCs)



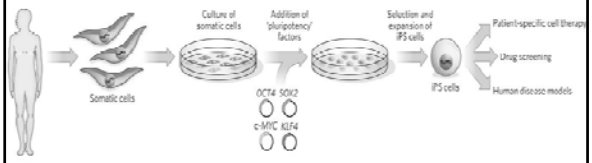
http://www.ucl.ac.uk/stemcells

Induced Pluripotent Stem Cells (iPSCs)

- These are 'artificial' pluripotent stem cells
- Derived from somatic cells (i.e. are reprogrammed from non-pluripotent, differentiated cells)
- Express similar stem cells genes and other characteristics to other pluripotent stem cells (Embryonic Stem cells etc)
- Generated by forced expression of 3 or 4 key genes (Oct-3/4, SOX2, c-Myc, and Klf4) on somatic cells
- These genes are introduced via retroviruses or treatment of the cells in culture with these proteins

Applications of iPSCs

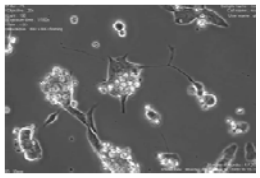
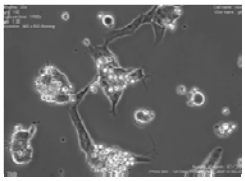
Shinya Yamanaka and Helen M. Blau. *Nature. 2010 465(7299)*:704–712



- To generate iPS cells, adult somatic cells are transduced with retroviruses encoding four pluripotency factors (SOX2, KLF4, c-MYC and OCT4)
- Fully reprogrammed iPSCs have similar properties to ES cells-Form teratomas on injection into mice and can generate progeny
- Patient's cells used to derive iPS cells, which can be differentiated into various somatic cell types, all with the same genetic information as patient
- Differentiated cells used in disease models for studying the molecular basis of a diseases and for screening drugs to treat these diseases

Mouse iPS Cells Differentiating into Neurons

1 2

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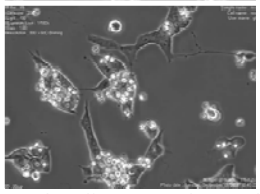
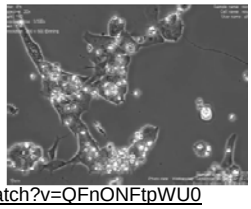
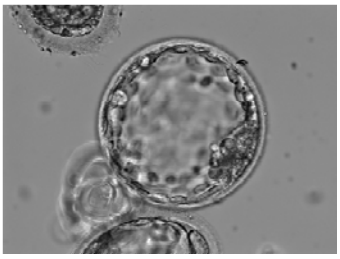



http://www.youtube.com/watch?v=QFnONFtpWU0

REGE
Reproduction and Genetics

Introduction to Epigenetics



What is Epigenetics?

- The term **epigenetics** refers to changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence
- *Think of it as another 'higher' level or layer of information, that is additional to that presented in the gene sequence*
- Pluripotency is maintained by strict epigenetic control

Epigenetic 'Marks'

•**Epigenetic marks**: molecular modifications regulating gene expression and genome function that can lead to heritable changes in gene expression without changes in DNA sequence

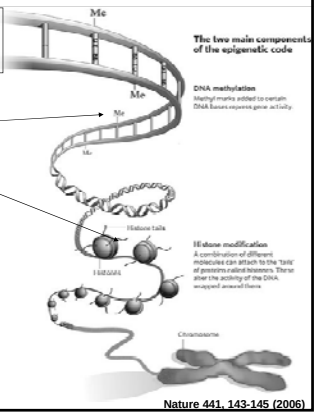
•The major epigenetic marks are modifications of histone proteins and DNA methylation

•Can affect gene expression

•Epigenetic marks are extensively remodelled during development

Epigenetic Modifications

Epigenetic information is a layer of information additional to that which is presented by the gene sequence



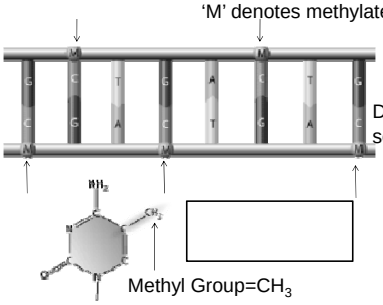
DNA methylation

Histone modification (Methylation, acetylation)

Nature 441, 143-145 (2006)

Types of Epigenetic 'Marks': DNA Methylation

'M' denotes methylated cytosine



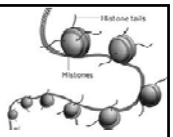
DNA sequence

Methyl Group=CH_3

•**DNA methylation:** covalent modification of DNA with methyl group at position C5 of the pyrimidine ring of cytosines, often in CpG dinucleotides

•**DNA methylation** is usually associated with gene silencing

Types of Epigenetic 'Marks'
Histone modifications



•Histones are the protein component of nucleosomes which, together with DNA and additional proteins, form chromatin

•Specific amino acid residues in histones can be modified post-translationally

•Modifications include methyl, phosphate, acetyl, ubiquitin groups

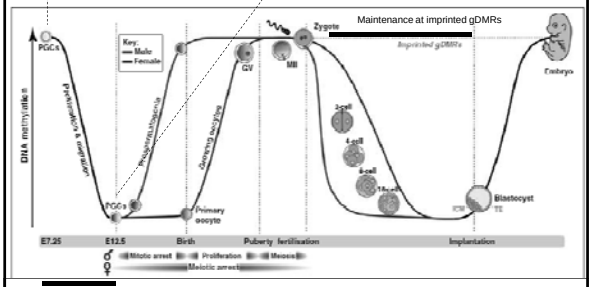
•Histone modifications affect chromatin state and gene expression

•H3K4me3 refers to tri-methylation on lysine (K) residue 4 in the tail of histone H3

Epignetic Reprogramming During Normal Development
Smallwood and Kelsey, Trends in Genetics, 2012, Vol. 28, No. 1

PGCs. DNA methylation globally erased during proliferation and migration to genital ridge

After sex-determination: DNA-methylation established in germ-cell precursors

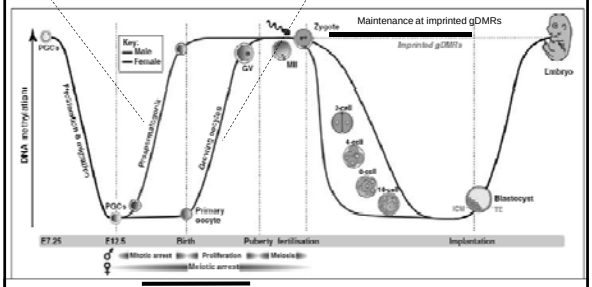


Erasure (of methylation)

Epignetic Reprogramming During Normal Development
Smallwood and Kelsey, Trends in Genetics, 2012, Vol. 28, No. 1

Male Embryo: *de novo* methylation before meiosis in mitotically arrested cells (G1-phase; prospermatogonia) completed before birth.

Female Embryo: primary oocytes enter meiosis and arrest in prophase-I (diplotene stage); DNA methylation established after birth at oocyte growth phase.



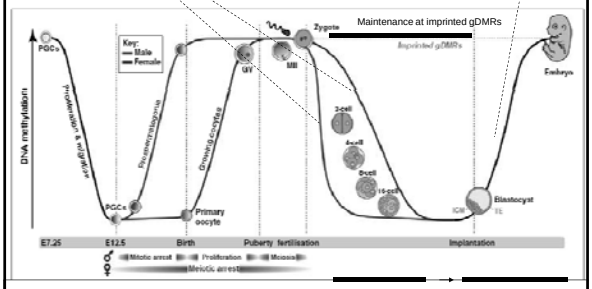
Establishment

Epignetic Reprogramming During Normal Development

Smallwood and Kelsey, Trends in Genetics, 2012, Vol. 28, No. 1

Post-Fertilisation: New wave of DNA demethylation takes place that is distinct on the parental genomes.

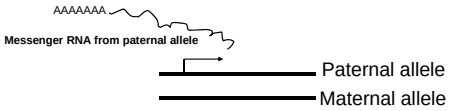
Blastocyst Stage: new methylation established, associated with cellular differentiation.



