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Haploide Stammzellen und CRISPR-Cas9 vermittelte Mutation
Herstellung von iPSC Zellen und Anwendungsmöglichkeiten

Haploide mESCs

Parthenogenese bis zum Blastozystenstadium→Nur einige ICM Zellen sind haploid.

Diese können mittels Durchfluss-Zytometrie mit Hoechst33342-DNA Färbung isoliert werden.

20 Chromosomen der Maus sind für die Selbsterneuerung ausreichend.

Differenzierung setzt voraus und / oder bewirkt Diploidisierung. (Mechansismus???)

Genetische Screens mit Hilfe von Gene-Trap Vektoren können so nur in undifferenzierten mESCs gemacht werden.

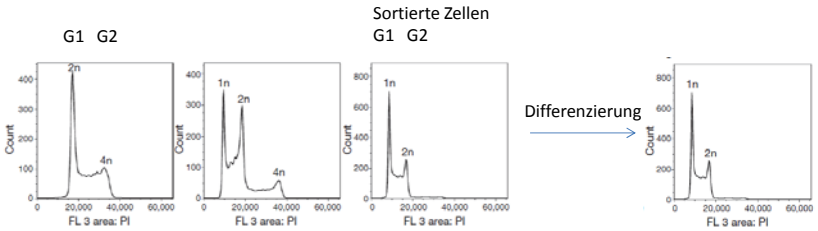
→ **Erste Möglichkeit Mutationen mit rezessive Phänotypen zu identifizieren.**

Da humane Zellen viel schlechter mutiert werden könne als murine Zellen:

CRISPR-RNA-guided CAS9 Nuklease vermittelte gezielte Mutationen.

Haploide mESCs durch parthenogenetische Aktivierung von Oozyten

Leeb &Wutz bzw. Elling …. & Penninger 2011



2n mESCs 1+2n m ESCs 1n ESCs

Cell Cycle. 2013 Jan 15;12(2):302-11. doi: 10.4161/cc.23103. Epub 2012 Jan 15.
Diploid, but not haploid, human embryonic stem cells can be derived from microsurgically repaired tripronuclear human zygotes. Fan Y[1], Li R, Huang J, Yu Y, Qiao J.

Abstract
Human embryonic stem cells have shown tremendous potential in regenerative medicine, and the recent progress in haploid embryonic stem cells provides new insights for future applications of embryonic stem cells. Disruption of normal fertilized embryos remains controversial; thus, the development of a new source for human embryonic stem cells is important for their usefulness. Here, we investigated the feasibility of haploid and diploid embryo reconstruction and embryonic stem cell derivation using microsurgically repaired tripronuclear human zygotes. Diploid and haploid zygotes were successfully reconstructed, but a large proportion of them still had a tripolar spindle assembly. The reconstructed embryos developed to the blastocyst stage, although the loss of chromosomes was observed in these zygotes. Finally, triploid and diploid human embryonic stem cells were derived from tripronuclear and reconstructed zygotes (from which only one pronucleus was removed), but **haploid human embryonic stem cells were not successfully derived from the reconstructed zygotes** when two pronuclei were removed. Both triploid and diploid human embryonic stem cells showed the general characteristics of human embryonic stem cells. These results indicate that the lower embryo quality resulting from abnormal spindle assembly contributed to the failure of the haploid embryonic stem cell derivation. However, the successful derivation of diploid embryonic stem cells demonstrated that microsurgical tripronuclear zygotes are an alternative source of human embryonic stem cells. In the future, improving spindle assembly will facilitate the application of triploid zygotes to the field of haploid embryonic stem cells.

