6th Workshop on Mammalian folliculogenesis and oogenesis
from basic science to the clinic

Epigenetic marks in offspring of

cryopreserved immature ovary of mouse

Nadine Binart
Inserm U845, Paris , France

Postdam Germany, 8-10 October 2009

Cancer Incidence in 2008

Major advances in oncological treatments and diagnosis have resulted in a marked improvement in the survival of children and young adults with cancer over the last decade

182 000 women with breast cancer in USA

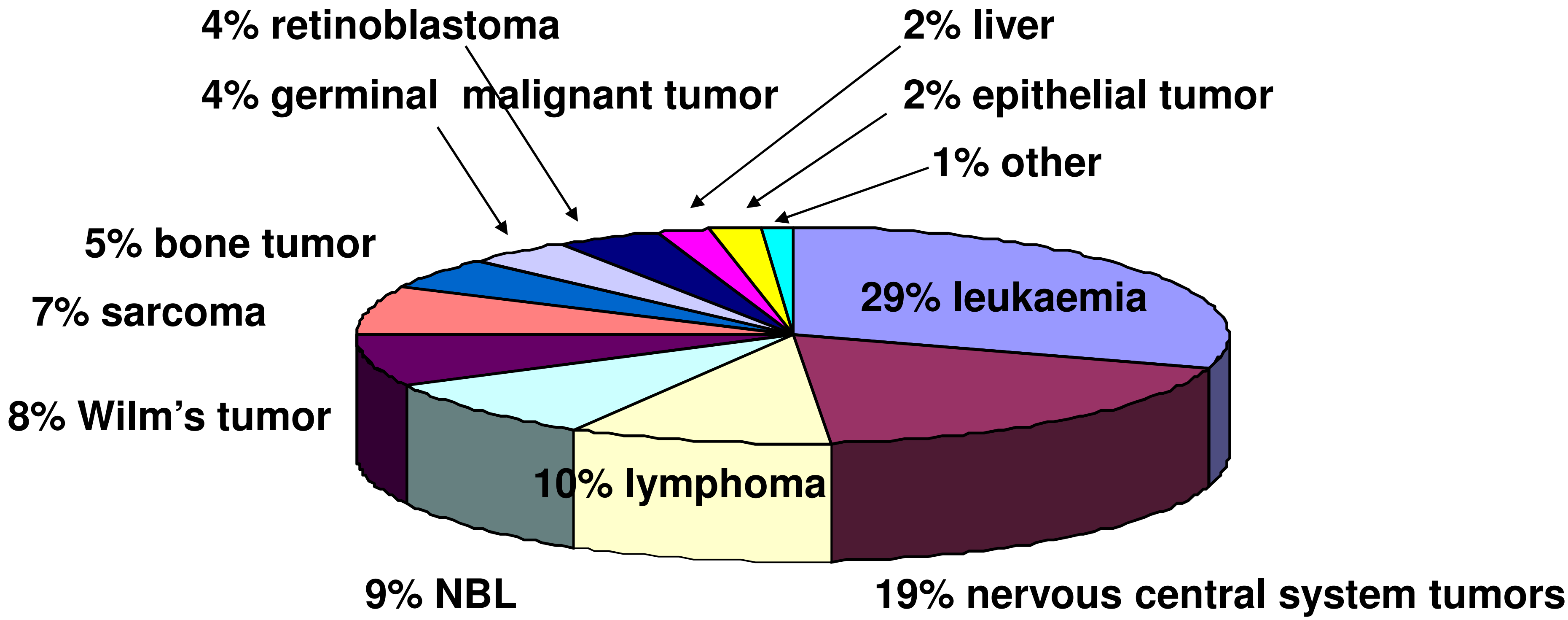
16 000 women under the age of 45

Options to preserve fertility have been explored before therapy

Incidence of Malignant Childhood Cancers

122 / million in USA or 1700 new cases / year in France

(half of cases before 5)



80% of children and teenagers become long term survivors

-> 1/600 adult between 20 and 39

Infertility Risk after Chemotherapy

Girl

- Alkylating agents: chloramblucil, cyclophosphamide, ifosfamid, melphalan, busulphan and procarbazine

→Direct ovarian lesions : follicular apoptosis both in growing follicles and in the dormant primordial follicle population

- Myeloablative chemotherapy used as preparation for stem cell transplantation:
  - busulphan-melphalan
  - cyclophosphamide + busulphan

Risks : - no spontaneous puberty

- sterility

- acute ovarian failure increasing with age of patient

Infertility Risk after Radiation Therapy

Girl

- **Ovary :**
  - **Doses (10-20 Gy) act on dividing and non-dividing cells, block cellular division associated with permanent ovarian failure**
  - **Total body irradiation 14.4 Gy: 90% infertility**

- **Uterus :**
  - **Risk on fœtal development : premature birth and hypotrophy**
  - **Miscarriage**

The chance of spontaneous pregnancy in women treated after 25 years of age has been estimated to be only 5%

Lobo RA, NEJM 2005

Preservation of Ovarian Function

before treatment

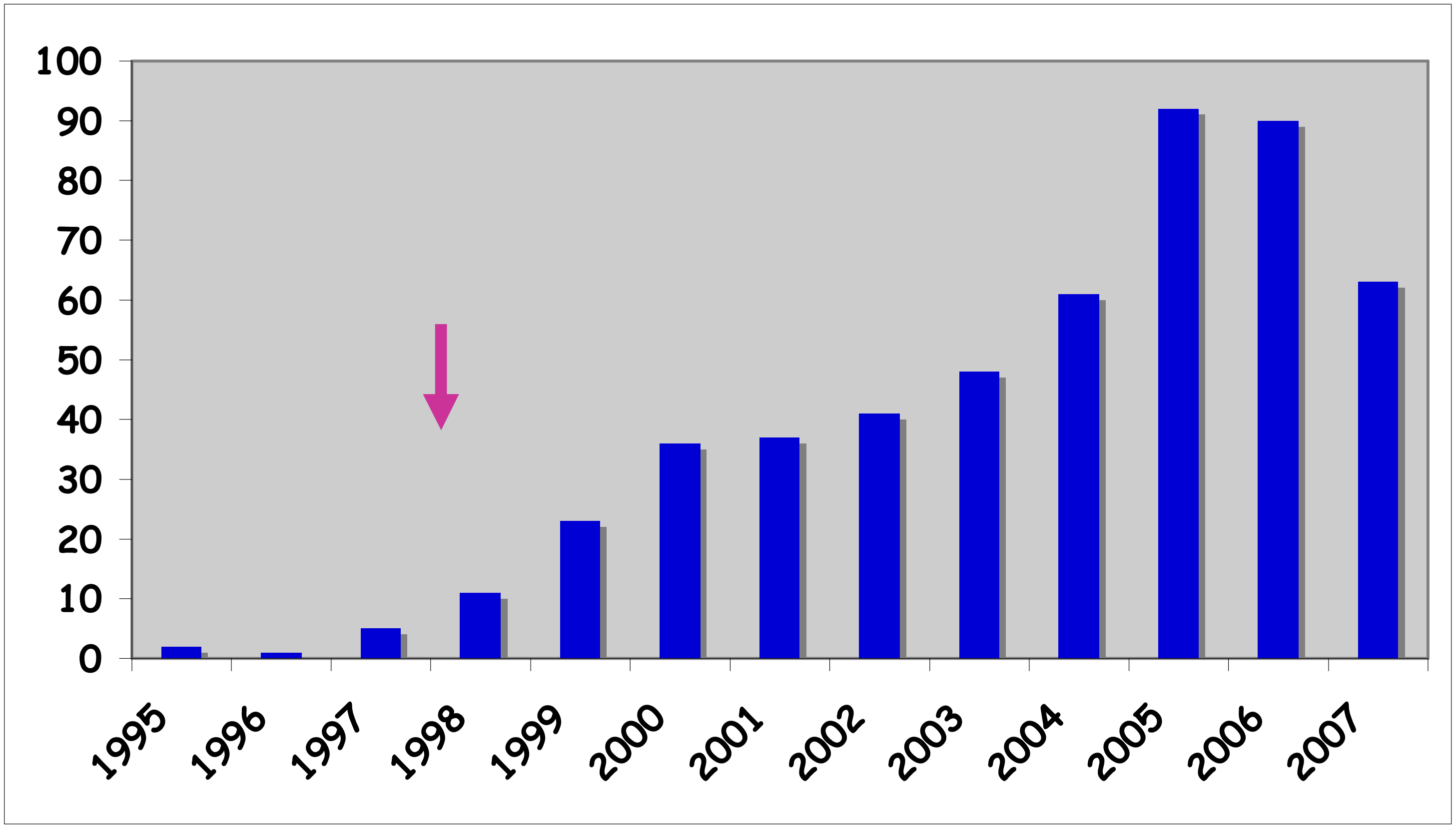
- Embryo freezing or oocyte freezing:

  >>impossible before puberty

- Ovary cryopreservation is a promising experimental technology :
  - Whole ovary in child
  - During abdominal tumor surgery, can be rapidly performed
  - Small fragments of ovarian cortex (histology)
  - Slow freezing protocol (DMSO)

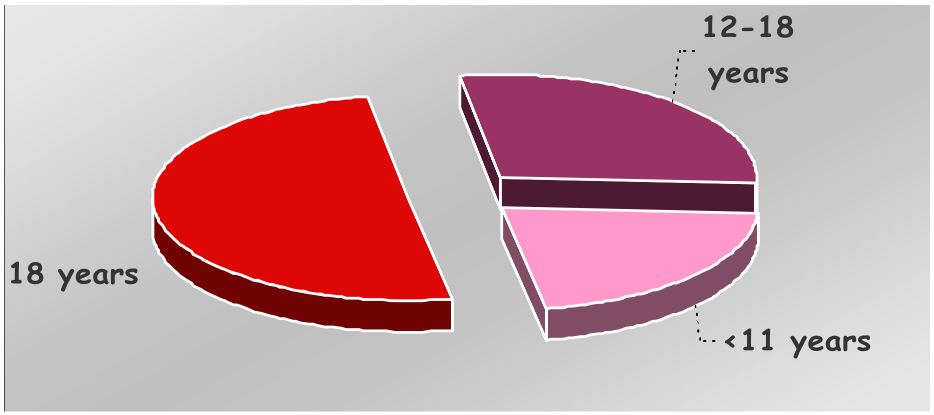
Ovarian Cryopreservation in France 1995-2007

n=510

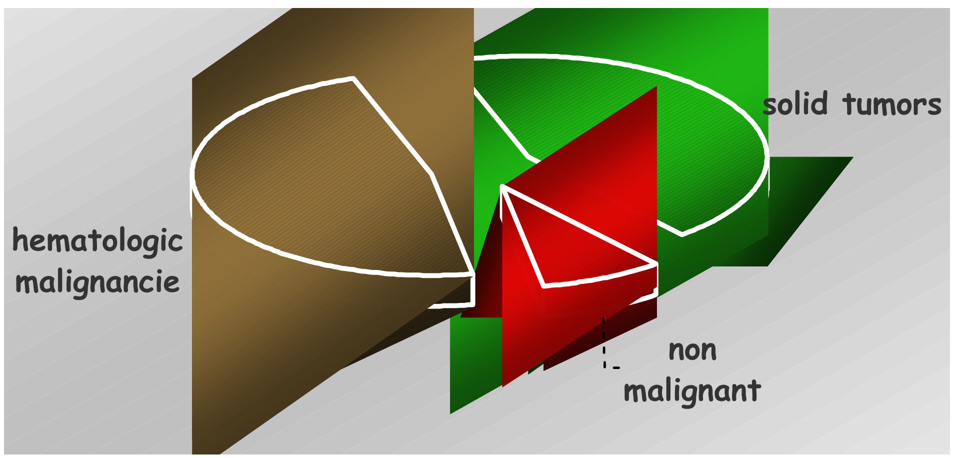


Poirot C, GRECOT 2008

Ovarian Cryopreservation in France (GRECOT)



Mean age: 17.8 year old



Solid tumors and hematologic malignancies represent the most frequent part in pediatric surgery

Poirot C, GRECOT 2008

Cryopreservation before Puberty

- Survival improvement after cancer treatment during childhood
- Ovarian function impairment: fertility / puberty

⟶ Ovarian cryopreservation as only therapeutic option

In France: 100 children under 12 years, since 1998

⇨ Fertility ?