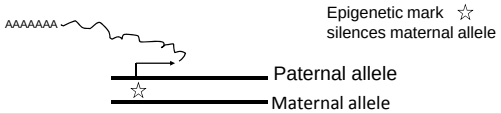
Erasure **Establishment**

Genomic Imprinting

- Delicately poised epigenetic system of regulating gene expression from parental alleles. Many imprinted genes have a role in growth, development placental function
- Genomic imprinting- Exclusive expression of one **allele** of the gene.

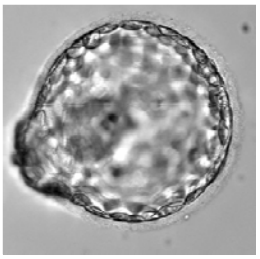


- Epigenetic information is used to regulate imprinting



REGE
Reproduction and Genetics

Control of the Pluripotent State



Pluripotency in Early Embryo/ES Cells

Requires:

- External regulators of pluripotency: several signalling pathways LIF, BMP4,TGF, activin A, Nodal, bFGF (FGF2)
- Internal regulators of pluripotency: Transcription Factors OCT4, NANOG, SOX2
- Epigenetic level control
- microRNAs

Control of the Embryonic Stem Cell 'State'

Young (2011), Cell 144, 940-954

Table 1. Transcriptional Regulators Implicated in Control of ESC State

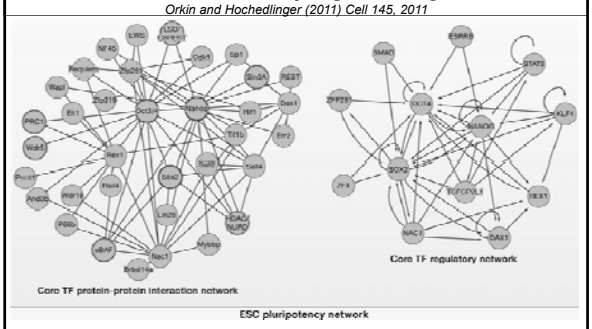
Type of Regulator	Function	References
Transcription Factors		
Oct4	Core circuitry	1
Sox2	Core circuitry	2
Nanog	Core circuitry	3
Tcf3	Wnt signaling to core circuitry	4
Stat3	Lif signaling to core circuitry	5
Smad1	BMP signaling to core circuitry	6
Smad2/3	TGF-β/Activin/Nodal signaling	7
c-Myc	Proliferation	8
Esrrb	Steroid hormone receptor	9
Sall4	Embryonic regulator	10
Tbx3	Mediates LIF signaling	11
Zfx	Self-renewal	12
Ronin	Metabolism	13
Klf4	LIF signaling	14
Prdm14	ESC identity	15

The most important regulatory inputs in ESCs come from a small number of ''core'' transcription factors acting with other transcription factors, some of which are terminal components of developmental signaling pathways

The ES cell ''state'' is the product of all the regulatory inputs that produce the gene expression program of pluripotent, self-renewing cells

Transcription Factor Network in ES cell Pluripotency & Cellular Reprogramming

Orkin and Hochedlinger (2011) Cell 145, 2011



Protein-protein interactions in ESCs

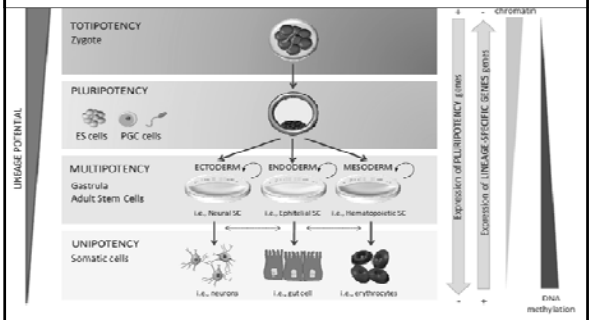
The 3 core pluripotency factors, Oct4, Nanog, and Sox2 in red

Epigenetic Control of Pluripotency

- Pluripotent cells must contain epigenetic information that allows the maintenance of self-renewal programs whilst **also** allowing the retention of multilineage differentiation potential
- Therefore, pluripotent cells must have unique chromatin features, including bivalent promoters, poised enhancers, and unique DNA modification patterns when compared with differentiated cells

Epigenetic Marks and Developmental Potency

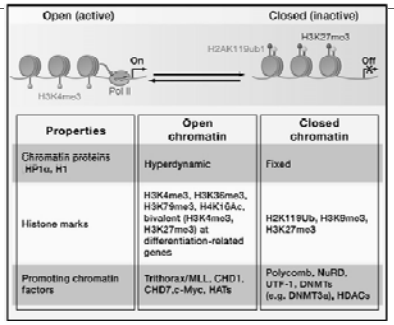
Berdasco and Esteller. *Stem Cell Research & Therapy* 2011, **2**:42



During differentiation: Lineage potential decreases
Open chromatin decreases
DNA methylation and heterochromatin increases

Simplified views of open and closed chromatin

Orkin and Hochedlinger Cell 145, 2011

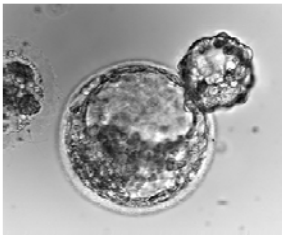


Properties	Open chromatin	Closed chromatin
Chromatin proteins HP1α, H1	Hyperdynamic	Fixed
Histone marks	H3K4me3, H3K36me3, H3K79me3, H4K16Ac, bivalent (H3K4me3, H3K27me3) at differentiation-related genes	H2K119Ub, H3K9me3, H3K27me3
Promoting chromatin factors	Trithorax/MLL, CHD1, CHD7,c-Myc, HATs	Polycomb, NuRD, UTF-1, DNMTs (e.g. DNMT3a), HDACs

- **As differentiation proceeds, chromatin becomes closed**
- **Cellular reprogramming reverses the chromatin state**

Unique Epigenetic Features in Pluripotent Cells

1) Chromatin Configuration and Histone Modifications



Bivalent Domains at Promoters

Rada-Iglesias and Wysocka *Genome Medicine 2011, **3:3***

- Chromatin regions marked by **H3K4me3** (associated with transcriptional initiation), **and H3K27me3 (**associated with Polycomb-mediated gene silencing)
- Bivalent domains are present in mouse ESCs (mESCs) and hESCs
- Bivalent domains mark transcription start sites of **key developmental genes** that are poorly expressed in ESCs, but induced upon differentiation
- Upon differentiation, bivalent domains change to either a transcriptionally active state, or a transcriptionally silent state
- *Some* bivalent domains retained on differentiation give epigenetic plasticity
- Promoter bivalency less abundant in differentiated cells

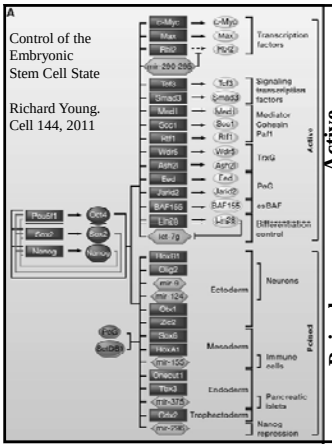
Poised Enhancers

Rada-Iglesias and Wysocka *Genome Medicine 2011, **3:36***

- Enhancers, play a central role in cell-type and signalling-dependent gene regulation
- Epigenomic profiling of histone modifications and chromatin regulators reveals 2 distinguished enhancer classes in hESCs: **active** and **poised**
- **Active class** is enriched in acetylation of lysine 27 of histone H3 (**H3K27ac**), and are associated with genes expressed in hESCs and in the epiblast
- The **poised class** is marked by **H3K27me3**. Found near genes that are inactive in hESCs, but which play critical roles during early post-implantation development (gastrulation, neurulation)
- Upon signalling stimuli, **poised** enhancers switch to active chromatin state in a lineage-specific manner and drive cell-type-specific gene expression patterns

Control of the Embryonic Stem Cell State

Richard Young. Cell 144, 2011



Selected Components of ESC Core Regulatory Circuitry selected protein coding and miRNA target genes **on Active and poised genes**

Oct4, Sox2, and Nanog directly activate spectrum of transcription factors, cofactors, chromatin regulators, and miRNAs that are known to contribute to ESC state

Oct4, Sox2, and Nanog associate with SetDB1- and PcG-repressed protein-coding and miRNA genes that are poised for differentiation

