ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses

Dr. Verena Nordhoff
Reproduktionsbiologin (AGRBM)
Senior Clinical Embryologist (ESHRE)

Centre of Reproductive Medicine and Andrology,
University Hospital of Münster

Assisted Reproductive Technologies (ART)

- ART techniques: *in-vitro*-fertilization (IVF), intracytoplasmic sperm injection (ICSI), cryopreservation of Embryos / fertilized oocytes

- ART infants in Europe 2008: 1.7 % of all born children[1]

- In 2012: 12 047 IVF- and 38 897 ICSI-treatments in Germany
  - 29,6 % led to pregnancies
  - 18,67% Baby take home rate

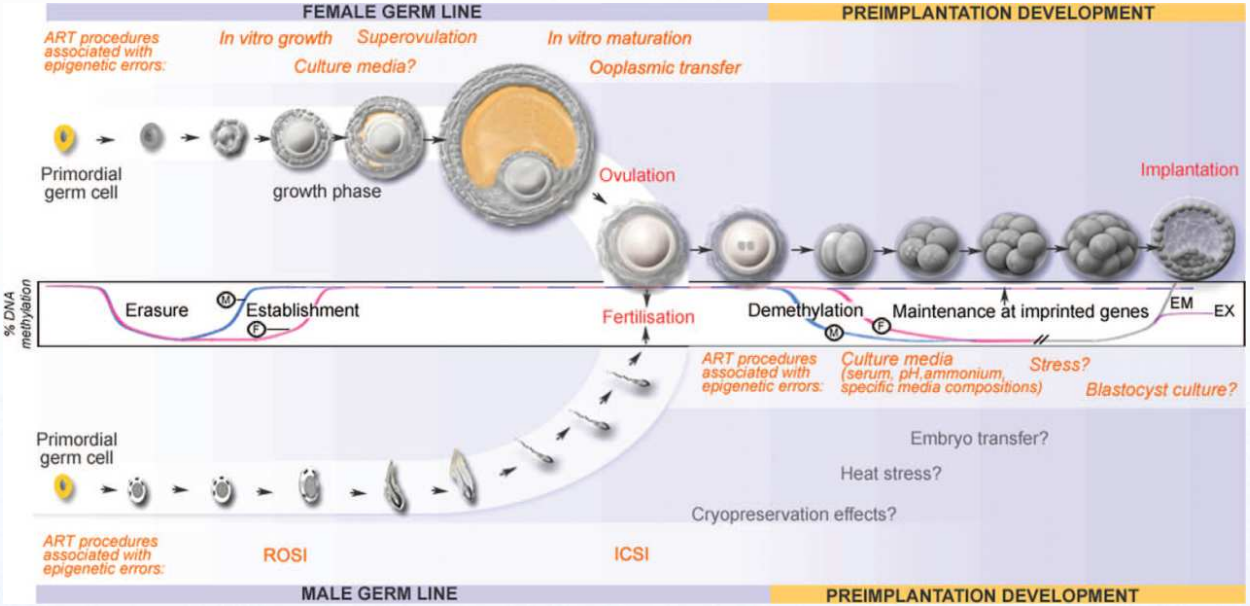
What are possible reasons for this low pregnancy and birth rates?

[1] Ferraretti et al. 2012; [2] Deutsches IVF Register 2013

Influences on ART embryos

- Lifestyle of the couple
- Diseases associated with subfertility or infertility
- suboptimal *in vitro* culture conditions ?

Epigenetics in preimplantation embryos



Huntriss and Picton, 2008

- ART procedures coincide with critical periods in genomic imprinting during germ cell and preimplantation embryo development
- Errors that result in loss-of-imprinting are often associated with diseases, e.g. Prader-Willi-, Beckwith-Wiedemann (BWS) or Angelman syndrome[1,2]
- Different culture conditions are supposed to change the methylation status of imprinted genes like H19, Lit1 or Mest[3]

([1] Denomme and Mann, 2012, [2] Huntriss and Picton, 2008, [3] deWaal et al. 2013)

Influence of culture media on human embryos

- Influence on birth weight after ART[1,2]
- No difference on birth weight or length[3,4,5]

BUT: all studies used different media, mostly retrospective

- therefore it is difficult to compare the outcome
- no thorough analysis regarding molecular features possible

Solution: use the mouse as model (!)

([1] Dumoulin et al. 2010, [2] Nelissen et al. 2012,
[3] 2013 Lin et al. 2013, [4] de Vos et al. 2014, [5] Vergouw et al. 2014)

Questions:

- Are mouse embryos cultured under different culture conditions susceptible to changes in gene expression, epigentetic imprinting or apoptotic events?

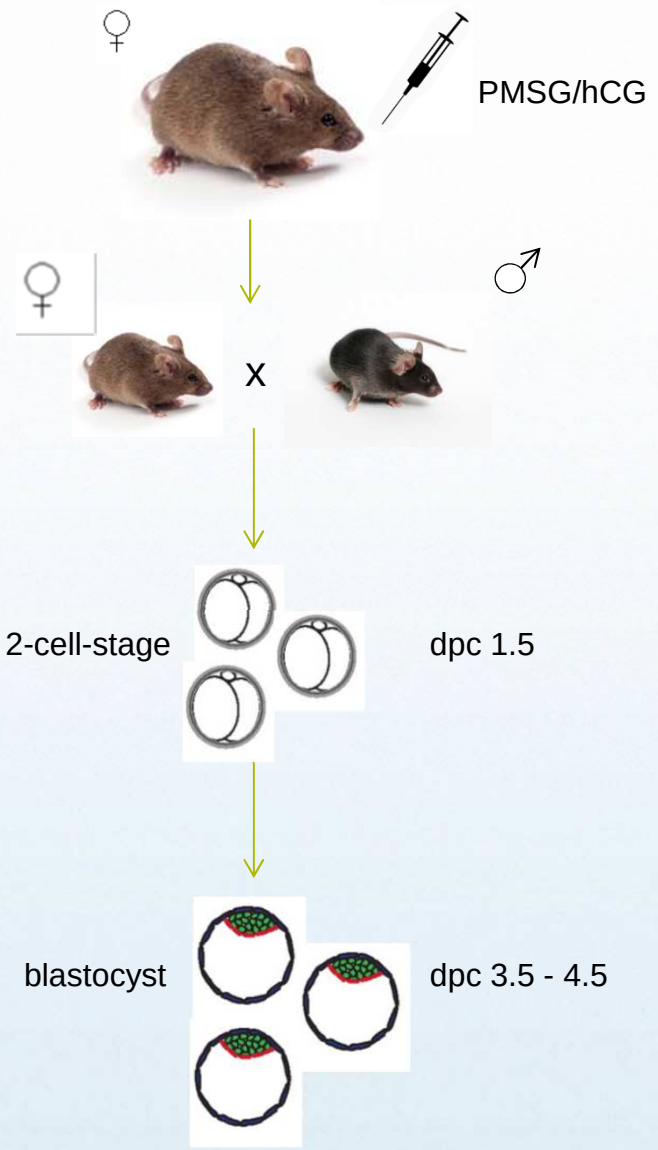
- Does the condition how the embryos were produced (naturally, IVF or ICSI) have an addtional effect on these events?

- Do these in vitro cultured embryos have the same implantation and developmental rates as in vivo produced embryos?

- Do the resulting fetuses (and their placentae) show changes in gene expression, imprinting or apoptosis in different organs/cells?

Mouse embryo assay - MEA

- Widely used bioassay for screening of equipment, of media components and especially of human culture media
- > 80 % of embryos have reached the blastocyst stage, the tested medium or equipment is considered as suitable for clinical use
- BUT: no long-term effects predictable, is there an impact on the fetal development, the rate of apoptosis or on epigenetics?

