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

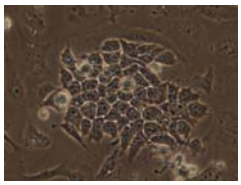
2.2.1. Isolierung von Blastozysten aus trächtigen Mäuseweibchen

2.2.2. Kultivierung der Blastozysten auf „feeder cells“

2.2.3. Kultur der embryonalen Stammzellen (ESCs)

2.2.4. Pluripotenzbeweis und Herstellung von transgenen Mäusen

2.2.3. Kultur der embryonalen Stammzellen (ESCs) –
Teil 2: Signalübertragunswege oder die
Molekulare Grundlage der Selbsterneuerung von Stammzellen in



mESCs:	hESC:
LIF (STAT3)	FGF2 (MEK)
Bmp2/4	TGFß / Activin / Nodal, Noggin (a Bmp antagonist)
Wnt (ß-Catenin)	Wnt (ß-Catenin)
Insulin	Insulin

mESC ←→ hESc

Warum dieser Unterschied?

Weil zwei verschiedene Spezies, die entwicklungsgeschichtlich zu weit weg sind?

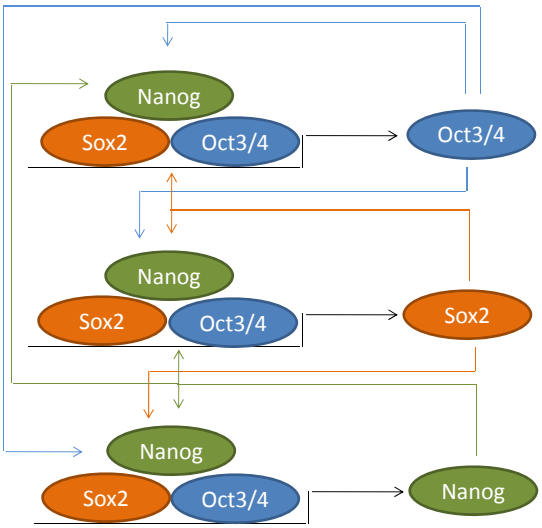
Sind ESC, durch die Isolierungsmethode bedingte Artefakte?