Reduced 'Repressive' Histone Modifications

Rada-Iglesias and Wysocka *Genome Medicine 2011, **3:36***

•In differentiated cells, genome enriched in histone modifications associated with heterochromatin formation and gene repression (H3K9me2/3, H3K27me3)

•These two histone methylation marks cover only 4% of the hESC genome, but well over 10% of the human fibroblast genome

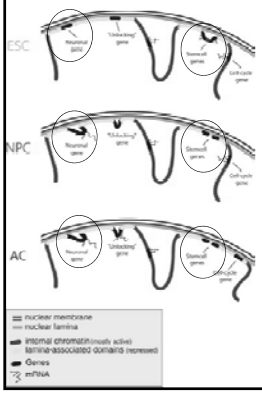
•H3K9me2-marked regions overlap with the recently described nuclear lamina-associated domains

•Therefore expansion of the repressive histone methylation marks might reflect a three-dimensional reorganization of chromatin during differentiation

•An 'open', hyperdynamic chromatin structure is a crucial component in pluripotency maintenance

Nuclear Lamina Interactions During Differentiation

Peric-Hupkes et al., Molecular Cell Volume 38, Issue 4 2010 603 - 613



Dynamic Reshaping of NL-Genome Interactions During Differentiation

•During differentiation, hundreds of genes change their lamina interactions

•Changes in lamina interactions reflect cell identity

•Release from the lamina may 'unlock' some genes for activation

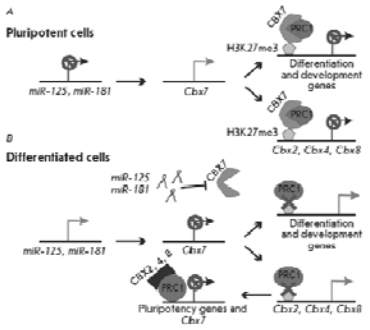
Key:
NPC: multipotent neural precursor cells

AC: terminally differentiated astrocytes

Interaction of pluripotency transcription factors and regulators of chromatin

- Open chromatin requires interaction of main pluripotency factors and proteins that regulates chromatin remodelling and modifications
- Components of Polycomb Repressive Complex 1 (PRC1) are required for stem cell function
- Binding of PRC1 to promoters depends on OCT4
- PRC1 component RNF2 interacts with Nanog
- PRC1 component CBX7 co-localizes with H3K27me3 in pluripotent cells & represses expression of development and differentiation genes
- Upon differentiation, microRNAs miR-125 and miR-181 represses CBX7 so development and differentiation genes are activated

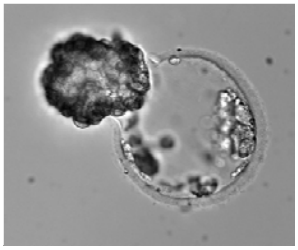
Interaction of pluripotency transcription factors and regulators of chromatin



Epigenetics of Pluripotent Cells. Medvedev et al., Acta naturae Vol. 4 № 4 (15) 2012

Unique Epigenetic Features in Pluripotent Cells

2) DNA Methylation in Pluripotent Cells



Pluripotency and DNA Methylation

- In addition to covalent histone modifications DNA methylation is also important in regulating pluripotency
- DNA methlytransferase DNMT1, DNMT3A, DNMT3B needed for differentiation
- Upon differentiation DNMTs methylate promoters of genes needed for maintaining self-renewal
- Pluripotent cells have reduced methylation in CpG rich promoters and increased methylation at CpG poor promoters

DNA Methylation and Stemness

*María Berdasco, Manel Esteller Stem Cell Research & Therapy 2011, **2**:42*

- Maintenance of pluripotency is given by occupancy of transcription factors OCT4, NANOG, and SOX2 on promoters of genes associated with self-renewal
- Expression of these transcription regulators is controlled by CpG promoter methylation (hypomethylated = activated, hypermethylated upon differentiation)
- ES cells have unique signatures of CpG methylation and histone modifications
- **Differentiation** of ES cells is accomplished by partial or full methylation of pluripotency-associated genes (*Oct4*, *Nanog*), resulting in their downregulation
- **Reprogramming** from differentiated cells to induced pluripotent stem (iPS) cells produces unmethylated active promoters of ES cell-specific genes

Unique DNA Methylation Patterns

*María Berdasco, Manel Esteller Stem Cell Research & Therapy 2011, **2**:42*

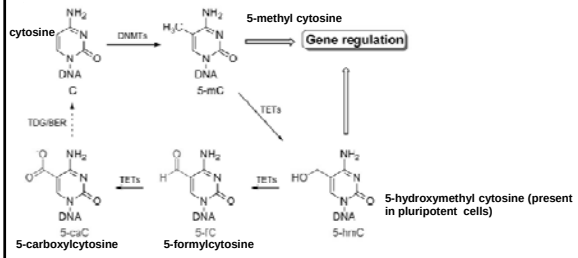
- Mammalian DNA methylation occurs at position 5 of cytosine residues, generally within CpG dinucleotides, and has been associated with transcriptional silencing
- DNA methylation studies of mESCs revealed most CpG-island-rich gene promoters (in house-keeping and developmental genes) are DNA hypomethylated
- CpG-island-poor promoters, typically in tissue-specific genes, are hypermethylated
- In hESCs, but not in differentiated cells, a significant proportion (~25%) of methylated cytosines are found in a non-CpG context, often in exons
- **Emphasizes unique epigenetic properties of pluripotent cell genome**

DNA Hydroxymethylation, Demethylation and Stemness

*Rada-Iglesias and Wysocka Genome Medicine 2011, **3:36***

- 5hmC is another epigenetic modification important in pluripotent cells
- Normally only in a limited number of cell types –e.g. Purkinje neurons
- Mediated by the ten-eleven translocation (TET) family enzymes which convert 5mC to 5hmC, **essential** for self-renewal of mouse ESCs, involved in regulating *Nanog* promoter methylation
- mESCs high levels of TET proteins, and their chromatin is 5hmC-rich
- In mESCs 5hmC occurs within gene bodies of transcriptionally active genes and at CpG-rich promoters
- 5hmC is 1st step in **removal** of DNA methylation from genomic loci

DNA Hydroxymethylation



DEMETHYLATION STEPS

(1) 5mC hydroxylated by TET enzymes to 5hmC or further oxidized to 5fC and 5caC

(2) UDG family of base excision repair (BER) glycosylases replaces the intermediates culminating in DNA demethylation

(3) Also, 5mC (or 5hmC) deaminated by AID/APOBEC

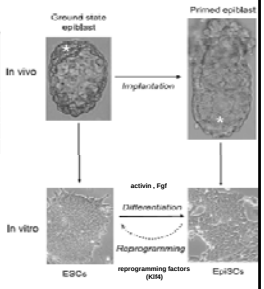
Naive and Primed ESCs

- In mESCs in serum, the ESC state is founded on the core regulatory transcription factors for pluripotency, OCT4, SOX2, and NANOG
- This is **stabilized** by leukemia inhibitory factor (LIF) and WNT signalling
- This system is **destabilized leading to differentiation** by FGF signalling
- OCT4, SOX2, and NANOG, while maintaining the pluripotent state of ESCs, activate FGF4, which is a key trigger for differentiation.
- ESCs in serum show heterogeneity and consist of at least two populations
  - **-One population with gene expression similar to the naive ICM state**
  - **-Other population has gene expression similar to primed epiblast state**
- They interchange identity during culture, so ESC state exhibits metastability and dynamic equilibrium, regulated by transcriptional circuitry and signalling inputs

Naive and Primed ESCs

Nichols and Smith, Cell Stem Cell, Volume 4, Issue 6, 2009

Property	Ground State	Primed State
Embryonic tissue	early epiblast	egg cylinder or embryonic disc
Culture stem cell	rodent ESCs	rodent EpiSCs; primate "ESCs"
Blastocyst chimaerax	yes	no*
Teratomas	yes	yes
Differentiation bias	none	variable
Pluripotency factors	Oct4, Nanog, Sox2, Klf2, Klf4	Oct4, Sox2, Nanog
Naive markers[5]	Rex1, Nr0b1, Fgf4	absent
Specification markers	absent	Fgf5, T
Response to Lif/Stat3	self-renewal	none
Response to Fgf/Erk	differentiation	self-renewal
Clonogenicity	high	low
XX status	XaXa	XaXi
Response to 2i	self-renewal	differentiation/death



•Ground state 'naïve' pluripotency in epiblast of blastocyst. ESCs are *in vitro* counterpart of this

•After implantation, epiblast primed for lineage specification. EpiSCs are the *in vitro* counterpart

PRDM14 Controls Naive Pluripotency, Connecting Signalling and Epigenetic Pathways in Mouse ES Cells

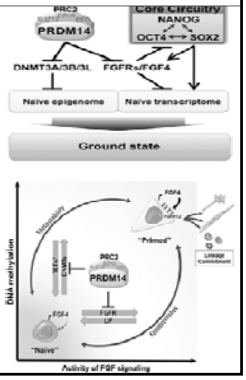
Yamaji et al., 2013. Cell Stem Cell 12, 1–15.