⇨ Puberty induction ?

Graft of Ovarian Cortex

Etiologies:	**- POF after chemotherapy (11)** **- Homozygous twins, POF unknown etiology (7)** **- Bilateral ovariectomy (5)**
Graft:	**fresh (10) / frozen (13)**
Site:	**heterotopic (7) / orthotopic (12) / combined (4)**

Bedaiwy MA, Human Reprod 2008

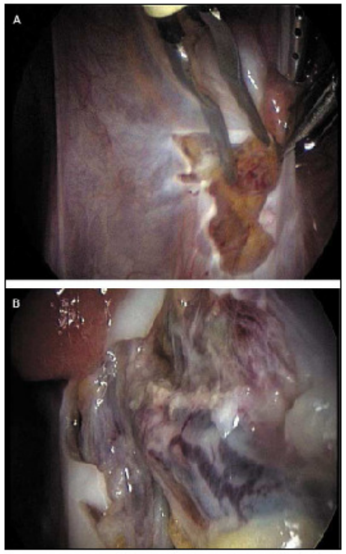
Results of Adult Ovarian Cryopreserved Grafting

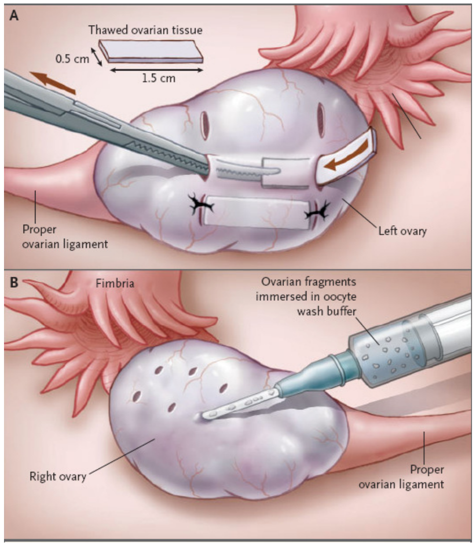
To date, 43 women who underwent cryopreserved or fresh ovarian transplantation have been reported, leading to the restoration of spontaneous cycles for several months

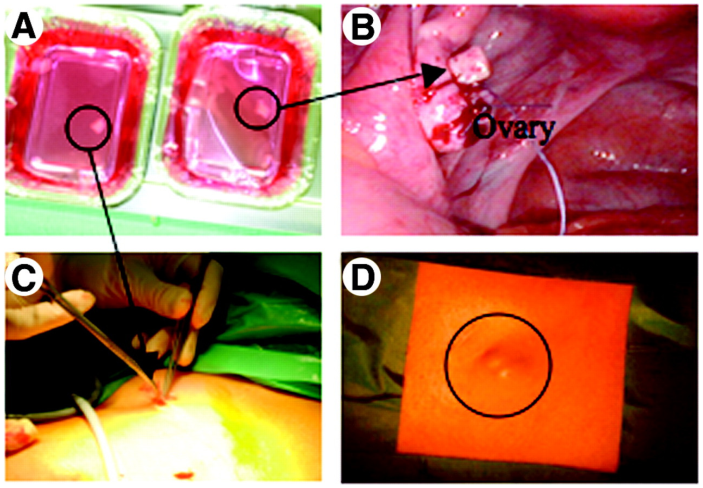
Bedaiwy MA et al , Hum Reprod 2008

Results of Adult Ovarian Cryopreserved Grafting

- Heterotopic reimplantation : 0
- Orthotopic reimplantation : 5 births







Donnez J et al, Lancet 2004 *Meirow D et al, NEJM 2005* *Demeestere et al, Oncologist 2007*

Andersen CY et al, Human Reprod 2008

Pregnancies after Heterologous Cortex Ovarian Graft

- Fresh cortex and one from frozen tissue: births

  Silber SJ, NEJM 2005 and 2007

  Silber SJ, Hum Reprod 2008

Concept of ovarian cortical grafting is based on the fact that all follicles containing eggs are located in the outer 1 mm of the ovary which can be sutured to the recipient's medulla

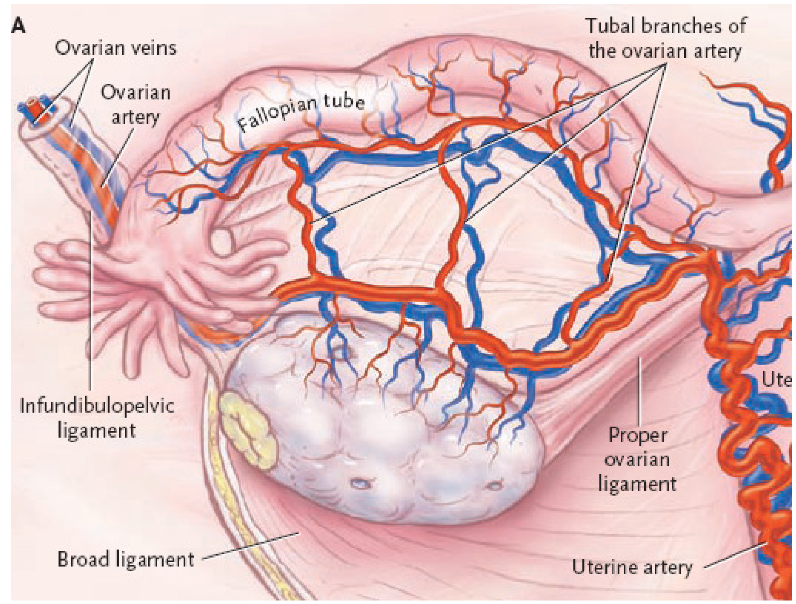
Limitation: loss of two thirds of follicles due to ischemia during revascularization

Data from studies in animal suggest that microvascular whole-ovary transplantation could avoid this problem!

Pregnancy after Microsurgical Transplantation of an Intact Ovary

Pair of 38-year-old monozygotic twins

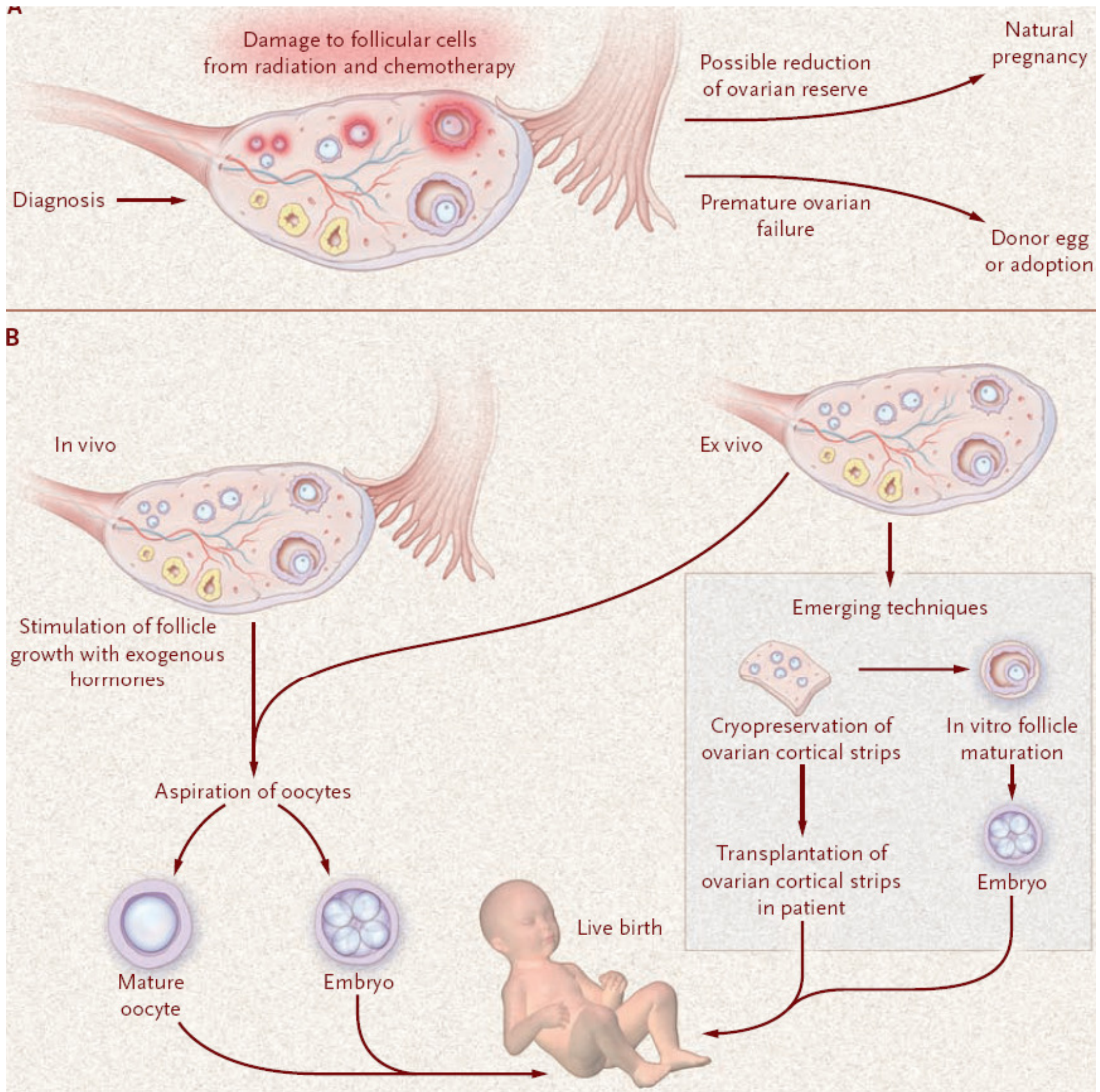
Donor : 2 children , normal ovarian function
Recipient: POF at 15 years (FSH 81 mIU/mL)



Donor's ovarian veins were anastomosed to the recipient's ovarian arteries

Immediate normal blood flow after an ischemic period of 100 min….
First cycle day 101 after transplantation, 11 regular menstrual cycles
Then, pregnancy!

