ISFP International Society for Fertility Preservation

Fertility restoration in prepubertal boys: perspectives with testicular tissue

C Wyns
MD, PhD
Gynecology and Andrology
Head of Department

Cliniques universitaires
SAINT-LUC
UCL BRUXELLES

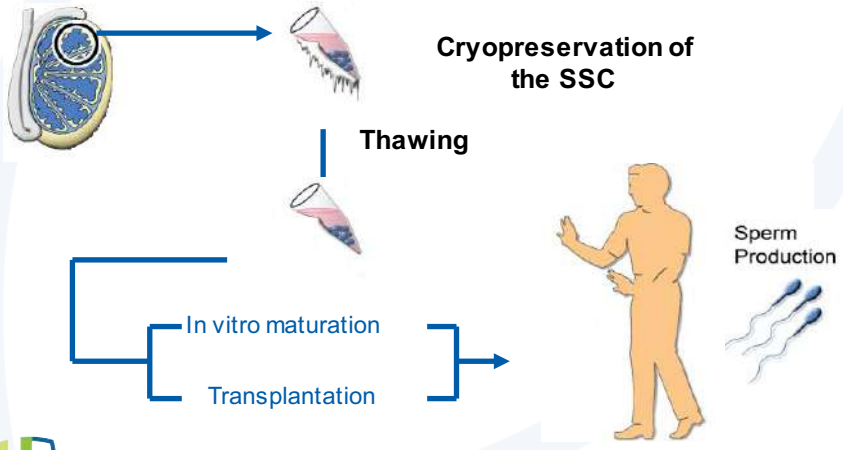
ISFP The 5th World Congress of the INTERNATIONAL SOCIETY FOR FERTILITY PRESERVATION
November 16-18, 2017 | Vienna, Austria

MD, PhD, Christine Wyns
Gynecology & Andrology

Fertility restoration in prepubertal boys: Perspectives with testicular tissue

Disclosure information: Nothing to declare related to the topic

Introduction
Fertility preservation for prepubertal boys
Preserving the spermatogonial stem cell



Introduction
Fertility preservation for prepubertal boys
Who is candidate?

Oncological	Non-oncological
Before gonatotoxic therapies	
Debatable	
• Low risk of infertility	• Risk of testicular degeneration: — Cryptorchidy — Klinefelter — Y-microdeletions

BUT … No complications following testicular biopsy

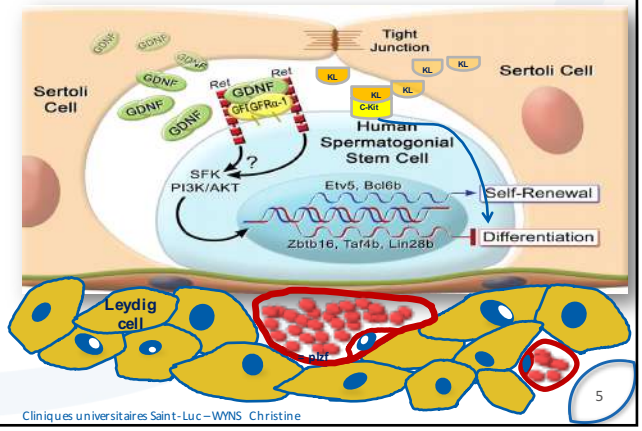
Wyns et al., 2010, 2011; Picton et al., 2015; Uijldert et al., 2017

Introduction

Fertility preservation for prepubertal boys

Cryopreserving the SSC in its niche

- Maintenance of somatic cells needed for subsequent SSC maturation
- Modification of epigenetic patterns of germ cells in case of SSC niche disruption *(Goosens et al., 2011)*
- Possibility of later cell isolation



Adapted from Wu and al; 2009

The fertility restoration approach depends on the disease:

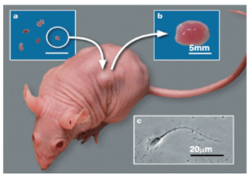
- **Risk of testicular tissue contamination with cancer cells**
- **Competent niche**
- **Competent SSCs**

Fertility restoration options with prepubertal TT (animals)

Where are we now?

Autografting of
- Tissue pieces
- Cell agregates

Offspring
Mouse (tissue and cell agregates)
Rabbit (tissue)
Pig (tissue xenografts)
Quail
Monkey ***(Liu et al., 2016)***



Honaramooz et al., 2002

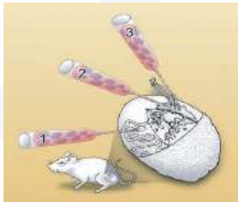
In vitro maturation of germ cells

Offspring
Mouse (organ culture method)
Sato et al., 2011

Transplantation of cell suspensions

Preimplantation embryo development
NH primates ***(Hermann et al., 2012)***

Offspring
Mouse
Rat
Goat
Chicken
Lamb
Zebrafish


Brinster et al., 1994

Fertility restoration with prepubertal tissue (humans)

Where are we now?

