

## 6. Voresungseinheit / 5. Doppelstunden

## 2. Herstellung von embryonalen Stammzellen

## 2.1. Die Entstehung von Stammzellen im Laufe der Ontogenese

2.1.1. Die frühe Embryonalentwicklung der Eutheria (Placentales) am Beispiel der Maus

(später 2.1.2. Entstehung der somatischen Stammzellen im adulten Organismus)

## 2.2. Die Herstellung von embryonalen Stammzelllinien

2.2.1. Isolierung von Blastozysten aus trächtigen Mäuseweibchen

2.2.2. Kultivierung der Blastozysten auf „feeder cells“

2.2.3. Kultur der embryonalen Stammzellen (ESCs)

2.2.4. Pluripotenzbeweise und Herstellung von transgenen Mäusen

2.2.4.1. Blastozyst ESC Injection

2.2.4.2. Tetraploidaggregation

2.2.4.3. Teratomaformation

2.2.4.4. Chimeraformation (Stammzell-Xenotransplantation)

2.2.4.5. Embryoid body formation and Organoid culture, siehe Kapitel 6. Wie macht man somatische Zellen aus Stammzellen? Stammzellen in der Medizin und die damit verbundene ethische Problematik.

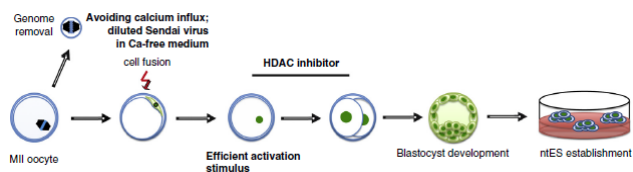
## 2.3. Herstellen von geklonten Embryonen für die Herstellung von ESCs

## 3. Wie macht man induzierte pluripotente Stammzellen?

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## 2.3 Herstellen von geklonten Embryonen für die Herstellung von ESCs

Wie IVF aber unter Bedingungen, die Mitose des somatischen Kernes in der Zygote erlauben und Zytokinese der Blastomere unterstützen.


<http://dx.doi.org/10.1016/j.gde.2015.06.007>

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## 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 dieser Möglichkeit durch Spindel- out/in Experimente. Jedoch:  
Möglichst wenig Zytoplasma aus der Eizelle entfernen!
3. Cytoplastenaktivierung bei *H. sapiens* nicht ausreichend.  
bei Tieren reicht  $Sr^{++}$ , 7% EtOH, Ionomycin ( $Ca^{++}$ ), Stromstoß, ...
4. Meiose II Metaphase in *H. sapiens* ist instabil. – „nicht genug Zeit“  
→ Stabilisierung mittels Inhibitoren notwendig.
5. Meiose spezifische Faktoren sind für die Reprogrammierung notwendig.
6. Phase des Zellzyklus des somatischen Kernen entscheidend?  
G0 / G1 (z.B. durch Arrest mit 3-5 % FCS für 3Tage.)
7. Chromosomen assoziiertes Material (Spindelapparat) ist für die Reprogrammierung bzw. Aktivierung des embryonalen genetischen Programmes mitverantwortlich.
8. Befruchtung / künstliche Aktivierung von Oocyten: Aktivierung = Aufheben des Metaphase II Blocks und Ausbildung eines weiblichen / somatischen Pronucleus –Im eingebrachten 2n somatischen Kern wird bei Aufheben des Metaphase II Blocks die Mitose ermöglicht.

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

Inaktivierter **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+ **Elektrostimulation** → 10% Blastozysten, ICM stirbt zu 100%.

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

→Steigerung der Reprogrammierungsausbeute mittels

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

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

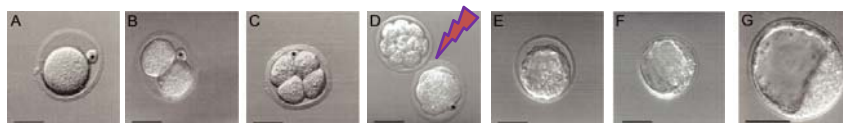
➡ Geht das beim Menschen auch? → Wie bekommt man Oozyten von *H. sapiens*?

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## Vorgeschichte

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



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## Oozytenaufbereitung und Kerntransfer in humanen Oozyten (Stand vor 2013)

- N=63
1. **Hyaluronidase** (Hyalase) Behandlung – um die Cumulus Zellen zu entfernen
  2. Kerntransfer in Puffer mit 5µg/ml **Cytochalasin B**; inhibiert die F-Aktin bildung.
  3. Entkernen / Spindel Absaugen (95%)
  4. **HVJ-E** vermittelte Fusion der Hautfibroblasten von humanen Föten (100%)
  5. Sofortige **Aktivierung mittels Elektroporation** (2.7kV7cm, 50µsec + 4h 2mM 6-**DMAP**).
  6. Inhibierung der Histondeacetylaseaktivität mittels 10nM **Trichostantin A** für 12 h.

→ 83% Pronucleus Ausbildung und 87% entwickelten sich zum Zwei-Blastomeren Stadium.  
 → 62% 8-Zell-Stadium aber nur 14% bildeten eine kompaktierte Morula  
 und nur 12% einen Blastozysten. → Reprogrammieren funktioniert.  
 Aber 0% (N=6) der Blastozysten hatten ICM und ergaben so keine NT-ESCs.

→ **Aktivierung des embr. Expressionsprogrammes im somatischen Kern funktioniert nicht.**

→ Was tun?

Tachibana et al., 2013; Cell 153, 1228-1238.

