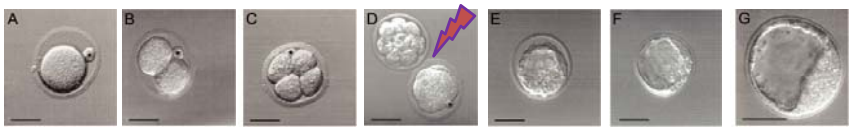
ESF-II / 5 WS2014 3. Dst 21.10.2014

In vitro Herstellung von Blastozysten von Macaca mullata und Homo sapiens

Somatischer Zellkern Transfer / Somatic cell nuclear transfer (SCNT)

Vorgeschichte

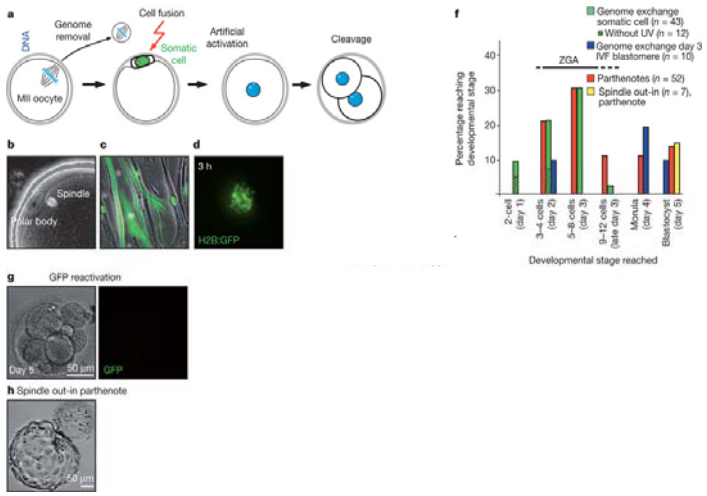
Im Gegensatz zum erfolgreichen SCNT bei Macaca mullata (Rhesusaffe), starben Homo sapiens Embryonen immer im Morulastadium.



Kritische Parameter für den Kerntransfer

1. Extranukleare Bestandteile stören (somatischer Kern ist giftig)
   Intermediärfilamente, die an der äußeren Kernmembran über Plectin und Nesprin 3 haften, ebenso Endoplasmatisches Retikulum
2. Mechanische Beschädigung des Oozyten
   Ausschluß durch Spindel- out/in Experimente
   Möglichst wenig Zytoplasma aus der Eizelle entfernen
3. Cytoplastenaktivierung bei H. sapiens nicht ausreichend.
   bei Tieren reicht Sr^{++}, 7% EtOH, Ionomycin (Ca^{++}), Stromstoß, …
4. Meose II Metaphase in H. sapiens ist instabli. – „nicht genug Zeit“
   → Stabilisierung mittels Inhibitoren
5. Meose spezifische Faktoren sind für die Reprogrammierung notwendig.
6. Phase des Zellzyklus des somatischen Kernen entscheidend?
   G0 / G1 Arrest mit 3-5 % FCS für 3Tage.
7. Chromosomen assoziiertes Material (Spindelapparat) ist für die Reprogrammierung bzw. Aktivierung des embryonalen genetischen Programmes mitverantwortlich.

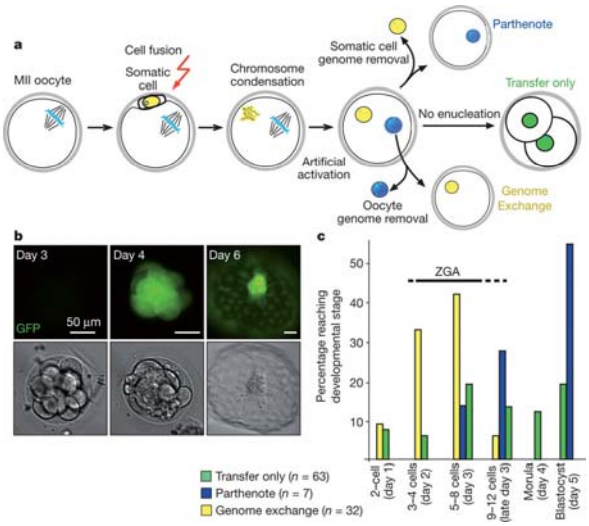
Add Punkte 1 und 2: Developmental and transcriptional defects after genome exchange.