Silber SJ et al. NEJM 2008



No report using pre pubertal ovaries !

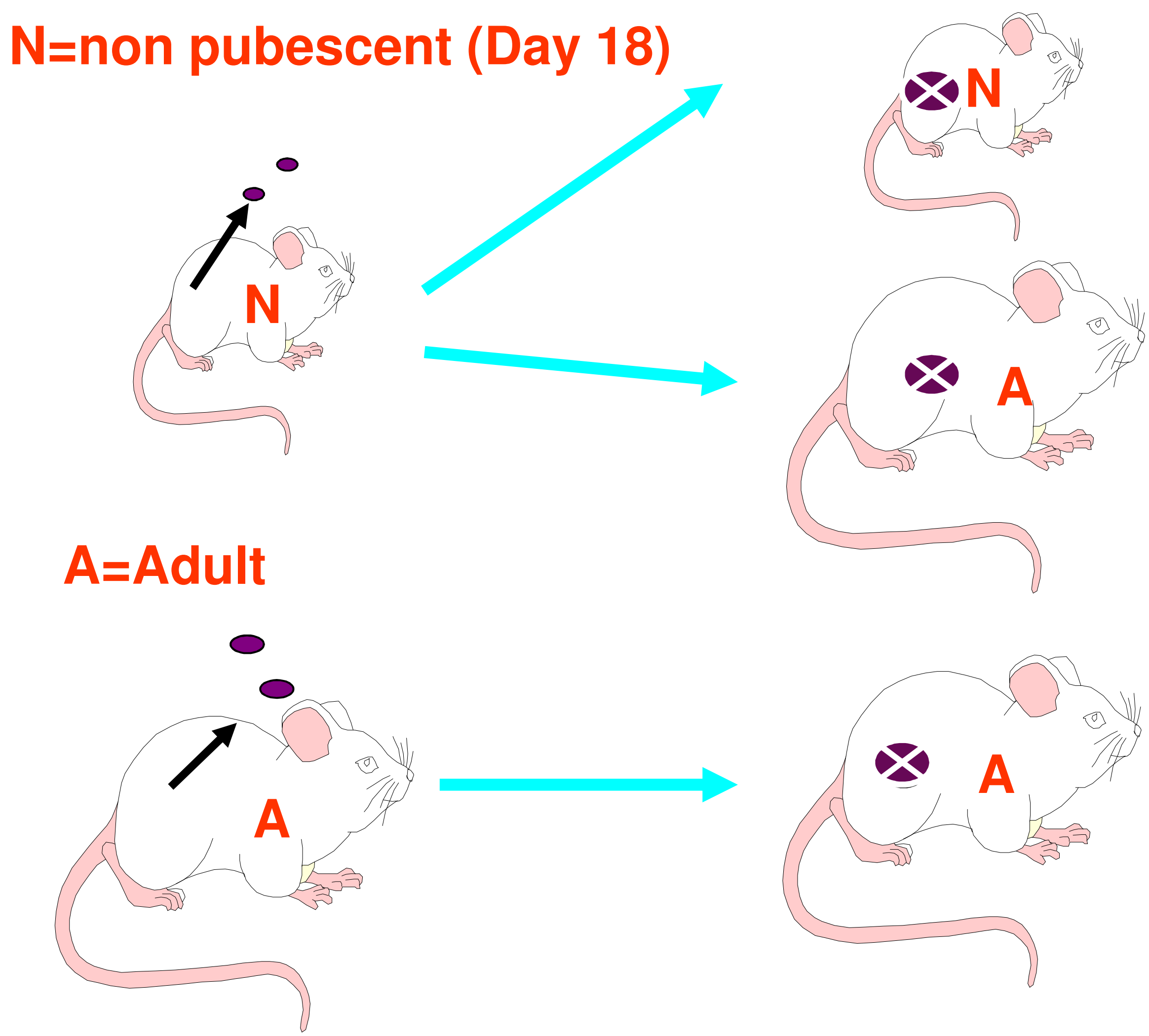
Jeruss JS, NEJM 2009

Ovarian Cryopreservation in Adult Mice

- Use of similar protocol of cryopreservation as in humans
- Spontaneous gestation
- Follicle loss > 50%
- No study with pre-pubertal grafting

We performed orthotopic transplantation using fresh and/or cryopreserved whole ovaries to restore both endocrine function and fertility

Animal Models



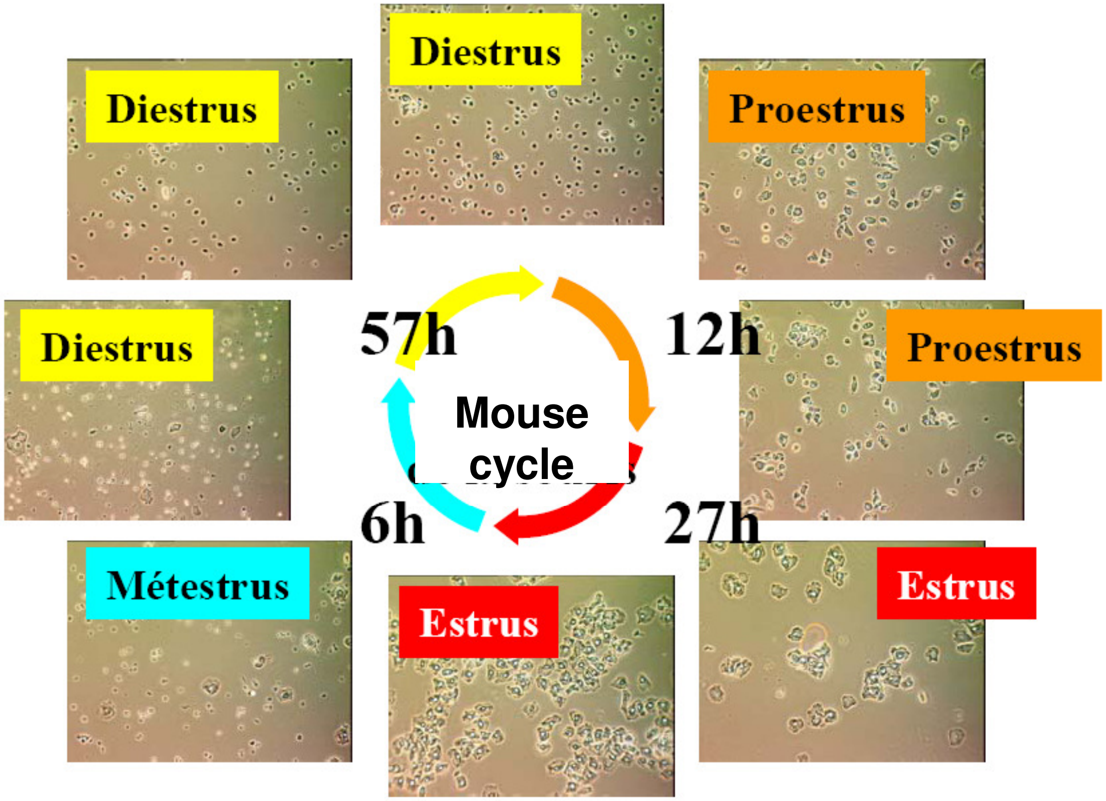
NNF or NNC
n=27 n=11

NAF or NAC
n=16 n=9

AAF or AAC
n=15 n=8

Total of 86 females

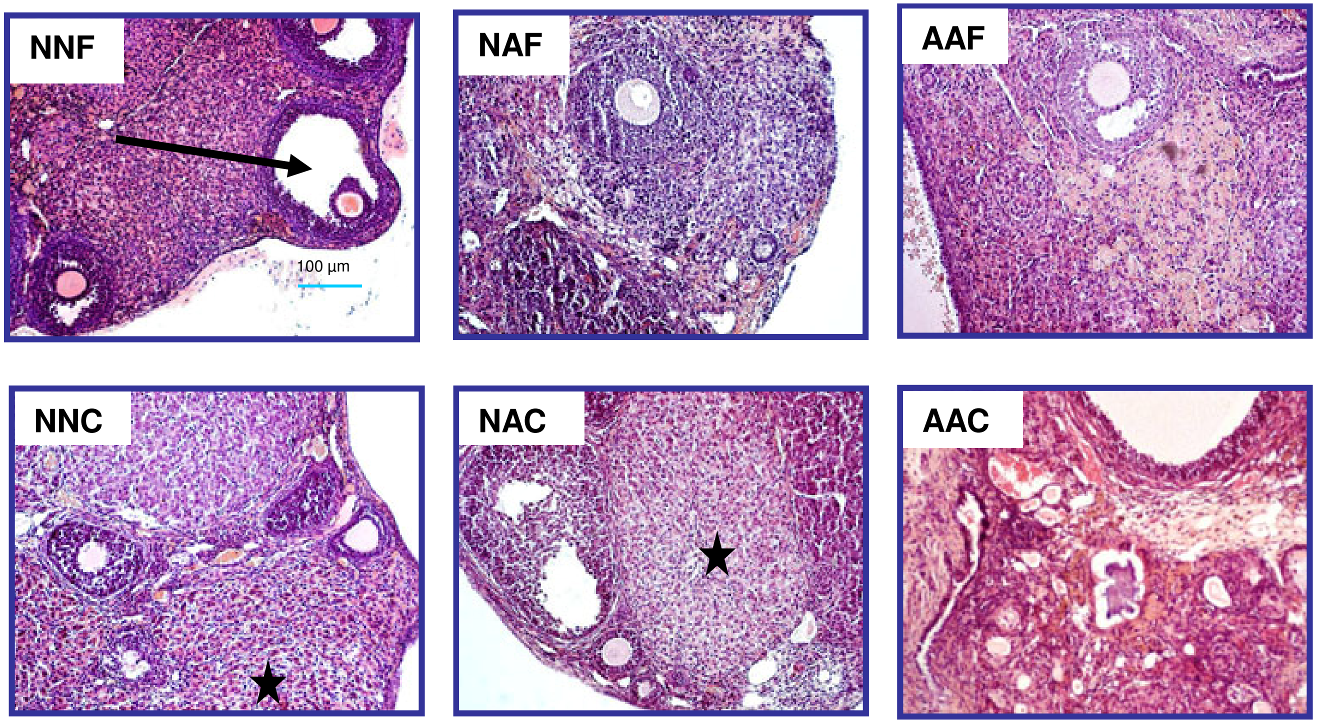
Cyclic Hormonal Activity

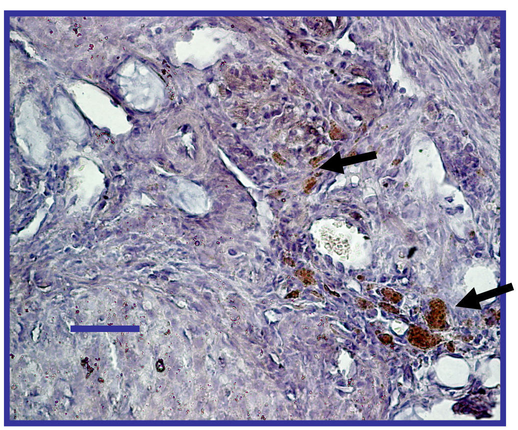


→ **Spontaneous puberty 15 days after grafting**

Cyclic activity (cycle: 5 days)