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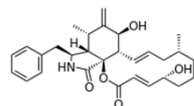
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Ingredienzien für den SCNT:

1. Hyaluronidase
2. Cytochalasin B
3. Ionomycin
4. 6-Dimethylaminopurine
5. Puromycin
6. Trichstatin A
7. Koffein

Cytochalasin B from [fungus, \*Helminthosporium dematioideum\*](#) or *Drechslera dematioidea*



- **Cytochalasin B**, substoichiometric concentrations of cytochalasin B (CB) strongly inhibit network formation by [actin](#) filaments.
- It inhibits [cytoplasmic division](#) by blocking the formation of [contractile microfilaments](#).
- It is also used in cloning through [nuclear transfer](#). Here enucleated recipient cells are treated with cytochalasin B. Cytochalasin B makes the cytoplasm of the [oocytes](#) more fluid and makes it possible to aspirate the nuclear genome of the oocyte within a small vesicle of [plasma membrane](#) into a micro-needle. Thereby, the oocyte genome is removed from the oocyte, while preventing rupture of the plasma membrane.
  - → spindle aspiration

**Ionomycin calcium salt  
from *Streptomyces globatus***

Catalog Number **I0634**

Storage Temperature 2–8 °C

CAS RN: 56092-82-1

Synonym: Calcium Ionomycin

**Product Description**

Molecular formula: C<sub>41</sub>H<sub>70</sub>O<sub>9</sub>Ca

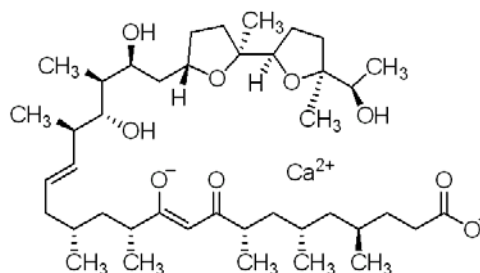
Formula weight: 747.07

Calcium Ionomycin is a polyether antibiotic produced by *Streptomyces globatus* (ATCC 31005). Calcium ionomycin is capable of extracting Ca<sup>2+</sup> and other divalent cations from an aqueous into an organic phase. Ion selectivity is as follows:

Ca<sup>2+</sup> > Mg<sup>2+</sup> >> Sr<sup>2+</sup> = Ba<sup>2+</sup>

Binding of Sr<sup>2+</sup> and Ba<sup>2+</sup> is insignificant and binding to monovalent cations or rubidium is negligible. La<sup>2+</sup> is also bound to some extent. Complexation with a cation is always in a 1:1 stoichiometry and pH dependent.

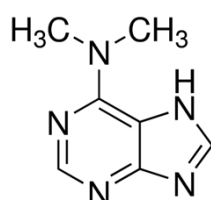
Essentially no binding of Ca<sup>2+</sup> occurs below pH 7.0 maximum binding takes place at pH 9.5.1



Giftig weil:

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

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## 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.”

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[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<sup>1</sup>](#), [Neant J](#), [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.

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[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.**

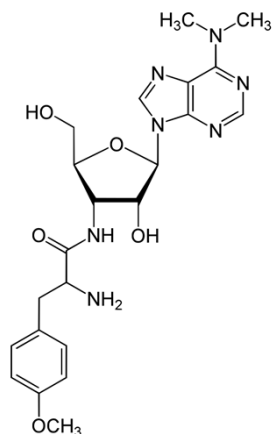
[Lan GC<sup>1</sup>](#), [Han D](#), [Wu YG](#), [Han ZB](#), [Ma SF](#), [Liu XY](#), [Chang CL](#), [Tan JH](#).

- 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.

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## Puromycin (blockiert Proteinsynthese)

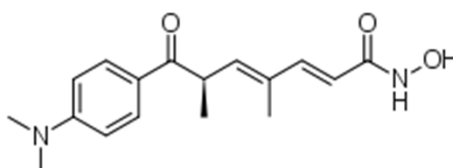


Puromycin wirkt auf zweierlei Weisen:  
*Termination der Translation:* Puromycin inhibiert die Translation, indem es eine verfrühte Termination einleitet. Dies geschieht dadurch, dass sich das Puromycin während des Peptidyltransfers anstatt einer Tyrosyl-tRNA an die Eingangsstelle (A-Stelle) des Ribosoms bindet. Ein Teil des Moleküls ähnelt dabei dem 3'-Ende (CCA) der Aminoacyl-tRNAs. Aus diesem Grund wird die sich in der Elongation befindliche Polypeptidkette auf den Pseudoamino säurearm des Puromycins übertragen und es kommt zu einer verfrühten Termination der Translation.  
*Inhibierung des Proteinimportes:* Puromycin hemmt den Proteinimport in Mitochondrien, indem es eine intramitochondriale, ATP-abhängige Reaktion behindert. (Wikipedia)

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## Trichostatin A (TSA) aus dem Bakterium *Streptomyces platensis*<sup>1</sup>



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

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## Problem: Spindel Entnahme macht Meiose II instabil?

### Fusion von Fibroblasten mit Oozyten

- In 17/17 Oozyten bildeten sich Spindeln in den somatischen Kernen innerhalb von 30 Minuten

### → Humane Oozyten werden durch den Spindelentzug aktiviert.

D.h. Meiose II wird beendet, ohne dass sich eine mitotische Spindel im somatische Kern ausbilden kann.

Wie kann man dies unterdrücken?

2007: Koffein verbessert die Erfolgsrate in Rhesusaffen. →

### Fusion von Fibroblasten mit Oozyten nach Spindelabsaugen

- 0/3 : keine Spindelausbildung

### Fusion von Fibroblasten mit Oozyten nach Spindelabsaugen in Gegenwart von 1,25 mM Koffein

- 83% (10/12): Spindelausbildung in den somatischen Kernen

Tachibana et al., 2013; Cell 153, 1228-1238.