SSC Niche

DISSOCIATED	3D culture	ND
	Isolated cells' transplantation	ND
PRESERVED	Organotypic culture	Spermatogonial proliferation, Sertoli cells and Leydig cells maturation (De Michele et al., 2018)
	Tissue transplantation	ND
	Tissue xenotransplantation	Survival and proliferation of spermatogonia (Wyns C et al. 2007; Goossens E et al., 2008; Poels J et al. 2012, 2014, 2016) Differentiation to pachytene spermatocytes and spermatid-like cells (Wyns et al., 2008)
RECONSTITUTED	Organoids	ND

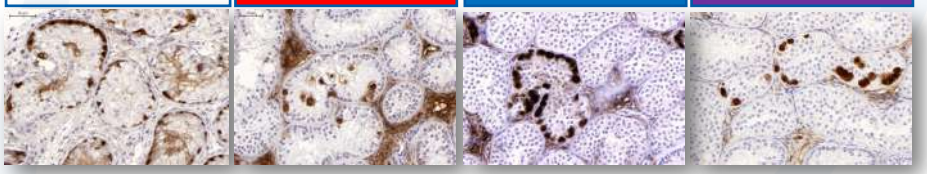
De Michele, Vermeulen & Wyns, 2017

Tissue transplantation

XenoTransplants of fresh/slow-frozen /vitrified human ITT

Spermatogonia: MAGE-A4

Control non-grafted	Fresh grafted	Slow-frozen grafted	Vitrified grafted



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

a and b differ significantly ($p < 0.001$)

→ Recovery of spermatogonia

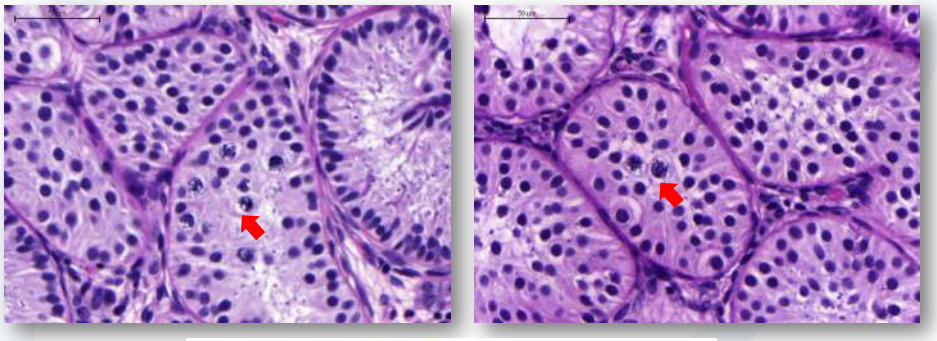
→ No difference for fresh or cryopreserved grafts

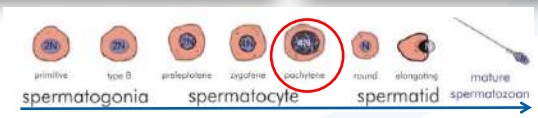
Poels et al., 2013

Tissue transplantation

XenoTransplants of fresh/slow-frozen /vitrified human ITT

Slow-freezing | **Vitrification**





→ Spermatogonial differentiation to pachytene stage

Poels et al., 2013

Transplantation of cryopreserved ITT: challenges …

Spermatogonial recovery rates after xenografting of human ITT

5 days	3 weeks	6 months
53% to 67%	14.5%	4.1%

→Most of spermatogonial loss occurs before 3 weeks

Intact seminiferous tubules after xenografting of human ITT

5 days	3 weeks	6 months
18.6% to 21%	82.2±16.5%	89.7±17.9%

→ST integrity did not worsen over time concomitantly with SG loss
→ST partially recover from initial insult → only empty SSCs niches remain

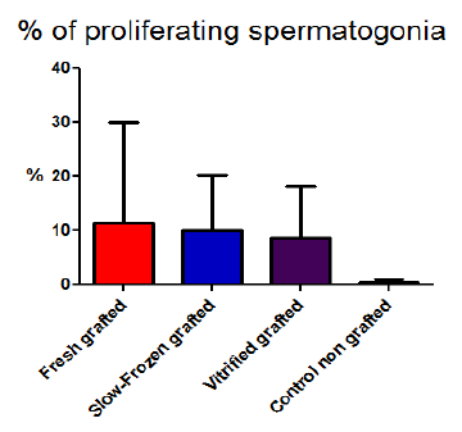
Wyns et al., 2007, 2008; Poels et al., 2013, 2014

Tissue transplantation

Why spermatogonial loss?

- Spermatogonial proliferation capacity maintained

% 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

Poels et al., 2013

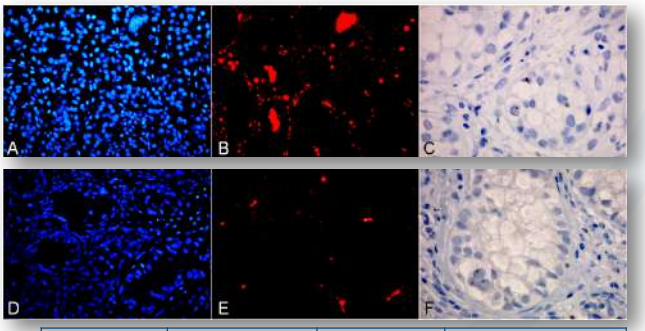
Tissue transplantation

Why spermatogonial loss?

- Inadequate recipient environment or ischemic stress before revascularization responsible for increased apoptosis/necrosis ?

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

Wyns (personal data)

Tissue transplantation

Why spermatogonial loss?

- Negative impact of the grafting procedure
  Early ischemia due to avascular grafting?