Ovaries after one Month post Grafting



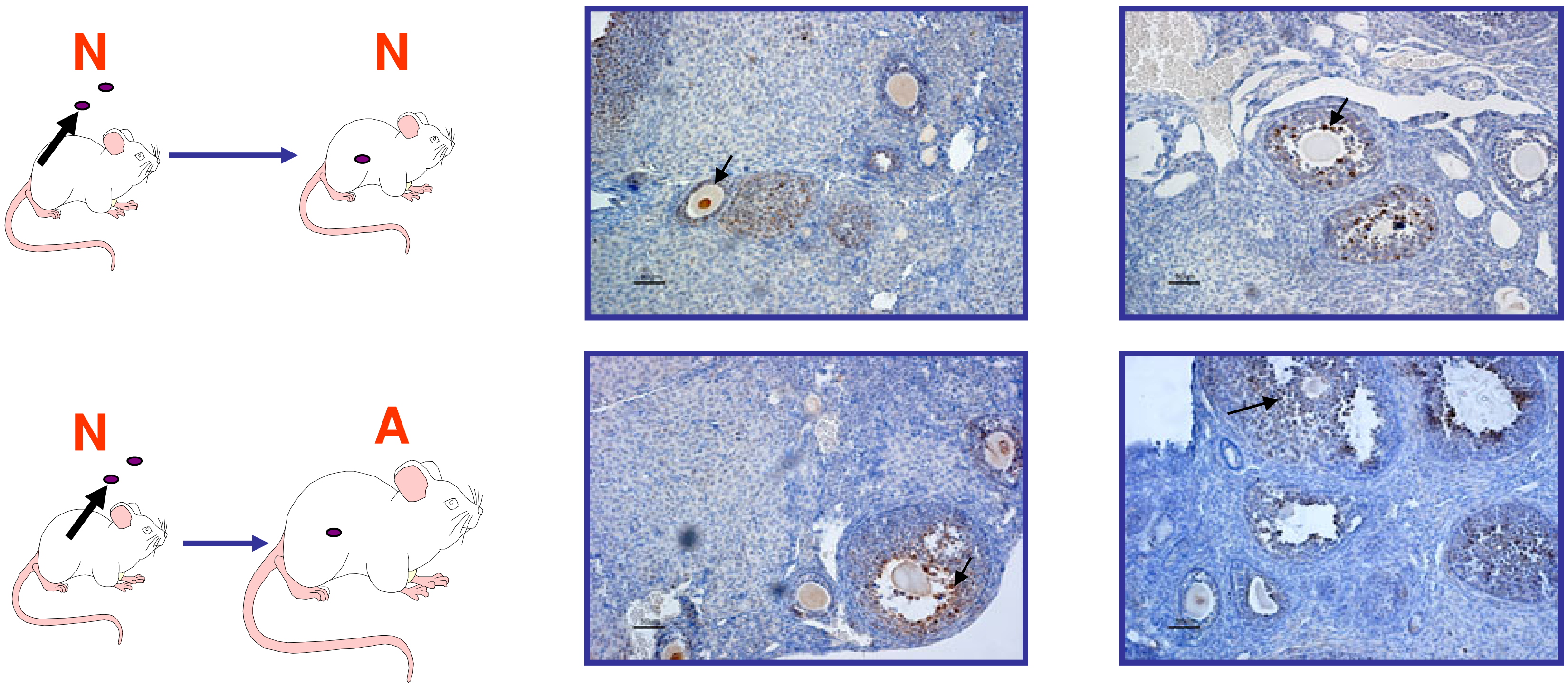


Antibody α-actin muscle

Neovascularization

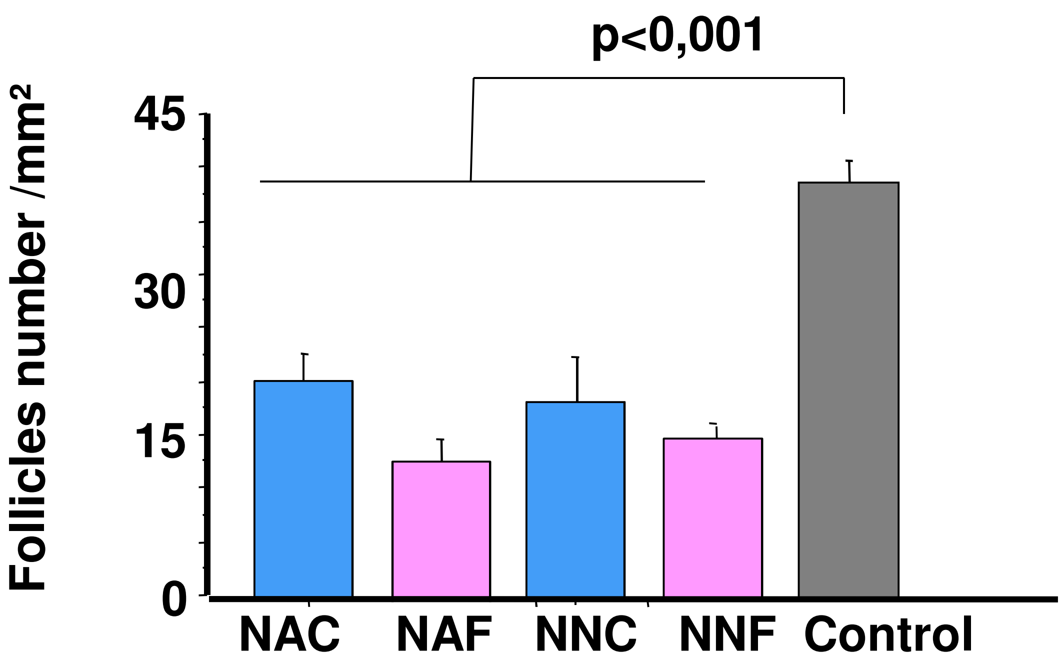
Ovarian Graft after one Month

Fresh **Cryopreserved**



Proliferation study : antibody anti-PCNA

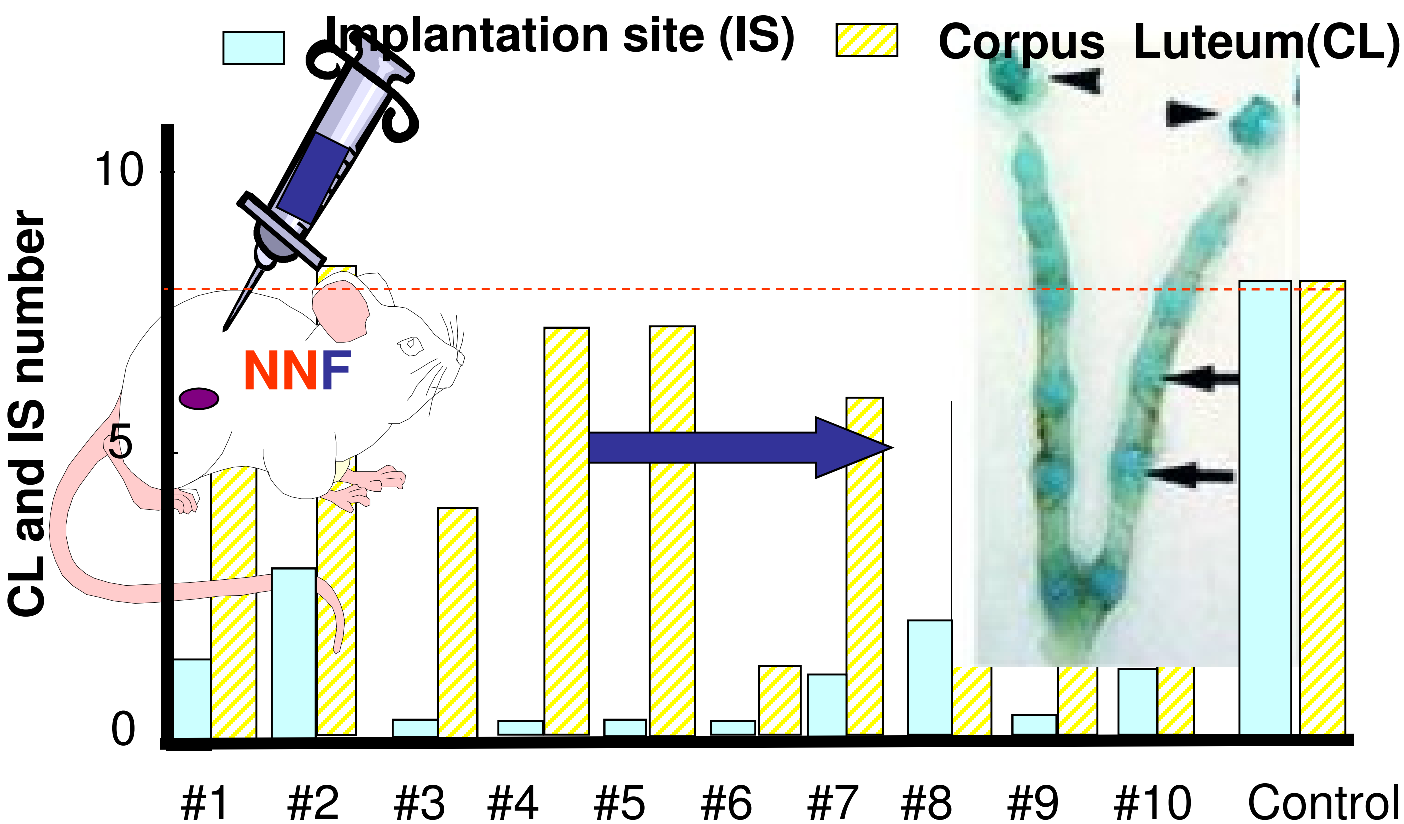
Follicular Density



→ **Follicle loss about 50%**

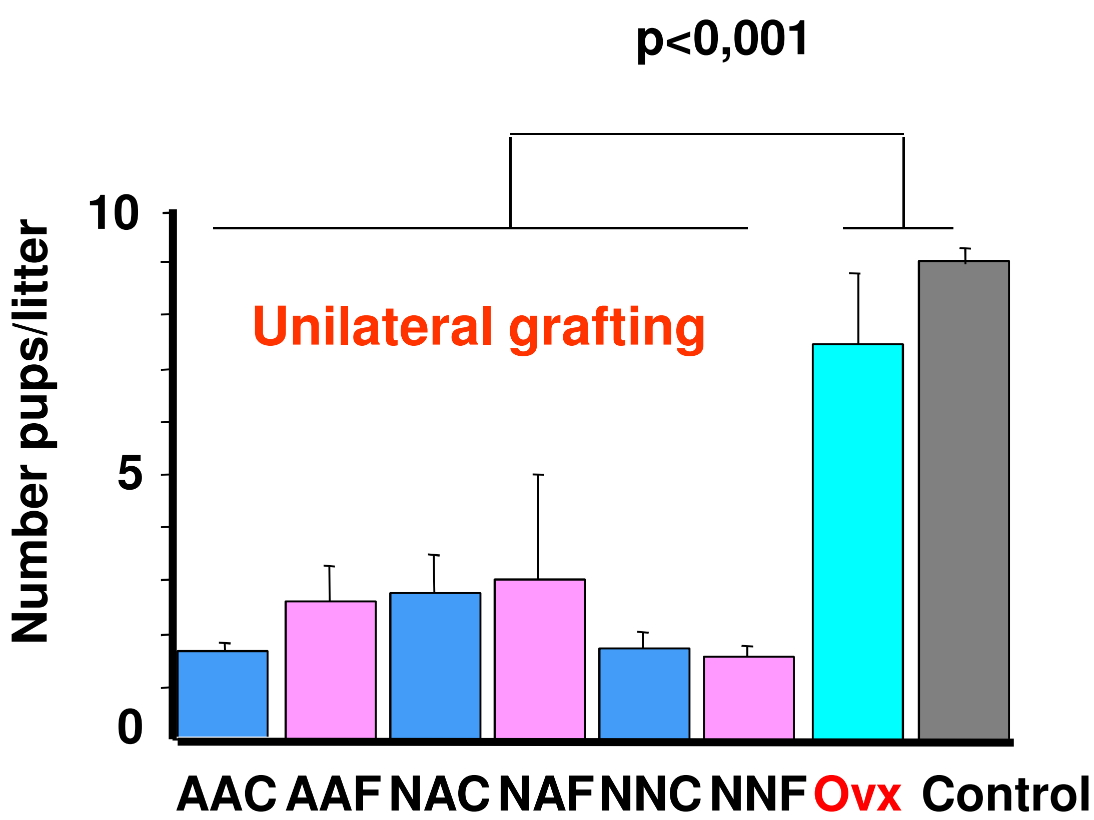
No influence of cryopreservation

Fertility



Anatomic problem
Implantation defect

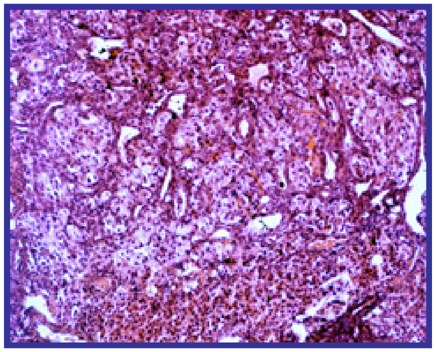
Litter Size



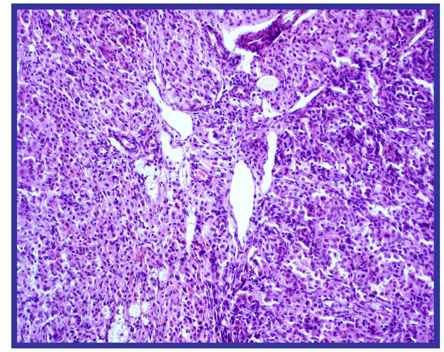
Normal ovulation rate
Anatomical myslocation
Implantation failure
Embryonic loss

Depletion of Functional Follicle

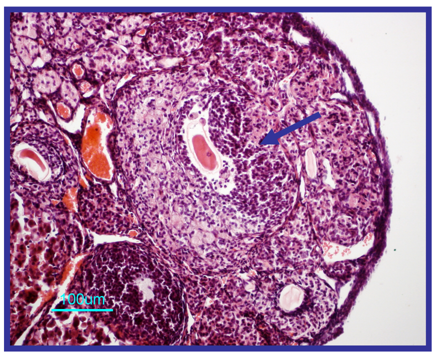
NNF



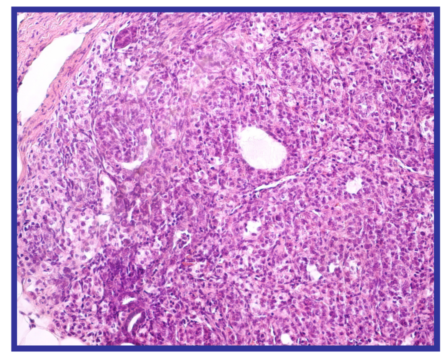
NNC



NAF



NAC



3 months after the ultimate gestation

Conclusion (1)

- Possibility of spontaneous puberty
- Follicle loss unrelated to cryopreservation process
- Gestation in all groups
- Decreased litter size
