

C. Anwendungen der Stammzellbiologie – Was kann man mit Stammzellen machen?

1. In der Forschung (Molekularbiologie und Entwicklungsbiologie)
 - 1.1 In vitro Differenzierung von Stammzellen - Wie macht man somatische Zellen aus Stammzellen?
 - 1.1.1 Was sind Embryoid Bodies?
 - 1.1.2 Was sind Organoide?
 - 1.1.3 Was sind Gastruloide? - Autonome Morphogenese
 - 1.2. Was sind chimäre und transgene Mäuse?
 - 1.3. Beweis der Stammzeleigenschaften – Welche Experimente erlauben es Stammzeleigenschaften zu definieren?
2. In der Biotechnologie und Medizin
 - 2.1 Stammzellen für die Diagnostik
 - 2.2. Stammzellen-Therapien
 - 2.3. Die damit verbundene ethische Problematik

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1.1. Wie macht man somatische Zellen aus Stammzellen?

...aus ESCs, iPSCs und ntESCs und vielleicht einmal aus adulten somatischen SCs

1.1.1. Embryoid Bodies

1.1.2. Organoids und Assembloide

1.1.3. Gastruloide

1.1.4. Direkte Re-Programmierung von somatischen Zellen

1.1.5. Differenzierung aus iPSC- Zwischenstufen

Mit den Ziel

1. Entwicklungsprozesse auf zellulärer-, gewebs- und molekularer Ebene ex vivo untersuchen zu können und
2. durch gezielte Einführung von Mutationen in Stammzellen, Krankheitsursachen zu erforschen.

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Methoden der Induktion der Stammzellendifferenzierung

Induktion der Selbst-Organisation von entstehenden somatischen Zellen durch Zell-Zelle und Zell-Oberflächen Wechselwirkungen.

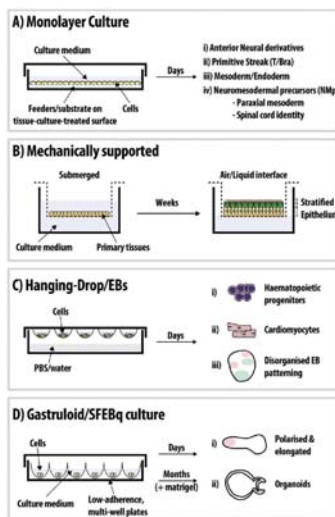
Definition of Terms

Genetic Program: In Developmental Biology, a genetic program is a temporal sequence of changes of state of a cell or cell population, brought about by the decoding of a temporal order of gene expression scripted in the genome.

Self-Assembly: The formation of an ordered structure from non-equivalent parts as a system moves towards equilibrium.

Self-Organization: The spontaneous emergence of order or asymmetry from an initially homogeneous starting population that occurs in an energy-dependent manner.

Genetically-Encoded Self-Assembly: A genetic program that contains cell autonomous instructions as well as signalling events which can induce emergent properties.



<http://onlinelibrary.wiley.com/doi/10.1002/bies.201500111/abstract>
 SFEBq, serum-free culture of embryoid body-like aggregates

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1.1.1. Embryoid bodies aus ESCs, iPSCs und ntESCs

- Aggregation von ca. 800 ESCs zu Embryoid Bodies (EBs) in Tropfen für 4.5 Tage löst Gastrulations-ähnliche Prozesse aus.
- Transfer der EBs auf extrazelluläre Matrix (EMC)- Surrogat = Gelatine am Tag 4.5 löst Implantations-ähnliche Prozesse und weitere Differenzierung der ectodermalen, mesodermalen und endodermalen Zelltypen aus.
- Alle Zellarten des Säugetierkörpers können ab Tag 6 entstehen.

ZB: Herzzellen entstehen ab Tag 7

glatte Muskelzellen ab Tag 15

Die in vitro Differenzierung von Stammzellen zu Embryoid Bodies

– ein Modell für die frühe Embryogenese?

Wie entstehen somatische Zellen in Embryoid Bodies?

Ist die Gastrulation chaotisch, oder gibt es reproduzierbare morphologische Strukturen?

Vergleich der Entwicklungsabschnitte in vivo und in vitro

In der Maus

6.1.1 Pre-implantations Entwicklung: Tag 0 - 4

6.1.2. Pre-gastrulations Entwicklung : Tag 4 - 7

6.1.3. Gastrulation / Keimblattbildung: Tag 7 - 9...

Herstellung von Embryoid Bodies

ESCs

Tag 1-3

20 min. Trypsin

Tag 4,5

24 Stunden

Tag 5.5

4,5 Tage

Tag 6.5

Embryoid Bodies

A d2

B d5

C d7

D d7

E d2

Anna Wobus, Gatersleben, D
Erfinderin der von ESC abstammenden Embryoid Bodies (1985)

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ESC Aggregat Tag 1

Kompaktierung Tag 1-2

Embryoid Body Tag 3

Embryoid Body Tag 4

Embryoid Body Tag 4,5

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6.1.1 Pre-Implantations Entwicklung: Tag 0 – 4 Morphologische Evidenzen

In vivo Zygote → 6-7 cell divisions → Blastocyst

In vitro Day 1 Day 1.5 Day 2 -3 Day 4

Labels: Trophectoderm, Inner Cell Mass, Primitive Endoderm (Hypoblast)

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Quelle: CDvor2000

EB d4.7 EB d4.7 Querschnitt

Der Hypoblast und Epiblast bildet sich, aber kein Trophektoderm.

Embryoid Bodies verhalten sich wie die Innere Zellmasse.

Kompaktierung der ESCs kann nicht wirklich mit der Kompaktierung der Blastomere verglichen werden.