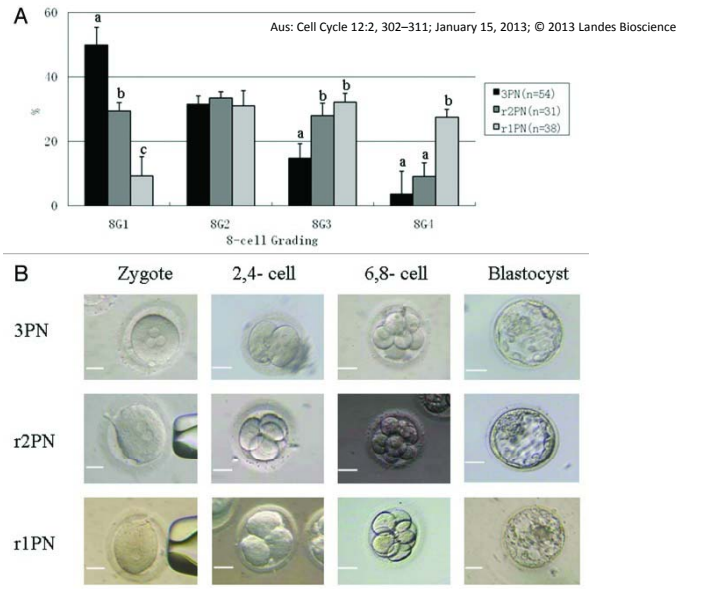
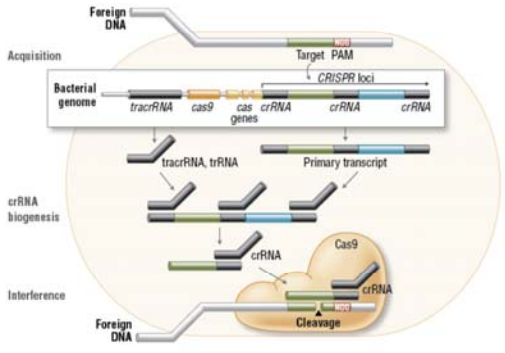


Figure 1. Effects of PN removal on the quality of reconstructed eight-cell embryos and the representative image of embryos in 3PN, r2PN and r1PN groups. (A) The quality of embryos at the eight-cell stage tended to decrease with the increasing number of removed PN. The ratio of high-quality embryos was (B) significantly decreased in the r1PN group. Means with "a" or "b" are significantly different ($p < 0.05$). (**B**) No significant morphological differences (C) were observed among the three groups before blastocyst formation; however, no high-quality blastocysts were formed in the r1PN group.

Gezieltes Einführen von Mutationen in ein Genom
wenn Mutagenese durch Homologe Recombination nicht möglich ist.

CRISPR /CAS9 CRISPR = **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats
CAS9 = CRISPR-associated protein 9

Figure 1. Cas9 *in vivo*: Bacterial Adaptive Immunity



In the acquisition phase, foreign DNA is incorporated into the bacterial genome at the CRISPR loci. CRISPR loci is then transcribed and processed into crRNA during crRNA biogenesis. During interference, Cas9 endonuclease complexed with a crRNA and separate tracrRNA cleaves foreign DNA containing a 20-nucleotide crRNA complementary sequence adjacent to the PAM sequence. (Figure not drawn to scale.)

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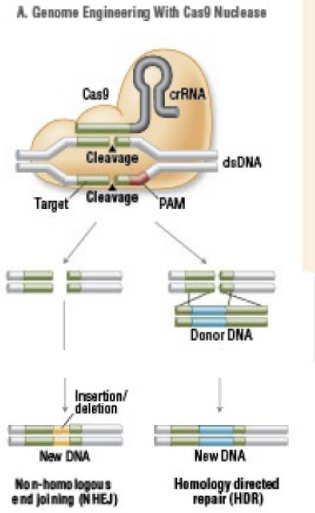
CRISPR/Cas9 System Applications



Genome Editing Glossary

Cas = CRISPR-associated genes

Cas9, Csn1 = a CRISPR-associated protein containing two nuclease domains, that is programmed by small RNAs to cleave DNA

crRNA = CRISPR RNA

dCAS9 = nuclease-deficient Cas9

DSB = Double-Stranded Break

gRNA = guide RNA

HDR = Homology-Directed Repair

HNH = an endonuclease domain named for characteristic histidine and asparagine residues

Indel = insertion and/or deletion

NHEJ = Non-Homologous End Joining

PAM = Protospacer-Adjacent Motif

RuvC = an endonuclease domain named for an *E. coli* protein involved in DNA repair

sgRNA = single guide RNA

tracrRNA, trRNA = trans-activating crRNA

TALEN = Transcription-Activator Like Effector Nuclease

ZFN = Zinc-Finger Nuclease

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CRISPR/Cas9 and Targeted Genome Editing: A New Era in Molecular Biology