- Embryonic loss
- Premature ovarian failure

Sauvat F et al PLoS ONE 2008

Is ovarian grafting safe for progeny?

Is genomic imprinting correctly set ?

Genomic Imprinting

Both maternal and paternal genomes required in mammals

Imprinting is due to epigenetic marks and leads to monoallelic expression according to the parental allele

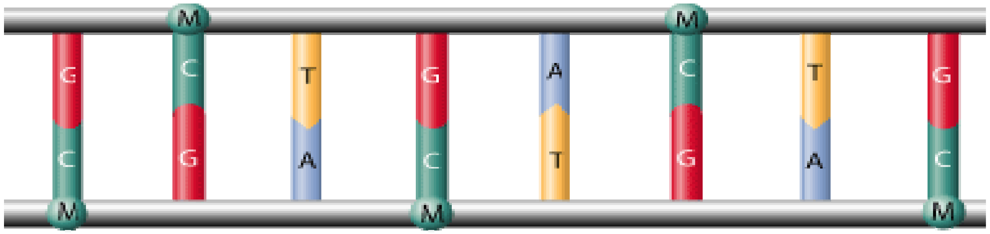
Characteristics:

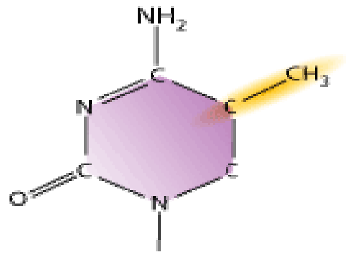
- **during gametogenesis**
- **clusters**
- **DNA methylation**

Control of embryonic growth

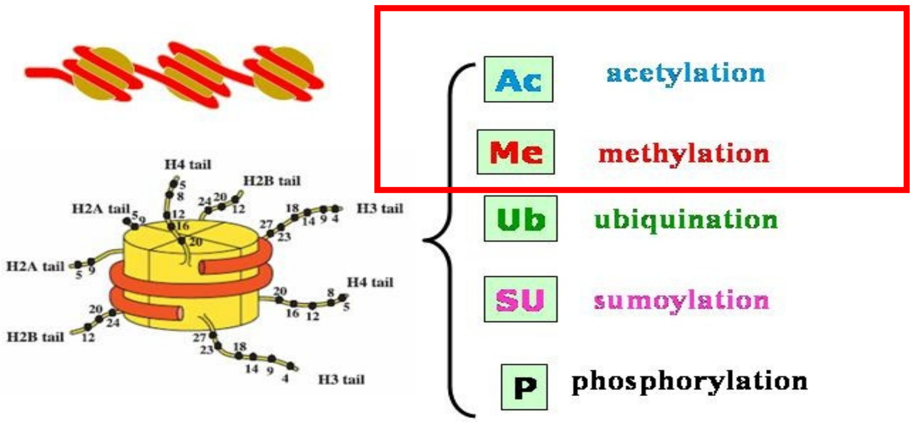
Epigenetic Modifications

- **DNA methylation (CG rich regions)**





- **Acetylation/methylation of histones associated to DNA**



Characteristics of Imprinted Genes

Organization in domains

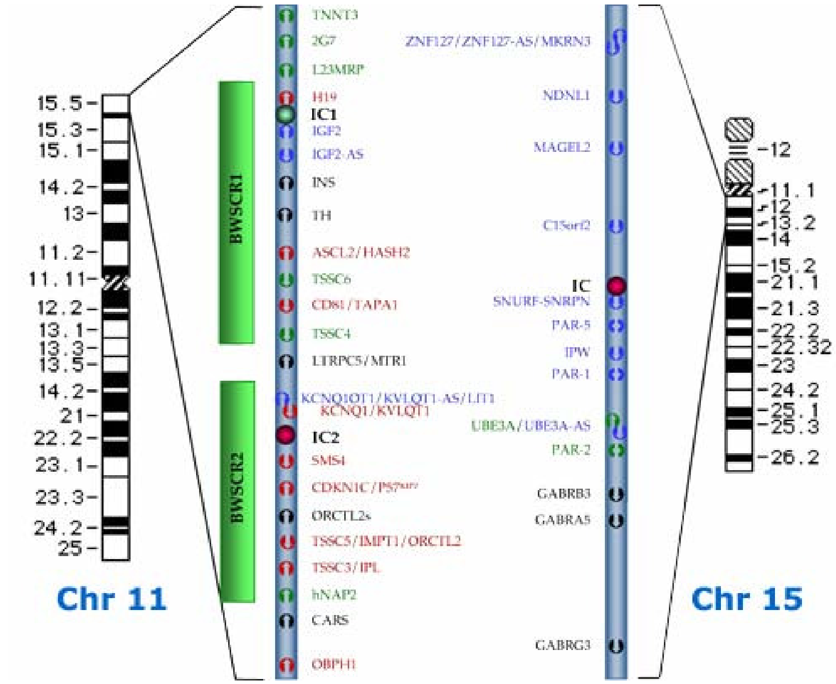
CG rich regions (DMR) and repeated sequences

Existence of Imprinting Center

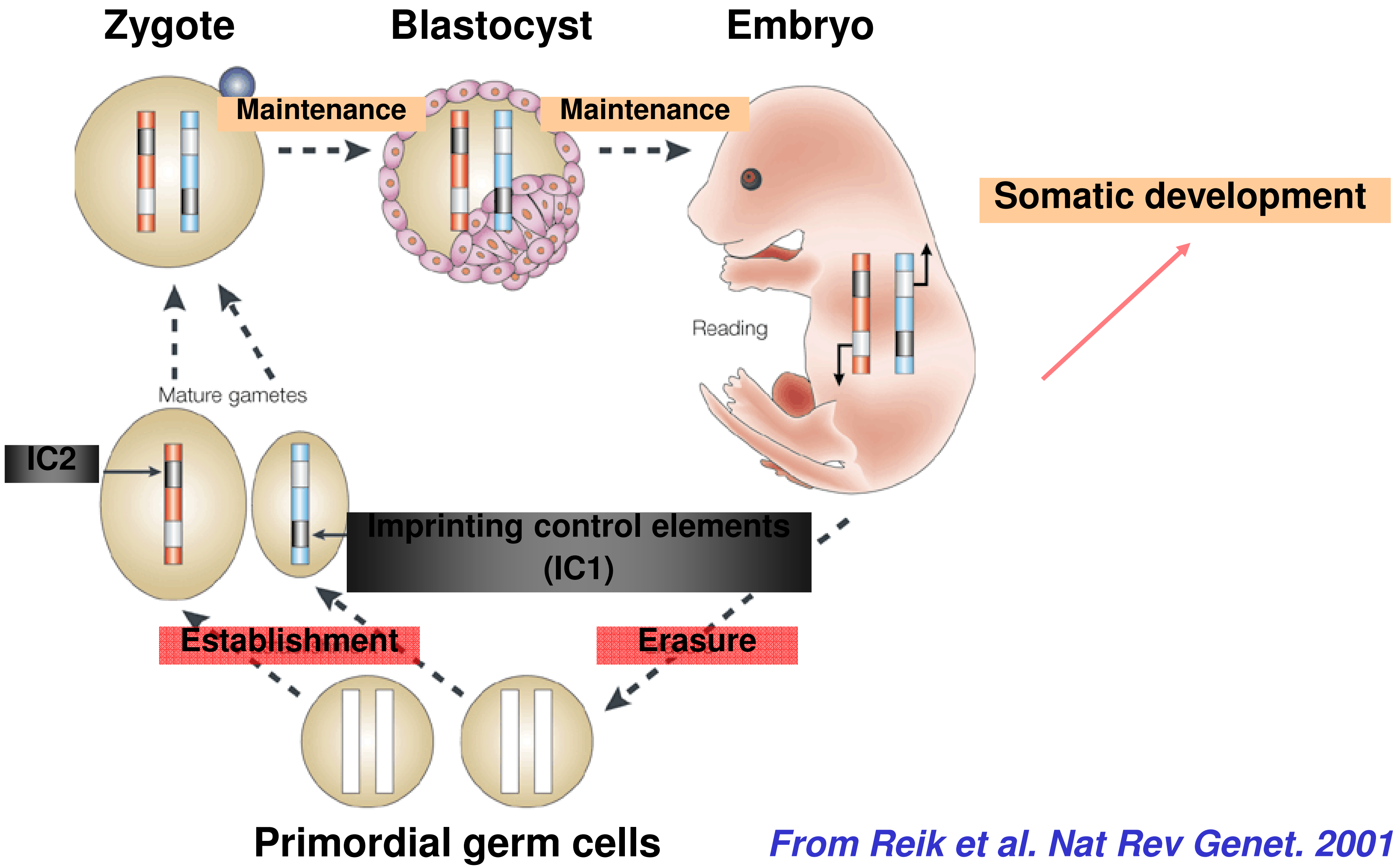
(ICR, acquisition methylation on specific allele during gametogenesis)