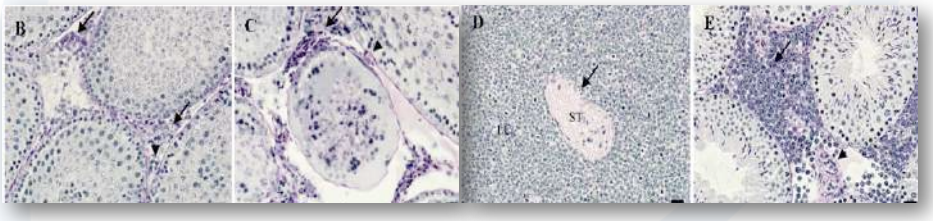
Tissue culture with VEGF: increased number of tubules with elongating spermatids in bovine grafts *(Schmidt et al, 2006)*

→ Need for improvement in early graft revascularization

Perspectives with transplantation of cryopserved ITT

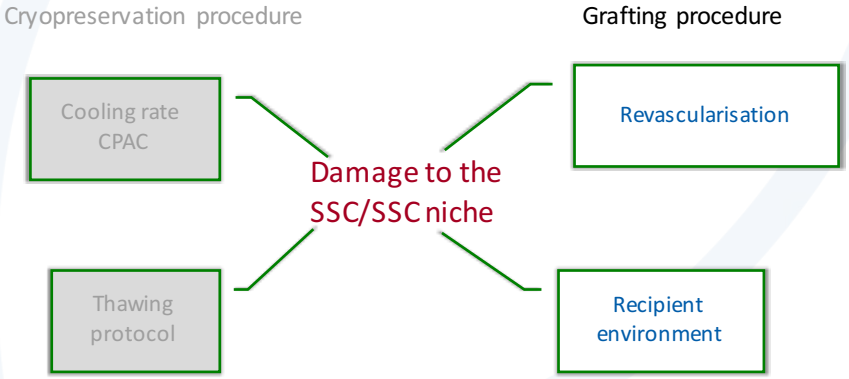
➢ **Safety issue**

- As few as 20 leukemic cells injected into a testis can induce disease relapse *(Jahnukainen et al., 2001)*
- Leukemic cells can survive cryopreservation/xenotransplantation and increase generalized leukemia in the nude mouse host *(Hou et al., 2007)*



→Procedure not applicable if risk of cancer cells' contamination

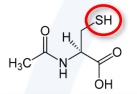
Transplantation of tissue pieces: the cryopreservation procedure is only partially responsible for spermatogonial stem cell loss and/or impairment



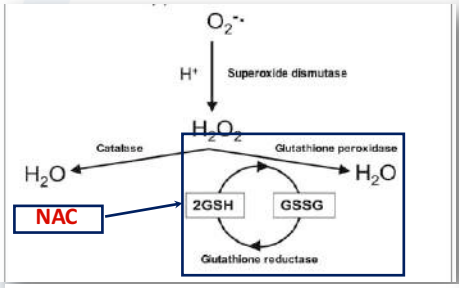
→ **Need for studies on graft revascularisation**
→ **Need for a preclinical model**

Perspectives with transplantation of cyropreserved ITT

➢ **Reducing tissue damage due to ischemic stress before revascularization**



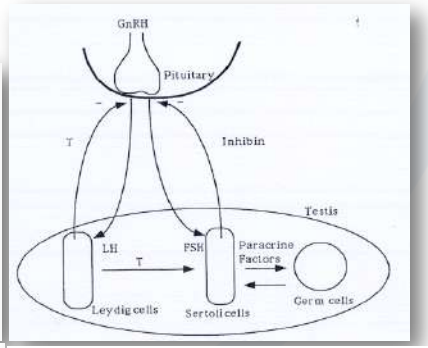
N-Acetyl Cysteine



→ Protective effect in germ cell cultures by apoptosis inhibition *(Erkkila et al., 1998)*

→ Protective effect on tissues in case of testicular torsion/distortion *(Turkmen et al., 2012)*

Testosterone



→ Suppresion of apoptosis in human GC and crucial role in GC survival *(Erkkila et al., 1997)*

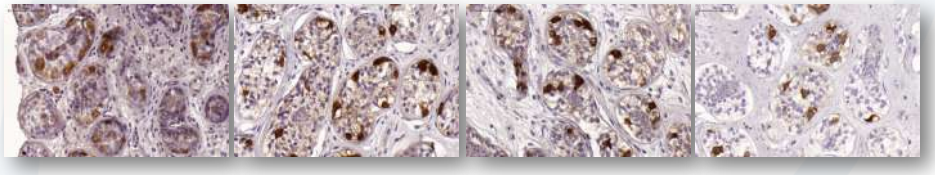
→ Testo withdrawal → progressive ↑ apoptosis and DNA fragmentation *(Tesarik et al., 2002)*

Perspectives with transplantation of cyropreserved ITT

➢ **Reducing tissue damage due to ischemic stress before revascularization**

Spermatogonia: MAGE A4

Control non-grafted	Thawed	Thawed+NAC	Thawed+Testo



	Non grafted tissue	Grafted tissue 5 days		
	Control	Thawed	Thawed+NAC	Thawed+Testo
Mage A4 positive cells/ST	3.02 ± 2.09	2.22 ± 1.67	1.91 ± 1.47	1.6 ± 1.77
% Recovery	100%	67%	63%	53%