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6.1.2. Pre-gastrulations Entwicklung: Day 4-7 Morphologische Evidenzen

In vivo **Blastocyst** → **Implantation** →

In vitro **Embryoid Bodies** → **Pseudo-Implantation** →

Primitive Endoderm Visceral Endoderm Parietal Endoderm

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Pseudo-Implantation von Embryoid Bodies

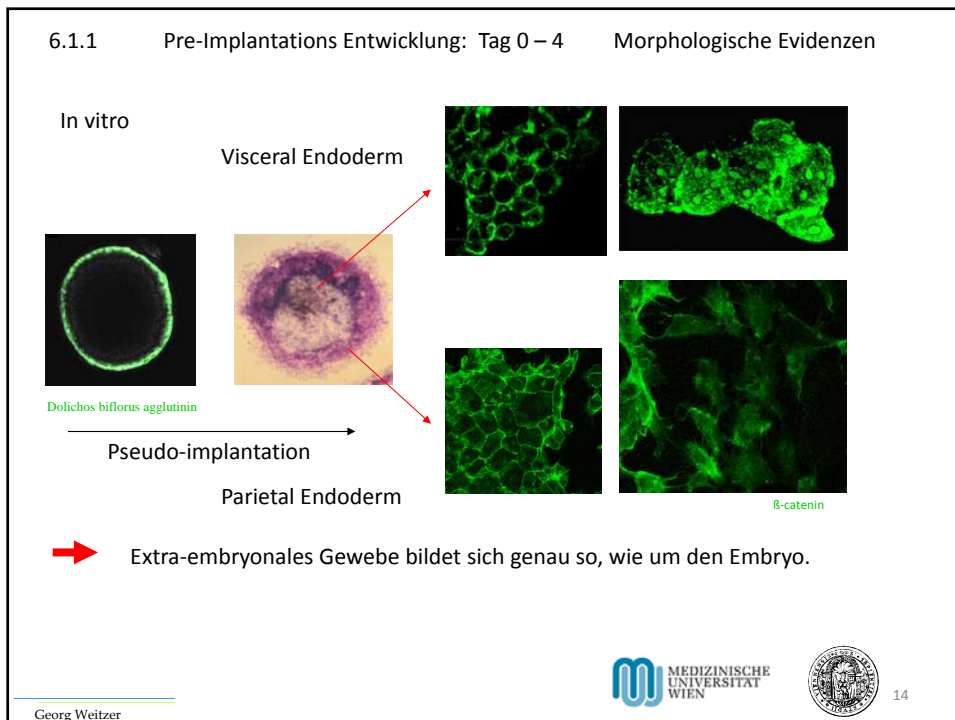
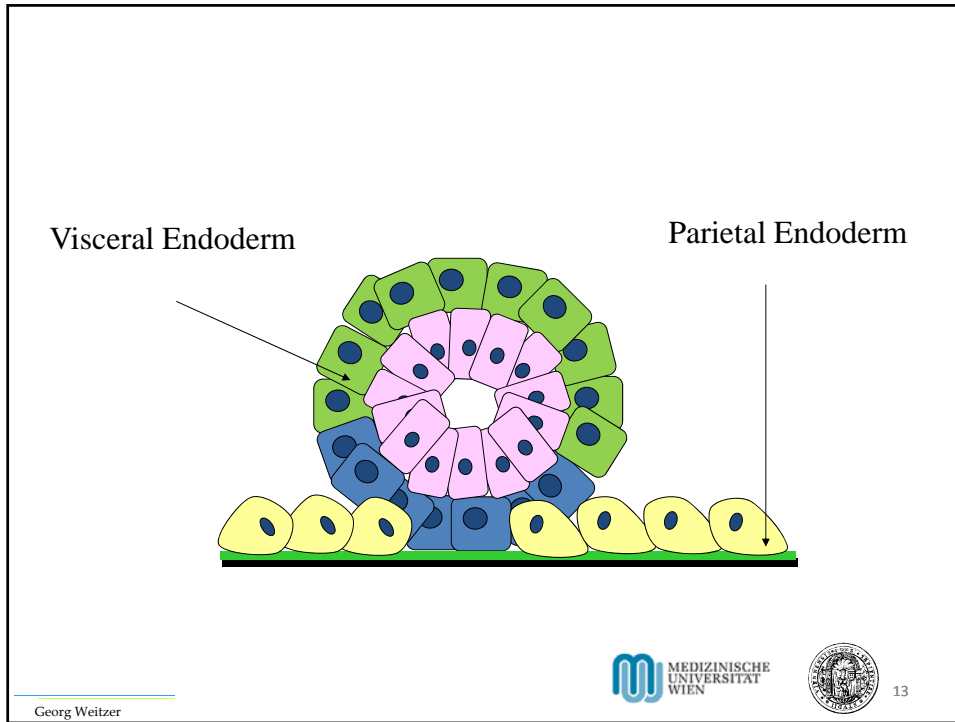
Primitives Endoderm