## Erfolgreicher SCNT bei Homo sapiens: Oozytenaufbereitung, Kerntransfer in humanen Oozyten und Blastozystenentwicklung

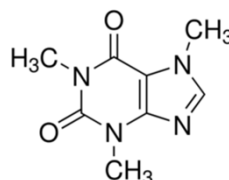
- Hyaluronidase (Hyalase) Behandlung – um die Cumulus Zellen zu entfernen N=42
- Transfer in Puffer mit 5µg/ml Cytochalasin B + 1.25 mM Koffein
- Entkernen / Spindel Absaugen (95%)
- HVJ-E vermittelte Fusion der Hautfibroblasten von humanen Föten (100%)
- Wenn < 60 Minuten: keine spontane Ausbildung einer Spindel im somatischen Kern = Metaphase der Meiose II bleibt stabil.
- Sofortige Aktivierung mittels Elektroporation (2.7kV/cm, 50µsec + 4h 2mM 6-DMPAP.
- Inhibierung der Histondeacetylaseaktivität mittels 10nM Trichostantin A für 12 h.
- 83% (74%) Pronucleus Ausbildung und 87% (81%) entwickelten sich zum
- Zwei-Blastomerenstadium.
- 62% (68%) 8-Zell-Stadium aber nur 14% (32%) bildeten eine kompaktierte Morula und nur 12% (24%) einen Blastozysten.
- 0% (50%, N=8) der Blastozysten hatten ICM und ergaben so 4 NT-ESC Zelllinien.
- Effizienz vergleichbar mit IVF-ESC Herstellungsausbeute.

Tachibana et al., 2013; Cell 153, 1228-1238.



## Koffein; 1,3,7-Trimethylxanthine ( Protein phosphatase Inhibitor)

- „Early experimentations on mouse SCNT described structural changes in the donor nucleus occurring soon after introduction into the MII cytoplasm due to high activity of maturation promoting factor (MPF) that is a complex of two subunits: a catalytic subunit, CDC2, a homologue of the yeast cdc2 protein kinase; and a regulatory subunit, cyclin B (Szollosi et al., 1988).“
- CDC2 + cyclin B wenn phosphoryliert = „**Maturation promoting factor**“
- Caffeine can abrogate the cell cycle checkpoints, including the G2 arrest that occurs in response to DNA damage.
- Caffeine inhibits dephosphorylation of cyclin B (?)



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## Reproduzierbarkeit der Methode (1)

- Alle 4 NT-ESC Linien stammen von einer Eispenderin (A)
- Statt fötale Hautfibroblasten ← → Fibroblasten von Kind mit Leigh Syndrom.
- Spenderinnen B (15) und C (5) Oocyten
- → 27% (4/15) und 60%(3/5) Blastozysten
- → je 1 stabile NT-ESC Linie (7% bzw 20%) Tachibana et al., 2013; Cell 153, 1228-1238.

## Reproduzierbarkeit der Methodemit adulten somatischen Kernen (2).

- 4 Spenderinnen
- nur 2 „erfolgreich“ nachdem die die Zeit zwischen Fusion und Aktivierung der Zygoten von 30 auf 120 Minuten verlängert wurde.
- D (12) und G (5) Oozyten
- Statt fötale Hautfibroblasten ← → von 35 jährigen und 75 jährigen Männern.
- → 17% (2/12) und 20%(1/5) Blastozysten
- → Je 1 stabile NT-ESC Linie (8% bzw 20%) vom 35 jährigen und 75 jährigen Mann. Chung et al., 2014; Cell Stem Cell 14, 1-4.

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### Bestätigung des SCNT (3)

- 54 Spenderinnen, 3NT-hESCs von Vorhaut-Fibroblasten neugeborener Knaben und 1 NT-hESC von einer **Frau** mit Diabetes Typ 1
- Methodische Verbesserungen:
- +Translations- und Kinase Inhibitoren, Ca<sup>++</sup>-freies Medium und stark verdünnte Sendai Virus Präparation
- Je kürzer die Stimulation mit Gonadotropinen (zur Follikelreifung), um so besser die Qualität der Oozyten.
- 21-26 jährige Frauen sind viel bessere Spenderinnen als 27- 32 jährige Frauen.

doi:10.1038/nature12127

**Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells**

Mitsutoshi Yamada<sup>1\*</sup>, Bjarki Johannsson<sup>2\*</sup>, Ido Sagal<sup>3</sup>, Elin Cole Barnett<sup>3</sup>, Daniel H. Kier<sup>4,5</sup>, Robert W. Prosser<sup>6,7</sup>, Daniel Prall<sup>8</sup>, Michael W. Nienke<sup>9</sup>, Matthew Freely<sup>9</sup>, Ellen Greenberg<sup>9</sup>, Robin S. Goland<sup>9</sup>, Rachael L. Leibel<sup>9</sup>, Susan L. Schmeiss<sup>9</sup>, Noam Benvenisty<sup>1</sup>, Mark V. Sussel<sup>10</sup> & Dieter Egli<sup>1</sup>

Yamada et al., 2014; Nature 510, 533-536.