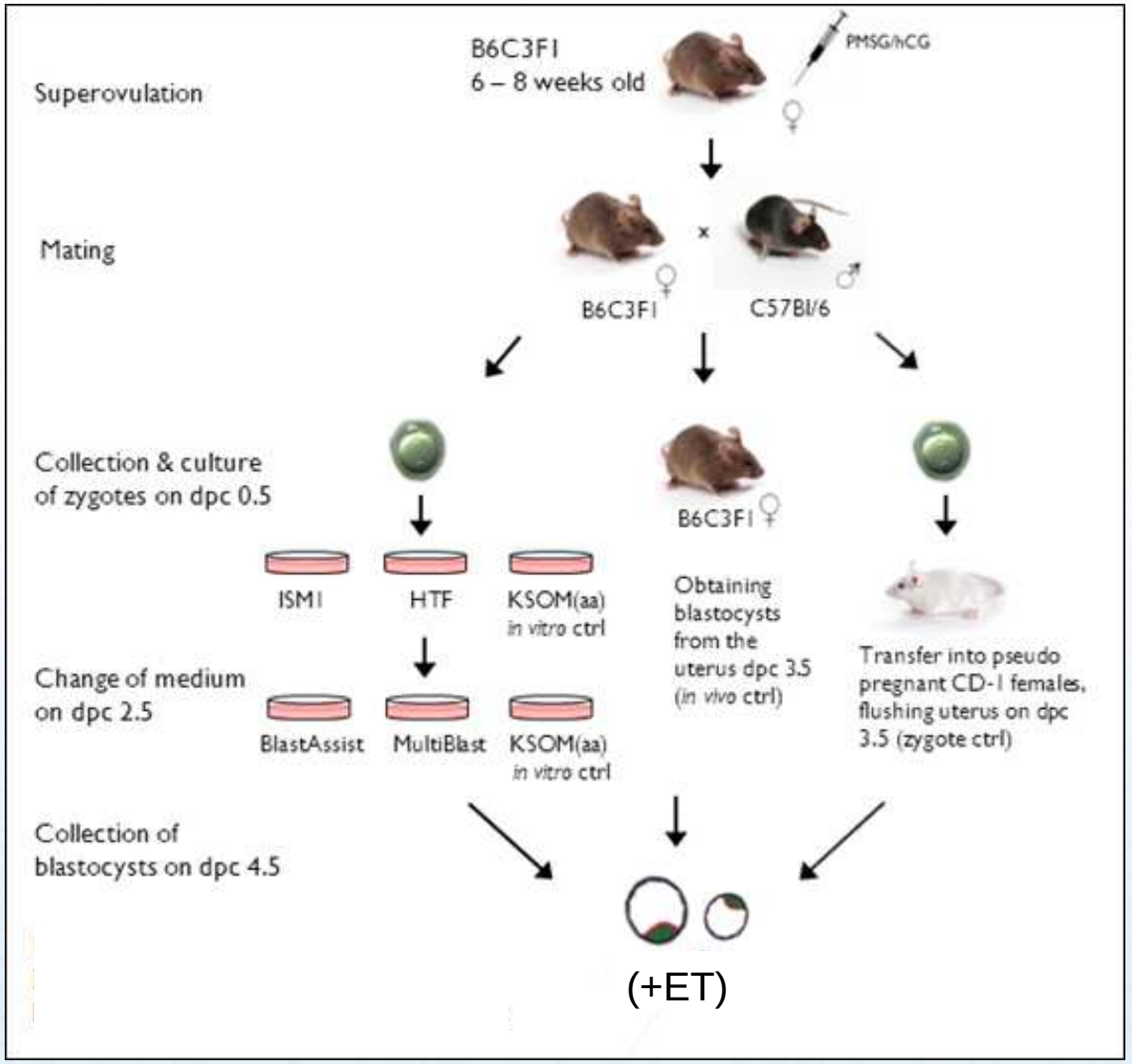


Human IVF media for incubation of mouse embryos

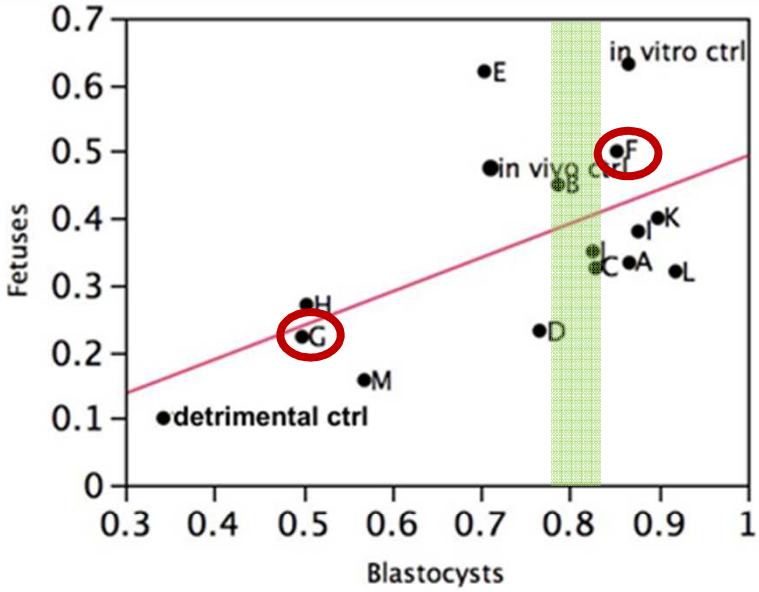
Protocol	Monoculture	Sequential culture	Manufacturer
A		Early Cleavage Medium (ECM)→ Multiblast	Irvine Scientific
B		Ferticult IVF → G3	Fertipro, Ferticult
C		G-1 Plus → G-2 Plus	Vitrolife
D	GM 501 gentamycin		Gynemed
E	GM501 pen/strep		Gynemed
F		Human tubal fluid (HTF) medium → Multiblast	Irvine Scientific
G		ISM1→ISM2	Origio
H		ISM1→Blastassist	Origio
I		P1 → Multiblast	Irvine Scientific
J		Quinn's advantage cleavage → blastocyst	SAGE
K	Single Step Medium (SSM)		Irvine Scientific
L		Sydney cleavage → Sydney blastocyst	Cook medical
M	Universal		Origio
Detrimental control	ISM1 not switched to ISM2		Origio
In vitro control	KSOM(aa)		Made by M.B.
In vivo control	oviduct		CD1 mouse

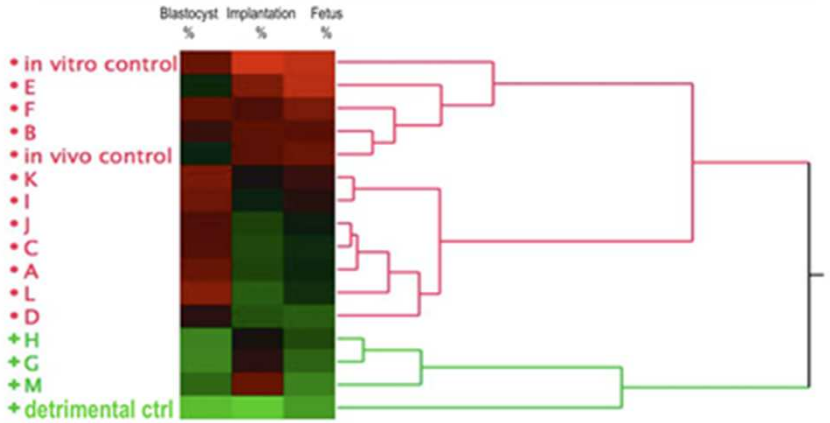
Schwarzer et al. 2012

Experimental design



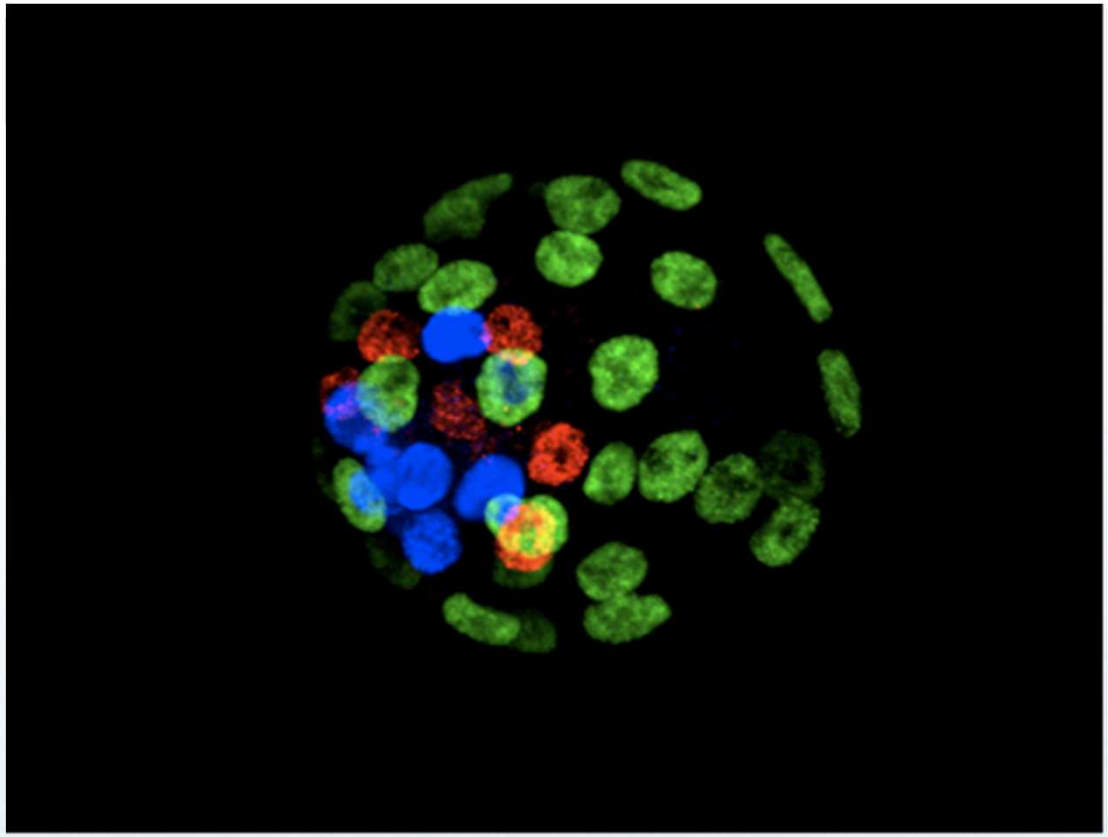
Human IVF media display different blastocyst and fetal rates