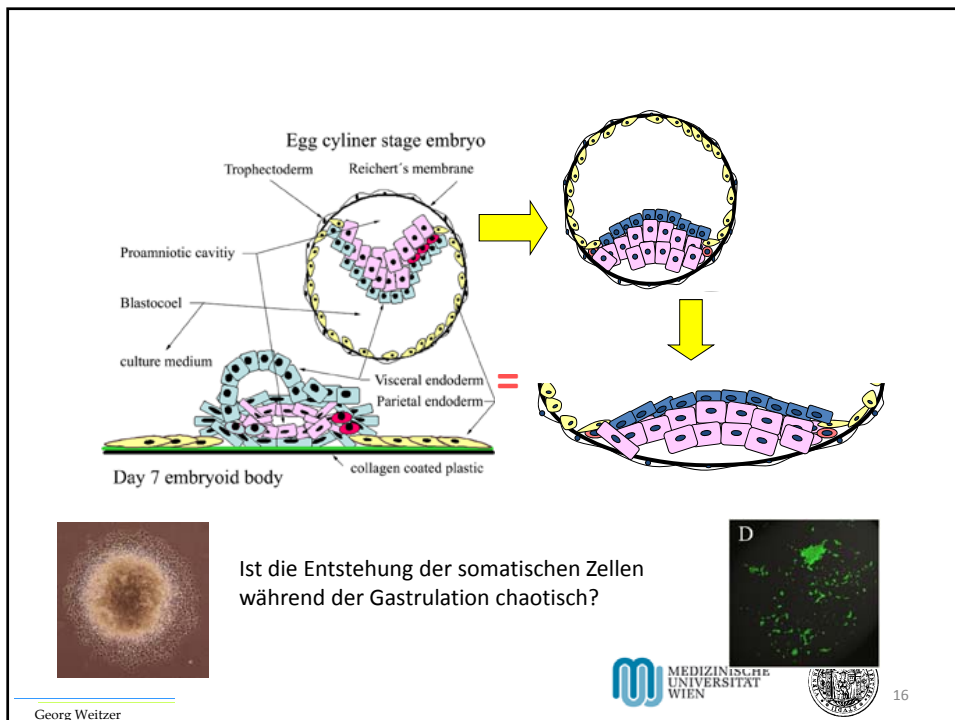
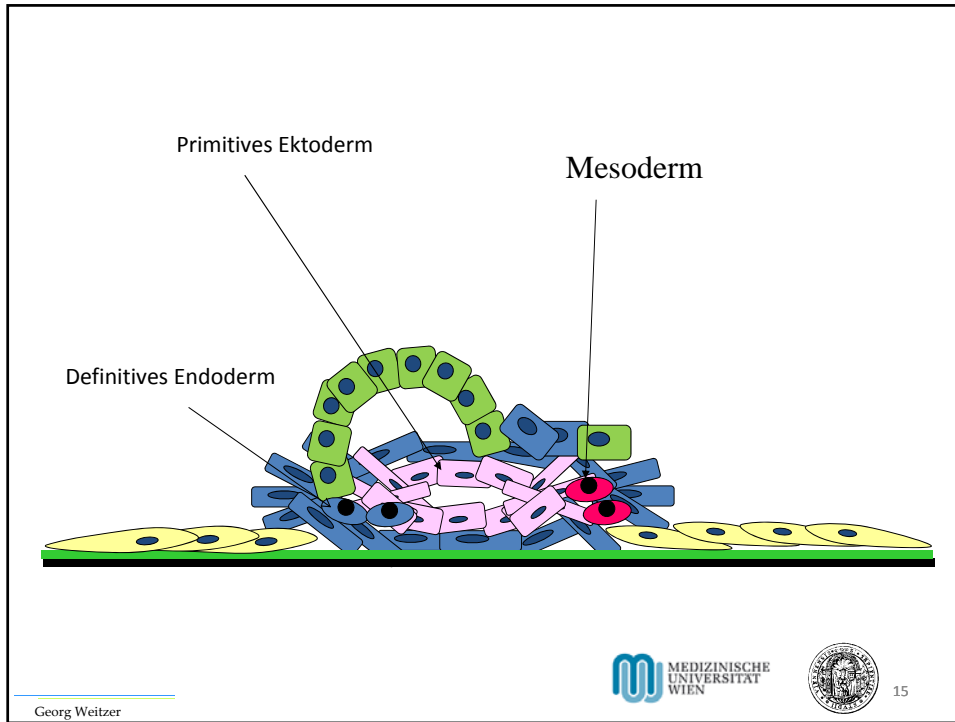
Primitives Ektoderm

Collagen Matrix

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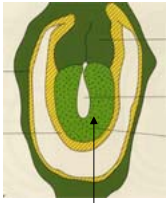





6.1.3. Gastrulation : Tag 7-9... Morphologische Evidenzen

In vivo

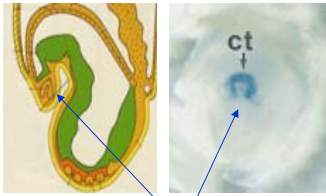
Egg cylinder stage



Primitive streak stage




Head fold stage



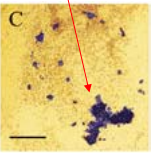
In vitro

Prim. Ectoderm



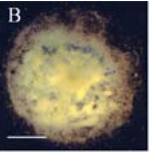
Embryoid Body Day 5

Primitive Mesoderm

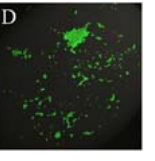


Day 6 (T⁺ Zellen)

Erythrozyten




Cardiocytes




Day 8

Der zeitliche Ablauf der Keimblattentwicklung in vitro ist gleich wie bei der Gastrulation in vivo.

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6.1.3. Gastrulation : Tag 7-9... Molekulare Evidenzen

Genexpression in Embryoid Bodies typisch für:

Days 0 4

AFP	+	+
tPA	+	+
Thbd	+	+
Follistatin	+	+
Epc3-26	+	+
Oct-3/4	+	+
Rex-1	+	+

Primitives Endoderm

Primitives Ektoderm

Days 0 4 6 9 12

AFP	+	+	+	+
tPA	+	+	+	+
Thbd	+	+	+	+
Follistatin	+	+	+	+
Brachyury	+	+	+	+
Goosecoid	+	+	+	+
Desmin	+	+	+	+
GAPDH	+	+	+	+

Definitive Endoderm

Primitive Mesoderm


Definitive Mesoderm

Kardiomyozyten


Day 0 4 6 8 11 15

Nkx 2.5	+	+	+	+	+	+
Mef 2C	+	+	+	+	+	+
MLC1v	+	+	+	+	+	+
MHC α	+	+	+	+	+	+
Connexin 43	+	+	+	+	+	+
GAPDH	+	+	+	+	+	+

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Further development of "implanted" embryoid bodies

Point symmetry \longrightarrow Line symmetry

Day 6 Day 6.5 Day 7.0 Day 8.0 Day 9.0

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Mesodermbildung in Embryoid Bodies

Primitive Nuclei Cardiomyocyt

Brachyury MHC α

Braking line symmetry

Area where mesodermal cells emerge

left right

upper

lower

In 65 +/- 7 % der Embryoid Bodies beginnen die ersten Kardiomyozyten „links unten“ zu schlagen! (N= 349)

\longrightarrow Embryoid Bodies sind asymmetrisch!

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Anwendungsmöglichkeiten:
Murine Embryo, day 7

In vivo

In vitro

Embryoid bodies erlauben eine relative einfache Bestimmung der Potentialität von Stammzellen

Die Untersuchung von molekularen und zellulären Prozessen während der Embryogenese, die experimentell im Embryo nicht erfassbar sind.

Herstellung somatischer Zellen für die Therapie

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Ad 1.1. Wie macht man somatische Zellen aus Stammzellen?

z.B.: Herstellung von Herzzellen aus ESCs, iPSCs oder ntESC.