S Noggle *et al. Nature* **478**, 70-75 (2011) doi:10.1038/nature10397

nature

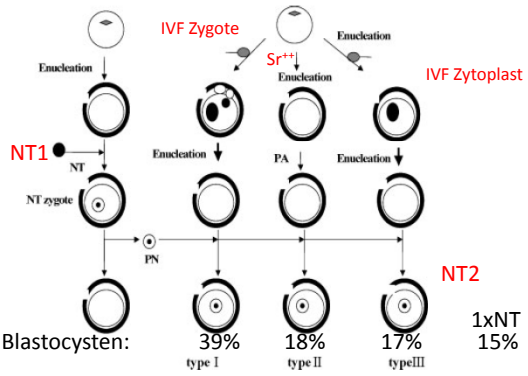
Add Punkte 1, 5 und 7: Development after somatic cell genome transfer with retention of the oocyte genome.



S Noggle *et al. Nature* **478**, 70-75 (2011) doi:10.1038/nature10397

nature

Add Punkt 1,3 und 4: Serial Nuclear Transfer in Mäusen



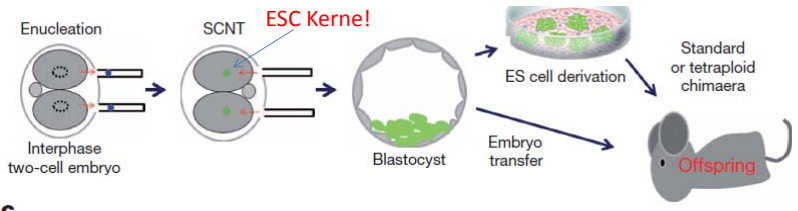
Ausbeute an Blastocysten: 39% type I, 18% type II, 17% typeIII; 1xNT 15%; in vivo Fertilisation 58%

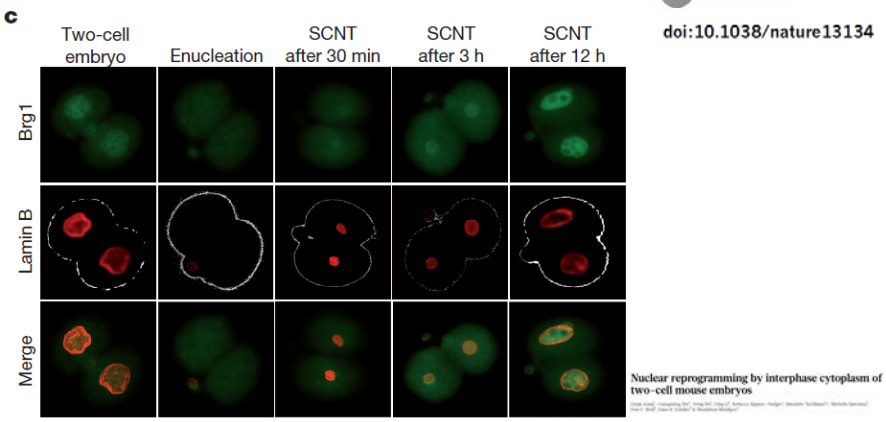
Fig. 1. Schematic representation of experiments on the construction of serial NT embryos. Fibroblasts as the donor cell were transferred into enucleated oocytes from maturation in mHTF. After activation, the pronuclei were transferred again into three different activated cytoplasts from oocyte matured in vitro. Type I: The IVF zygote. Type II: Preactivated cytoplast (activated oocyte following enucleation by Sr^{2+}). Type III: IVF cytoplast (activated oocyte following enucleation by sperm).

→Zytoplasma von Zygoten Reprogrammiert am besten

Aus:**Serial** nuclear transfer improves the developmental potential of mouse embryos cloned from oocytes matured in a protein-free medium.
Bai Z, Yong J, Qing T, Cheng J, Shen W, Ding M, **Deng** H.
Mol Reprod Dev. **2007** May;74(5):560-7.