Schwarzer et al. 2012

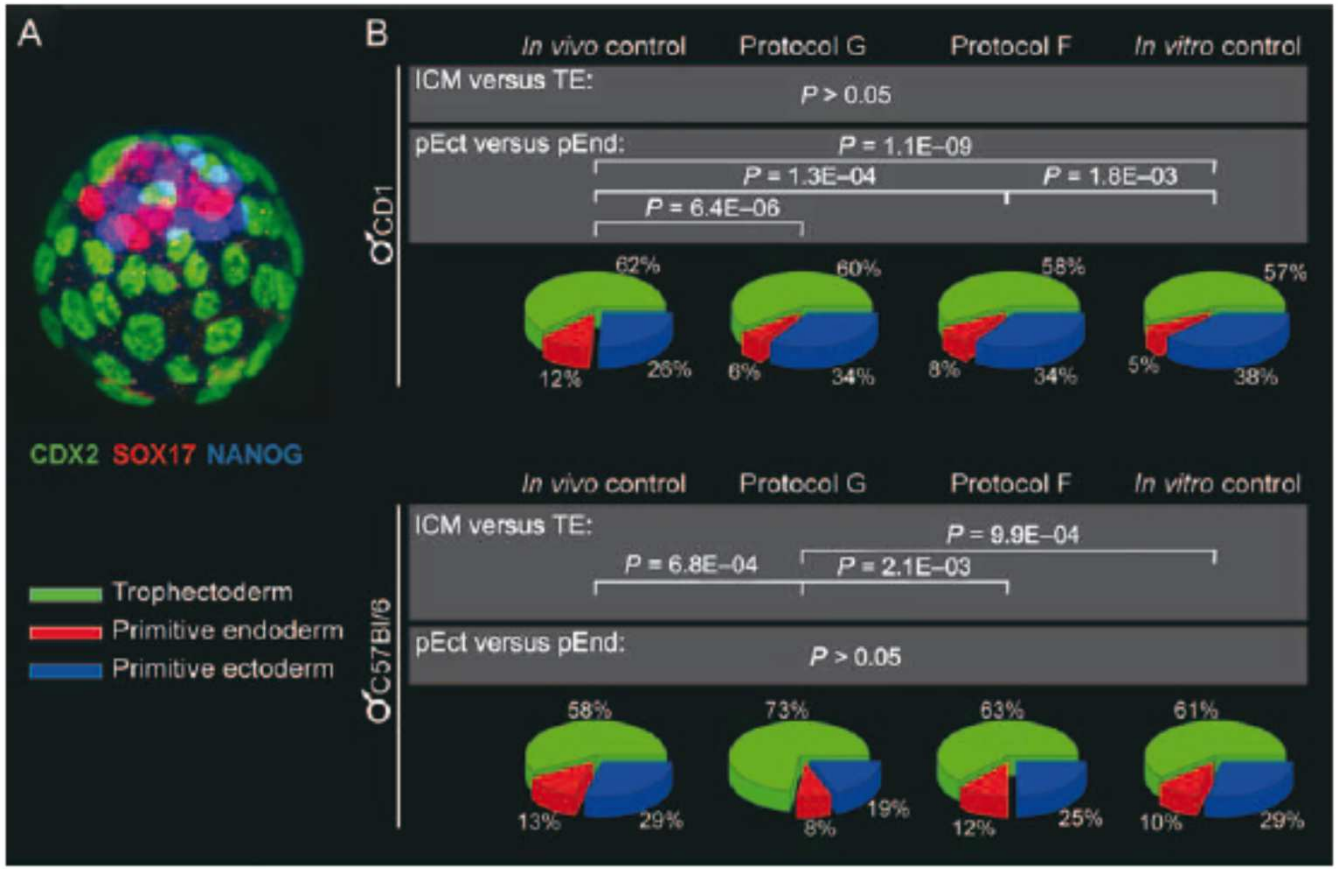
Immunostaining of blastocysts



Stack of z-confocal series of a representative blastocyst after immunostaining (green, trophectoderm (Cdx2); blue, epiblast (Nanog); red, primitive endoderm (Sox17)).

Schwarzer et al. 2012

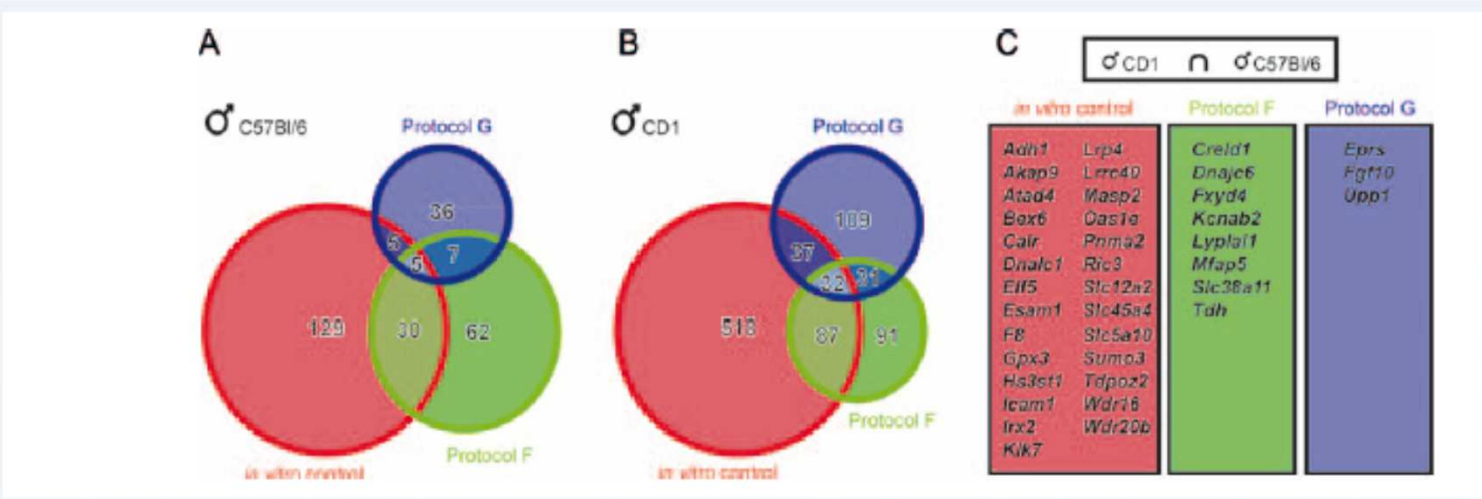
Human IVF media can change the proportion of the three cell lineages in the developing mouse blastocyst



Schwarzer et al. 2012

Human IVF media are able to change expression of genes

- Also dependent on different mouse strains



Schwarzer et al. 2012

BUT: we did not find any behavioral changes in the resulting offspring!

Schwarzer et al. 2014 submitted

Which influence has ICSI on the proportion of the three cell lineages?

- Already at the 2-cell stage (24 h post-ICSI), cleavage rates differed between HTF and ISM1 ($p<0.05$), continuation at the 4-cell stage.