Fig. 2. Specification and progression of the cardiac cell lineage during development. The stepwise commitment of pluripotent cells via various intermediate stages towards mature cardiac cell types within the heart during development. The intermediate stages can be characterized by specific molecular signatures and the progression of differentiation is influenced by various signaling pathways. EPDCs, epicardium-derived cells; EMT, endothelial-to-mesenchymal transition; SAN, sinoatrial node; RBB, right bundle branch; LBB, left bundle branch; PF, Purkinje fibers; AV, atrioventricular.

© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 4418-4431 doi:10.1242/dev.091538

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2.1.4. Gerichtete in vitro Differenzierung von Stammzellen → Auf dem Weg zu Organoiden

Ohne Beeinflussung entstehen alle Zelltypen.

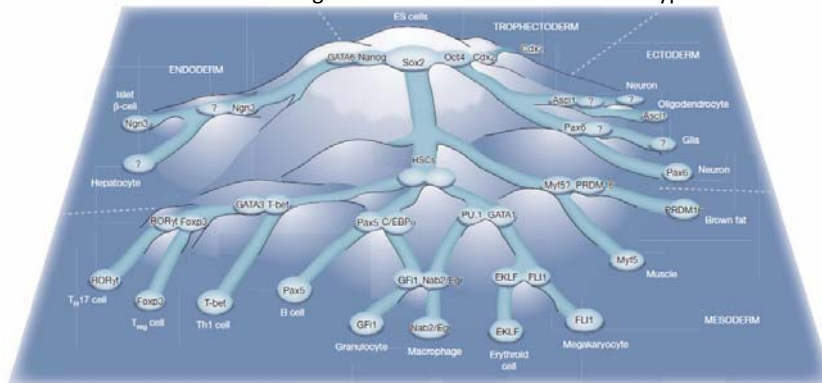


Figure 5 | Transcription factor cross-antagonisms in a cascading landscape of unstable and stable cell states. The territory, represented as a mountain range, depicts all possible solutions of a single regulatory network that specifies cell identity. Robust network states correspond to stably differentiated cell types (deep basins in the low-lying plains) whereas unstable solutions correspond to ridges and slopes in the landscape. The latter are only fleetingly occupied during development and thus unlikely to correspond to observable cell types. ES cells, embryonic stem cells; HSCs, haematopoietic stem cells.

Nach Konrad H. Waddington



1.1.2. Organoids

... Entstehen aus EBs unter speziellen Kulturbedingungen (sind eigentlich nur ein neuer Name für die seit 1986 bekannten und publizierten (Anna Wobus, Gatersleben) EBs.

1. Künstliche Darmstücke 2006
2. Künstliche Augen 2011
3. Künstliche Hirnstücke 2013
4. Endometrium Organoide aus „endometrialen adulten Stammzellen“ auf dem Weg zur künstlichen Plazenta. Siehe <https://www.nature.com/articles/nb3516>

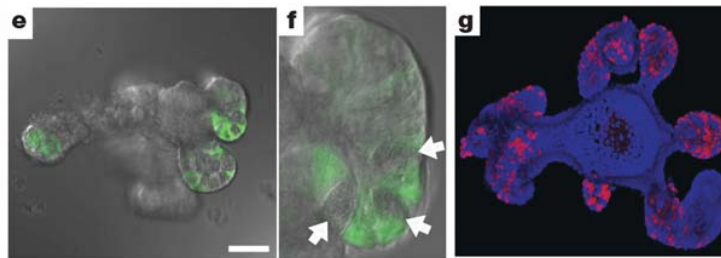
Oder nur Zelltypen ohne strukturierte Gewebe, wie

1. Pancreatic β-cells
2. Oligodendrozyten
3. Retinazellen



1. Mini Darm (Erstes Organoid) - Single Lgr5⁺ cells generate crypt-villus structures.

The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. We have recently demonstrated the presence of about six cycling Lgr5⁺ stem cells at the bottoms of small-intestinal crypts⁴. Here we describe the establishment of long-term culture conditions under which single crypts undergo multiple crypt fission events, while simultaneously generating villus-like epithelial domains in which all differentiated cell types are present. Single sorted Lgr5⁺ stem cells can also initiate these crypt-villus organoids. Tracing experiments indicate that the Lgr5⁺ stem-cell hierarchy is maintained in organoids. We conclude that intestinal crypt-villus units are self-organizing structures, which can be built from a single stem cell in the absence of a non-epithelial cellular niche.



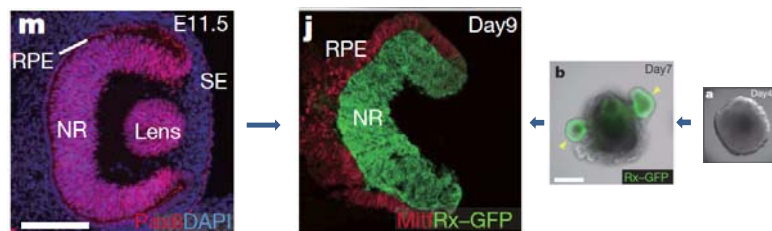
e, f, Fourteen days after sorting, single GFP^{hi} cells form crypt organoids, with Lgr5-GFP⁺ cells and Paneth cells (white arrows) located at crypt bottoms. Scale bar, 50 μ m. f, Higher magnification of e. g, Organoids cultured with the thymidine analogue EdU (red) for 1 h. Note that only crypt domains incorporate EdU. Counterstain, 4,6-diamidino-2-phenylindole (DAPI; blue).