### Spekulationen was man mit SCNT beim Menschen alles machen können wird.

- Austausch von Mitochondrien mit defekten Genom.
- (Mt haben 37 Gene und über 250 Mutationen sind bereits bekannt.)
  - Durch Spindeltransfer
  - Durch Pronukleustransfer („Drei-Patienten-IVF“)\*
  - Durch Polarkörperchentransfer
- Zeugung von Kindern durch zwei Männer
- Zeugung von Mädchen durch zwei Frauen

\* „Kinder für alte (<45a) Frauen aus jungen Oozyten“

### Pronukleitransfer

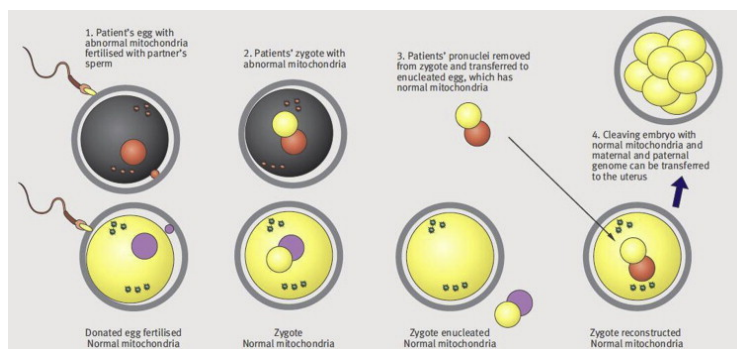


Figure 1 Pronuclear transfer technique. Reproduced from Bredenoord and Braude with permission from BMJ Publishing Group Ltd

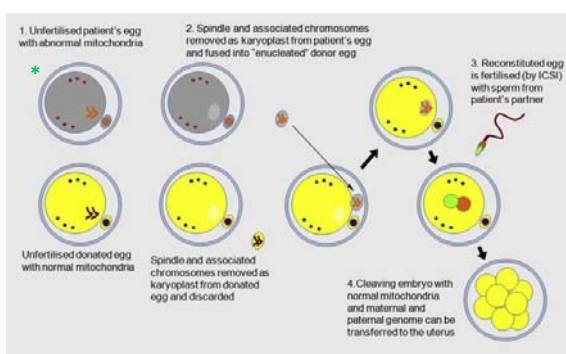
#### Three-parent in vitro fertilization: gene replacement for the prevention of inherited mitochondrial diseases

Paula Amato , Masahito Tachibana , Michelle Sparman , Shoukhrat Mitalipov

Fertility and Sterility, Volume 101, Issue 1, 2014, 31 - 35

<http://dx.doi.org/10.1016/j.fertnstert.2013.11.030>

### Spindeltransfer = Transfer of a karyoplast from an foreign egg Versus SCNT = Somatic Cell Nuclear Transfer



Spindle transfer technique

\* Beim SCNT wird statt dem Ei eine somatische Zelle genommen.

Problem:  
Heteroplasmie  
und genetischer Drift  
der mt DNA!

#### Three-parent in vitro fertilization: gene replacement for the prevention of inherited mitochondrial diseases

Paula Amato , Masahito Tachibana , Michelle Sparman , Shoukhrat Mitalipov

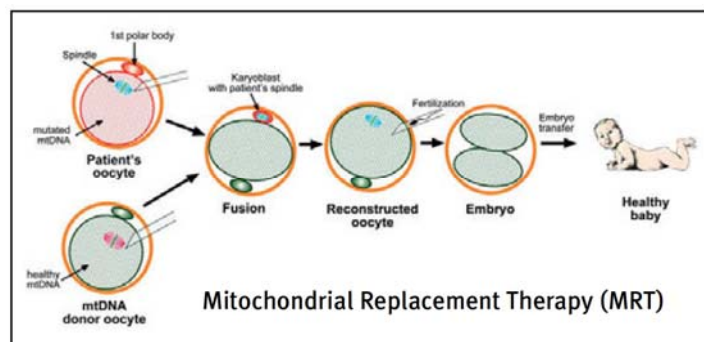
Fertility and Sterility, Volume 101, Issue 1, 2014, 31 – 35. <http://dx.doi.org/10.1016/j.fertnstert.2013.11.030>

SCNT = Somatic Cell Nuclear Transfer

Spindeltransfer = Transfer of a karyoblast from an foreign egg

Umbenannt in :

Mitochondrial replacement therapy (MRT) = Reproduktives Klonen von Menschen



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## Gefahrenquellen bei der Zeugung von Kindern mit Hilfe von SCNT

- Ist das Genom der Oozyte identisch mit dem in den 1. und 2. Polkörperchen ?
- Welche vielleicht einzigartigen Faktoren im Zytoplasma der gespendeten Oozyte beeinflussen die Embryogenese des Kindes und somit dessen Individualität?
- Fehlen dem Keimbläschen vielleicht essentielle Faktoren?
- Ist die Reprogrammierung des Genoms wirklich vollständig?
- Bewirkt SCNT das gehäufte Auftreten von Mutationen?

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### Zusatz 1: „Superwoman“

- Hohe endogene Östrogen-Werte vor Eisprung führen zu vielen Oozyten aber zu geringerer Qualität dieser bezüglich der Entstehung von lebensfähigen Blastozysten.
- Unterdrückung der Hypophysenaktivität durch GnRH-Antagonisten (Ganirexil) fördert das Überleben der Blastozysten.
  - Ganirexil hemmt die LH und FSH Ausschüttung durch die Hypophyse und senkt dadurch die Östrogenkonzentrationen.
- Je kürzer die Stimulation mit Gonadotropinen (zur Follikelreifung), um so besser die Qualität der Oozyten.