Induzierte pluripotente Stammzellen (iPSCs)

Herstellung, Eigenschaften und Anwendungsmöglichkeiten

Herstellung von iPSCs

- Lentivirale polycistronische OKSM Vektoren

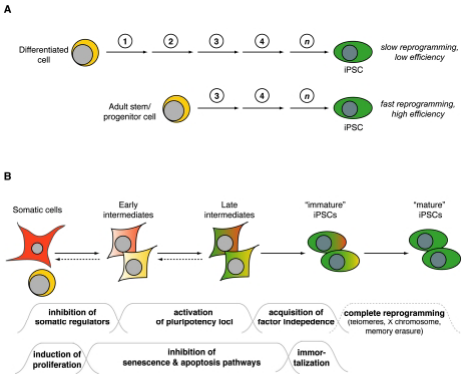
O…Oct4
K…Klf4
S…Sox2
M…c-Myc

- FBx15::EFGP Reporter-Fibroblasten
- BAC: Nanog::GFP/Puro^R/Nanog Reporter-Fibroblasten → Nanog Expression erlaubt iPSC Mäuse herzustellen.
- Lentivirale OSNL Vektoren

N…Nanog
L…Lin28

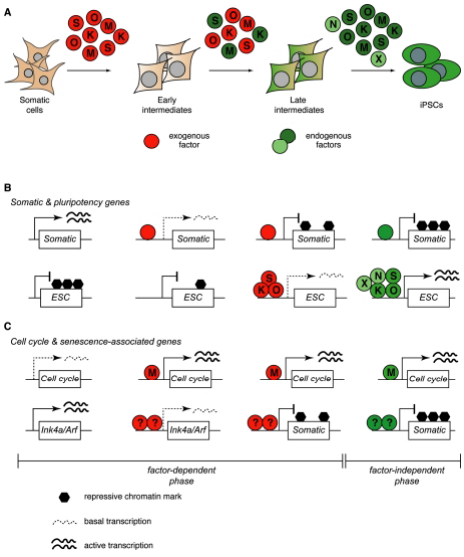
- Reprogrammieren nur mit Proteinen, RNA, kleinen synthetischen Molekülen.→ RepSox; STAP-Zellen, Mycobacterium laprae induzierte Reprogrammierung ……alles sehr ineffizient!

Herstellung von iPSCs



Aus Genes Dev. Oct 15, 2010; 24(20): 2239–2263.

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.



Putative role of reprogramming factors during iPSC formation. (*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-unidentified 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.

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Vektoren für die Reprogrammierung

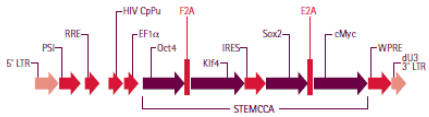
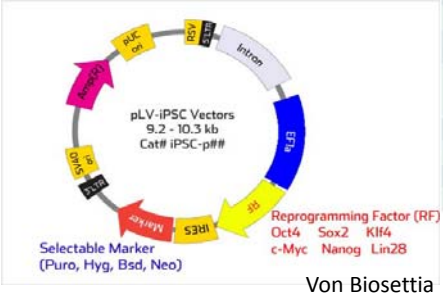


Figure 2. The STEMCCA vector is comprised of the transcription factors Oct-4, Klf4, SOX-2, and c-Myc (OKSM), separated by the self-cleaving 2A peptide and IRES sequences driven by the EF-1α constitutive promoter[12]. It is also available with flanking LoxP sites incorporated for Cre-mediated excision of the exogenous reprogramming transgenes.