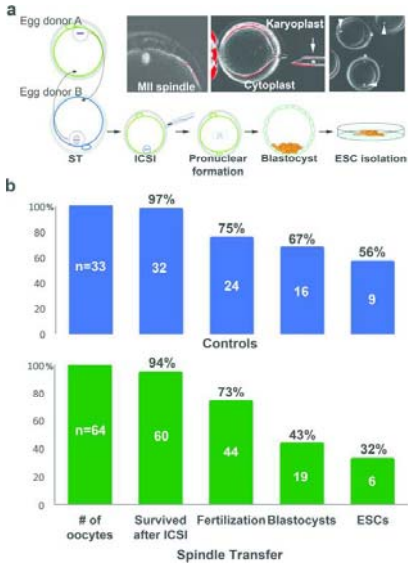
Wider Punkt 5: Zytoplasma von Blastomeren reicht aus für die Reprogrammierung





Zytoplasma der ersten Blastomere hat reprogrammierende Aktivität.

Wider Punkte 1 und 3: Spindeltransfer in H. sapiens Oozyten erfolgreich. (Vorarbeit für SCNT)



ST = spindel transfer
ICSI = Intracellular sperm injection

Nature. 2013 Jan 31;493(7434):627-31. doi: 10.1038/nature11647. Epub 2012 Oct 24.

Towards germline gene therapy of inherited mitochondrial diseases.

Tachibana M[1], Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, Gutierrez NM, Tippner-Hedges R, Kang E, Lee HS, Ramsey C, Masterson K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer R, Mitalipov S.

Add Punkte 2, 3 und 6: Fusion von somatischen Zellen mit Oozyten erhöht Überlebensfähigkeit von M. mullata Embryonen und Ionomycin Aktivierung ist zu giftig.

Inactivierter Hemagglutinating virus of Japan type E (HVJ-E) führt zur Fusion von Somatischen Zellen und M. mullata Oozyten (100%). – wenn Kerne in G0/G1 Phase!

Aktivierung mit
Ionomycin + 6DMAP → 50% Morula → 100% Morula sterben ab.

Ionomycin + 6DMAP+ Elektrostimmulation → 10% Blastozysten, ICM stirbt zu 100%.

6DMAP+ Elektrostimmulation → 17,5% Blastozysten, ICM stirbt zu 100%.

→Steigerung der Reprogrammierungsausbeute mittels

Histondeacetylase InhibitorTrichostantin A (37,5 nM 24 h) → ICM stirbt zu 100%.

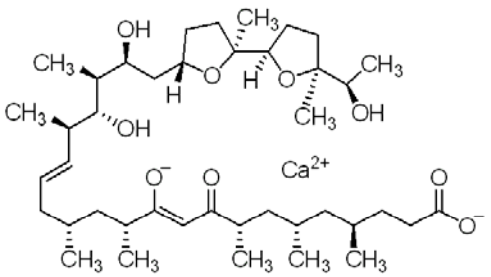
Trichostantin A (10 nM 12 h) → **13% der Blastozysten ergaben NT-ESCs.**

⇨ Geht das beim Menschen auch? → Wie bekommt man Oozyten von H. sapiens?

Befruchtung / künstliche Aktivierung

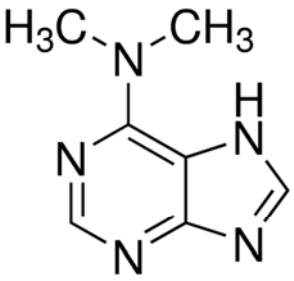
Aktivierung = Aufheben des Metaphase II Blocks und Ausbildung eines weiblichen / somatischen Pronucleus

Ionomycin calcium salt
from *Streptomyces conglobatus*
Catalog Number **I0634**
Storage Temperature 2–8 °C
CAS RN: 56092-82-1
Synonym: Calcium Ionomycin