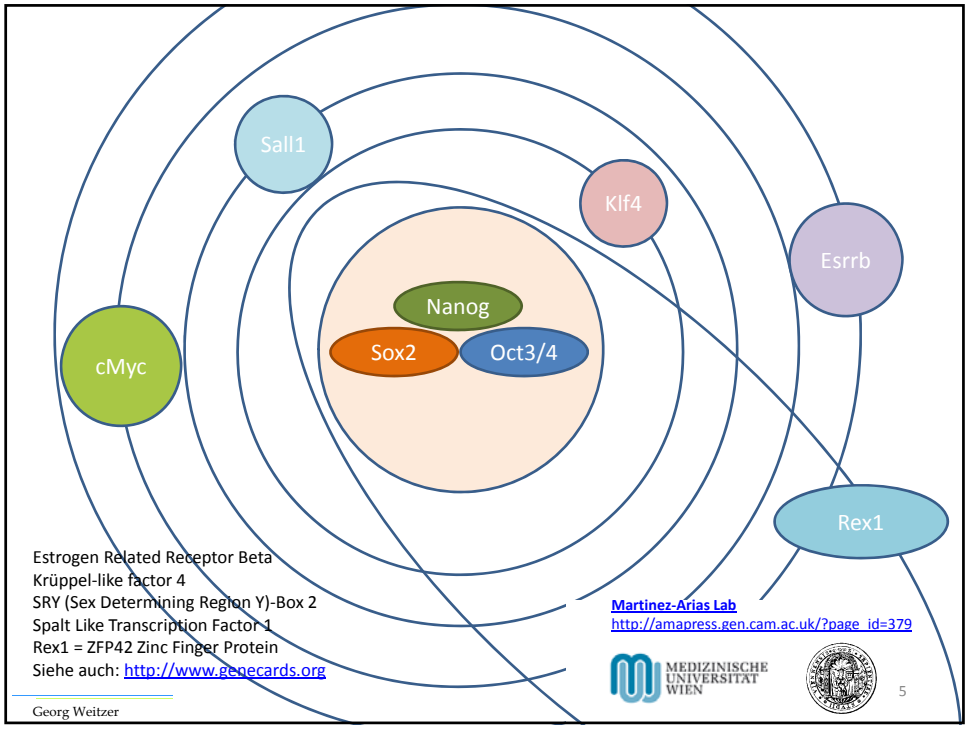
…

… haben doch alle Stammzellen die gleichen „stemness“ oder „ trinity“ Transkriptionsfaktoren Gene als Ziel Gene dieser Signalübertragungswege.

Oct4	Hans Schöller
Sox2	?
Nanog	Austin Smith

Das Netzwerk der „trinity factors“ zur Erhaltung der Pluripotenz

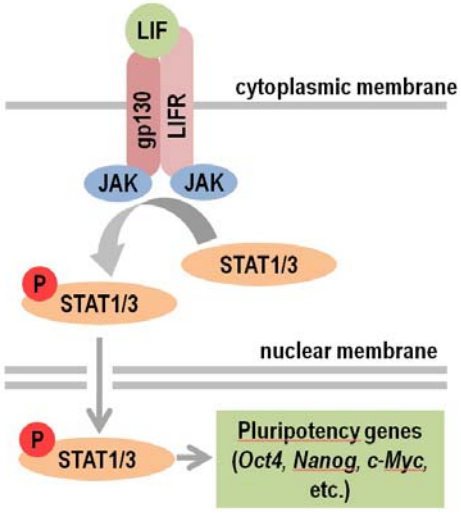




Estrogen Related Receptor Beta
Krüppel-like factor 4
SRY (Sex Determining Region Y)-Box 2
Spalt Like Transcription Factor 1
Rex1 = ZFP42 Zinc Finger Protein
Siehe auch: http://www.genecards.org

Martinez-Arias Lab
http://amapress.gen.cam.ac.uk/?page_id=379

Maus ESCs:

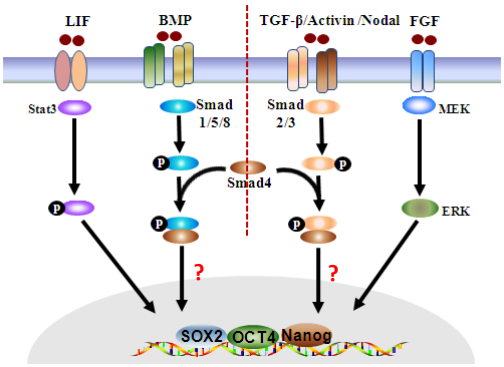


http://www.biodiscoveryjournal.co.uk/Archive/A9.htm

Zusammenfassung der unterschiede zwischen mESCs und hESCs

Maus:
mESCs

+ LIF (~ FGF2)



Mensch:
hESCs

+ FGF2

ICM-like cells

primitive Ectoderm-like cells

Ground state (naive state) →

primed (poised) state

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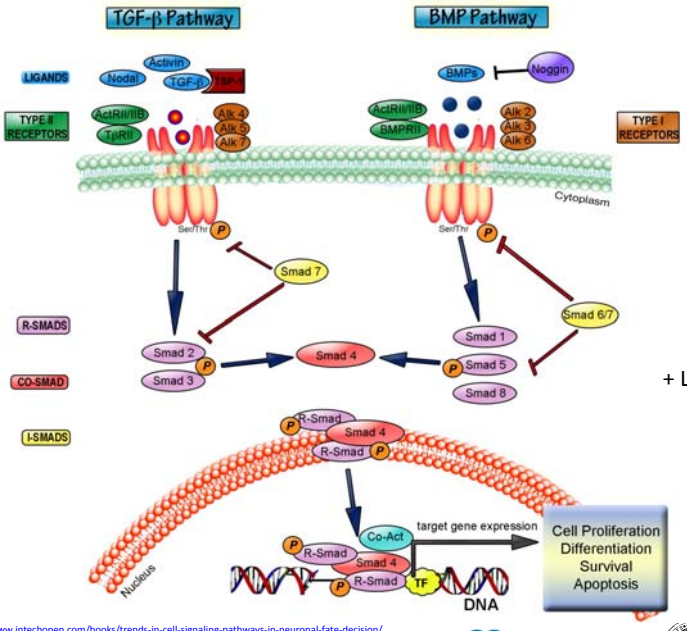