→ **Presence of spermatogonial cells in all grafting groups**

→ **Number of SG/ST not statistically different between groups**

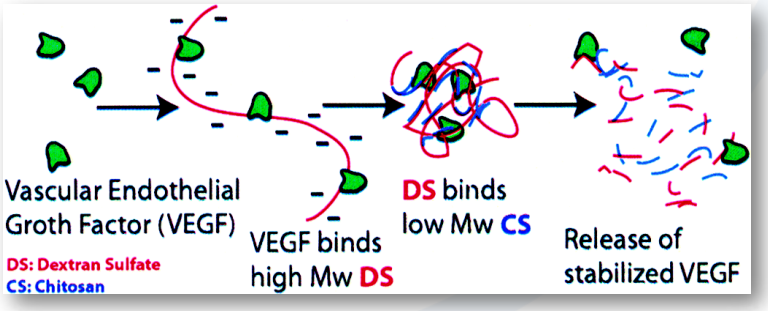
Poels et al., 2014

Perspectives with transplantation of cryopserved ITT

- **Use of VEGF nanoparticles to promote early graft revascularisation**

Drug attached to nanoparticles in hydrogels

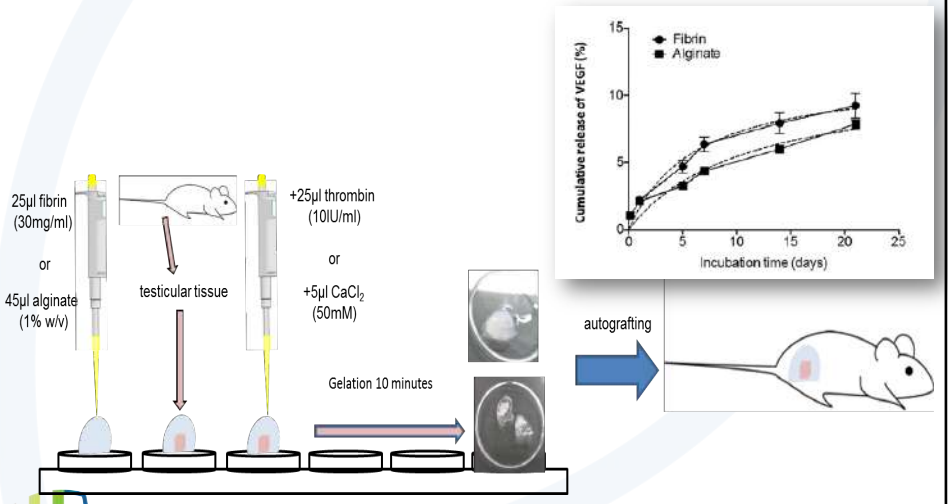
Constant and controlled concentration, reduced dispersion



Min Huang et al., 2007

Perspectives with transplantation of cryopserved ITT

- **Development of appropriate embedding matrices for tissue engraftment**

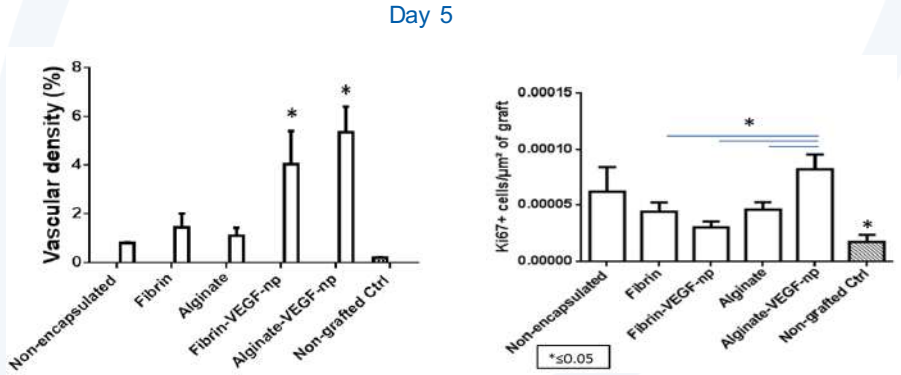


Compromise between solidity and tissue integration into the host

Poels et al., 2016

Perspectives with transplantation of cryopserved ITT

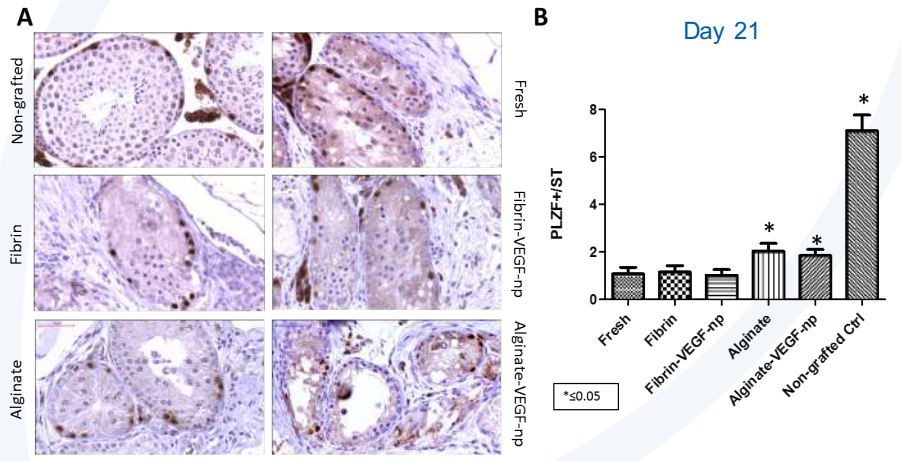
- VEGF nanoparticles loaded in two different matrices to optimize graft outcome