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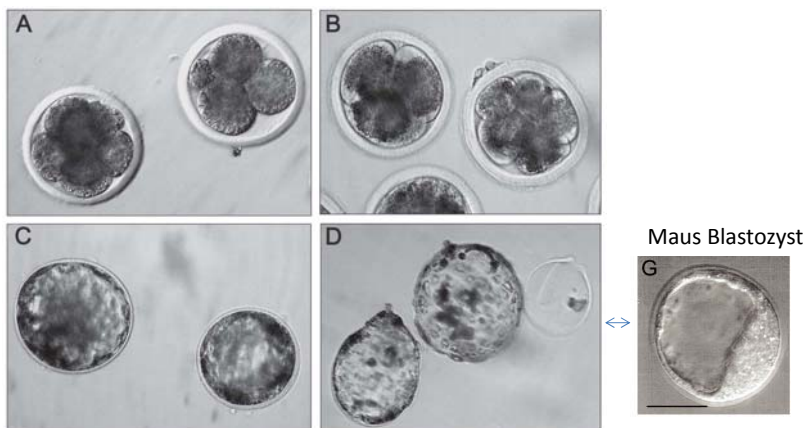
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### Zusatz 2: Rind-Mensch SCNT Blastozysten

Interspecies-SCNT preimplantation embryos derived from **human granulosa cells** fused with enucleated **bovine oocytes**. Cleavage embryos (A) and blastocysts (C) derived from SCNT. Parthenogenetically developed cleavage embryos (B) and hatching blastocysts (D) as controls. (aus Illmensee K., *J. Reproduktionsmed. Endokrinol* 2007; 4 (1), 6-16)



Qualität extrem schlecht!

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### Zusatz 3: Frühere Versuche:

- **Stem cell technologies 2006 in Boston : Blastozysten implantieren nicht**

Indien 2007: 33 NT ESC Linien 8?). Keine Details publiziert

- **Derivation of Cloned Human Blastocysts by Histone Deacetylase Inhibitor Treatment After Somatic Cell Nuclear Transfer with  $\beta$ -Thalassemia Fibroblasts**

Yong Fan, Yonghua Jiang, Xinjie Chen, Zhanhui Ou, Yifei Yin, Shengchang Huang, Zhaohui Kou, Qing Li, Xiaolin Long, Jianqiao Liu, Yuemei Luo, Baoping Liao, Shaorong Gao, and Xiaofang Sun.

Stem Cells and Development. November 2011, 20(11): 1951-1959. doi:10.1089/scd.2010.0451.

Derivation of embryonic stem cells from patient-specific cloned blastocysts by somatic cell nuclear transfer (SCNT) holds promise for both regenerative medicine and cell-based drug discovery. However, the efficiency of blastocyst formation after human SCNT is very low. The developmental competence of SCNT embryos has been previously demonstrated in several species to be enhanced by treatment with histone deacetylase inhibitors, such as trichostatin A (TSA), to increase histone acetylation. In this study, we report that treatment of SCNT embryos with 5 nM TSA for 10 h following activation incubation increased the developmental competence of human SCNT embryos constructed from  $\beta$ -thalassemia fibroblast cells. The efficiency of blastocyst formation from SCNT human embryos treated with TSA was approximately 2 times greater than that from untreated embryos. Cloned blastocysts were confirmed to be generated through SCNT by DNA and mitochondrial DNA fingerprinting analyses. Further, treatment of SCNT embryos with TSA improved the acetylation of histone H3 at lysine 9 in a manner similar to that observed in in vitro fertilized embryos.

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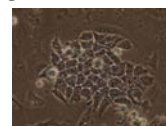
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### 3. Herstellen von induzierten pluripotenten Stammzellen aus somatischen Zellen

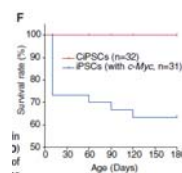
- Lentivirale polycistronische OKSM Vektoren
- Fbx15\*::EFGP Reporter-Fibroblasten
- BAC: Nanog::GFP/Puro<sup>R</sup>/Nanog Reporter-Fibroblasten → Nanog Expression erlaubt iPSC Mäuse herzustellen.
- Lentivirale OSNL Vektoren
- Reprogrammieren nur mit Proteinen, RNA, kleinen synthetischen Molekülen. → RepSox;
- S-iPSCs (stress induced) oder STAP (stimulus-triggered acquisition of pluripotency)-Zellen; Nature 2014
- Mycobacterium laprae induzierte Reprogrammierung zu Stammzellen
- C-iPSCs (pure chemically induced): 10.000 compounds tested; Efficacy 1:50.000 Hou et al., Science Aug. 2013
- .....alles sehr ineffizient! → stochastische Ereignisse?

\* Fbx15 = F-Box Protein 15 (siehe <http://www.uniprot.org/uniprot/Q8NCQ5>)

O...Oct4  
K...Klf4  
S...Sox2  
M...c-Myc



N...Nanog  
L...Lin28



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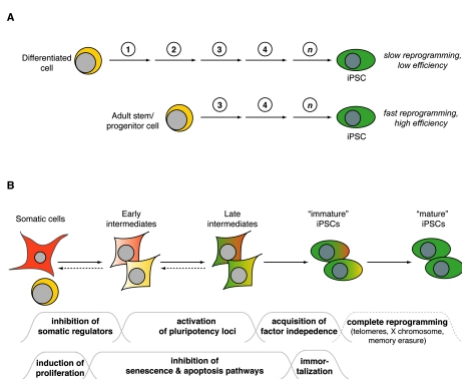
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Herstellung von iPSCs – Dauert ca. 3-4 Wochen! – Warum?

Hypothese I:  
Models of cellular reprogramming.



(A) Mature cells, such as lymphocytes, reprogram into iPSCs at lower efficiencies than immature cells, such as hematopoietic stem cells. This may be due to a lower number of stochastic epigenetic events (represented by circled numbers and arrows) that are required in immature cells to acquire pluripotency. The precise number and nature of such changes is unclear (represented by "n").