Antisense RNA not translated

Asynchronous replication

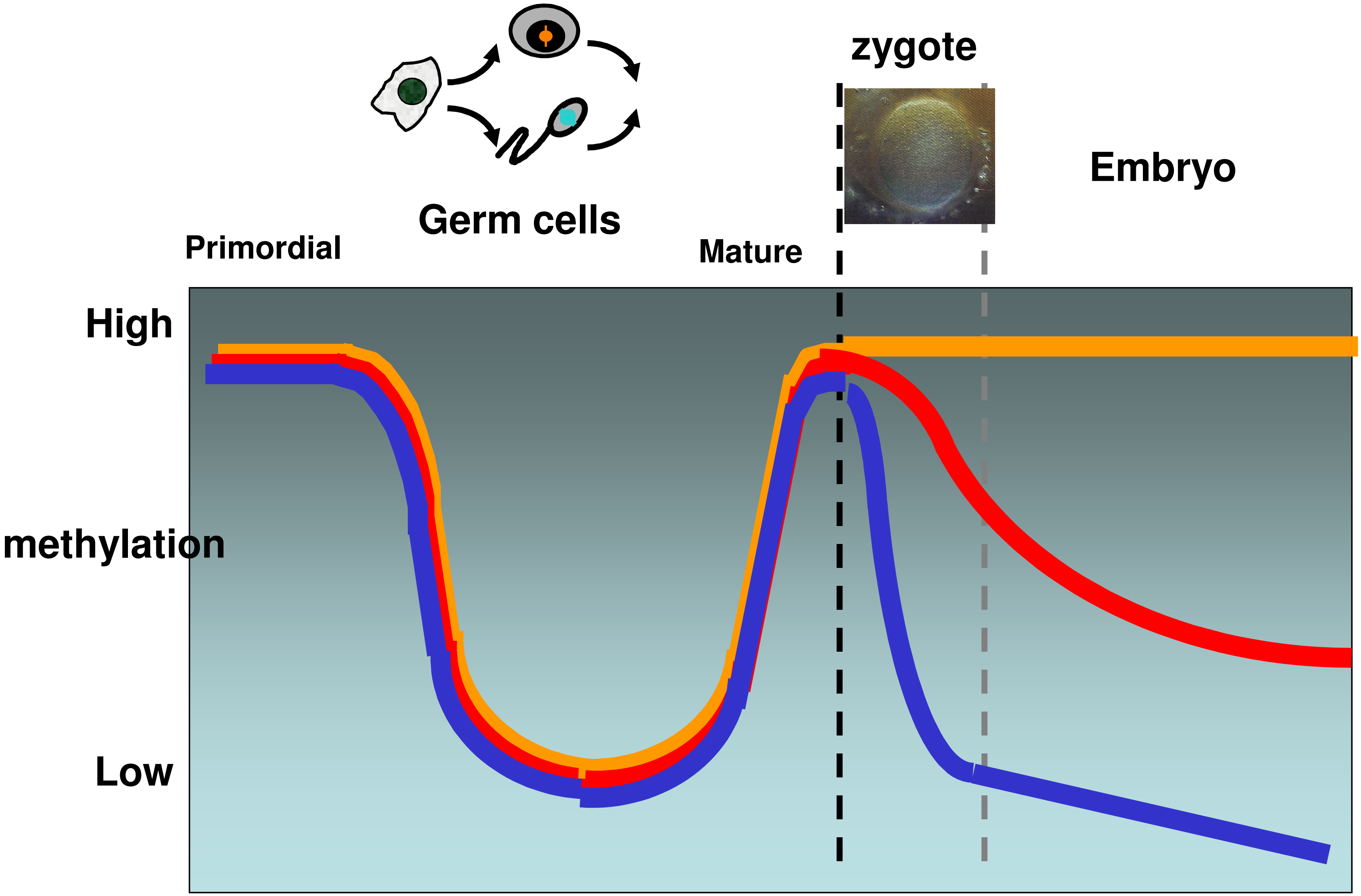


Life Cycle of Methylation Imprints



From Reik et al. Nat Rev Genet. 2001

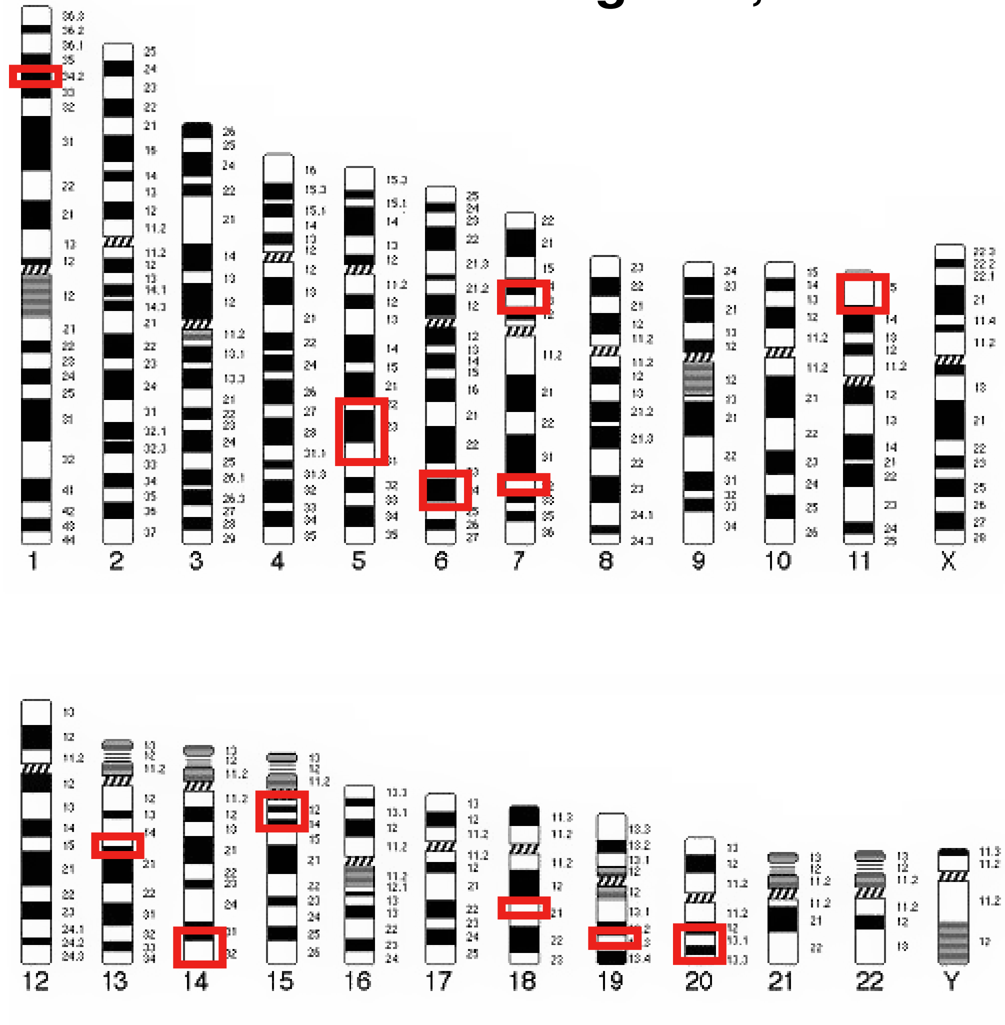
Methylation Reprogramming in the Germ Line and Embryo