Poels et al., 2016

Perspectives with transplantation of cryopserved ITT

- VEGF nanoparticles loaded in two different matrices to optimize graft outcome

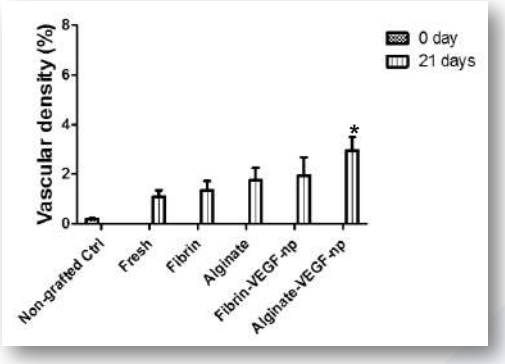


→Higher preservation of undifferentiated spermatogonia (x 2)

Poels et al., 2016

Perspectives with cryopreserved ITT

- VEGF nanoparticles loaded in two different matrices to optimize graft outcome



* p ≤ 0.05 compared to non grafted control

→Insufficient stabilization of the vasculature by day 21 ?
(new blood vessels could be leaky and rupture easily)
→ Suboptimal exposure to VEGF?

Transplantation of isolated SSCs: challenges …

- Safety issue: cancer cell decontamination with cell sorting

Table 1. Studies reporting cell sorting aiming at elimination of neoplastic cell contamination from testicular cell suspensions

References	Species	Cell-sorting technique	Markers	Evaluation after cell sorting	Outcome (% of residual contamination/number of contaminated samples or xenografted mice)
Fujita *et al.* [48]	Mouse	FACS	H2Kb/H2Db$^-$ (MHC cl I) CD45$^-$	Cell transplantation histology: testis, bone marrow, peritoneal exudate of recipient mice	No tumor
Fujita *et al.* [49]	Human	FACS	MHC cl I$^-$CD45$^-$	RT-PCR for germ cell markers (DAZL, HIWI, VASA, NANOG, STELLAR, OCT4)	1.45% K562 cells (CML), 0% K562 cells after IFγ (for induction of MHC cl I)
Geens *et al.* [50]	Mouse	MACS + FACS	H2Kb$^-$ (MHC cl I) CD49f$^+$	FACS; in-vitro culture; cell transplantation	0.39% H2Kb$^+$ cells; 3.1% (1/32) contaminated cultures
	Human	FACS	H2Kb$^-$ (MHC cl I)	FACS; in-vitro culture; PCR for B cell receptor	0.58% SB$^+$ cells 1/11 contaminated samples
Hermann *et al.* [51]	Nonhuman primates	FACS	CD90$^+$ /CD45$^-$	Xenografts in mice + epifluorescent microscopy	0,1% contamination + tumors
			+ SD replicates	Postsorting purity	No tumor
Dovey *et al.* [52]	Human	FACS	EpCAM$^+$/CD49f$^-$/ HLA-ABC$^-$	Xenografts in mice	0% contamination; (vs 23–55% for cancer cell fraction)
				Postsorting purity	98.8–99.8%

CD45, surface marker of leukemic cells; CD49 (α6 integrin), marker of spermatogonial stem cells; CD90 (Thy-1), marker of spermatogonial stem cells; CML, chronic myelogenous leukemia; DAZL, deleted in azoospermia-like (expressed in germ cells); EpCAM, epithelial cell adhesion molecule; germ cells marker; FACS, fluorescence-activated cell sorting; HIWI, human homolog of PIWI, family of genes essential in stem cell self - renewal; HLA, human leukocyte antigen; H2Kb/H2Db, mice MHC haplotype antigens; IFγ, interferon-γ; K562, CML marker; MACS, magnetic-activated cell sorting; MHC cl I, major histocompatibility complex class I (marker of somatic cells); NANOG, transcription factor in embryonic stem cells; OCT4, octamer-binding transcription factor 4; SD, singlet discrimination; STELLAR, marker of germline differentiation; VASA, marker of germline differentiation.

F de Michele, M Vermeulen, C Wyns, Current opinion, 2017

Transplantation of isolated SSCs: challenges …

- Low number of SSCs contained in a small testicular biopsy

Propagation of Human Spermatogonial Stem Cells In Vitro

Hooman Sadri-Ardekani, MD
Sefika C. Mizrak, DVM, PhD
Saskia K. M. van Daalen, BSc
Cindy M. Korver, BSc
Hermien L. Roepers-Gajadien, BSc
Morteza Koruji, PhD
Suzanne Hovingh, MSc
Theo M. de Reijke, MD, PhD

Context Young boys treated with high-dose chemotherapy are often confronted with infertility once they reach adulthood. Cryopreserving testicular tissue before chemotherapy and autotransplantation of spermatogonial stem cells at a later stage could theoretically allow for restoration of fertility.

Objective To establish in vitro propagation of human spermatogonial stem cells from small testicular biopsies to obtain an adequate number of cells for successful transplantation.

Design, Setting, and Participants Study performed from April 2007 to July 2009 using testis material donated by 6 adult men who underwent orchidectomy as part of prostate cancer treatment. Testicular cells were isolated and cultured in supplemented StemPro medium; germline stem cell clusters that arose were subcultured on human pla-

Conclusion Long-term culture and propagation of human spermatogonial stem cells in vitro is achievable.