PRDM14 ensures naive pluripotency by:

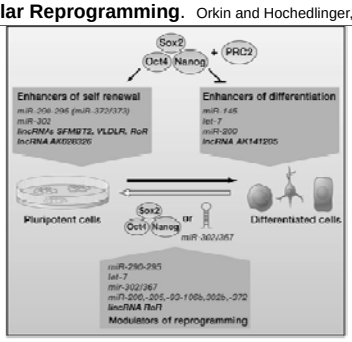
1) Antagonizes activation of FGFR signalling by the core pluripotency transcriptional circuitry

2) Represses expression of *de novo* DNMTs that modify the epigenome to a primed epiblast-like state

PRDM14 recruits PRC2 specifically to key targets, repressing their expression



Noncoding RNAs Modulate ESC Self-Renewal, Differentiation, and Cellular Reprogramming. Orkin and Hochedlinger, Cell 145, 2011

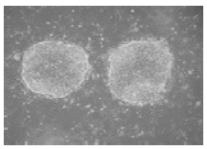


•microRNAs (in red) and lncRNAs (in black) that are either activated by Oct4, Sox2, and Nanog or silenced by the same factors in combination with PRC2 in pluripotent cells

•Manipulation of several noncoding RNAs enhances cellular reprogramming in iPSCs

Epigenetic Problems with Stem Cells

- Epigenetic defects:
  - Culture induced
  - Inefficient reprogramming
  - Founder cell problems



Tumorigenicity of human ESCs and iPSCs

Ben-David U, Benvenisty N. Nat Rev Cancer. 2011 Apr;11(4):268-77.

• The potential tumorigenicity in HESC- and HiPSCs needs to be addessed in order to develop safe treatments.

Epigenetic abnormalities		
Factors influencing tumorigenicity	**HESCs**	**HiPSCs**
Cell of origin	Similarity of global gene expression with some cancers (onco-fetal genes are highly expressed) *	• Similarity of global gene expression with some cancers (onco-fetal genes are highly expressed) • Epigenetic memory of somatic transformations and/or of susceptible traits of the somatic tissue‡
Derivation process	No substantial epigenetic aberrations are known to occur in the process*	• Cancer-related epigenetic abnormalities arise during reprogramming‡ • Relaxation of imprinting might also occur in the process‡
Cellular adaptation to culture	Relaxation of imprinting might occur in culture*	Relaxation of imprinting might occur in culture*

‡ High risk of tumour generation, * Medium risk of tumour generation

Epigenetic Defects in Embryonic Stem Cells

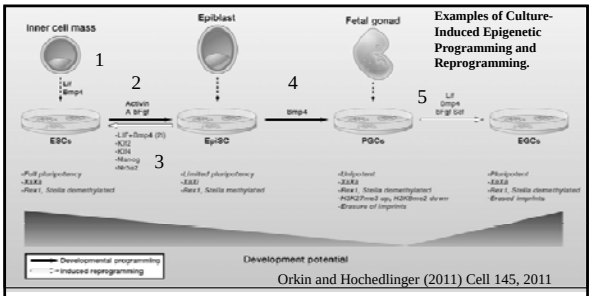
Huntriss and Picton Current Stem Cell Research & Therapy, 2008

• Defective imprinting states are observed in ES cells from various species
• Extended culture exacerbates problems

Species	**Imprinted Gene**	**Aberrant Event in Embryonic Stem Cells**
Mouse	*H19, Igf2r,Igf2, U2af1-rs1*	Altered methylation and imprinting status of several genes
Mouse	*H19*	Variable *H19* expression and methylation
Rhesus Monkey	*H19, IGF2*	Biallelic expression in ES cells
Rhesus Monkey	*H19/ IGF2 ICR CTCF site*	Aberrant hypermethylation on maternal allele
Human	*H19*	*H19* biallelic, increase in DMR methylation
Human	*IGF2, MEG3*	Variable allelic expression of *IGF2. MEG3* Biallelic
Human	*IPW, H19, MEG3, MEST, PEG10,MESTIT, GNAS, ATP10A, PHLDA2, IGF2*	Variable allelic expression of 10 genes in 22 hESC lines

Epigenetic Defects in Human Induced Pluripotent Stem Cells

- **Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells** Ohi *et al*., Nature Cell Biology Volume: 13, Pages: 541–549. (2011)
- **Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells.** Lister *et al*., Nature Volume: 471, Pages: 68–73 (2011)
- 5 human iPSC lines show significant reprogramming variability, including somatic memory and aberrant reprogramming of DNA methylation
- iPSCs have regions near centromeres & telomeres with incomplete reprogramming
- Errors persist after differentiation



Examples of Culture-Induced Epigenetic Programming and Reprogramming.

Orkin and Hochedlinger (2011) Cell 145, 2011

•There are extensive epigenetic changes associated with stem cell derivation, and programming / reprogramming to achieve the final desired cell type:

•Major changes with:

-X chromosome activation/silencing

-Imprint erasure and establishment

Comprehensive Methylome Analysis in Pluripotent Cells

Review: Anton Wutz, Cell Stem Cell 11, 2012

- Gene expression and DNA methylation patterns assessed in over 200 hESC and hiPSC lines (Nazor *et al*., 2012)
- Identified two groups of genes with reciprocal epigenetic patterns in the undifferentiated versus differentiated state were
- (1) a set of genes that is consistently methylated in hiPSCs and hESCs and unmethylated in all examined tissues
- (2) a group of genes that is methylated in hiPSCs and hESCs and is unmethylated only in specific tissues
- **Most variation at imprinted genes and genes and the X chromosome in female hiPSCs-culture induced**

Are hESCs and iPSCs Epigenetically Equivalent?

Rada-Iglesias and Wysocka *Genome Medicine 2011, **3:36***

•iPSCs share properties with ESCs but are they functionally equivalent?

•The most stringent pluripotency assay, tetraploid embryo complementation, shows that mouse iPSCs can give rise to all tissues of the embryo proper but many iPSC lines do not support this assay on or are less efficient at it

•Differences reported in DNA methylation and gene expression patterns

•Sources of differences :
(i) variability in cell line derivation and culture
(ii) genetic variation among cell lines
(iii) hotspots of aberrant epigenomic reprogramming
(iv) incomplete erasure of somatic marks during iPSC reprogramming
(v) defects in the re-establishment of hESC-like patterns in iPSCs
(vi) selective pressure during reprogramming

• **Therefore, attempts at artificial pluripotency are not yet optimal**

Conclusions on Epigenetic Errors in Pluripotent Cells

- Pluripotent state is maintained by intricate mechanisms with epigenetic factors and cell signalling linked to the pluripotency transcription factor network
- Epigenetic aberrations occur frequently in hiPSCs and hESCs and this limits their use for potential clinical applications
- Tumorgenicity is a concern for applications of stem cells
- Reprogramming stem cells involves major epigenetic changes
- Studies on pluripotent stem cells to date suggest extreme caution should be taken when considering the use of human gonadal stem cells (or any stem cells) for fertility treatment

Stimulation protocols in cancer patients

Juan A Garcia-Velasco, MD and Carlos Iglesias, MD
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Learning objectives

- To understand the different option for fertility preservation, with special emphasis on ovarian stimulation protocols
- To acknowledge the difficulties of some patients undergoing COs for fertiilty preservation (time, ovarian response…)
- To review the results of 5-years experience with fertility preservation with different COS protocols

Conflict of interest

- I declare that I have no commercial or financial interests pertaining to the subject of this presentation or its content

Key issues

- To whom offer treatment
- What to do
- How to do it
- Results

Introduction

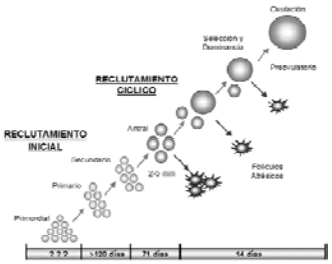
- Spain, 2008: 185.000 cancer patients
- 2003: 95.000 patients died of cancer disease
- 56% are women
- 16.000 are breast cancer patients
- 78% women overcome breast cancer

Ministerio de Sanidad y Consumo - 2005

Diagnosis: cancer… and so what?