STEMCCA™

E2A selbst-spaltenden Protease
Aus Adenoviridae



Von Biosettia

Zellarten Stand 2011

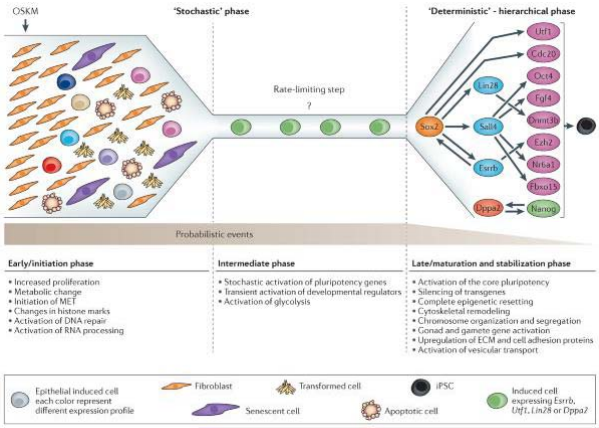
Table 2 | **Summary of iPSC derivation methods***

Cell types	Reprogramming factors and methods			
	Integrating	Excisable	Non-integrating	Chemicals
Fibroblasts	• Retroviral KSOM or KSO[109] • Lentiviral KSOM • Inducible lentiviral KSOM[110] • Lentiviral OSNL[9] • Lentiviral mir-302 cluster[105]	• Floxed lentiviral KSOM or KSO[97] • Transposon KSOM[98]	• Adenoviral[104] • Plasmids[94,107] • Protein[98] • mRNA[94] • RNA virus[92] • miRNA mimics[106]	• KSO + valproic acid[110] • KSOM + vitamin C[108] • KSOM + SB431542, PD0325901 and thiazovivin[103] • KSOM + sodium butyrate (NaB)[81]
Bone marrow (mobilized) or peripheral blood cells	• Retroviral KSOM[98,107] • Inducible lentiviral KSOM[94]	Not applicable	• RNA virus[92] • Plasmids[93,94]	Not applicable
Cord blood cells	• Lentiviral OSNL[102] • KSOM[103] • Retroviral OCT4 and SOX2 (REF. 164)	Not applicable	• Plasmids[93,94]	Not applicable
EBV-immortalized blood cells	-	Not applicable	• Plasmids[95,99]	Not applicable
HUVECs	• Retroviral KSOM[109]	Not applicable	Not applicable	• OCT4 + PS48, NaB, A-83-01 and PD0325901 (REF. 161)
Adipose-derived stem cells	• Lentiviral KSOM[104] • Retroviral KSO[97]	Not applicable	• Plasmid minicircles[97] • miRNA mimics[106]	Not applicable
Keratinocytes	• Retroviral KSOM and KSO[104] • Inducible lentiviral KSOM cassette[99]	Not applicable	Not applicable	• OCT4 and KLF4 + tranylcypromine and CHIR99021 (REF. 170) • OCT4 + PS48, NaB, A-83-01 and PD0325901 (REF. 61)
Neural stem cells	• Retroviral OCT4 (REF. 171)	Not applicable	Not applicable	Not applicable
Astrocytes	• Retroviral KSOM[172]	Not applicable	Not applicable	Not applicable
Hepatocytes	• Retroviral KSOM[173]	Not applicable	Not applicable	Not applicable
Amniotic cells	• Retroviral KSOM[174] • Lentiviral OSN[175]	Not applicable	Not applicable	• OCT4 + PS48, NaB, A-83-01 and PD0325901 (REF. 61)

A-83-01, 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide; CHIR99021, 6-[[2-[[4-(2,4-dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile; HUVECs, human umbilical vein endothelial cells; iPSC, induced pluripotent stem cell; KLF4, SOX2 and OCT4 and MYC; miRNA, microRNA; OCT4, octamer binding protein 4; OSN, a lentivirus containing transcription factors OCT4, SOX2 and Nanog; OSNL, a lentivirus containing transcription factors OCT4, SOX2, Nanog and LIN28; PD0325901, N-[(2R)-2,3-dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-benzamide; PS48, (2Z)-5-[(4-chlorophenyl)-3-phenyl-2-pentenoic acid; SB431542, 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]benzamide. *For simplicity, only the first reports of a given method on a given cell type are listed.

iPSC - Probleme

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



Nat Rev Genet. 2013 Jun;14(6):427-39. doi: 10.1038/nrg3473.
Mechanisms and models of somatic cell reprogramming.
Buganim Y[1], Faddah DA, Jaenisch R.