T Sato *et al. Nature* **000**, 1-4 (2009) doi:10.1038/nature07935 Hans Clevers Lab

2. Augen

Self-organizing optic-cup morphogenesis in three-dimensional culture

Mototsugu Eiraku, Nozomu Takata, Hiroki Ishibashi, Masako Kawada, Eriko Sakakura, Satoru Okuda, Kiyotoshi Sekiguchi, Taiji Adachi & Yoshiki Sasai



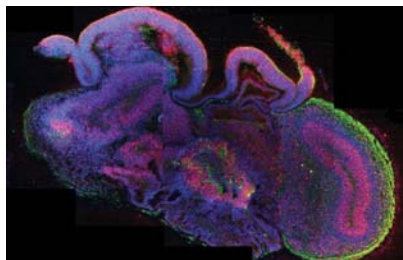
Maus

Embryoid bodies

doi:10.1038/nature09941

7 APRIL 2011 | VOL 472 | NATURE | 51

3. Hirn



Cerebral organoids model human brain development and microcephaly

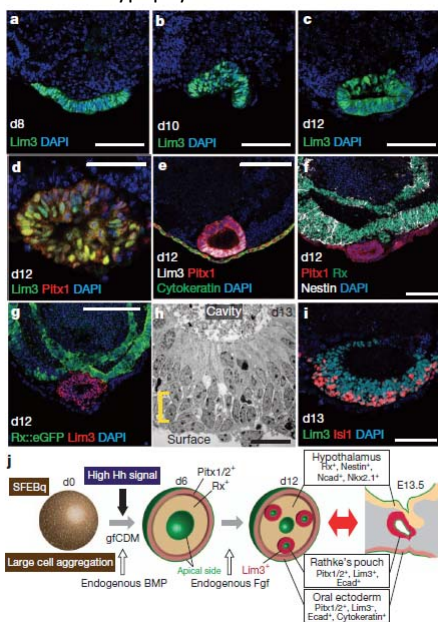
[Madeline A. Lancaster](#)¹ [Magdalena Renner](#)¹ [Carol-Anne Martin](#)² [Daniel Wenzel](#)¹ [Louise S. Bicknell](#)² [Matthew E. Hurler](#)³ [Tessa Homfray](#)⁴ [Josef M. Penninger](#)¹ [Andrew P. Jackson](#)² & [Juergen A. Knoblich](#)¹
 Nature Volume: 501, Pages: 373–379 Date published: (19 September 2013) DOI: doi:10.1038/nature12517
 Published online 28 August 2013

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4. Adenohypophyse



Self-formation of functional adenohypophysis in three-dimensional culture
[Hidetaka Suga](#), [Taisuke Kadoshima](#), [Maki Minaguchi](#), [Masatoshi Ohgushi](#), [Mika Soeno](#), [Tokushige Nakano](#), [Nozomu Takata](#), [Takafumi Wataya](#), [Keiko Muguruma](#), [Hiroyuki Miyoshi](#), [Shigenobu Yonemura](#), [Yutaka Oiso](#) & [Yoshiki Sasaki](#)
 Nature volume 480, pages 57–62 (01 December 2011)

Figure 2 Spontaneous generation of Rathke's pouch-like vesicles in ES cell culture. a–c, Morphogenesis of Lim31 epithelia. d–g, Immunostaining of day-12 pouch vesicles and surrounding tissues for Pitx1 (red, d–f), Lim3 (green, d; white, e; red, g), pancytokeratin (green, e), nestin (white, f) and Rx (green, f, g) in ES cell culture. h, Electron microscopy of the day-13 pouch. Delaminating cells on the basal side (bracket). i, Islet11 cells in the basal zone of the day-13 pouch. j, Schematic of in vitro generation of Rathke's pouches. Scale bars, 100 μm (a–c, e–g); 50 μm (d, i); 20 μm (h).

Self-formation of functional adenohypophysis in three-dimensional culture
 Suga et al., 2011 | VOL 480 | NATURE | 57 doi:10.1038/nature10637
 The adenohypophysis (anterior pituitary) is a major centre for systemic hormones. At present, no efficient stem-cell culture for its generation is available, partly because of insufficient knowledge about how the pituitary primordium (Rathke's pouch) is induced in the embryonic head ectoderm. Here we report efficient self-formation of three-dimensional adenohypophysis tissues in an aggregate culture of mouse embryonic stem (ES) cells. ES cells were stimulated to differentiate into non-neural head ectoderm and hypothalamic neuroectoderm in adjacent layers within the aggregate, and treated with hedgehog signalling. Self-organization of Rathke's-pouch-like three-dimensional structures occurred at the interface of these two epithelia, as seen *in vivo*, and various endocrine cells including corticotrophs and somatotrophs were subsequently produced. The corticotrophs efficiently secreted adrenocorticotrophic hormone in response to corticotrophin releasing hormone and, when grafted *in vivo*, these cells rescued the systemic glucocorticoid level in hypopituitary mice. Thus, functional anterior pituitary tissue self-forms in ES cell culture, recapitulating local tissue interactions.

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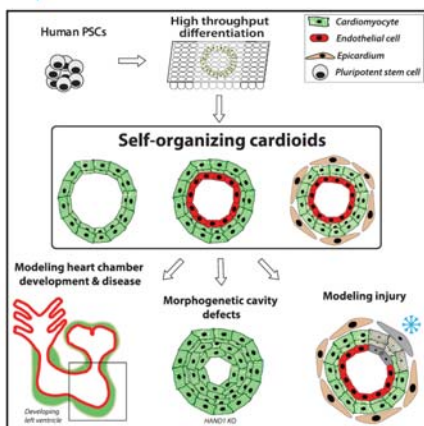


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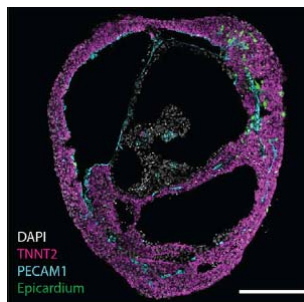
5. Herz-ähnliche Organoide - Cardioids reveal self-organizing principles of human cardiogenesis.

Hofbauer P, Jahnel SM, Papai N, Giesshammer M, Deyett A, Schmidt C, Penc M, Tavernini K, Grdseloff N, Meledeth C, Ginistrelli LC, Clortecka C, Šalic Š, Novatchkova M, Mendjan S. Cell. 2021 Jun 10;184(12):3299-3317.e22. doi: 10.1016/j.cell.2021.04.034. Epub 2021 May 20. PMID: 34019794

Graphical abstract



In diesen Fall:
Selbstassemblierung und
nicht Selbstorganisation!