Product Description
Molecular formula: C41H70O9Ca
Formula weight: 747.07
Calcium Ionomycin is a polyether antibiotic produced by *Streptomyces conglobatus (ATCC 31005). Calcium* Ionomycin is capable of extracting Ca2+ and other divalent cations from an aqueous into an organic phase. Ion selectivity is as follows:
Ca2+ > Mg2+ >> Sr2+ = Ba2+
Binding of Sr2+ and Ba2+ is insignificant and binding to monovalent cations or rubidium is negligible. La2+ is also bound to some extent. Complexation with a cation is always in a 1:1 stoichiometry and pH dependent. Essentially no binding of Ca2+ occurs below pH 7.0 and maximum binding takes place at pH 9.5.1

Giftig weil:
"Calcium Ionomycin can serve as an inducer of apoptosis, which was suggested to act by activation of a latent, calcium-responsive endonuclease."



6-Dimethylaminopurine (Kinase Inhibitor)

- "6-DMAP-sensitive kinase(s) is (are) involved in the control of post-fertilization events such as the formation of the interphase network of microtubules, the remodelling of sperm chromatin and pronucleus formation."

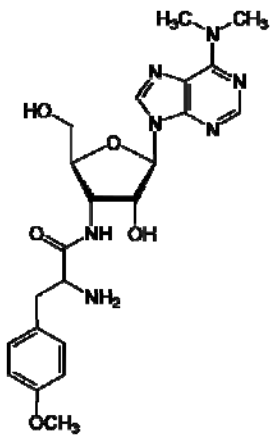
Dev Biol. 1989 May;133(1):169-79.

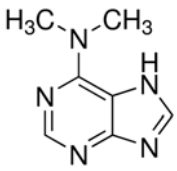
6-Dimethylaminopurine (6-DMAP), a reversible inhibitor of the transition to metaphase during the first meiotic cell division of the mouse oocyte.

Rime H[1], Neant I, Guerrier P, Ozon R.

- The first meiotic cell division (meiotic maturation) of dictyate stage mouse oocytes removed from the follicle resumes spontaneously in vitro. We used the puromycin analog 6-dimethylaminopurine (6-DMAP) to test the respective roles of protein synthesis and protein phosphorylation in driving this process. While protein synthesis inhibitors do not block meiosis resumption, 6-DMAP was found to inhibit germinal vesicle breakdown (GVBD), by inhibiting the burst of protein phosphorylation without changing the rate of incorporation of [35S]methionine into proteins. This effect is reversible; it depends both upon drug concentration and the particular female. When added after GVBD and before the emission of the first polar body, 6-DMAP decreases the level of protein phosphorylation and induces decondensation of the chromosomes and reformation of the nuclear envelope. In contrast, 6-DMAP did not trigger these processes in metaphase II oocytes which only produce resting nuclei when treated by protein synthesis inhibitors. From these data, we conclude that (1) the early appearance and stability of mouse MPF in Metaphase I oocytes depend on protein phosphorylation rather than on protein synthesis, and (2) protein synthesis is necessary to maintain the condensation of the chromosomes in metaphase II oocytes.

Puromycin (blockiert Proteinsynthese)





6-Dimethylaminopurine
(Kinase Inhibitor)

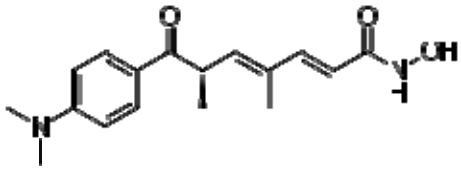
Mol Reprod Dev. 2005 Jul;71(3):380-8.

Effects of duration, concentration, and timing of ionomycin and 6-dimethylaminopurine (6-DMAP) treatment on activation of goat oocytes.

Lan GC[1], Han D, Wu YG, Han ZB, Ma SF, Liu XY, Chang CL, Tan JH.