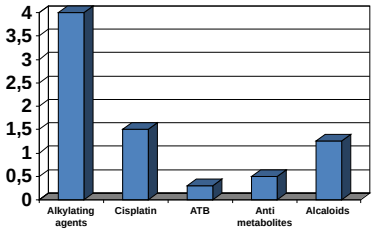
- Irregular menstruation
- POF
- …infertility?

Gonadotoxic effect



Chemotherapy harms preantral follicles

Chemotherapy



Meirow et al. 2001

Key issues

- To whom offer treatment
- What to do
- How to do it
- Results

Ovarian reserve tests

Clinical parameters

-Menstruations
-Regular menses
-Pregnancy

Laboratory parameters

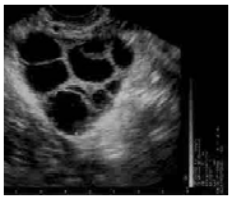
-FSH
-Estradiol
-AMH

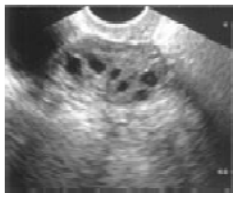
Ultrasound parameters

-AFC
-Ovarian volume

Ovarian reserve tests

Ultrasound scan: AFC





Ovarian reserve tests

Laboratory parameters

- **FSH**
- **Estradiol**
- **AMH**
  - No variability from cycle to cycle
  - No variability on the day of the cycle
  - No varibility in PCO

Nelson et al. 2011; La Marca et al 2010

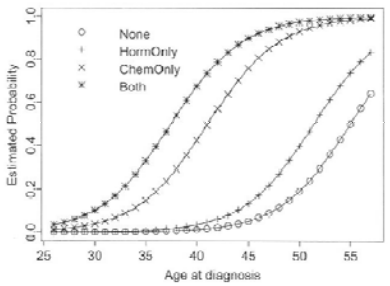
Evaluation of the ovary function

- 37 women– haematologyc cancer
- 1995- 2004
- Serum samples previous and post Ch/Tt

	Cancer	Control	
Age	**29.4**	**29.9**	
Regular menstruation	**23%**	**100%**	*
AMH (ng/l)	**0.3**	**1.3**	*
FSH	**64**	**5.8**	*
AFC	**1**	**14**	*

Lie Fong et al. 2008

What is happening?



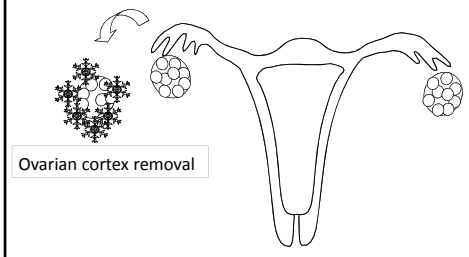
Goodwin et al. 1999

Options to preserve fertility

- GnRHa
- Ooforopexy
- Ovarian tissue freezing
- Embryo vitrification?
- Oocyte vitrification.

Ovarian cortex orthotransplant

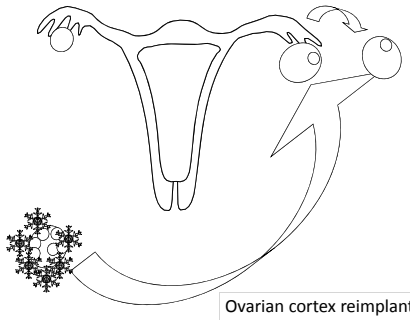
BEFORE CANCER TREATMENT BEGINS



Ovarian cortex removal

Ovarian cortex orthotransplant

AFTER CANCER IS CURED



Ovarian cortex reimplantation

Ovarian cortex orthotransplant

VALENCIA_ Program Ovarian Banking

- Started in2005 Ob/Gyn Dept, University Hospital Dr Peset, Valencia
- Open to all Ob/Gyn and Oncology Depts from Spain
- >400 ovarian cortex frozen
- 5 implants performed

Ovarian cortex orthotransplant

- Donnez et al, 2004
- Meirow et al, 2005 (FIV)
- Demeestere et al, 2007
- Yding Andersen et al, 2008 (2 emb FIV)
- …..17 pregnancies
- Failed transplants are not published
- Still is experimental

5th pregnancy/world & 1st Spain

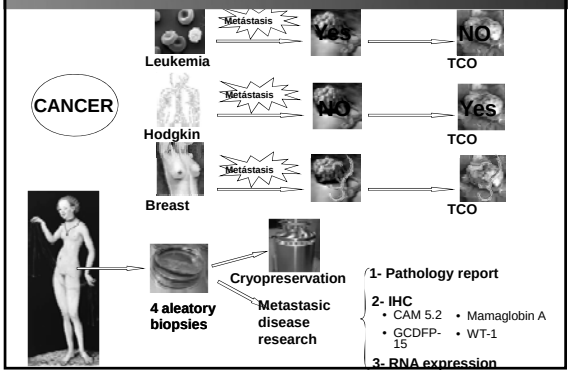
Twins born after transplantation of ovarian cortical tissue and oocyte vitrification

María Sánchez Serrano, M.D.,[a,b] Juana Crespo, M.D.,[a] Vicente Mirabet, Ph.D.,[c] Ana C. Cobo, Ph.D.,[a] María-José Escribá, Ph.D.,[a] Carlos Simón, M.D.,[a] and Antonio Pellicer, M.D.[a,b]

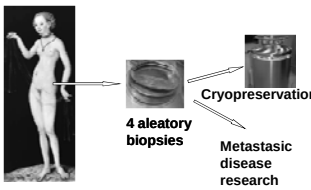
[a]Instituto Valenciano de Infertilidad, University of Valencia; [b]Hospital Universitario Dr. Peset; and [c]Centro de Transfusión de la Comunidad Valenciana, Valencia, Spain

Fertil Steril 2010

Metastasic disease in the ovarian cortex



Metastasic disease in the ovarian cortex



4 aleatory biopsies

Cryopreservation

Metastasic disease research

1- Pathology report

2- IHC

- CAM 5.2
- Mamaglobin A
- GCDFP-15
- WT-1

3- RNA expression

100 ovarian biopsies (63 breast ca pts)
No evidence of metastasic disease in any of them

Sánchez et al Hum Reprod 2009

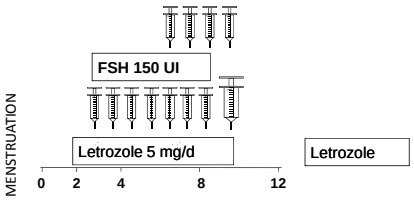
Controlled ovarian stimulation

Oocyte vitrification

Key issues

- To whom offer treatment
- What to do
- How to do it
- Results

COH: Letrozole protocol



- GnRH antagonist when leading follicle is ≥14 mm.
- hCG/GnRHa as soon as both follicles are 19-21 mm.
- Restart letrozole until next menstruation

Oktay et al, JCEM 2006

Natural cycle, monofollicular cycle and Letrozole

	Natural cycle	Monofollicular cycle	Letrozole+FSH	Tamoxifen	Tam+FSH
Estradiol (mean)	269,4	277.9	380	419	1.182
Estradiol (median)	224,5	251	---	---	---
	(1)	(2)	(3)	(3)	(3)
Nº oocytes	1	1	12,3±2,5	1,7±0,3	6,9±1,1
Nº MII	1*	1*	8,5±1,6	1,5±0,3	5,1±1,1

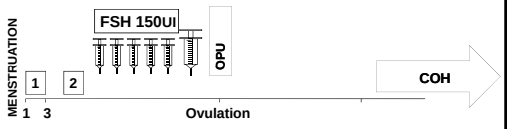
(1) Instituto Valenciano de Infertilidad. IVI Valencia.
(2) Hospital Universitario Materno Infantil. Las Palmas GC.
(3) Oktay et al, J Clin Oncol 2005