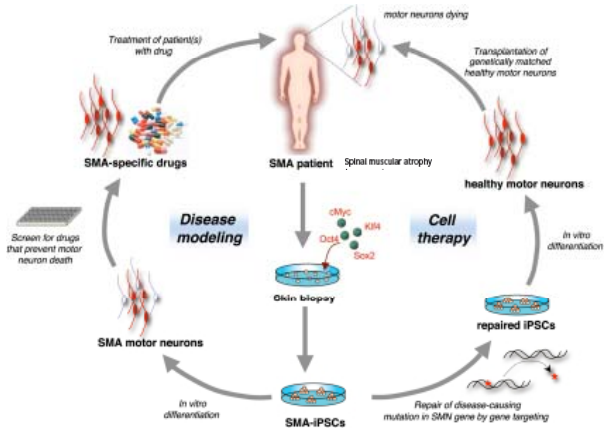
Phases of the reprogramming process
In the model we discuss in this review, the reprogramming process can broadly be divided into two phases: firstly, a long `**stochastic**' phase of gene activation; and secondly, a shorter hierarchical more `**deterministic**' phase of gene activation that begins with the activation of the *Sox2* locus. After a fibroblast is induced with OSKM, 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[23], a transient activation of developmental regulators[17], and activation of glycolysis[18]. 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 *Utf1*, *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[16–18,23]. In this model, probabilistic events decrease and hierarchical events increase as the cell progresses from a fibroblast to an iPSC.

iPSC-Anwendungsmöglichkeiten

- Herstellung von patientenspezifischen iPSCs

  → Erforschung von Ursachen und Therapiemöglichkeiten von Krankheiten polygenetischen Ursprungs.
- Verwendung dieser Zellen zum Auffinden neuer Krankheits- oder Patienten-spezifischer Medikamente.
- Entwicklung von alternative Strategien zur direkten programmierten Transdifferenzierung von einem somatischen Zelltyp in einem anderen. z.b. Herzzellen und Leberzellen aus Fibroblasten

Potential applications of iPSCs. Shown are the potential applications of iPSC technology for cell therapy and disease modeling using SMA as an example. In SMA patients, motor neurons are afflicted and die, causing the devastating symptoms of the disease. SMA-specific iPSCs could be coaxed into motor neurons in vitro in order to establish a culture model of the disease that may lead to the identification of novel drugs that prevent the abnormal death of motor neurons in patients. Alternatively, if known, the disease-causing mutation could be repaired (in this case the *SMA* gene) in iPSCs by gene targeting prior to their differentiation into healthy motor neurons, followed by transplantation into the patient's brain.



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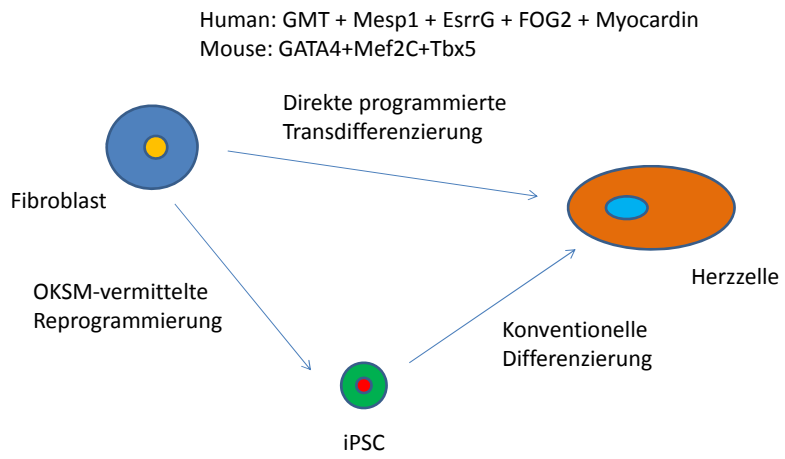
SMA = spinale Muskelatrophie



Weiterführende Literatur:

Siehe

- Shinya Yamanaka
- Marius Wernig
- Konrad Hochedlinger
- Rudolf Jänisch