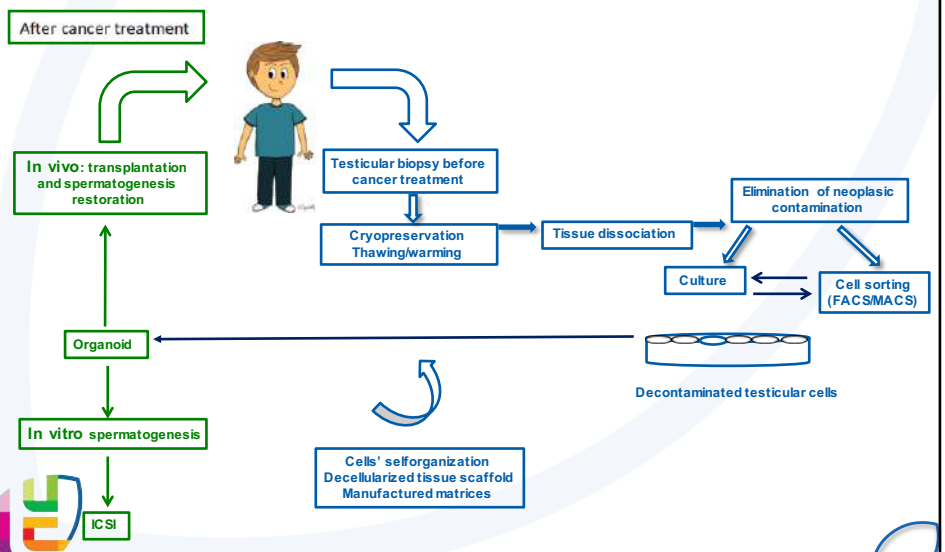
JAMA. 2009;302(19):2127-2134 www.jama.com

→ **Technique further applied to propagate prepubertal SSCs** *(Sadri-Ardekani et al., 2011)*
→ **Technique useful to eliminate cancer cells?** *(Sadri-Ardekani et al., 2011, 2014)*

- Damage to the SSC niche due to chemo- and/or radiotherapy *(Bar-Shira Maymon et al., 2004)*
- No valid study model for human SSC transplantation

Perspectives using reconstituted SSC niches

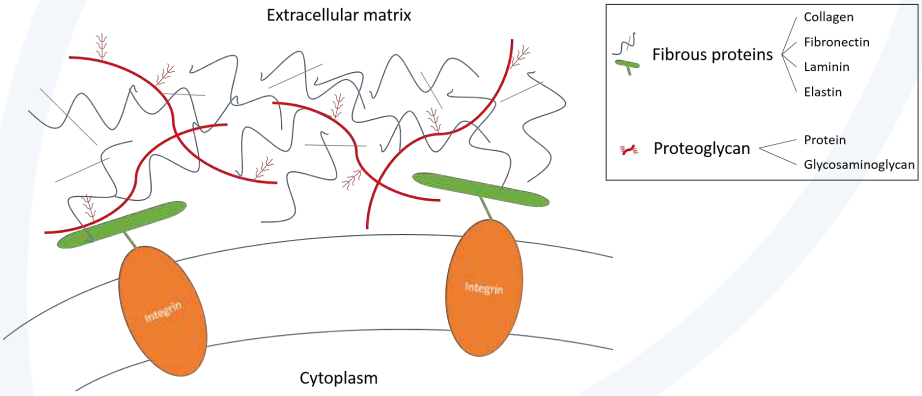
- **Development of organoids**



Perspectives using reconstituted SSC niches

Organoids using decellularized prepuberal testicular tissue

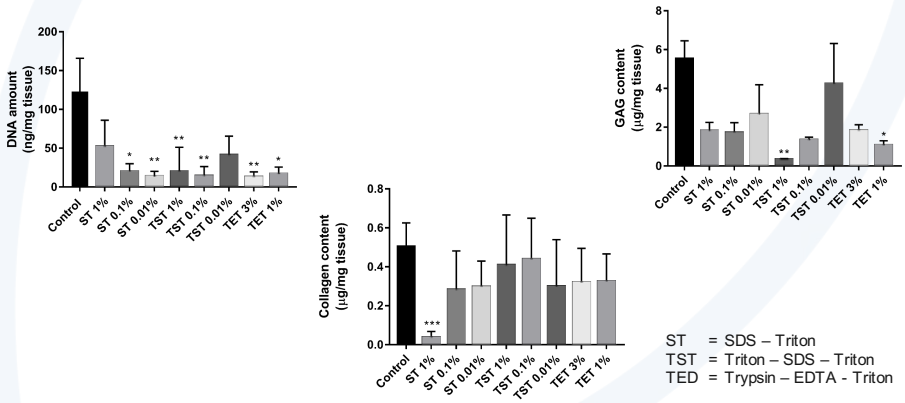
➢ **Importance of the ECM**



Perspectives using reconstituted SSC niches

Organoids using decellularized prepuberal testicular tissue

➢ **In search of an optimal and accessible scaffold**

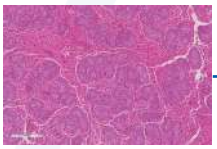


→ **Best compromise between DNA elimination and ECM preservation**

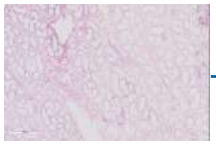
Vermeulen et al., 2017 (submitted)

Perspectives using reconstituted SSC niches

Organoids using decellularized prepuberal testicular tissue

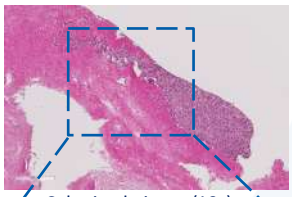


Native tissue (10x)

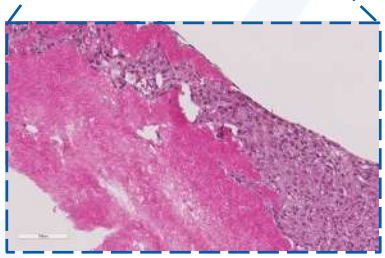


Decellularized tissue (10x)