Mensch
hESCs

+ FGF2

Maus
mESCs

+ LIF (~ FGF2)

Nanog
= ?
?

https://www.intechopen.com/books/trends-in-cell-signaling-pathways-in-neuronal-fate-decision/role-of-tgf-signaling-in-neurogenic-regions-after-brain-injury

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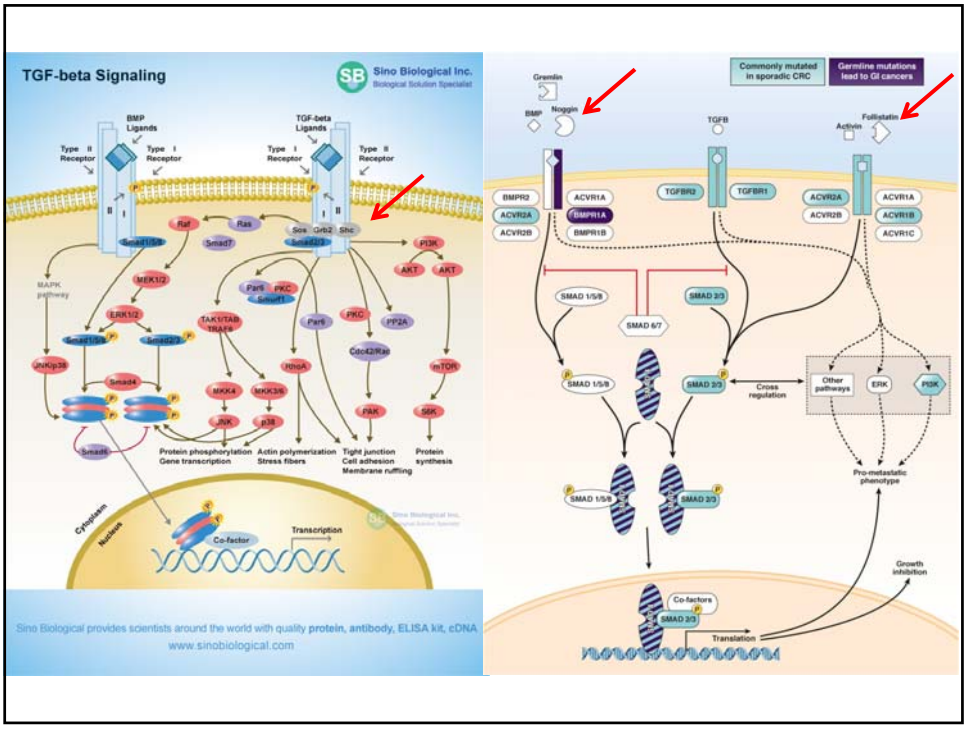
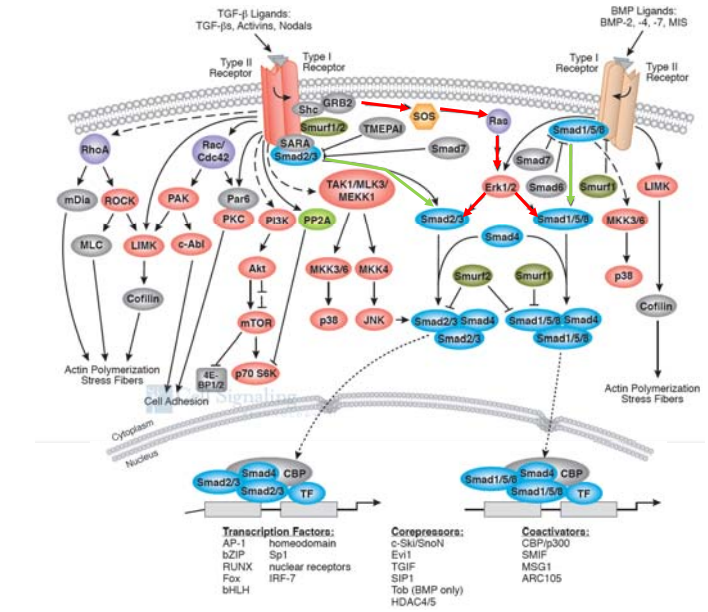
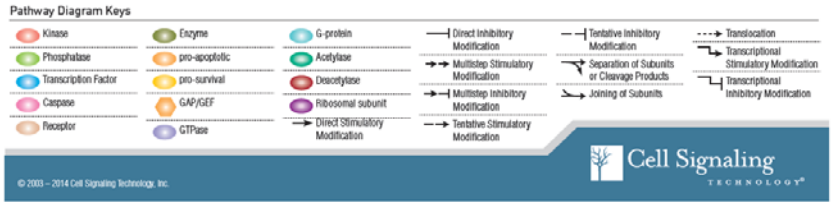