(B) Scheme summarizing major changes that characterize the transition of somatic cells into iPSCs. The early steps are reversible, as indicated by the dashed reverse arrows. "Immature iPSCs" are defined as cells that have already acquired pluripotency but still retain an epigenetic memory of their cell type of origin, while "mature iPSCs" have lost this memory. The wavelines below indicate assumed reprogramming roadblocks that cells are facing at different stages. Failure to pass any of these roadblocks may result in cells that arrest at that stage or, alternatively, undergo senescence or apoptosis.

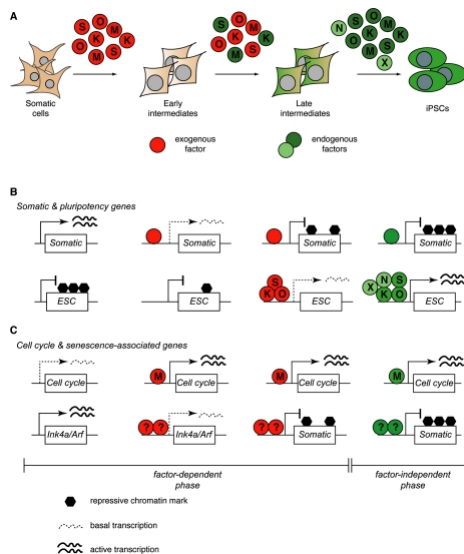
Aus [Genes Dev. Oct 15, 2010; 24\(20\): 2239-2263.](http://genesdev.cup.edu/2010/24/20/2239-2263)

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Putative role of reprogramming factors during iPSC formation.

Hypothese II:



(A) Scheme depicting the expression of exogenous (red circles) and endogenous (dark-green circles) pluripotency factors at the protein level during different stages of reprogramming. The reprogramming process is initiated predominantly by the exogenous factors, which are gradually replaced by endogenous proteins as well as their targets, such as Nanog (N) or as-yet-undefined factors (X) (light-green circles). The endogenous loci of some reprogramming factors (such as *c-Myc*, *Klf4*, and *Sox2*) are expressed in some somatic cell types, and the corresponding endogenous proteins might thus become available before activation of the *Oct4* locus.

(B) Scheme illustrating how the reprogramming factors may exert the rapid repression of somatic genes and the gradual activation of pluripotency (ESC) genes, two processes assumed to be mediated largely by *Klf4*, *Sox2*, and *Oct4*. Somatic gene silencing is associated by single-factor binding to promoter regions, while ESC gene activation involves the establishment of multi-protein complexes. The initial loss of repressive marks (such as DNA methylation and H3K27 histone trimethylation) at ESC promoters might be a passive process driven by multiple rounds of cell division.

(C) Scheme showing activation of genes promoting cell division (such as cyclins) by *c-Myc* and repression of the *Ink4a/Arf* tumor suppressor locus conferring immortality by an as-yet-undefined combination of reprogramming factors.

Aus [Genes Dev. Oct 15, 2010; 24\(20\): 2239-2263.](http://genesdev.cup.edu/2010/24/20/2239-2263)

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**Nat Rev Genet.** 2013 Jun;14(6):427-39. doi: 10.1038/nrg3473. Mechanisms and models of somatic cell reprogramming. Buganim Y, Faddah DA, Jaenisch R.

**Hypothesis III: Phases of the reprogramming process**  
In the model we discuss in this review, the reprogramming process can broadly be divided into two phases: firstly, a long 'stochastic' phase of gene activation; and secondly, a shorter hierarchical more 'deterministic' phase of gene activation that begins with the activation of the Sox2 locus. After a fibroblast is induced with OSMK, it will initiate stochastic gene expression and assume one of several possible fates (such as, apoptosis, senescence, transformation, transdifferentiation or reprogramming). In the early phase, reprogrammable cells will increase proliferation, undergo changes in histone modifications at somatic genes, initiate mesenchymal to epithelial transition, and activate DNA repair and RNA processing. Then the reprogrammable cells will enter an intermediate phase with an unknown rate-limiting step that delays the conversion to iPSCs and contributes to the long latency of the process. In this phase, cells undergo a stochastic activation of pluripotency markers<sup>23</sup>, a transient activation of developmental regulators<sup>24</sup>, and activation of glycolysis<sup>25</sup>. In general the transcriptional changes in this phase are small. In some rare cases, the stochastic gene expression will lead to the activation of 'predictive markers' such as Ulf1, Esrrb, Dppa2, and Lin28, which then will instigate the second phase that starts with the activation of Sox2. Activation of Sox2 by the 'predictive markers' can be direct or indirect and will trigger a series of deterministic events that will lead to an iPSC. In this late phase, the cells eventually stabilize into the pluripotent state in which the transgenes are silenced, the cytoskeleton is remodeled to an ESC-like state, the epigenome is reset and the core pluripotency circuitry is activated<sup>14-18,21</sup>.

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**Vektoren für die Reprogrammierung**