GnRHa vs hCG triggering

	hCG n=47	GnRHa n=27
Peak E2 (pg(mL)	472	695*
total# oocytes	12.8	16.4
MII oocytes	7.4	11.9*
2 PN	6.3	9.3*
Fertilization rate	74%	84%*

Oktay et al. 2010

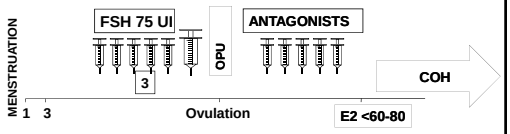
How to start a cycle (1)



1 - Before menstruation
-1º-3º cycle day.
- Without dominant follicle → Start COH

2 Dominant follicle (<14 mm). → Aspiration and start COH

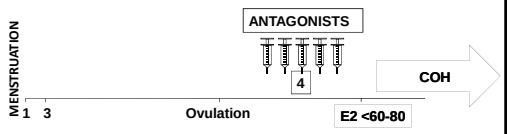
How to start a cycle (2)c



3 Follicle >14 mm:

- Growing dominant follicle with 75 IU FSH.
- Aspiration and vitrification the oocyte obtained
- Inicitate GnRH antagonist and COH

How to start a cycle (3)



4 -Fase:

- GnRH antagonist during 3-5 days
- When E2<60-80 pg/ml, start COH appart from bleeding

Random start

	#1	#2	#3
Age	29	26	26
COH start day	14	11	17
E2 (pg/mL)	62	269	50
P4 (ng/mL)	1.2	0.4	2.5
AFC	11	20 d	20 cl
peak E2 (pg/mL)	499	988	478
Oocytes obtained	9	17	16
MII	7	10	11
Fertility rate	87.5	83.3	69.2
Frozen embryos	7	10	9

Sönmezer et al. 2011

Oocyte vitrification

Cryotop®

Key issues

- To whom offer treatment
- What to do
- How to do it
- Results

Patient demographics and characteristics of the stimulation cycles for both oncological and non-oncological FP patients.

	Non-oncological FP n=560	Oncological FP n=355
Age (years)	36.7 ± 4.2	31.9 ± 5.1*
Previous children	-	11.3%
Days from 1st visit to COS	-	9.4
Length of stimulation (days)	10.1 ± 2.1	9.5 ± 5.9 *
Cancellation rate (%)	2.7	6.7 *
Total # oocytes	7225	4104
Total MII (%)	5498 (76)	2939 (71.6) *
MII/patient	9.9	8.5
Total FSH/hMG with letrozole	-	1493 ± 940
Total FSH/hMG (IU)	3038 ± 337	1851 ± 979 *
Peak E2 at triggering with letrozole (pg/mL)	-	404 ± 676
Peak E2 at triggering (pg/mL)	2214 ± 566	1369 ± 1371 *

* p<0.001

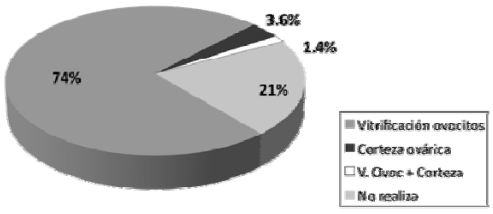
Garcia-Velasco et al Fertil Steril 2013

	Non-oncological	Oncological
N patients/warming cycles	26	4
"Fresh" embryo transfers (%)	24 (92.3)	4
N embryos transferred	37 (1.5 ± 0.6)	8(2)
CPR/patient (%)	11 (42.3)	1(25)
OPR/patient (%)	8 (30.7)	1(25)
N patients with surplus embryos	17 (65.3)	2(50)
N surplus embryos vitrified	49 (2.8 ± 4.2)	4(2)
N cryo transfers	15 (88.2)	1
N embryos transferred per cryo transfer	2.3 ± 0.7	2
CPR/patient (%)	7 (46.6)	1(100)
OPR/patient (%)	5 (33.3)	0
Total live birth	5	1
Mean birth weight (g)	3150 ± 0.3	3440

Unless otherwise indicated, values are mean ± SD.
CPR=clinical pregnancy rate, OPR= ongoing pregnancy rate.

Garcia-Velasco et al Fertil Steril 2013

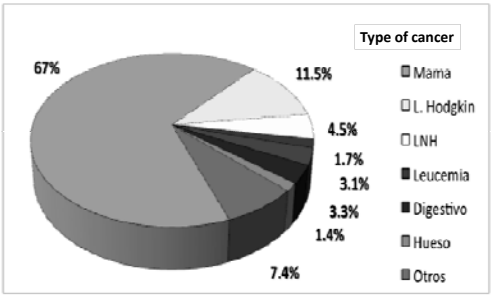
IVI_Fertility preservation programme



419 patients

Cycle cancellation rate: 6.5% (n: 21)

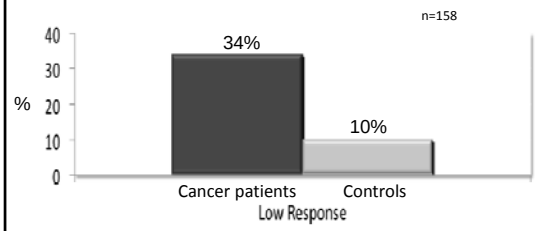
IVI_Fertility preservation programme



IVI_Fertility preservation programme

Age	**31.9 (16-42)**
Previous children	**48 (11.5%)**
Days since 1st visit till stimulation	**7.6 (0-77)**
Days of stimulation	**9.04**
Nº cycles	**324**
Previous natural cycle	**11**
Cancelled cycle	**21 (6.5%)**

Fertility preservation: low responder (≤5 oocytes)



Fertility preservation: low responder (≤5 oocytes)

Ovarian response to controlled ovarian hyperstimulation in cancer patients is diminished even before oncological treatment

Javier Domingo, M.D.,[a] Vicente Guillén, M.D.,[a] Yanira Ayllón, M.D.,[a] María Martínez, M.D.,[b] Elkin Muñoz, M.D.,[c] Antonio Pellicer, M.D.,[d] and Juan A. García-Velasco, M.D.[b]

[a] IVI Las Palmas, Las Palmas, [b] IVI Madrid, Rey Juan Carlos University, Madrid, [c] IVI Vigo, Vigo, and [d] IVI Valencia, Valencia University, Valencia, Spain

n=272

Domingo J et al. Fertil Steril 2012c

Fertility preservation: low responder (≤5 oocytes)

	Non HD,antagonist FSH n=66	HD, Letrozole FSH n=142	Control n=97
Age	30,6 +- 5,7	33,2 +- 4,3	31,9 +- 5,3
Days of stimulation	8,7 +- 1,7*	9,6 - 2,4	9,9 +- 1,6
Total FSH IU	1803 +- 889	1755+- 1114	1947 +- 808
Peak serum E2, pg/ml	1744+- 1242	381 +- 191*	2109 +- 1260
Retreived oocytes	12,2 +- 6,5	9,8 +- 7,1*	12,4 +- 5,4
% MII oocytes	75,3 +- 18,5	74,4 +- 22,1	72,2 +- 17,7

* P<0,05

Domingo J et al. Fertil Steril 2012

Conclusions

- Spontaneous pregnancy
  - Age
  - Intensive care
- ART
  - Before ChT/RT or without pregnancy
  - After ChT/RT
    - Low response to COH
    - Individualized protocols
    - Oocyte donation

Cancer stem cells and their role in male germline cancers

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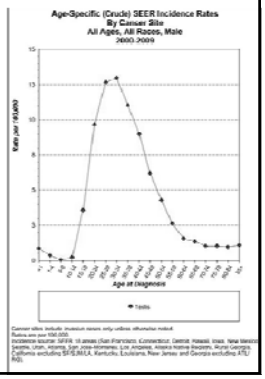
- I have no commercial relationships or other financial conflicts of interest to disclose

Learning Objectives

- Adult male germ cell tumor (GCT) etiology
- Stem cell nature of GCT and similarities to ES
- Implications for therapy
- Unifying features of genotype, phenotype, and clinical behavior

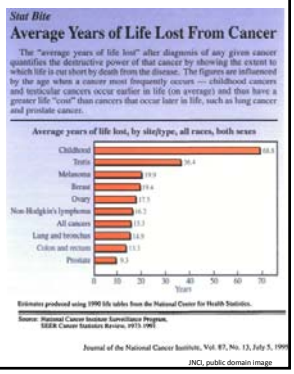
Germ Cell Tumors

- Most common solid tumor in young adult men (18-35)
- Excellent outcomes following treatment with cisplatin
- Overall cure rates are greater than 90%