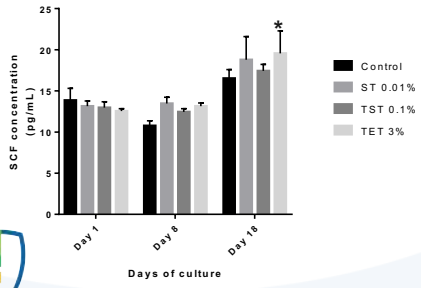
+ SCs



Colonized tissue (10x)



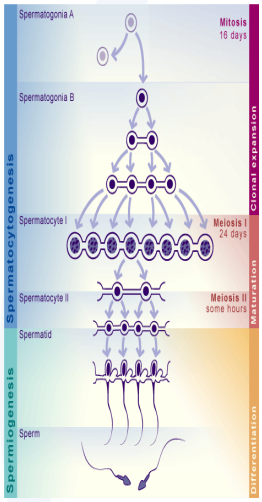
Colonized tissue (20x)



Vermeulen et al., 2017 (submitted)

Perspectives using intact SSC niches: in vitro maturation of ITT

In vitro maturation in humans: state of the art



Normal mature tissue

Tissue from transexual men after 2 years - hormonal therapy

Lee J. 2007

Lee D. 2006

Roulet 2006

Tesarik 1998

Perrard 2016

3D

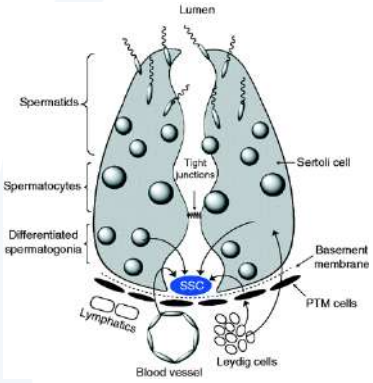
Organotypic culture

Partial digested tissue

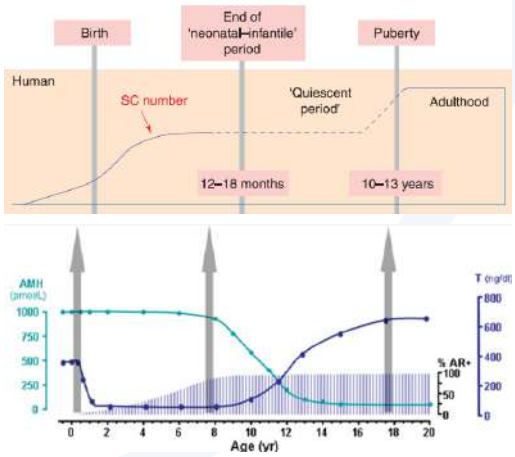
Isolated seminiferous tubules segments

In vitro maturation of prepubertal testicular tissue: challenges …

The SSC niche maturation process



- Sertoli cells:



- Leydig cells:
  Testosterone production

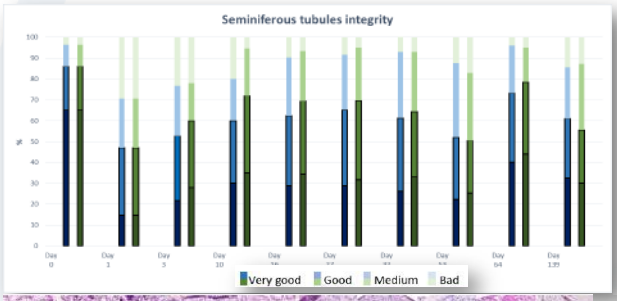
Caires et al., 2010; Sharpe et al., 2003

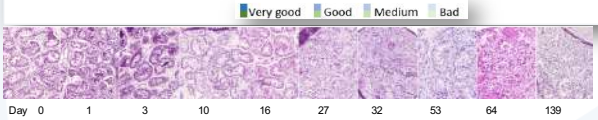
In vitro maturation of human prepubertal testicular tissue

➢ **Long term organotypic culture**

Tubules' integrity

Score 0 (bad) to 4 (very good)
- absence of cell–cell detachment
- absence of cell detachment from the BM
- no more than 5% of nuclear pyknosis
- identification of Sertoli and germ cells.





Day 0 1 3 10 16 27 32 53 64 139

→ STs are well preserved (score 3-4) during the culture without significant difference

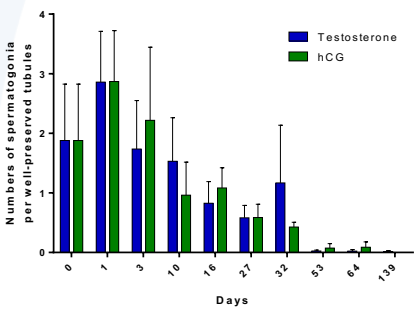
→ No difference between the two culture media

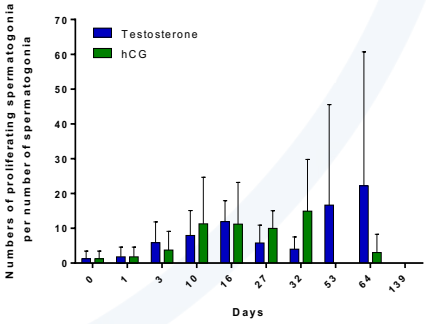
De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