- The protocol of ionomycin followed by 6-dimethylaminopurine (6-DMAP) is commonly used for activation of oocytes and reconstituted embryos. Since numerous abnormalities and impaired development were observed when oocytes were activated with 6-DMAP, this protocol needs optimization. Effects of concentration and treatment duration of both drugs on activation and development of goat oocytes were examined in this study. The best oocyte activation (87-95%), assessed by pronuclear formation, was obtained when oocytes matured in vitro for 27 hr were treated with 0.625-20 microM ionomycin for 1 min before 6-hr incubation in 2 mM 6-DMAP. Progressional reduction of time for 6-DMAP-exposure showed that the duration of 6-DMAP treatment can be reduced to 1 hr from the second up to the fourth hour after ionomycin, to produce activation rates greater than 85%. Activation rates of oocytes in vitro matured for 27, 30, and 33 hr were higher ($P < 0.05$) than that of oocytes matured for 24 hr when treated with ionomycin plus 1-hr (the third hour) 6-DMAP, but a 4-hr incubation in 6-DMAP enhanced activation of the 24-hr oocytes. Goat activated oocytes began pronuclear formation at 3 hr and completed it by 5-hr post ionomycin. An extended incubation in 6-DMAP (a) impaired the development of goat parthenotes, (b) quickened both the release from metaphase arrest and the pronuclear formation, and (c) inhibited the chromosome movement at anaphase II (A-II) and telophase II (T-II), leading to the formation of one pronucleus without extrusion of PB2. In conclusion, duration, concentration, and timing of ionomycin and 6-DMAP treatment had marked effects on goat oocyte activation, and to obtain better activation and development, goat oocytes matured in vitro for 27 hr should be activated by 1 min exposure to 2.5 microM ionomycin followed by 2 mM 6-DMAP treatment for the third hour.

Trichostatin A (TSA) aus dem Bakterium *Streptomyces platensis*[



blockiert selektiv die Klasse I- und Klasse II- Histon-Deacetylasen (HDAC) von Säugetieren

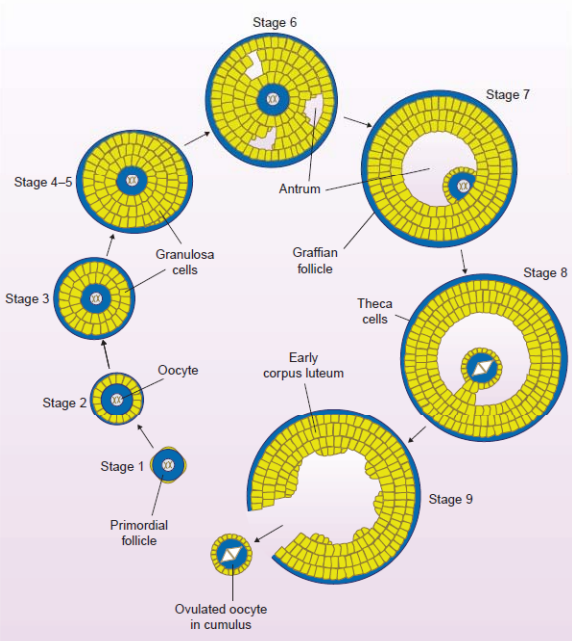
Wie bekommt man Oozyten von H. sapiens?

- Reifung der Oocyten
- Ablauf der Meose bei Frauen und Männern
- Befruchtung
- Superovulation