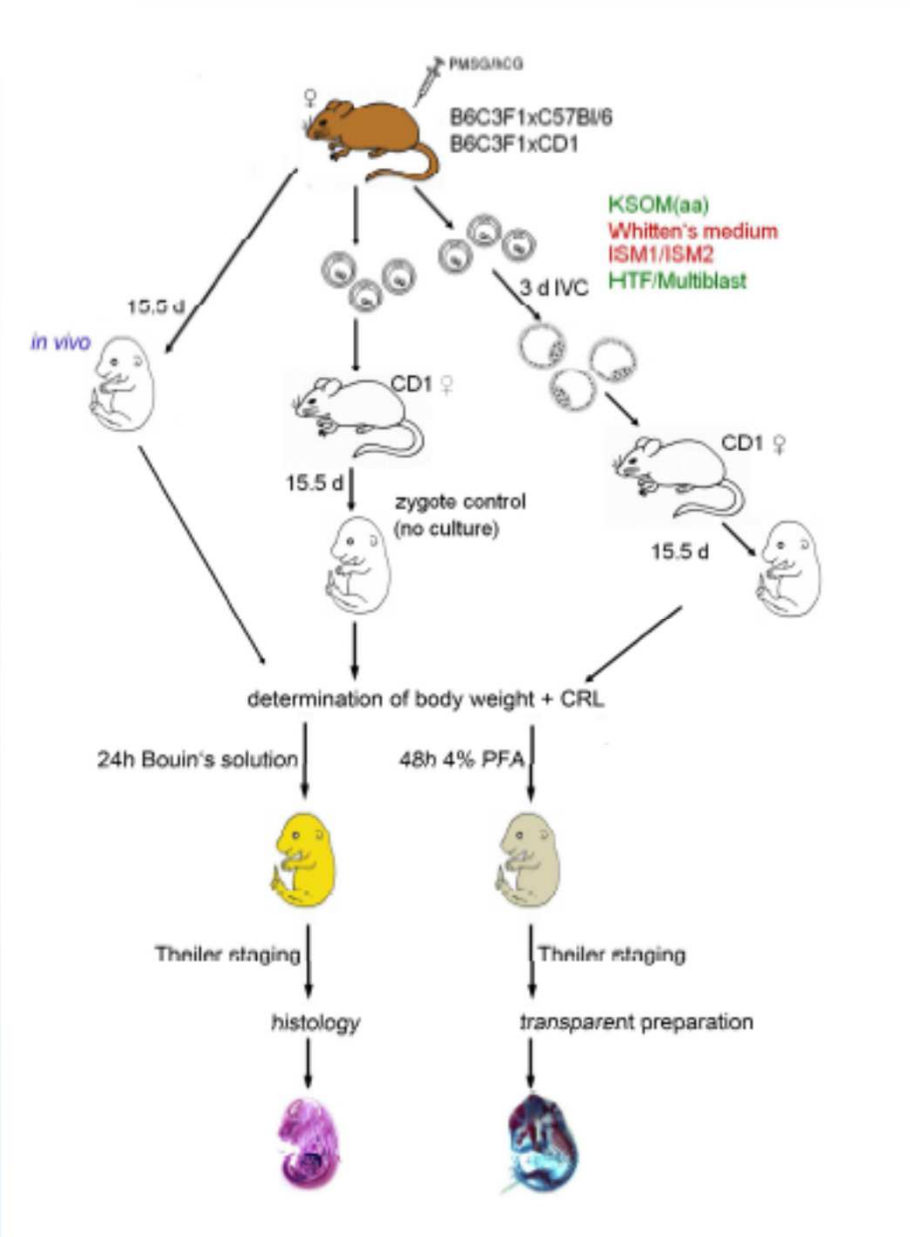
- The different cleavage progression that led to different blastocyst rates had already started at the 2-cell stage.

- Synchronous fertilization by ICSI: different rates ascribed to the different kinetics of cleavage in the different environments tested.

- In summary, these results show that blastocyst rates were affected more by the culture medium than by ICSI.

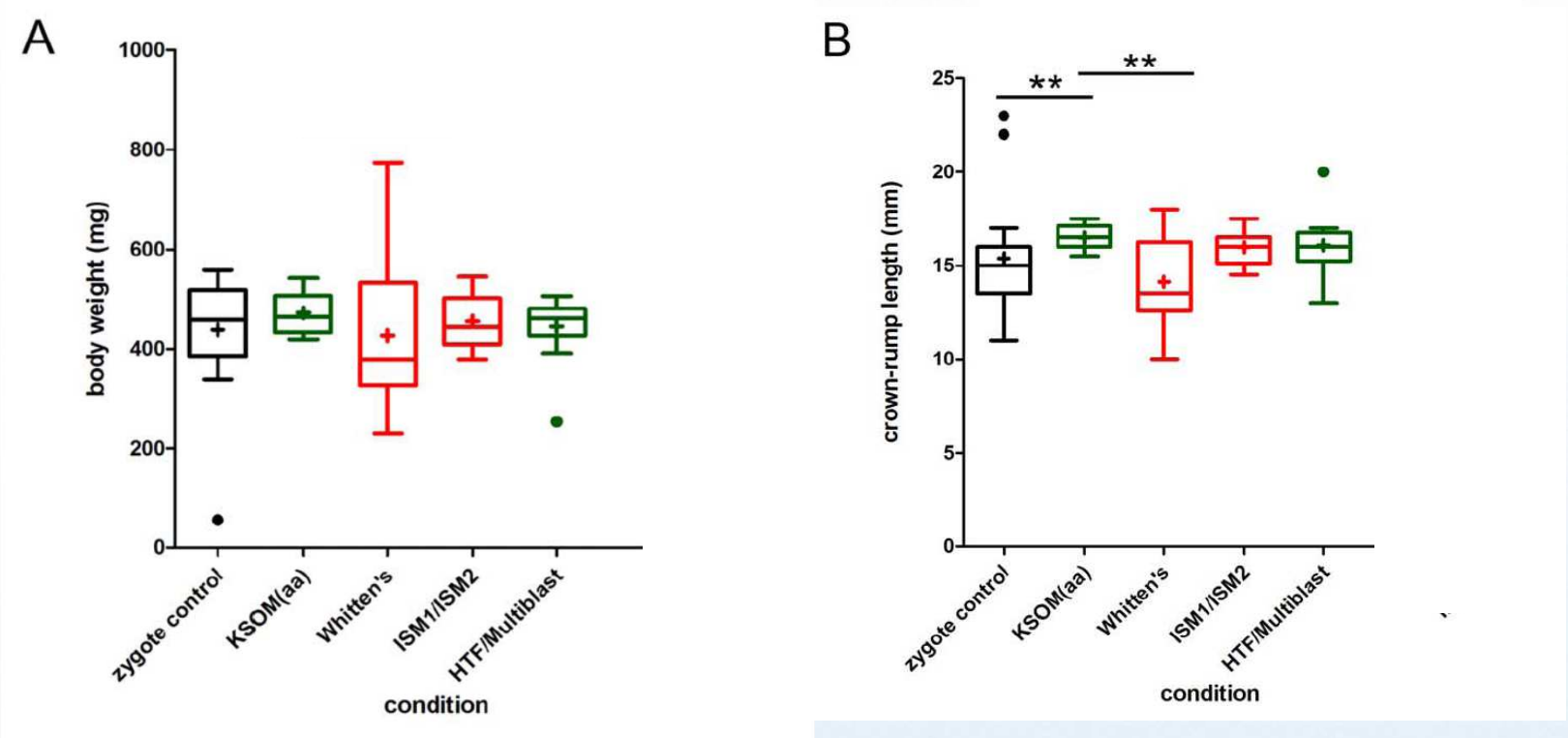
Schwarzer et al. 2014 submitted

But, do these changes lead to differences in post-implantation development?