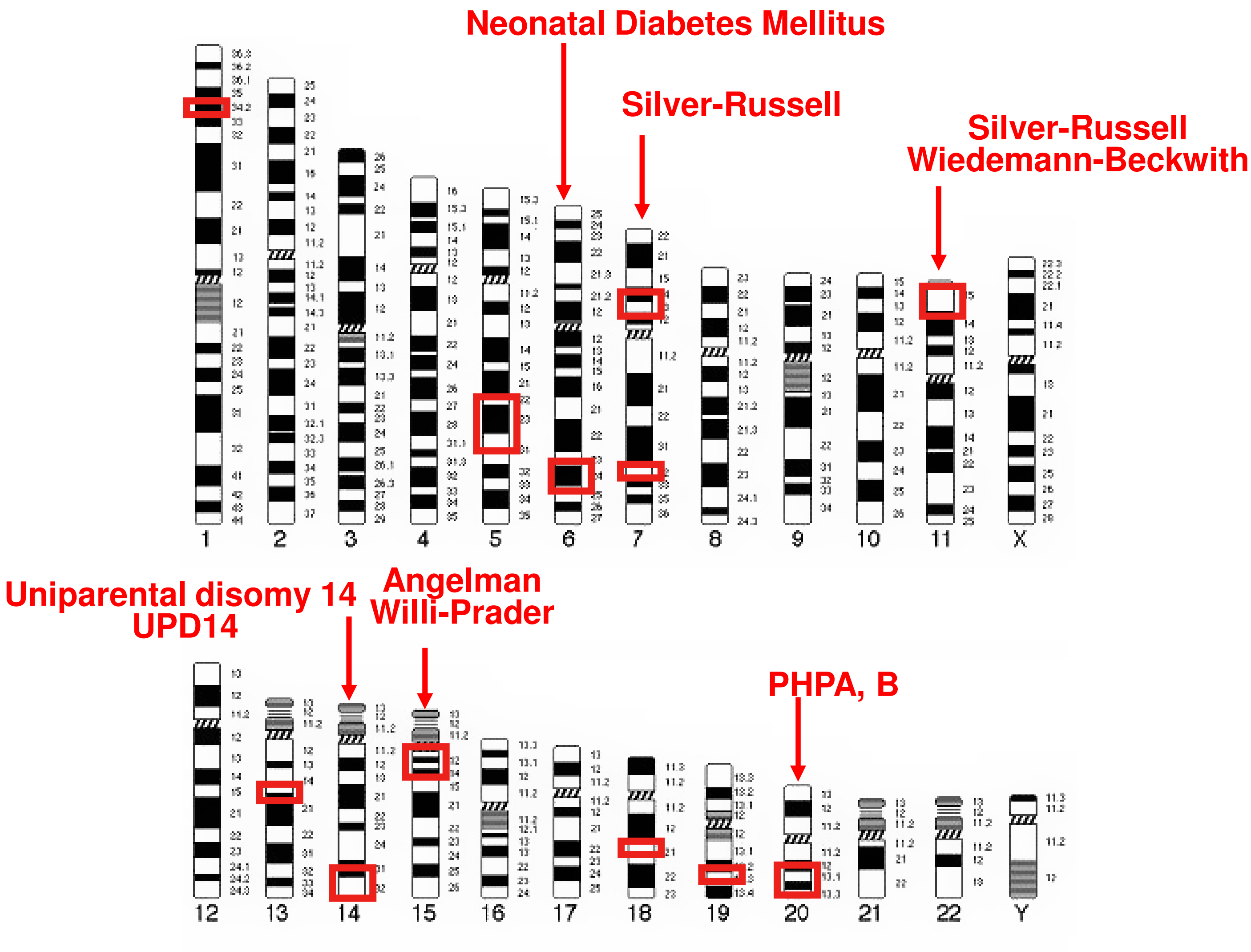
From Reik et al. Nat Rev Genet. 2001

Genomic Imprinting

~ 100 identified genes, cluster



Genomic Imprinting

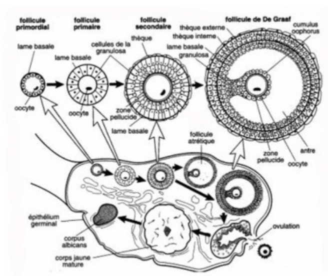


Assisted Reproductive Technologies (ART) and Diseases Related to Imprinting


Sperm cells: fresh or frozen

ICSI vs IVF

Frozen embryo



Ovarian stimulation

blastocyst transfert/ culture medium

No identification of specific procedure

Gicquel et al Am J Hum Genet 2003
Chang et al Fertil Steril 2005
Ludwig et al J Med Genet 2005

Assited Reproduction Technologies (ART) and Genomic Imprinting Disorders

Beckwith-Wiedemann syndrome : 4 series

Gicquel et al. Am J Hum Genet 2003
DeBaun et al. Am J Hum Genet 2003
Maher et al. J Med Genet 2003
Halliday et al. Am J Hum Genet 2004

Loss of methylation at maternal locus ICR2/KCNQ1OT1

Angelman syndrome: 5 cases (4 ICSI)

Cox et al. Am J Hum Genet 2002
Orstavik et al. Am J Hum Genet 2003
Ludwig et al. J Med Genet 2005

Loss of methylation at maternal locus SNRPN

Large Offspring Syndrome (Sheep)

Young et al. Nat Genet 2001

Loss of methylation at maternal locus DMR2 of IGF2R gene

Russell Silver Syndrome (RSS)

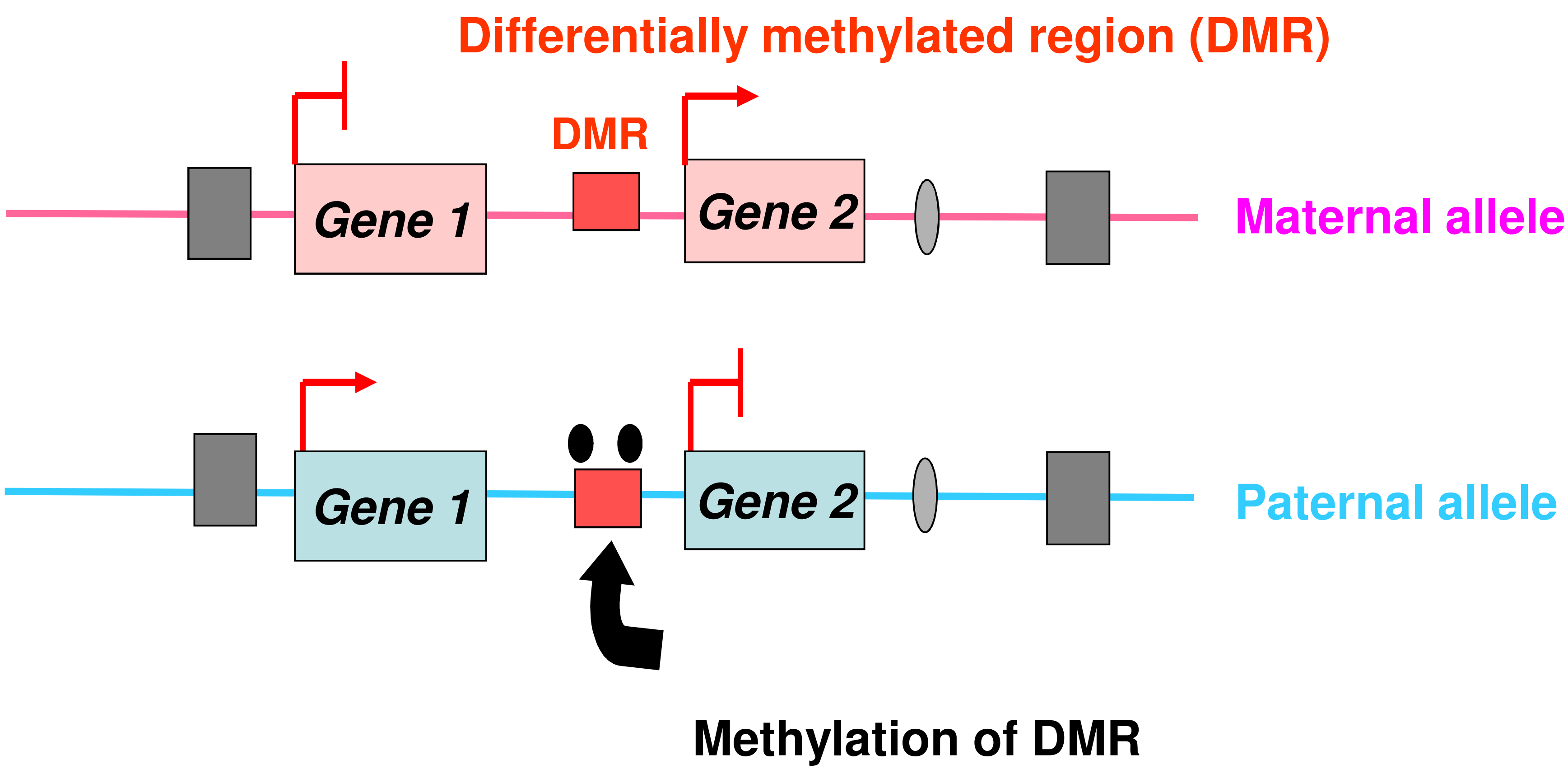
Prevalence (6%) of ART in a series of patients RSS (Trousseau)

Loss of methylation at paternal locus ICR1

Does ART interfere with establishment or maintenance of methylation marks in the imprinted regions?

Genomic Imprinting

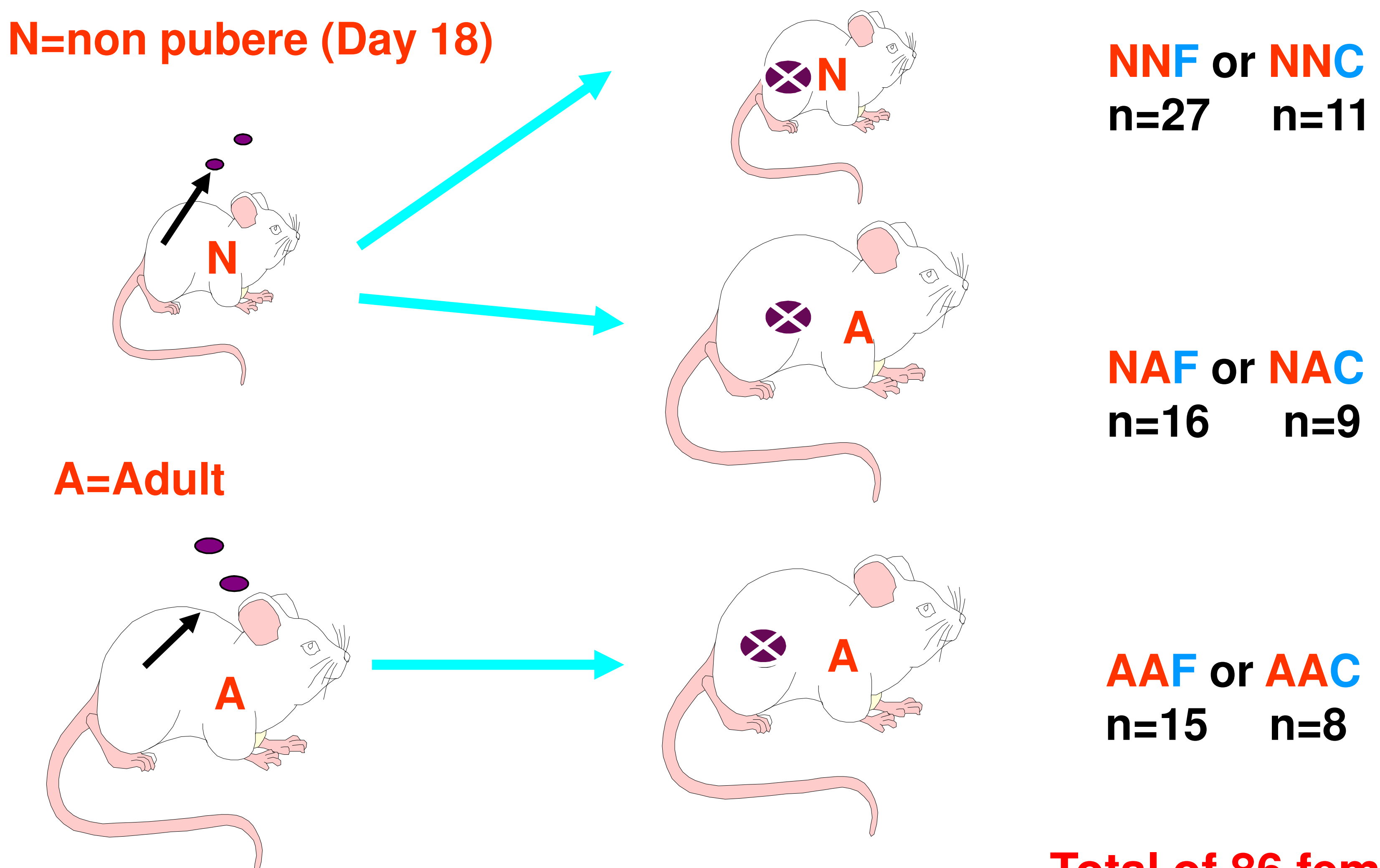
Both maternal and paternal genomes required in mammals