One link between TGFß and EGF signaling:



Copyrigth: Cellsignaling.com and Prof. Peter ten Dijke, Leiden University Medical Center, Leiden, The Netherlands,



Pathway Description:
Transforming growth factor-β (TGF-β) superfamily signaling plays a critical role in the regulation of cell growth, differentiation, and development in a wide range of biological systems. In general, signaling is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signaling molecules Smad2 and Smad3 for the TGF-β/activin pathway, or Smad1/5/9 for the bone morphogenetic protein (BMP) pathway. Carboxy-terminal phosphorylation of Smads by activated receptors results in their partnering with the common signaling transducer Smad4, and translocation to the nucleus. Activated Smads regulate diverse biological effects by partnering with transcription factors resulting in cell-state specific modulation of transcription. Inhibitory Smads, i.e. Smad6 and Smad7 antagonize activation of R-Smads. The expression of inhibitory Smads (I-Smads) 6 and 7 is induced by both activin/TGF-β and BMP signaling as part of a negative feedback loop. The stability of TGF-β family receptors and/or Smads are regulated by Smurf E3 ubiquitin ligases and USP4/11/15 deubiquitinases. TGF-β/activin and BMP pathways are modulated by MAPK signaling at a number of levels. Moreover, in certain contexts, TGF-β signaling can also affect Smadindependent pathways, including Erk, SAPK/JNK, and p38 MAPK pathways. Rho GTPase (RhoA) activates downstream target proteins, such as mDia and ROCK, to prompt rearrangement of the cytoskeletal elements associated with cell spreading, cell growth regulation, and cytokinesis. Cdc42/Rac regulates cell adhesion through downstream effector kinases PAK, PKC, and c-Abl following TGF-β activation.

Activation of Ras by receptor tyrosine kinases Margolis B[1], Skolnik EY. J Am Soc Nephrol. 1994 Dec;5(6):1288-99..

Ras, a small GTP-binding protein, is an important component of the signal transduction pathway used by growth factors to initiate cell growth and differentiation. Cell activation with growth factors such as epidermal growth factor (EGF) induces Ras to move from an inactive GDP-bound state to an active GTP-bound state. Recently, a combination of genetic and biochemical studies has resulted in the elucidation of a signaling pathway that leads from growth factor receptors to Ras. After binding EGF, the EGF receptor tyrosine kinase is activated, leading to receptor autophosphorylation on multiple tyrosine residues. Signaling proteins with Src homology 2 (SH2) domains then bind to these tyrosine-phosphorylated residues, initiating multiple signaling cascades. One of these SH2 domain proteins, Grb2, exists in the cytoplasm in a preformed complex with a second protein, Son of Sevenless (Sos), which can catalyze Ras GTP/GDP exchange. After growth factor stimulation, the tyrosine phosphorylated EGF receptor binds the Grb2/Sos complex, translocating it to the plasma membrane. This translocation is thought to bring Sos into close proximity with Ras, leading to the activation of Ras. In contrast, the insulin receptor does not bind Grb2 directly but rather induces the tyrosine phosphorylation of two proteins, insulin receptor substrate-1 and Shc, that bind the Grb2/Sos complex. Once Ras is activated, it proceeds to stimulate a cascade of protein kinases that are important in a myriad of growth factor responses.