**Zellarten**

**Stand 2011**

Table 2 | Summary of iPSC derivation methods\*

Cell types	Integrating	Excisable	Non-integrating	Chemicals
Fibroblasts	• Retroviral KSOM or KSO <sup>23</sup> • Lentiviral KSOM • Inducible lentiviral KSOM <sup>28</sup> • Lentiviral OSNL <sup>24</sup> • Lentiviral mi-302 cluster <sup>29</sup> • Retroviral KSOM <sup>28</sup>	• Flanked lentiviral KSOM • Prozac <sup>28</sup> • miRNA <sup>28</sup> • Transposon • RNA virus <sup>28</sup> • miRNA mimics <sup>28</sup> • Flamide <sup>30,31</sup>	• Adenoviral <sup>23</sup> • Plasmid <sup>23</sup> • Protease <sup>28</sup> • RNA virus <sup>28</sup> • miRNA mimics <sup>28</sup> • NaB <sup>32</sup>	• KSO + valproic acid <sup>23</sup> • KSO + vitamin C <sup>23</sup> • KSO + SB415286, PD032901 and thiazovivin <sup>23</sup> • KSO + sodium butyrate • NaB <sup>32</sup>
Bone marrow (isolated) or peripheral blood cells	• Retroviral KSOM <sup>23</sup> • Inducible lentiviral KSOM <sup>28</sup>	Not applicable	• RNA virus <sup>28</sup> • Flamide <sup>30,31</sup>	Not applicable
Cardiac blood cells	• Lentiviral OSNL <sup>24</sup> • KSO <sup>23</sup> • Retroviral OCT4 and SOX2 (REF. 164)	Not applicable	• Flamide <sup>30,31</sup>	Not applicable
EBV-immortalized	-	Not applicable	• Flamide <sup>30,31</sup>	Not applicable
Blood cells	• Retroviral KSOM <sup>23</sup>	Not applicable	Not applicable	• OCT4 + P546, NaB, A-83-01 and PD032901 (REF. 161)
HUVECs	• Retroviral KSOM <sup>23</sup>	Not applicable	Not applicable	• OCT4 + P546, NaB, A-83-01 and PD032901 (REF. 161)
Adipose-derived stem cells	• Lentiviral KSOM <sup>28</sup> • Retroviral KSO <sup>27</sup>	Not applicable	• Plasmid <sup>23</sup> • miRNA mimics <sup>28</sup>	Not applicable
Keratinocytes	• Retroviral KSOM and KSO <sup>23</sup> • Inducible lentiviral KSOM cassette <sup>28</sup>	Not applicable	Not applicable	• OCT4 and KLF4 + transcypromin and CHIR99031 (REF. 170) • OCT4 + P546, NaB, A-83-01 and PD032901 (REF. 61)
Neural stem cells	• Retroviral OCT4 (REF. 171)	Not applicable	Not applicable	Not applicable
Astrocytes	• Retroviral KSOM <sup>23</sup>	Not applicable	Not applicable	Not applicable
Hepatocytes	• Retroviral KSOM <sup>23</sup>	Not applicable	Not applicable	Not applicable
Amniotic cells	• Retroviral KSOM <sup>23</sup> • Lentiviral OSNL <sup>24</sup>	Not applicable	Not applicable	• OCT4 + P546, NaB, A-83-01 and PD032901 (REF. 61)

A-83-01, 3-(8-Methyl-2-pyrrolidinyl)-9-(4-phosphoryloxyphenyl)-5-(4-methyl-1H-imidazol-2-yl)-1H-benzotriazolium hexafluorophosphate  
CHIR99031, 2-(2-hydroxypropyl)-2-methyl-5-oxo-1,2,3,4-tetrahydropyridine-4-carboxamide  
KSO, Karyopherin small orthoheptamer  
KLF4, Krüppel-like factor 4  
miRNA, microRNA  
OSNL, Oct4 nuclear localization signal  
OCT4, Oct-3/1/2 transcription factor  
P546, 4-phenyl-2-(2-hydroxypropyl)-2-methyl-5-oxo-1,2,3,4-tetrahydropyridine-4-carboxamide  
PD032901, 1-(2,3-bis(4-chlorophenyl)phenoxy)-2-(2,3-bis(4-chlorophenyl)phenoxy)propan-2-ol  
Prozac, Fluoxetine  
REF, Reference  
RRE, Rev Responsive Element  
SB415286, 2-(3-(4-chlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)phenyl)-5-(4-chlorophenyl)-2,2-dimethyl-1,3,4-oxadiazole  
Sox2, Sry-related HMG box 2  
Ulf1, Ultron 1  
WPRE, Woodchuck Pre-microRNA Element

**E2A selbst-spaltenden Protease Aus Adenoviridae**

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### ciPSC Herstellung (auch nicht schneller als OSKM-Methode)

Table S1. (B) Small-molecule compounds used in reprogramming.

Full Name	Abbreviation	Concentration (μM)	Source	Molecular Weight	Structure
Valproic acid sodium salt	VPA, V	700	Sigma, cat. no. 34743	144.19	
CHIR99021	CHIR, C	10.20 *	synthesized by WUZI APFTEC	445.34	
81482	8	5.00	synthesized by WUZI APFTEC	409.34	
Demethylpropionamide	DM, T	5.00	Sigma, cat. no. BM8-8127-0003	122.23	
Forskolin	Fsk, F	10.00 **	Sigma, cat. no. BM8-05100-0100	418.59	
Edaravone	ED, E	100.00	synthesized by WUZI APFTEC	262.29	
2-Methyl-5-(hydroxymethyl)pyridine hydrochloride	2-Me-5HT, M	1	synthesized by WUZI APFTEC	213.09	
SB415286	SB	1	synthesized by WUZI APFTEC	399.41	

(continued)

Full Name	Abbreviation	Concentration (μM)	Source	Molecular Weight	Structure
SB415286	SB	1	synthesized by WUZI APFTEC	399.41	
4-Methylumbelliferone	4-MU	10	Sigma Chem. cat. no. 0214910	248.23	
DMX	DMX	50	Sigma, cat. no. 2347	222.24	
Diethylsuccinyl-CoA	DiEtCoA	50	Sigma Chem. cat. no. 021767	491.57	
2',3'-Dibutyryladenosine	2',3'-DBA	5.20	Sigma Chem. cat. no. 021762	319.29	
Phenylethylidene acetylacetone	PEAA	1	Sigma, cat. no. 14810	182.46	
Surfactant	S	20	Sigma, cat. no. 00107	118.09	