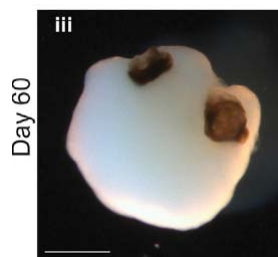
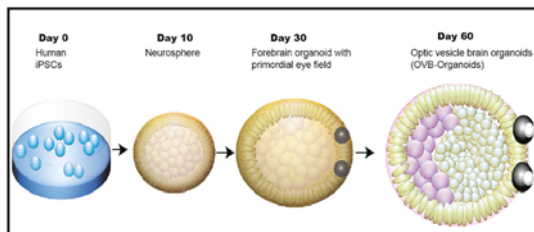


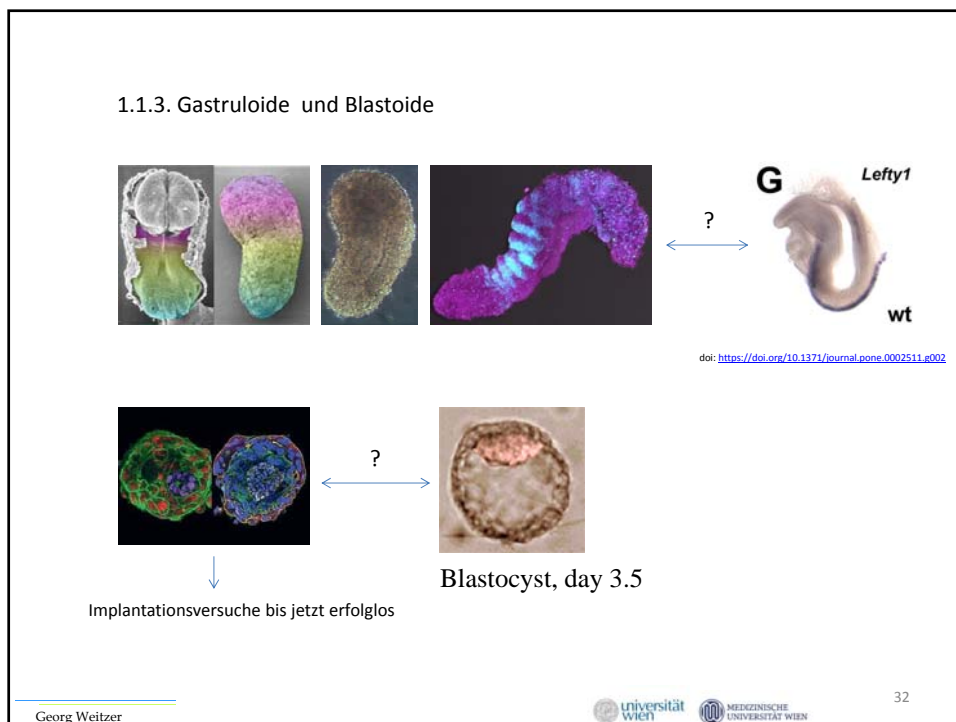
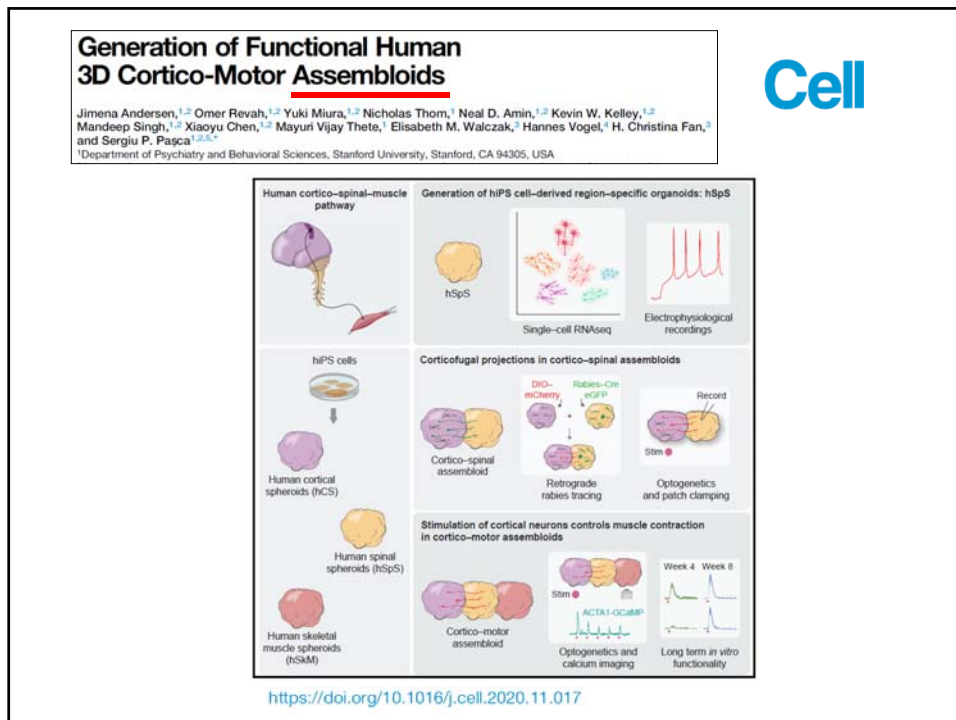
Cell Stem Cell

Human brain organoids assemble functionally integrated bilateral optic vesicles

Elke Gabriel,¹ Walid Albanna,^{2,3} Giovanni Pasquini,⁴ Anand Ramani,¹ Natasa Josipovic,^{5,13} Aruljothi Mariappan,¹ Friedrich Schinzel,¹ Celeste M. Karch,⁶ Guobin Bao,⁷ Marco Gottardo,¹ Ata Alp Suren,¹ Jürgen Hescheler,² Kerstin Nagel-Wolfrum,⁸ Veronica Persico,⁹ Silvio O. Rizzoli,⁷ Janine Altmüller,^{10,12} Maria Giovanna Riparbelli,⁹ Giuliano Callaini,⁹ Olivier Goureau,¹¹ Argyris Papanonis,⁶ Volker Busskamp,⁴ Toni Schneider,² and Jay Gopalakrishnan^{1,13,*}

¹Institute of Human Genetics, University Hospital, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany

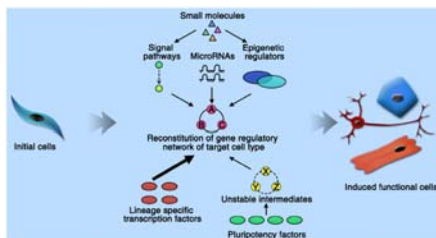




1.1.4. Direkte Re-Programmierung von somatischen Zellen

Quelle: hauptsächlich Fibroblasten

Methode: Kombination von Transkriptionsfaktoren, Wachstumsfaktoren, zwischenzeitliches FACS-en und SySMs (chemical compounds)



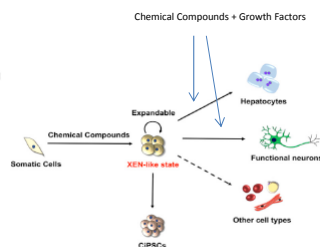
FACS, fluorescence activated cell sorting

SySMs, synthetic small molecules

1.1.5. Differenzierung aus iPSC- Zwischenstufen

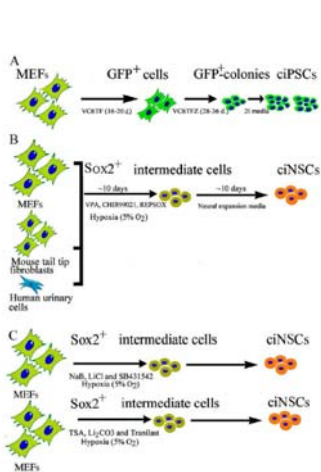
Direct Reprogramming of Fibroblasts via a Chemically Induced XEN-like State

Xiang Li,^{1,9} Defang Liu,^{1,2,9} Yantao Ma,^{1,4,9} Xiaomin Du,^{1,3,5,9} Junzhan Jing,^{3,9} Lipeng Wang,^{1,6} Bingqing Xie,² Da Sun,² Shaoqiang Sun,³ Xueqin Jin,³ Xu Zhang,¹ Ting Zhao,¹ Jingyang Guan,¹ Zexuan Yi,^{1,2} Weifeng Lai,^{1,4} Ping Zheng,⁷ Zhuo Huang,^{1,8} Yanzhong Chang,⁹ Zhen Chai,^{3,*} Jun Xu,¹ and Hongkui Deng^{1,2,10,*}



XEN-like state of ciPSCs allow the direct reprogramming of fbs to iNs (Neurons) and iHCs (Hepatocytes)

Ad 1.1.5. Differenzierung aus iPSC- Zwischenstufen



International Journal of Molecular Sciences

MDPI

Review

Chemically Induced Reprogramming of Somatic Cells to Pluripotent Stem Cells and Neural Cells

Dhruva Biswas¹ and Peng Jiang^{1,2,3,*}

Figure 1. Chemically induced reprogramming of ciPSCs and ciNSCs. (A) A schematic diagram showing direct iPSC reprogramming from MEF using the small-molecule cocktail, VC6TF (VPA, CHIR99021, E-616542, Tranylcypromine, Forskolin), and VC6TFZ (VPA, CHIR99021, E-616542, Tranylcypromine, Forskolin and DZNep) followed by application of two MEK and GSK3-β inhibitors, also known as “2i”, to finalize chemical reprogramming. Using a doxycycline (DOX)-inducible GFP-Oct4 expression screening system, ectopic GFP-Oct4 expression was induced during the first round, followed by DOX withdrawal and small molecule treatment. Epigenetic modulators, particularly 3-deazaneplanocin A (DZNep), an S-adenosylhomocysteine hydrolase inhibitor, were later added along with MEK and GSK3-β inhibitors (2i), to achieve complete reprogramming; (B) The scheme of direct ciNSC reprogramming from MEFs, mouse tail fibroblasts, and human urinary cells using the small-molecule cocktail VCR (VPA, CHIR99021, and Repsox) and physiological hypoxia; (C) A schematic diagram showing direct ciNSC reprogramming from MEF using alternative cocktails NLS (sodium butyrate (NaB), Lithium chloride (LiCl) and SB431542) and TLT (Trichostatin A (TSA), Lithium chloride (Li2CO3) and Trilastil) and physiological hypoxia (5% O2).

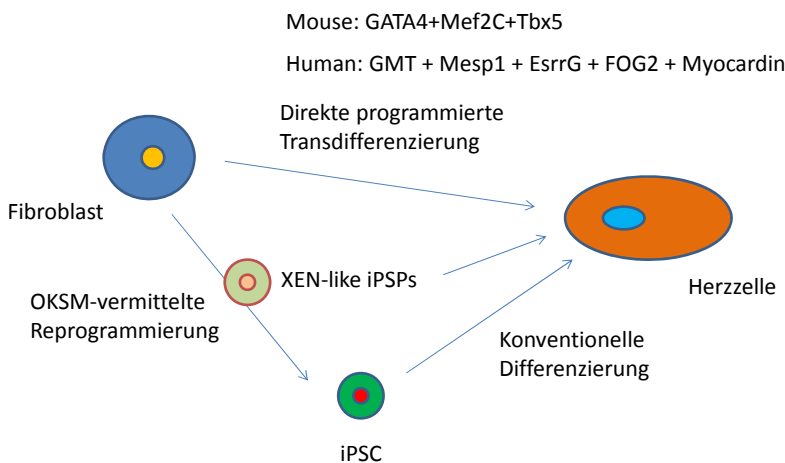
TSA = Trichostatin A

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1.1.4. und 1.1.5. Möglichkeiten der Herstellung von induzierten Herzzellen:



Mouse: GATA4+Mef2C+Tbx5

Human: GMT + Mesp1 + EsrrG + FOG2 + Myocardin

Direkte programmierte Transdifferenzierung

Fibroblast

OKSM-vermittelte Reprogrammierung

Konventionelle Differenzierung

iPSC

Herzzelle

XEN = extra-embryonic endoderm
 iPSPs = induced pluripotent progenitor cells
 EsrrG = Estrogen-Related Receptor Gamma
 FOG2 = Friend of GATA2

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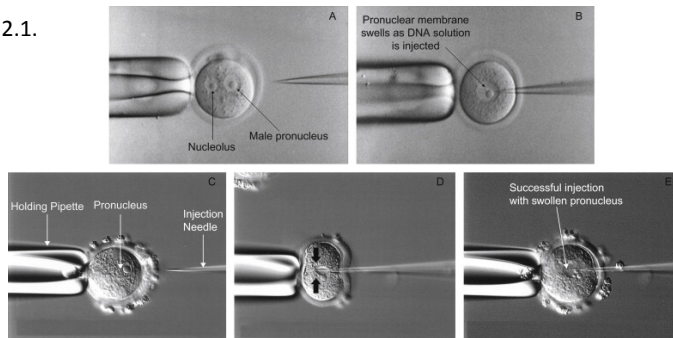
1.2. Was sind und wie macht man chimäre und transgene Mäuse?