Hemkemeyer et al. 2014

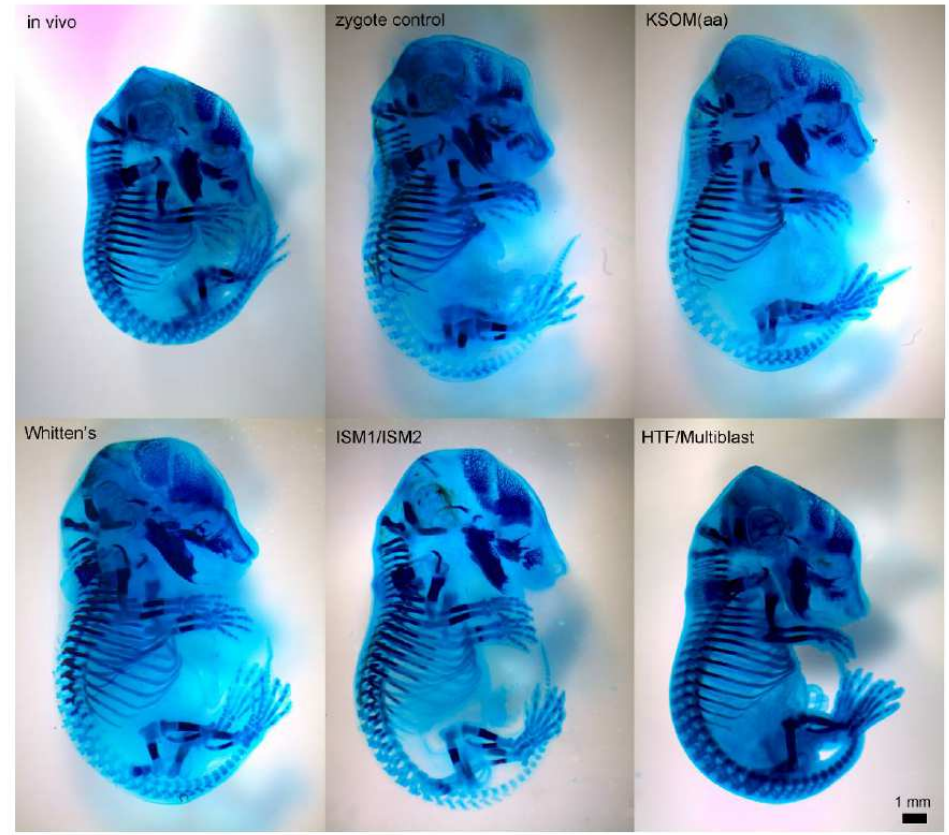
Human IVF media have no consequences on body weight or crown-rump length



Comparison of body weight and CRL of E15.5 fetuses cultured under different conditions. p ≤ 0,01 significant (**), p ≤ 0,001 highly significant (***)

Hemkemeyer et al. 2014

Human IVF media have no influence on cartilage and bones



Cartilage and bone staining of E15.5 fetuses of six different conditions.
Cartilage stained in blue (AlcianBlue), ossified bone in red (AlizarinRed).

In vivo control group differed significantly from ISM1/ISM2 group in the total bone length of fibia and tibula.

Hemkemeyer et al. 2014

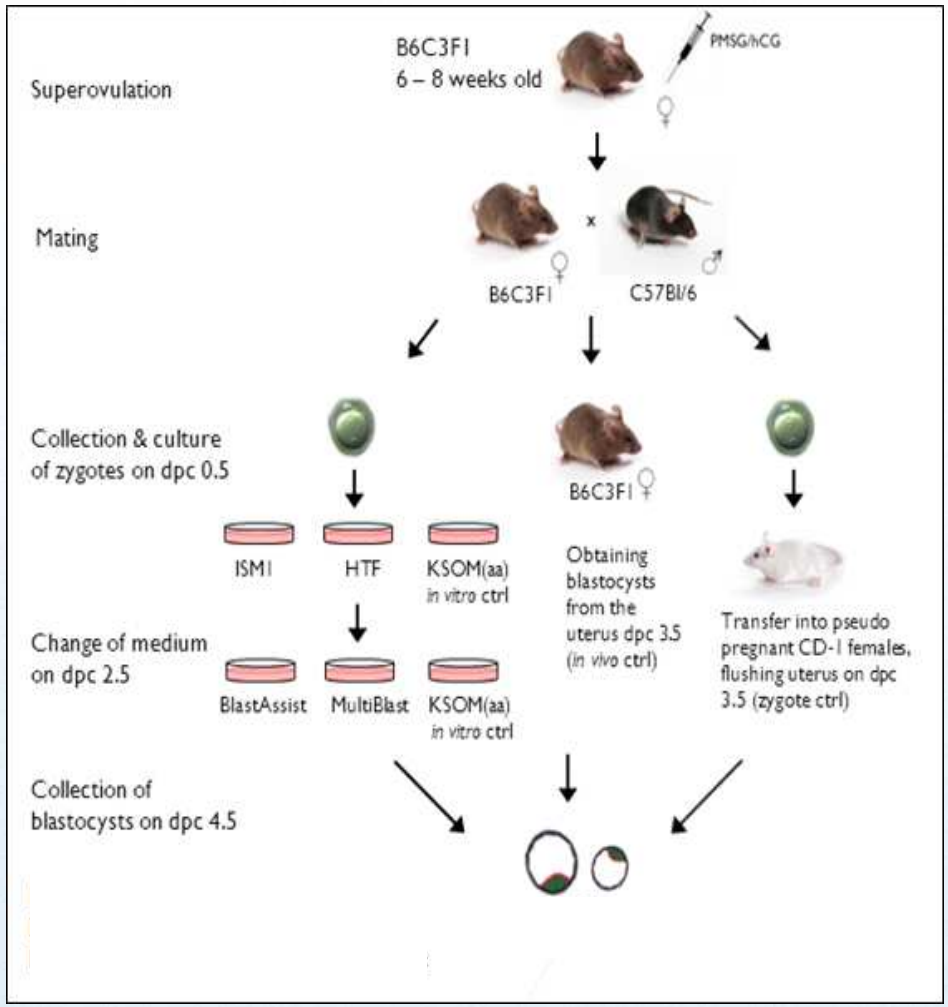
Conclusion:

- Human IVF media have an influence on cell lineage in early pre-implantation development in mice
- Human IVF media can change the gene expression (also in ICSI) in blastocysts

BUT:

- Once these embryos implant, the “normal” developmental program runs undisturbed (compensatory growth?)
- Although we do not know how selection at implantation works (! Lower fetal rates)

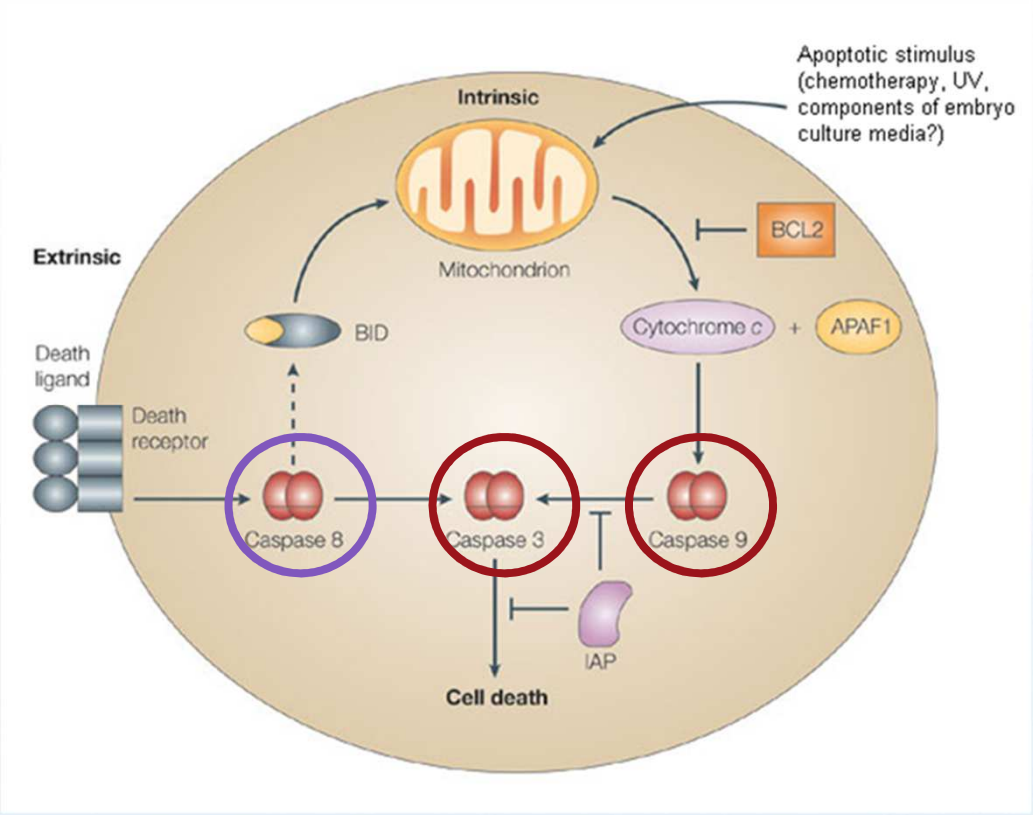
So where do these differences in total cell numbers arise from?