### ciPSC Herstellung: u.a. CHIR99021, Valproic Acid und Na-Butyrate, Fskolin, Ionomycin Wnt HDAC-I Adenylatcyclase-A Ca<sup>++</sup> Transport

Full Name	Abbreviation	Concentration (μM)	Source	Molecular Weight	Structure
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
4-Methylumbelliferone	4-MU	10	Sigma, cat. no. 0214910	248.23	
DMX	DMX	50	Sigma, cat. no. 2347	222.24	
Diethylsuccinyl-CoA	DiEtCoA	50	Sigma Chem. cat. no. 021767	491.57	
2',3'-Dibutyryladenosine	2',3'-DBA	5.20	Sigma Chem. cat. no. 021762	319.29	
Phenylethylidene acetylacetone	PEAA	1	Sigma, cat. no. 14810	182.46	
Surfactant	S	20	Sigma, cat. no. 00107	118.09	

Full Name	Abbreviation	Concentration (μM)	Source	Molecular Weight	Structure
2-aminoadenine	2-AA	5	Sigma, cat. no. 1642	244.29	
Adenosine	A	2	Calbiochem, cat. no. 1142	133.13	
Adenosine	A	2	Calbiochem, cat. no. 114030C	267.24	
SB415286	SB	1	Sigma Chem. cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	
SB415286	SB	1	Sigma, cat. no. 0214910	399.41	

## iPSC - Probleme

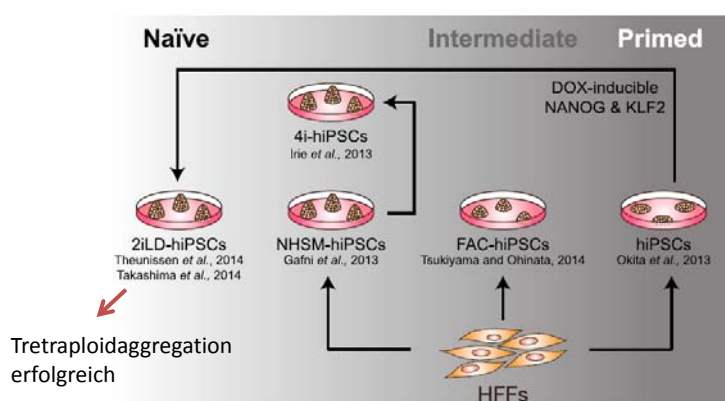
- Effizienz und Dauer
- Reprogrammierung nicht vollständig
- Reprogrammierung nicht stabil → Epigenetisches Gedächtnis der iPSCs
- Tumorbildung c-Myc, Lentivirus Reaktivierung,
- Reaktivierung der Transgene, ...

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## Herstellung von verschiedenen Stammzelltypen aus „rohen“ hiPSCs



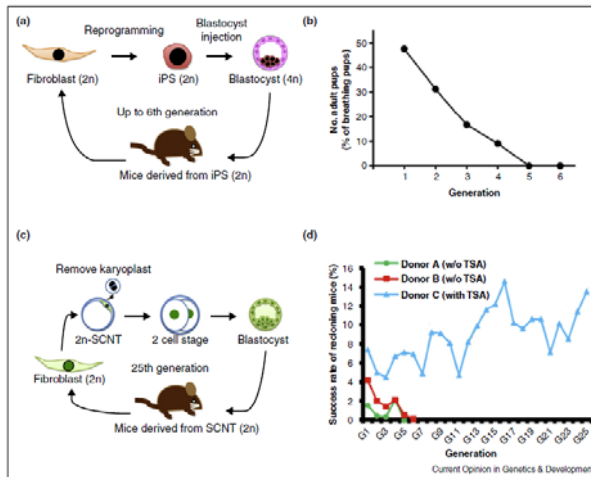
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Wie schädlich ist die iPSC und ntESC Herstellung?

Figure 2



Propagation of mice by reprogramming. (a) Schematic of successive rounds of iPSC reprogramming and injection into tetraploid blastocysts to generate all-iPSC mice. (b) The percentage of live-born all-iPSC pups that reached adulthood (from [30]). (c) Schematic of repeated rounds of SCNT, followed by foster embryo transfer and generation of cloned mice. (d) The success rate of mouse recloning in each generation with or without the use of trichostatin A (TSA), an HDAC inhibitor during nuclear transfer (adapted from [31]).

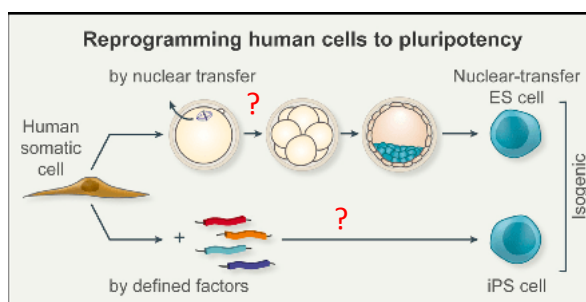
Article <http://dx.doi.org/10.1016/j.gde.2015.06.007>

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Resumé: Vergleich ntESCs mit iPSCs



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1. Entstehung der Stammzellforschung  
- Historischer Überblick
2. Herstellung von embryonalen Stammzellen
3. Wie macht man induzierte pluripotente Stammzellen?
4. **Wie isoliert man somatische adulte Stammzellen?**
5. Welche Eigenschaften haben Stammzellen?
6. Wie macht man somatische Zellen aus Stammzellen?  
Stammzellen in der Medizin und die damit verbundene  
ethische Problematik.
7. Neue Herausforderungen in der Grundlagenforschung  
zur Stammzellbiologie.