1.2.1. durch Pronucleus-Injektion von (mutierter) DNA

1.2.2. durch Injektion von ESCs in Blastozysten

1.2.3. durch Tetraploidaggregation (siehe Pluripotenzbeweise)

1.2.1.



<https://www.sciencedirect.com/topics/nursing-and-health-professions/microinjection>

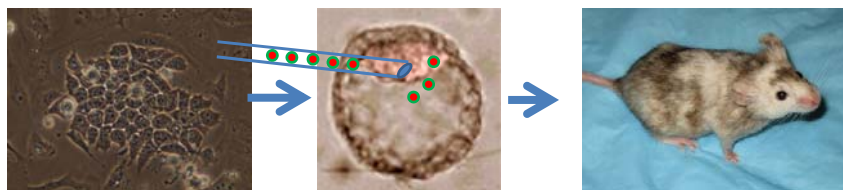
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1.2.2. ESC Injektion in Blastozysten

(siehe auch 1.3. Pluripotenzbeweise)



Injektion von
Transgenen
ESCs

Chimäre Maus:
Keimbahntransmission:
F1 = heterozygot
F2 = homozygot

Herstellung von transgenen ESCs durch

Homologe Rekombination mit Vektor DNA. → Heterozygot transgene ESCs

Intrachromosomale Rekombination in heterozygoten ESCs → homozygote transgene ESCs.

CRISPR/Cas9 Technologie

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Herstellen von transgenen Mäusen

In vivo

Durch Injektion von genetisch veränderten embryonalen Stammzellen in die innere Zellmasse von Blastozysten.

Genetisch Veränderung von embryonalen Stammzellen durch homologe Rekombination.



Chimäre Maus
F1 Generation kann das
Transgen in der Keimbahn tragen

→ Erforschung der Funktionen der einzelnen Gene wurde so möglich.

MAX
PERUTZ
LABS

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Für die Einführung von Mutationen in Gene der ESCs werden folgende Selektionsmarker verwendet:

neo^R: aminoglycoside 3'-phosphotransferase

G418 Sulfate: An aminoglycoside antibiotic similar to gentamycin. Toxic to bacteria, yeast, higher plant and mammalian cells in addition to protozoans and helminths.

hyg^R: aminocyclitol phosphotransferase

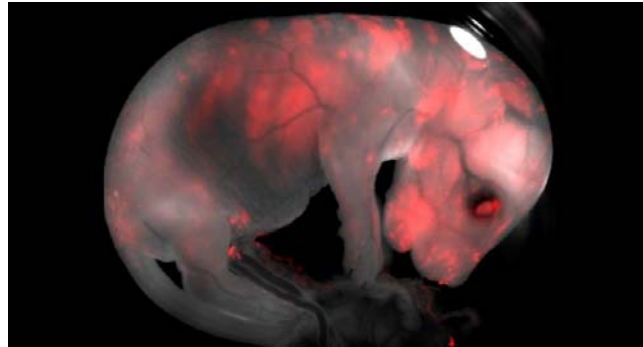
Hygromycin B: An aminoglycoside antibiotic that inhibits protein synthesis in bacteria, fungi and higher eukaryotes.

puro^R: puromycin-N-acetyltransferase

Puromycin dihydrochloride: A broad spectrum antibiotic that inhibits protein synthesis in both prokaryotic and eukaryotic organisms.

Ad 1.1.2. Chimeraformation

(Siehe auch 2.4. Pluripotenzbeweise, z.b. tierische Blastozysten Injektion mit hESCs)



1.3. Beweis der Stammzeleigenschaften

Welche Experimente erlauben es das Differenzierungspotenzial von Stammzellen zu beweisen?

1.3.1. Embryoid bodies und Gastruloide (siehe in vitro Differenzierung 1.1.)

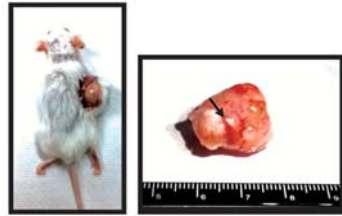
1.3.2. Teratoma Induktion in Mäusen

1.3.3. Chimäre und Transgene Mäuse (siehe Blastozyst ESC Injektion 1.2.)

1.3.4. Mäuse durch Tetraploid-Aggregation hergestellt

1.3. Pluripotenzbeweise

1.3.2. Teratomaformation: Subkutane Injektion von großen Mengen an ESCs.



<https://slideplayer.com/slide/10105550/>

Teratoma formation is a key indicator of pluripotency
cells from all three germ layers are formed

→ Histologische Analyse des Tumors
Nachweis der Zelltypen

1.3. Pluripotenzbeweise

1.3.4. Tetraploidaggregation

(eine weitere Methode des Herstellens von transgenen Mäusen, siehe 1.2.)

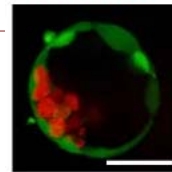
TS cells = 4N
ES cells = 2N

Zygoten → 2Blastomeren Stadium → Elektrofusion: 4n Zygote → Morula

Sandwich aus 4N-Molula - zu testende ESCs oder iPSCs - 4N-Molula

→ Blastocyst; besteht nur aus 4N Trophektoderm und 2N ICM

Einpflanzen in pseudo-schwangere Maus → 100%-ige ESC-abstammende Maus in F0



<https://www.catsmouse.de/warum-cats-mouse/inhaltsstoffe/>