Possible mechanism:

- Apoptosis?
- And what about epigentics?

Apoptosis – a crucial process in mammalian pre-implantation development

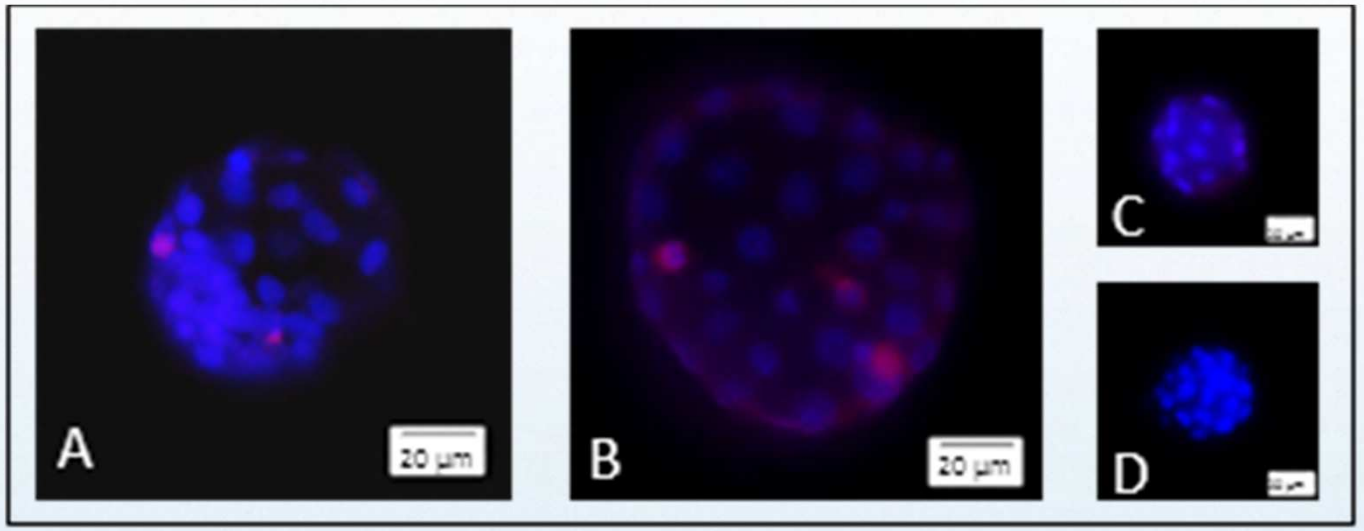


modified from Andersen et al. 2005

- Apoptosis plays a potential role in embryonic loss and in cellular responses to suboptimal developmental conditions and stress
- Regulated via two different pathways, e.g. intrinsic and extrinsic pathway

Positive control: Actinomycin D (10 mM)

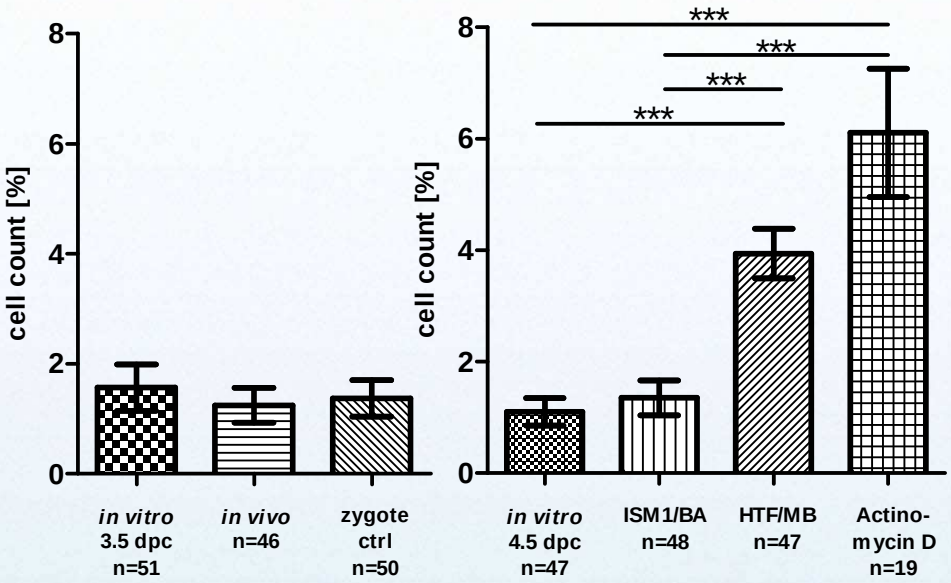
- Actinomycines belong to a class of polypeptide antibiotics, derived from *streptomyces* bacteria
- Promotes induction of apoptosis by specific stimuli, e.g. TRAIL and FAS, activation of caspase-3,-8,-9 and -12



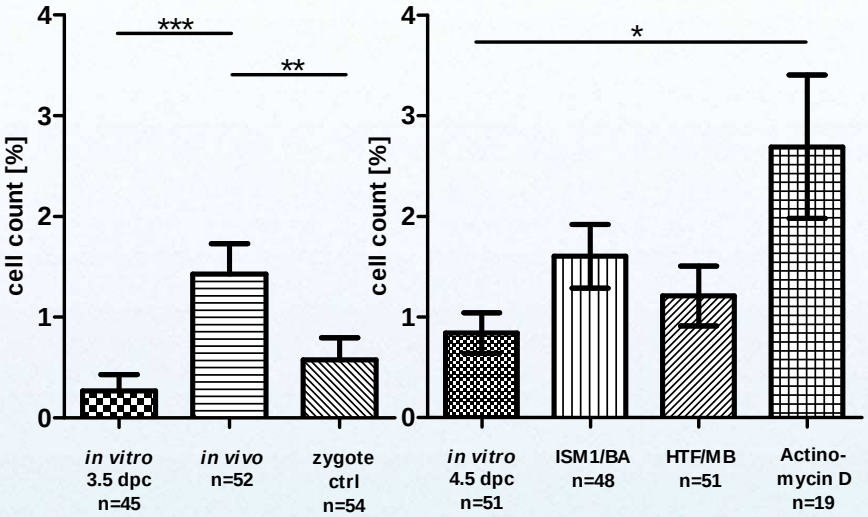
(A) Staining of blastocysts with caspase-9 (B) positive control with Actinomycin D; (C and D) IgG and negative control