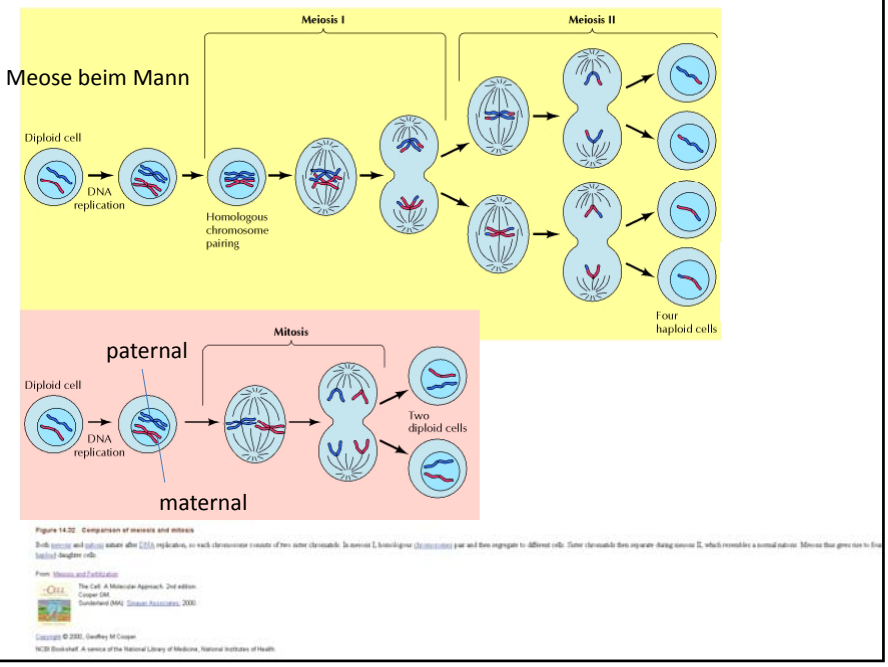
Reifung der Oocyten

Figure 1 **Stages of follicular growth in mammals.** Follicular growth begins with stage-1 primordial follicles in the ovary, which consist of a non-growing oocyte surrounded by a few epithelial-like somatic cells. As growth is initiated in stage-2 follicles, the somatic cells or granulosa cells become cuboidal. During stages 3–5, the granulosa cells proliferate while the oocyte continues to increase in diameter and lays down a zona pellucida. In stage 6, a fluid-filled cavity (the antrum) begins to form and by stage 8 the antrum is complete. Surrounded by a thick zona pellucida, the fully-grown oocyte sits at the end of a stalk of granulosa cells, which is in turn surrounded by several layers of cumulus cells. At stage 9, the oocyte, which has arrested at metaphase II of meiosis, is ovulated into the oviduct. The follicle that is left behind becomes a corpus luteum. In mice, it takes 2–3 weeks for this developmental process to be completed. This figure is adapted from ref. 11 with permission from Cambridge University Press.

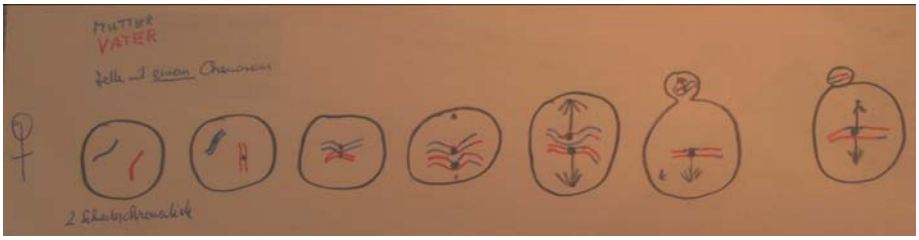
From:
Nat Cell Biol. 2002 Oct;4 Suppl:s7-9.
Channels of communication in the ovary.
Wassarman PM.



Ablauf der Meose bei Frauen und Männern:



Meose bei der Frau



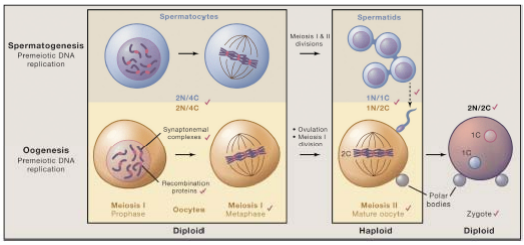
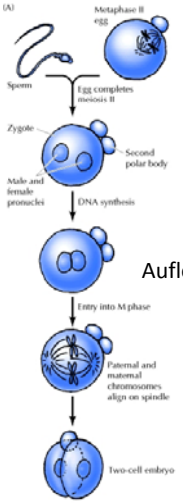