- **Long term organotypic culture**

Spermatogonial cells survival and proliferation capacity



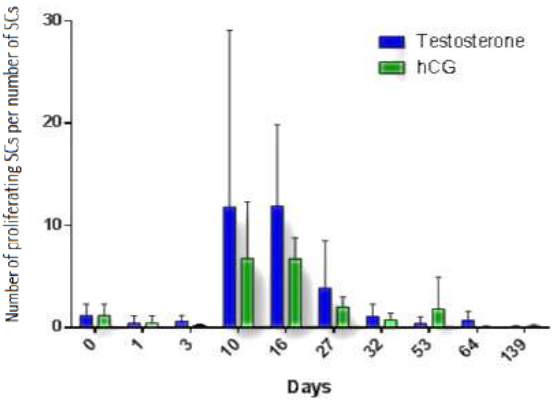


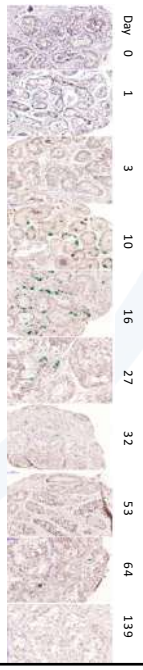
De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

- **Long term organotypic culture**

Sertoli cell maturation: Sertoli cell proliferation rate



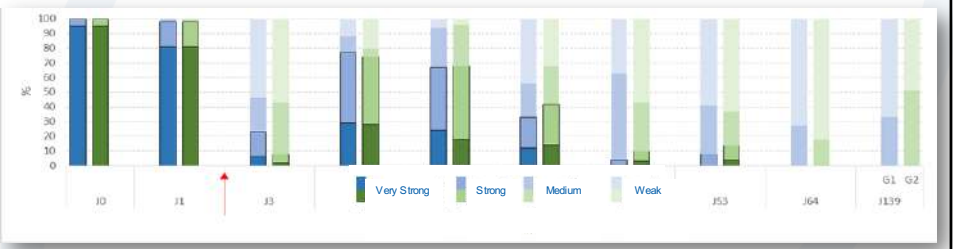


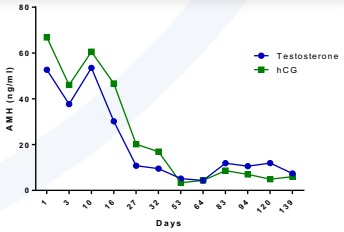
De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

➢ **Long term organotypic culture**

Sertoli cell maturation: evolution of AMH secretion





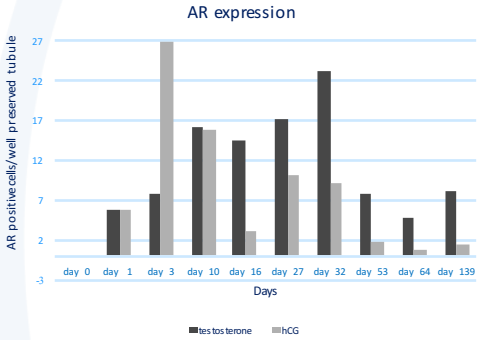
→ **Statistically significant decrease in AMH secretion**

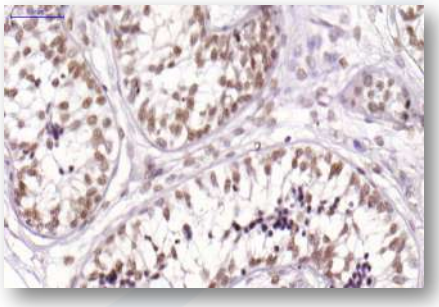
De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

➢ **Long term organotypic culture**

Androgen receptor expression
2 year-old boy



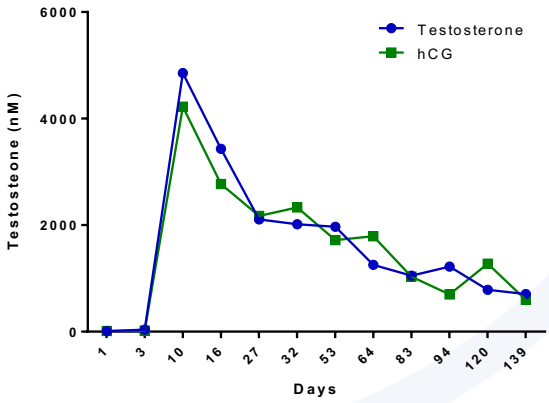


De Michele et al., 2017

In vitro maturation of human prepubertal testicular tissue

- **Long term organotypic culture**

Leydig cells survival and functionality



De Michele et al., 2017

Conclusions

- **Animals**: encouraging results with offspring in non-human primates (tissue transplantation)
- **Humans**:
  - Transplantation of ITT only for patients with benign diseases
    - → further improvement of avascular grafting technique
  - Transplantation of SSCs: need for preclinical models to evaluate the SSCs' differentiation potential
    - → perspectives with organoid development
  - IVM of ITT: research limited due to the scarcity of tissue

BUT achievement of the pubertal transition of the maturation phase of the SSC niche

THANK YOU FOR YOUR ATTENTION

Department of Gynecology-Andrology
Cliniques Universitaires Saint-Luc
Catholic University of Louvain, Brussels

Clinical Team

- C Wyns
- P Laurent
- MG Giudice
- J Poels
- M. Raeymaekers
- S Gantois
- E Huon
- A Kabova

Research Team

- C Wyns
- J Poels
- F De Michele
- M Vermeulen
- F Del Vento
- MG Giudice