The percentage of caspase positive cells is different among the culture media

Cleaved caspase - 3



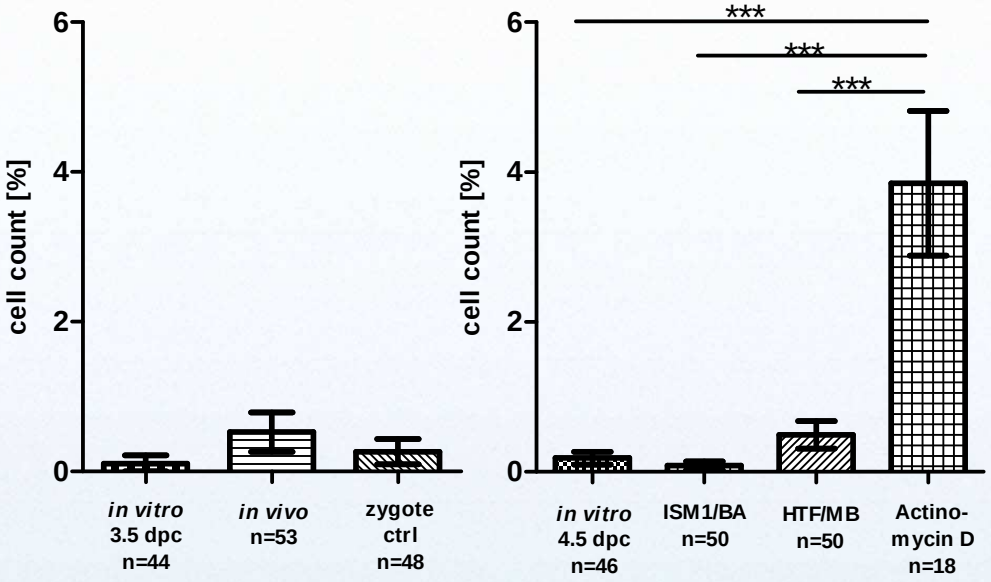
Cleaved caspase - 9



* p < 0.05; ** p<0.01; *** p<0.001

Schulte et al. in preparation

No differences in Cleaved Caspase 8 positive cells among the different media



*** p<0.001

Schulte et al. in preparation

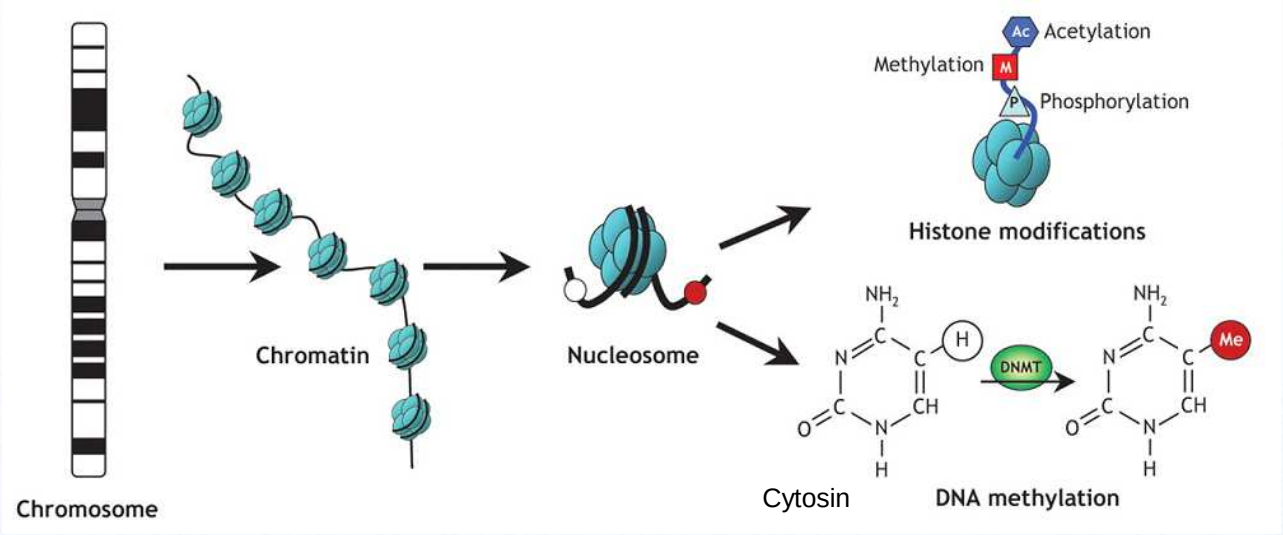
Conclusion:

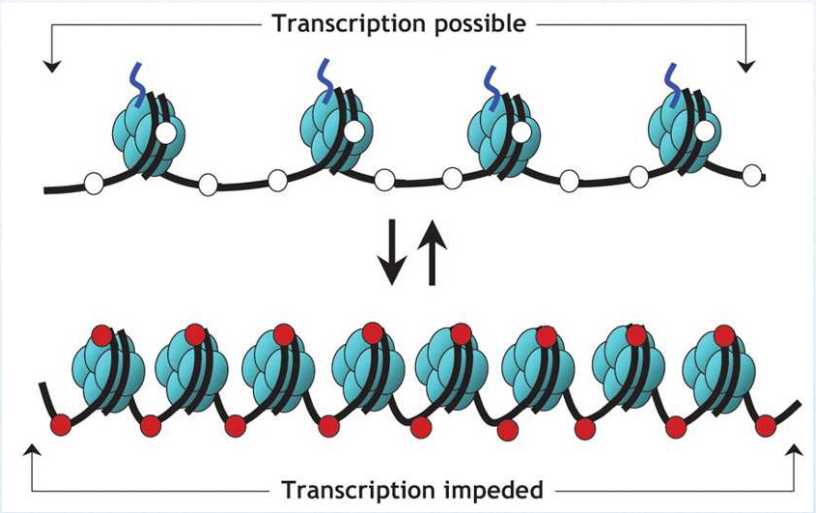
- all embryos exhibit caspase activity, as shown before (Fabian et al. 2007)
- Apoptosis seems not to be the main mechanism for the lower cell count
- The detected apoptosis is triggered more intrinsically than extrinsically
- Possible other caspase-9 independent pathways leading to activation of caspase-3

Outlook

- More detailed analysis regarding different pathways, expression patterns of particular apoptotic genes or protein synthesis
- Detailed analysis of cell lineages
  - Dissociation of ICM and TE to analyse the gene expression or rate of apoptosis in each compartment separately
- Mouse is not the perfect model for reproductive investigations related to the human physiology

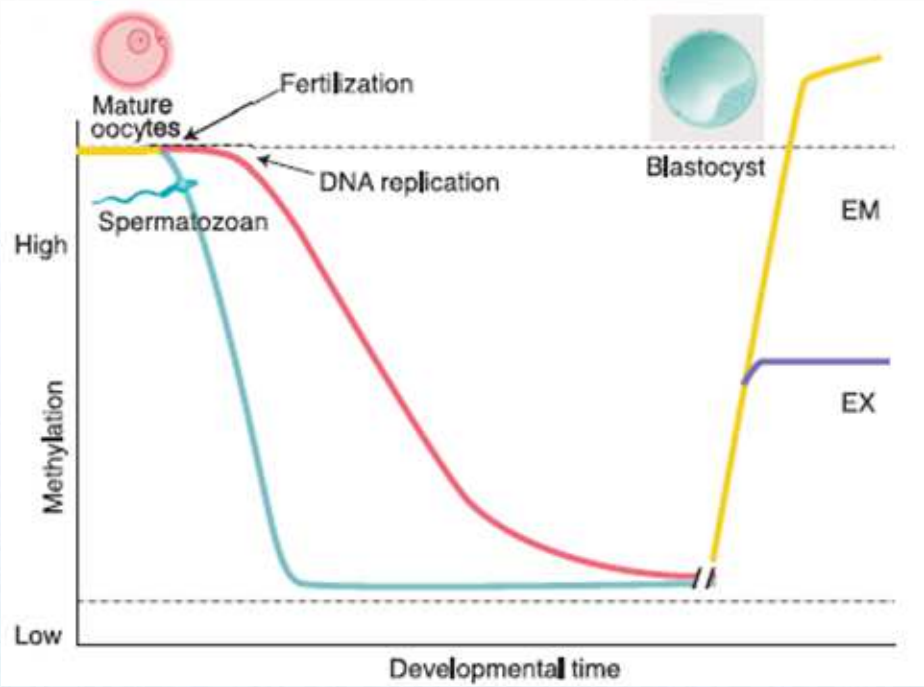
Epigenetics - Basics





cnx.org

Methylation reprogramming in Mammalian Embryos



- Paternal genome is actively demethylated directly after fertilization (blue line)
- Maternal genome is passively demethylated, depending on DNA replication (red line)
- Remethylation around implantation in embryonic (EM) and extraembryonic (EX) lineages
- Imprinted genes are excluded from these mechanisms (dashed line)