GCT Average Life Years Lost

- Mortality from GCT has the highest # of average life years lost of any adult cancer
- Incidence has more than doubled over the past 40 years

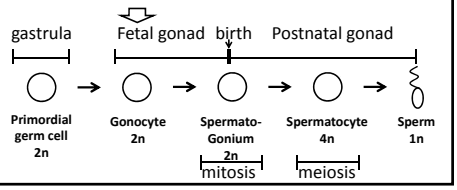


JNCI, public domain image

Cell of Origin of GCTs: Theory #1

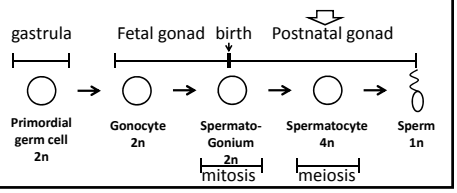
- gonocyte is cell of origin
- event occurs *in utero*
- based on: i) stains (e.g. PLAP, BLIMP1)
  ii) DNA damage response patterns
  iii) expression profiles
- Correlative rather than mechanistic model

Normal human sperm develop-ment



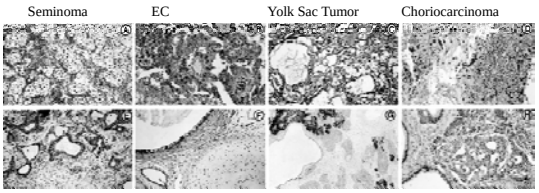
Cell of Origin of GCTs: Theory #2

- spermatocyte is cell of origin; event occurs post-puberty
- based on spermatocyte specific stains (DDX3Y, MAGEA4)
- Mechanistic: aberrant recombination during meiosis generates genomic aberrations that drive carcinogenesis

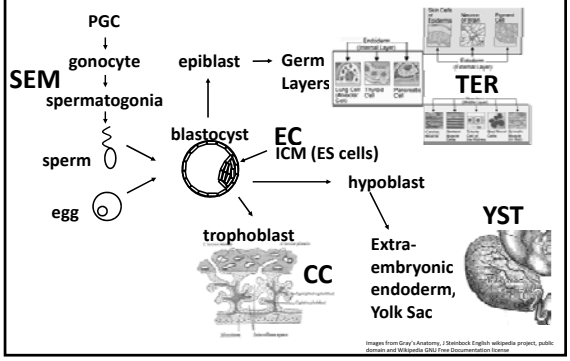


GCTs, Pluripotency, and Differentiation

- GCTs are pluripotent, capable of giving rise to all cell lineages
- GCTs are a model for transformed ES cells, and as such, share many of the same properties



GCT Etiology vs. Embryogenesis



Why is Pluripotency Important for GCT?

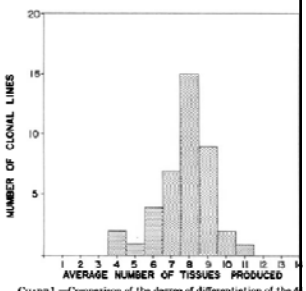
• Pluripotent stem cells share one important property with cancer cells: <u>unlimited replication potential</u>

EC are True Stem Cells

•***<u>SINGLE</u>*** cells from a murine EC were engrafted

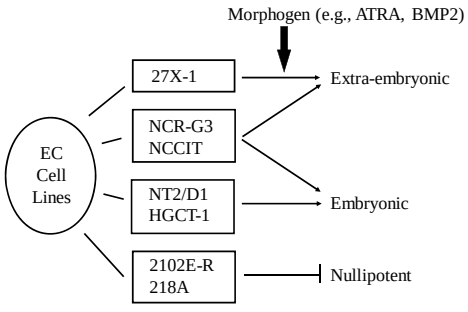
• Tumors in 11% of implants

• Tumors (teratocarcinomas) had multiple different cell types present



Reprinted by permission from the American Association for Cancer Research: Kleinsmith and Pierce, Cancer Research 1964; 24, 1544-1551

Differentiation of EC cell lines in vitro



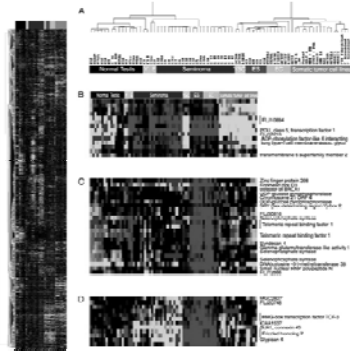
• EC share pluripotent phenotypic properties with ES cells
• What about at the molecular level?

Stem Cell Markers in EC vs ES

	Human ES	EC
Self renewal	Yes	Yes
pluripotency	Yes	Yes
SSEA3+	Yes	Yes
SSEA4+	Yes	Yes
TRA-1-81+	Yes	Yes

- Several of these classic stem cell markers were identified in EC cells
- Studies of cell surface markers demonstrated that particular markers were confined to cells with pluripotency (Andrews, 1996)

GCT vs. ES cells

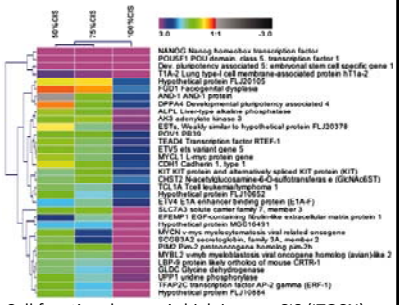


- expression profiling shows EC and ES cluster together and form a distinct cluster
- Sem share some expression patterns (B) but others are unique to ES/EC cells (C and D)

Sperger J M et al. PNAS 2003;100:13350-13355

Stem Cell Gene Expression in ITGCN (CIS)

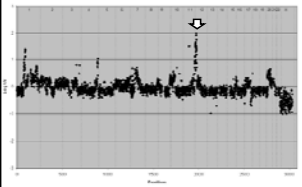
- NANOG
- POU5F1/OCT3/4
- DPPA5
- DPPA4
- KIT
- ETV4
- ETV5
- PIM2
- TFAP2C
- TEAD4



- expression of Stem Cell functional genes is high in pure CIS (ITGCN)
- Surface pluripotency marker expression was found to be more variable in CIS however (Andrews et al, 1996)

Chromosomal Alterations in GCTs

- Gain of 12p occurs in ~100% of GCTs
- Occurs as an isochromosome
- presence in ITGCN is more controversial



arrayCGH profile of GCT showing high level gain of 12p

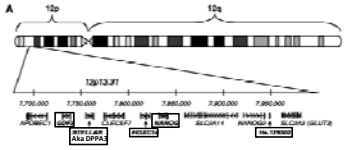
• **<u>What are the targets of 12p gain?</u>**

•*CCND2*?

•many other potential target genes (~440 total genes on 12p)

Expression Profiling of 12p gain in GCTs

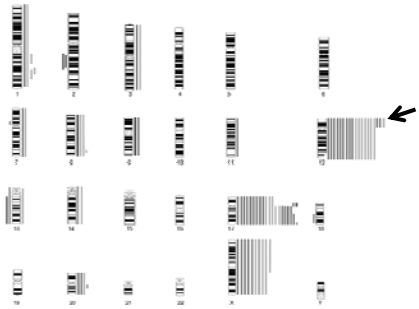
- 200 kb stem cell cluster on 12p is another target (region includes NANOG)
- Over-expression specifically in pluripotent EC cells and undifferentiated SEM



Denotes stem cell associated gene expression pattern

Korkola J E et al. Cancer Res 2006;66:820-827

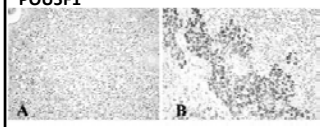
Importance in Normal Stem Cells?