BWS: genomic region of interest is 11p15.5 which contains two DMRs:

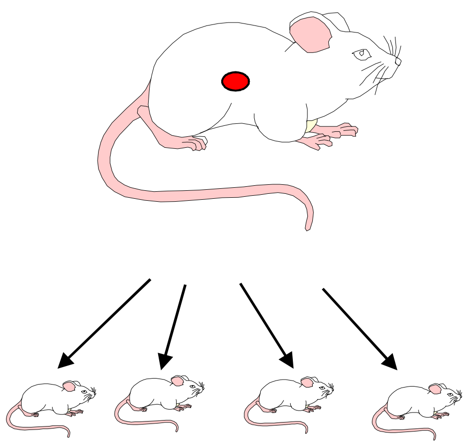
H19 and IGF2 genes (H19 ICR)°
CDKN1C gene(KvDMR1)

Animal Models



Total of 86 females

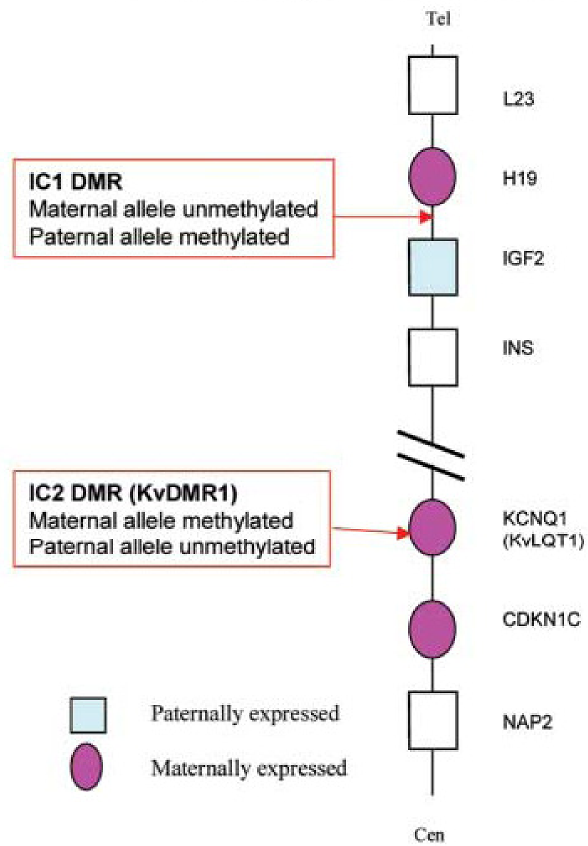
Methylation Status of H19 ICR and Lit1 KvDMR1 in Pups from Grafted Mice



21day-old

kidney
muscle
tongue

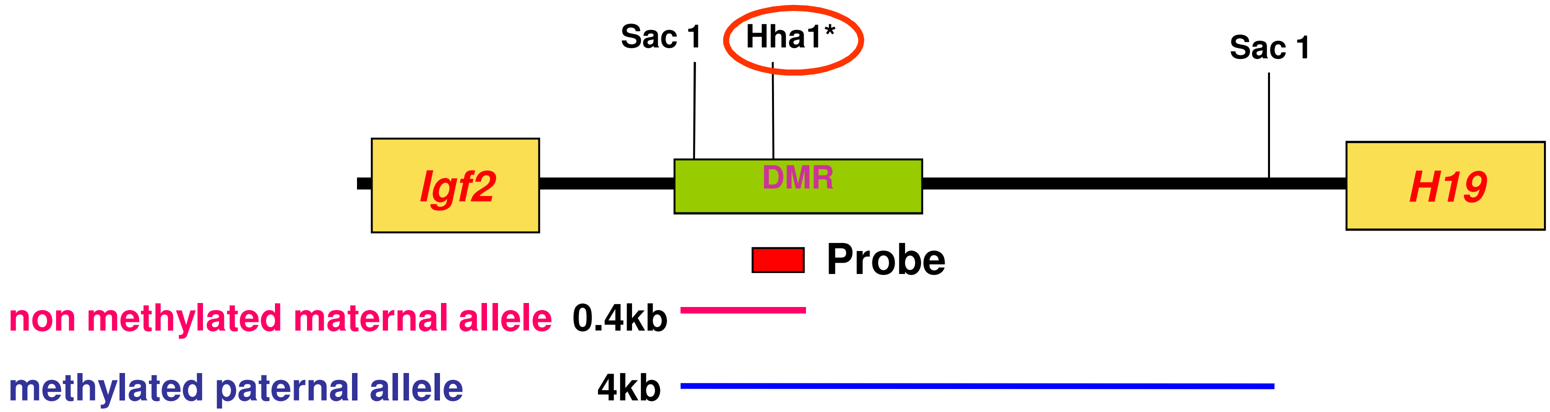
Imprinted gene cluster at 11p15.5

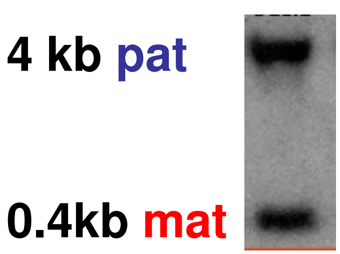


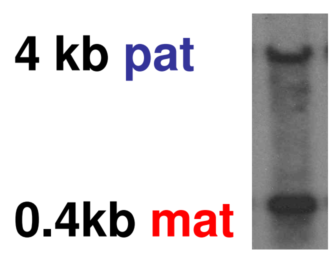
Aberrant genomic imprinting induces numerous genetic disorders (BWS) linked to ch 11p15.5

Maher E.R. Hum Mol Genet 2005

Methylation of *H19-Igf2* DMR





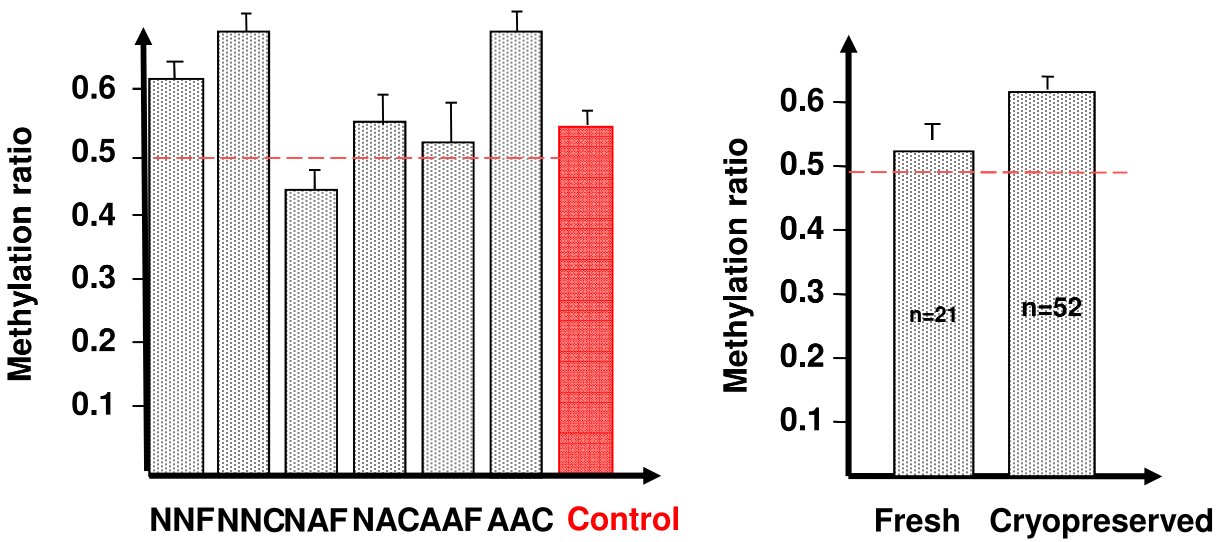


stœchiometric epigenotype

methylation defect on paternal allele

Methylation index = 4 kb intensity / 4+0.4kb intensity

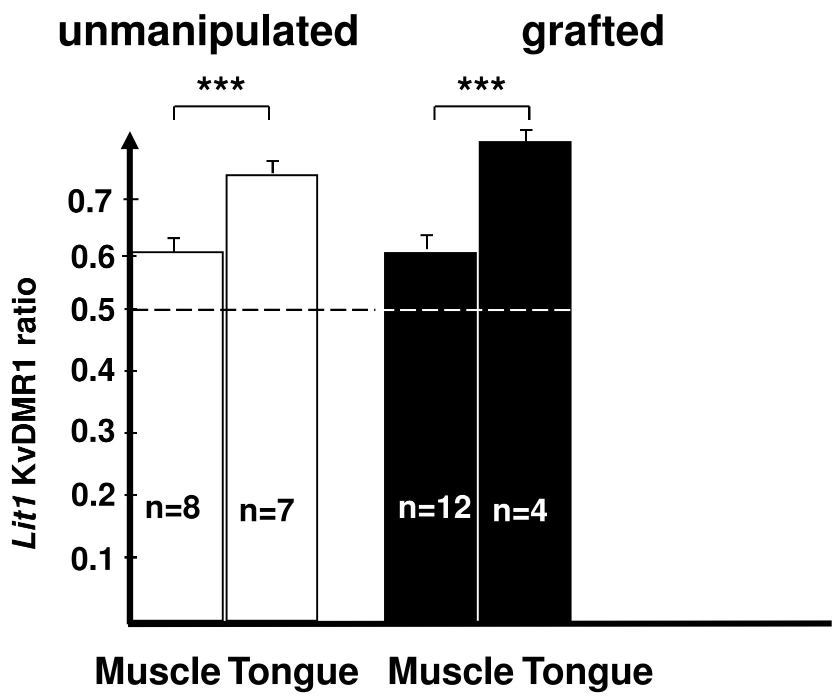
H19-Igf2 DMR Methylation Status



kidney + muscle + tongue

Manipulated versus Controls
Fresh versus Cryopreserved
No difference

KvDMR1 Methylation Status



No difference between groups

Sauvat F et al PLoS ONE 2008

Conclusion (2)

- **No significant imprinting alterations of pups from grafted mice as compared to control**
- **No effect of cryopreservation on imprinting mechanisms**
- **BUT reduced litter size maybe due to spontaneous abortion linked to malformations: Imprinting?**

Alternatives to cryoconservation

Transplantation of a whole cryopreserved ovary cannot be proposed for all patients, due to the risk of tumour cell transmission during the procedure

Research programme is needed to develop alternatives such as :

- **Isolate follicles from frozen ovarian cortex**
- ***in vitro* follicular culture to generate oocytes**

Yet to be tested

Conclusions

- Because all data indicate that the reproductive lifespan of grafted ovarian tissue is limited, the main target of ovarian grafting is restoration of fertility

- This model supports the legitimacy to propose cryopreservation in young girls before gonadotoxic treatment as a tool to restore fertility, as has been done in adult women

- Regarding our current knowledge concerning this procedure, one should remain cautious when delivering information to patients and their family at the time of cryopreservation, in terms of puberty induction and potential risks for children

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