Modified after Reik et al. 2001

Bisulfite Conversion and Pyrosequencing

DNA Isolation

↓

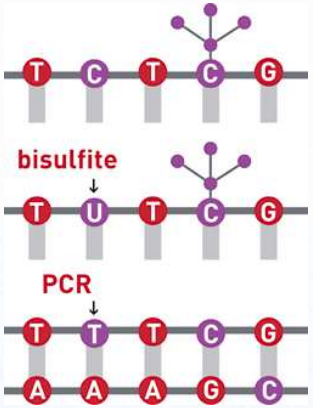
Bisulfite Treatment

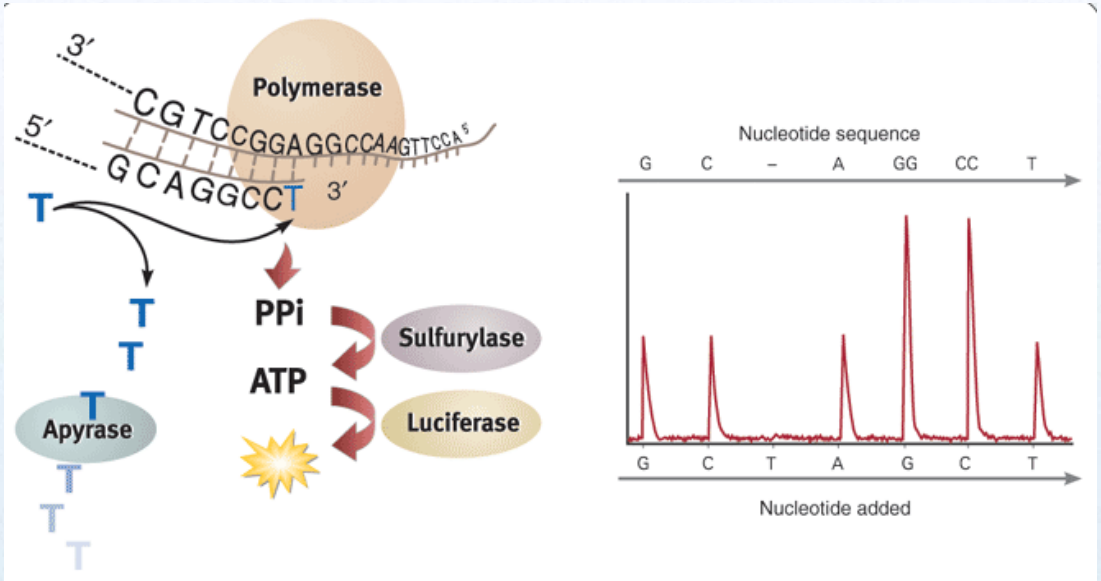
↓

PCR of desired gene sections

↓

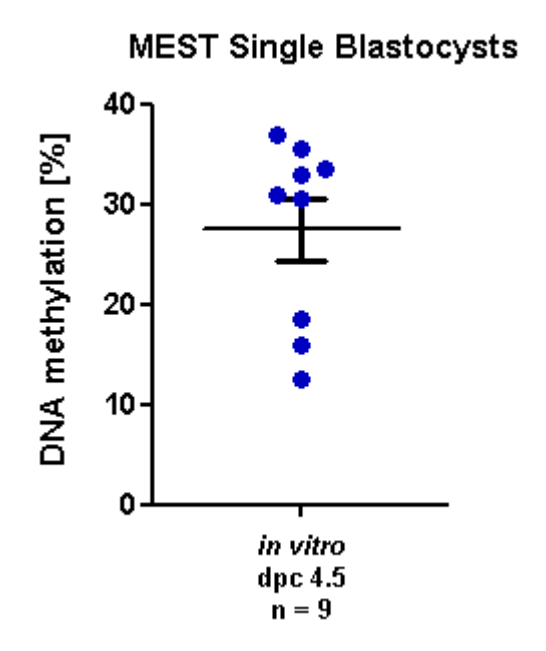
Pyrosequencing

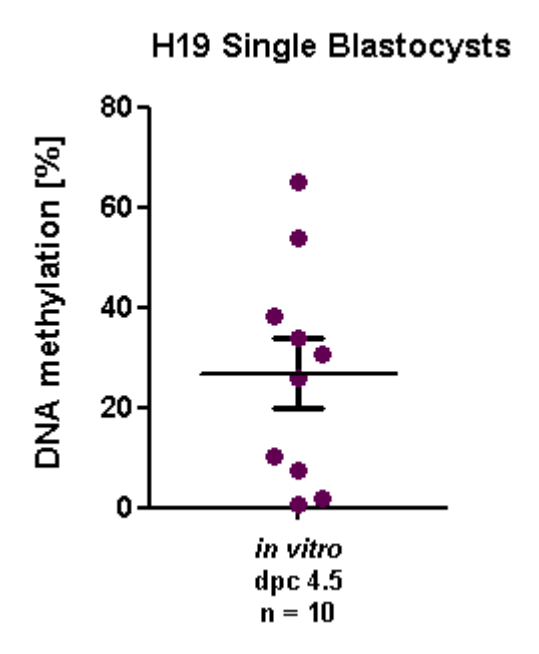




diagenode.com; Nature Methods 2005

First epigenetic results





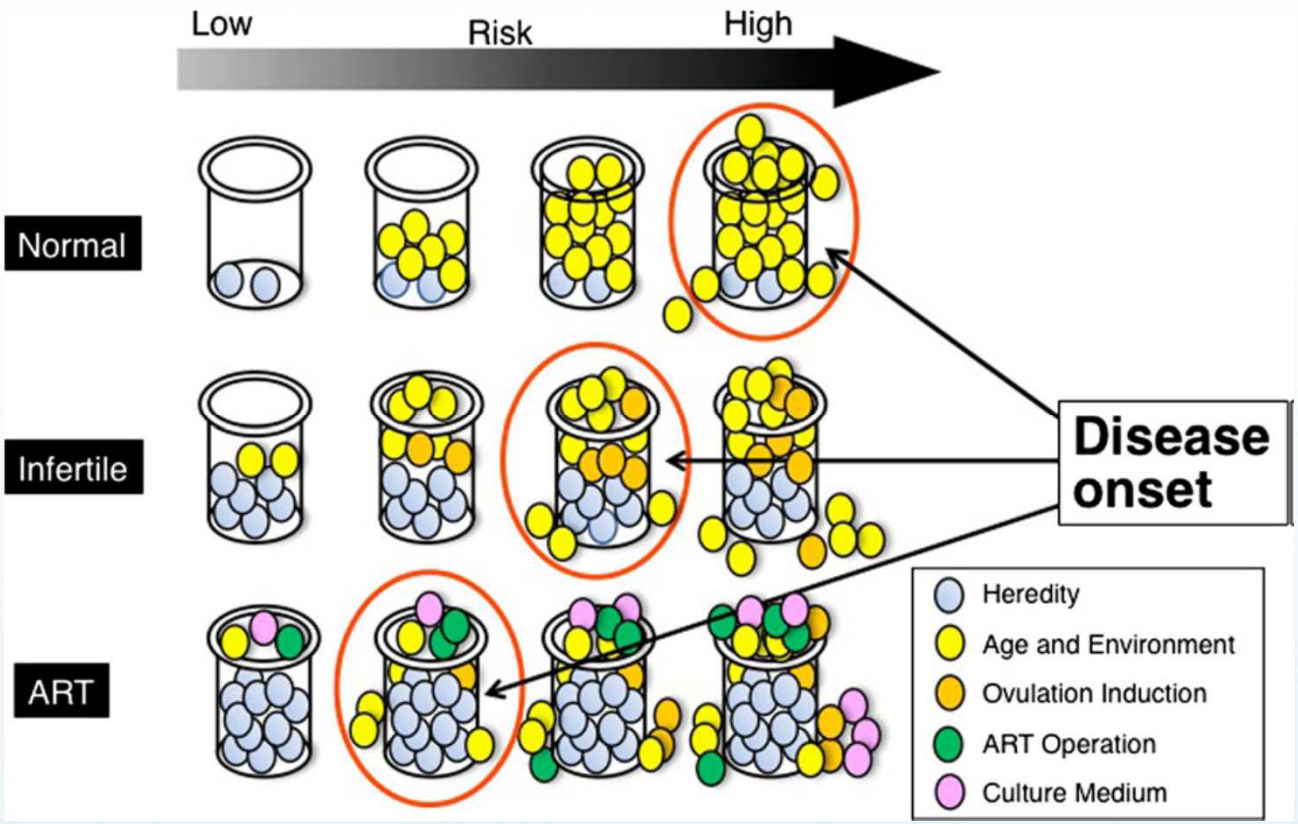
Schulte et al., unpublished

Outlook

- Include more blastocysts in the analysis
- What about the inner cell mass alone?
- Confirm results of pyrosequencing with conservative method
- Dynamic imprinting pattern in ES cell lines[1], oocytes and blastocysts of different stages[2]

([1]Humpherys et al. 2001, [2]Tomizawa et al. 2011)

Model of the onset of imprinting disorders after ART



Onset of imprint-associated disorders shows that a combination of factors such as the process of ART, infertility and advanced maternal age are likely to account for the increases in the diseases as synergy effects.

(Hiura et al 2014, Review)

Dank u voor uw aandacht

CeRA
Katharina Schulte
Stefan Schlatt

MPI Münster
Michele Boiani
Caroline Schwarzer

ZTE
Jens Ehmcke

Uni Enschede
Séverine Le Gac

Funding:
- Deutsche Forschungs-Gemeinschaft BO 2540/4-1 and SCHL 394/9
- Nederlandse Organisatie voor Wetenschappelijk Onderzoek (no. 63-258)
- Innovative Medizinsche Forschung (IMF) NO 111212