Figure 1. Checklist of Key Events during Mammalian Meiocyte Development

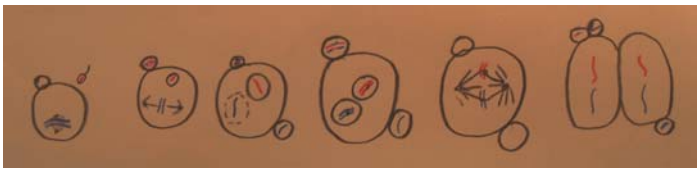
Diagrammed are the processes of spermatocyte and oocyte development and key events in these processes. Of particular importance for assessing accurate meiocyte development are features denoted by the red checkmarks. Synaptonemal complexes can be visualized by immunolabeling proteins such as SYCP1 or SYCP3. Markers of recombination include RAD51 (which is bound at hundreds of foci in early meiosis I prophase) and MLH1 (which binds 25 or so foci at sites of chiasmata in mice). Metaphase figures can be visualized in various ways cytologically (for example, by DAPI staining of DNA and a-tubulin immunolabeling of the spindle). Mature oocytes should have extruded a single polar body, and fertilized oocytes (zygotes) should have extruded another (occasionally the first polar body will undergo a division too). A final test is that any zygotes formed from in-vitro-derived gametes should be able to form viable progeny following transfer to pseudopregnant female hosts. C = DNA content; N = Anzahl der Chromosomen

Aus In-Vitro-Derived Germ Cells
Mary Ann Handel,1,* John J. Eppig,1 and John C. Schimenti2,*
1The Jackson Laboratory, Bar Harbor, ME 04609, USA
2Cornell University, Ithaca, NY 14853, USA
*Correspondence: maryann.handel@jax.org
Cell 157, June 5, 2014

Befruchtung:



Auflösung der Pronuklei



Superovulation bei Homo sapiens

1. Zyklus

„Pille“

Östrogene und Gestagene

Follicel stimmulierendes Hormon (FSH)
Lutenisierendes Hormon (LH)
(Gonadotropine der Hypophyse)

2. Zyklus

Gonadotropin freisetzendes Hormon
(GnRH) 0,5 mg täglich s.c.

225 IU FSH täglich
weiterhin GnRH

Humanes Choriongonadotropin
HCG 10 000 U i.m. abends

36 -45 Stunden später

Tag 2
Eisprung verhindernd

Tag 21
(14)

Tag 28+2

Tag 7-10

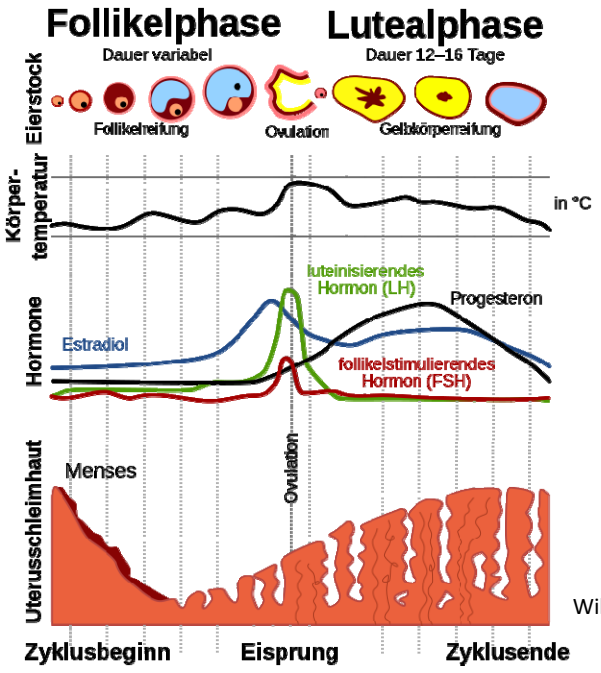
Intra-vaginales Ultraschall-monitoring →

Pregnant mare serum (PMS)
Trächtige Stuten Serum
Postmenopausales Frauenserum

Oder LH + FSH

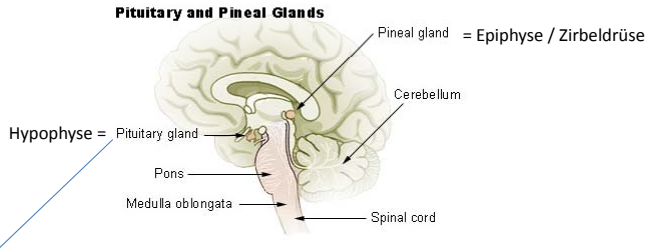
Laparoskopische
oder transvaginale
Oozyten Gewinnung

Ad Superovulation



Wikipedia

Ad Superovulation



gonadotropen Hormone:
follikelstimulierendes Hormon (FSH) und Luteinisierendes Hormon (LH)