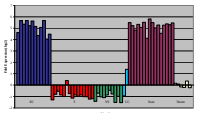


Gain of 12p is the second most common event in cultured HESCs, and can occur as i(12p) concomitant with over-expression of NANOG

Core Stem Cell TFs in EC vs SEM

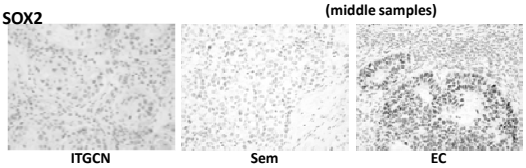
POU5F1





NANOG is one of the target genes; high in EC (left) and SEM (right), low in TER and YST (middle samples)

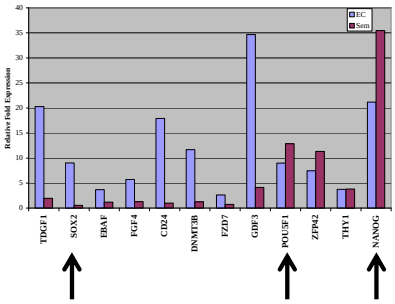
SOX2



ITGCN Sem EC

Korkola J E et al. Cancer Res 2006;66:820-827

Other Stem Cell Genes in EC vs Sem

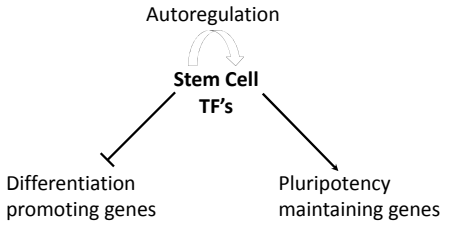


Korkola J E et al. Cancer Res 2006;66:820-827

Stem Cell Networks in ES

- ChIP of core stem cell TF's identified regulatory networks controlling pluripotency and differentiation (Boyer et al, 2005)
- TFs co-occupy regulatory regions of target genes and are auto-regulatory
- lineage specificity vs. maintenance of pluripotency controlled in part by partner TF's that co-occupy regulatory sites (POU5F1 for ESC, BRN2 for NSC; Lodata et al, 2013)

Stem Cell Networks in GCT and ES



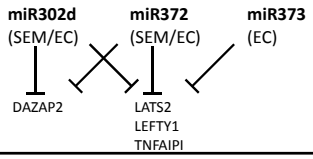
•Many of the target genes identified in ES are preserved in GCTs, and many of the same programs are functional

MicroRNA in GCT and ES

• Gillis et al performed microarray analysis in GCT and identified differentially expressed miRNA

•Inhibition of differentiation targets in EC/SEM (e.g. LEFTY1- identified originally as a gene necessary for differentiation)

• miR302d has also been shown to be highly expressed in hES cells (Suh et al. 2004)



EC vs ES

- GCTs and hESC share many of the same properties:
  - Pluripotency
  - Expression of core stem cell transcription factors
  - Maintenance of stem cell signaling networks
  - Expression of microRNA concomitant with repression of common targets

Stem Cell Properties: Implications for Therapy

- Relationship to clinical response:
  - Pluripotency and sensitivity of GCTs?
  - Is resistance associated with patterns of differentiation?
  - What role does DNA repair play?

Treatment of GCTs

• for patients with metastatic GCT, therapy consists of:

- 4 cycles EP (etoposide plus cisplatin)
- 3 cycles BEP (bleomycin, etoposide, plus cisplatin)
- more aggressive therapies (salvage therapy) for non-responsive cases

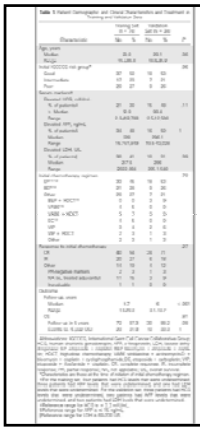
•These regimens result in cure rates >80% in combination with surgical resection of residual disease

Risk Assessment in NSGCT (IGCCCG)

Category	NSGCT	% Cured
Good	Low Serum Marker Levels No non-pulmonary visceral mets present Gonadal or retroperitoneal tumor	>90%
Int.	Moderate Serum Marker Levels No non-pulmonary visceral mets present Gonadal or retroperitoneal tumor	~70%
Poor	High Serum Marker Levels Non-pulmonary visceral mets (bone, brain, liver) Primary Mediastinal tumor	~40%

Characteristics of Serum Risk Markers

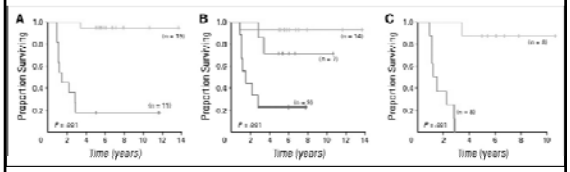
- **AFP**: alpha fetoprotein
  - Plasma protein produced by yolk sac/placenta
  - Low levels in adults
- **LDH**: lactate dehyrodgenase
  - Enzyme responsible for catalysis of lactate and pyruvate
  - Usually expressed in heart, liver, lungs, kidney, and placenta
- **HCG**: Human Chorionic Gonadotropin
  - Hormone produced by placenta



Outcome Prediction

- Expression profiling performed on a panel of mixed NSGCTs (N=74)
- Outcome predictor developed using computational approach
- Predictive utility of the gene signature in an independent tumour cohort (N=34)

Outcome Prediction Results



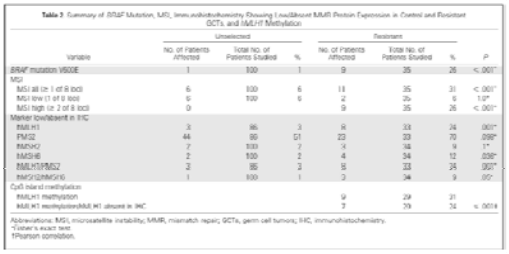
Kaplan-Meier survival curves of patients (A) who were predicted to have good (gold) or poor (blue) outcome based on gene expression; B) who had good (gold), intermediate (blue), and poor (gray) International Germ Cell Cancer Collaborative Group (IGCCCG) risk assessment; and (C) who had intermediate and poor IGCCCG risk assessment and good (gold) and poor (blue) PAM prediction.

Analysis of Predictive Genes

Function	Genes Associated with Good Outcome
immune regulation and function	*BLNK, IGHM, IGKC, IGJ, IGHA1, IGKV1-5, IGLV3-25, PTPRC, SYK, CXCL12, ITGB2, C1S, C1R, C7, IL6R, IFI16, MNDA, TNFSF13B, HLA-DPA1*
cell migration and motility	*CAPZB, CD97, CCL5,* and *CXCL12*
Function	**Genes Associated with Poor Outcome**
neural development and differentiation	*BMP7, MDK, NRCAM, OTX2, PCDHB14, PLXNA2, SOX11, and ZIC1*
left-right symmetry -pattern specification	*BMP7, ZIC1, and CFC1*
cell adhesion/ECM	*COL2A1, COL9A2, FLRT3, NRCAM, and PCDHB14*
Smoothened signaling	*OTX2, ZIC1*

Good Outcome: Immune signature and maintenance of pluripotency
Poor Outcome: genotypic differentiation into neural, renal, and skeletal pathways (even in the absence of phenotypic changes)

DNA Repair in GCT



- Looked in 35 cisplatin resistant and 100 unselected GCT for microsatellite instability (MSI), Mismatch Repair deficiency, and association with BRAF mutations
- Cisplatin resistant GCT had: MSI high; decreased expression of MMR proteins; high incidence of BRAF mutation V600E.

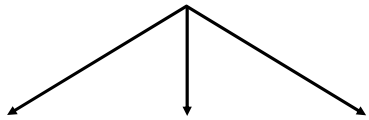
More DNA Repair

- Cavallo et al examined EC repair of intra-strand crosslinks
- saw evidence for a defect in homologous recombination (HR) repair (decrease in the number of RAD51 foci)
- These cells also demonstrated sensitivity to PARP inhibitors

DNA Repair Implications

- Resistance due to increased microsatellite instability and decreased MMR?
- Sensitivity of GCT to cisplatin is in part due to defects in HR repair?
- ESC are also sensitive to cisplatin; Csnk1a1 found to be important in mESC in this process (Puigvert 2013)

Germ Cell Tumors



Genotype	Phenotype	Clinical
Gain of 12p in ~100% of cases	**"Stemness"**	**~90-95% cure rates**
• Establishes and/or maintains pluripotency	• TFs maintain stem cell gene and repress lineage specific gene expression	•Differentiation into specific lineages associated with poor outcome
• provides oncogenic stimulus	•Defects in DNA repair and the ability to fix intra-strand crosslinks	

Summary

- GCT (EC) show strong similarities to ES cells
- Cells in this state appear to be more sensitive to toxic insult (consistent with DNA repair programs in ES/EC)
- Differentiation patterns may be associated with resistance to chemotherapy

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