Naive (ground) versus primed (poised) states of ESCs:

Tabula rasa versus richly set table (abgeschabte Schreibtafel; metaphorisch leergefegter Tisch versus reich gedeckter Tisch)

m6A mRNA methylation facilitates resolution of naïve pluripotency toward differentiation
Naïve and primed pluripotent states retain distinct molecular properties, yet limited knowledge exists on how their state transitions are regulated. Here, we identify **Mettl3**, an N6-methyladenosine (m6A) transferase, as a regulator for terminating murine naïve pluripotency. Mettl3 knockout preimplantation epiblasts and naïve embryonic stem cells are depleted for m6A in mRNAs, yet are viable. However, they fail to adequately terminate their naïve state and, subsequently, undergo aberrant and restricted lineage priming at the post-implantation stage, which leads to early embryonic lethality. m6A predominantly and directly reduces mRNA stability, including that of key naïve pluripotency-promoting transcripts. This study highlights a critical role for an mRNA epigenetic modification in vivo and identifies regulatory modules that functionally influence naïve and primed pluripotency in an opposing manner.

In summary, we identify m6A mRNA methylation as a regulator acting at molecular switches, during resolution of murine naïve pluripotency, to safeguard an authentic and timely downregulation of pluripotency factors, which is Needed for proper lineage priming and differentiation. These findings set the stage for dissecting the role of m6A in other developmental transitions (12, 13) and for exploring other potential regulatory roles for m6A and its **reader proteins.** (Geula et al., Science 2015)

Triple Knockout of Dnmt1, 3a, 3b stabilizes naive state of ESCs.

5-Hydroxymethylcytosin (hmC) → fC → acC →?→ dC –(Dnmts) → mC –(TET)→ hmC
(Münzel et al., Ang. Chem. Intl. Ed. 2011)

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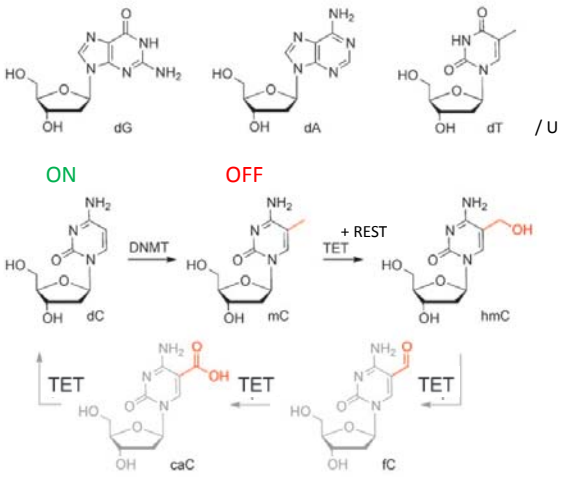
Die sechste Base des Genoms (hmC):



Schema 1. Die kanonischen DNA-Nukleoside dG, dA, dT und dC. Cytosin kann zu mC und hmC modifiziert werden. hmC könnte weiter zu den möglichen Demethylierungsintermediaten fC und caC oxidiert werden, diese wurden jedoch noch nie in vivo nachgewiesen.

Obligatorische Kofaktoren:
O_2
Glukose Glykolyse!
α-Ketoglutarat

Und weniger
oxidative Phosphorylierung

2011
DOI: 10.1002/ange.201101547
5-Hydroxymethylcytosin, die sechste Base des Genoms
Martin Münzel, Daniel Globisch und Thomas Carell*

TET 1-3: ten-eleven translocation hydroxylase 1-3

Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass (Guo et al., Stem Cell Report 2016)

Oct4 Gene has 2 enhanchers, one active in naive mESCs and the other in primed mESCs. (Choi et al., Stem Cell Report 2016)

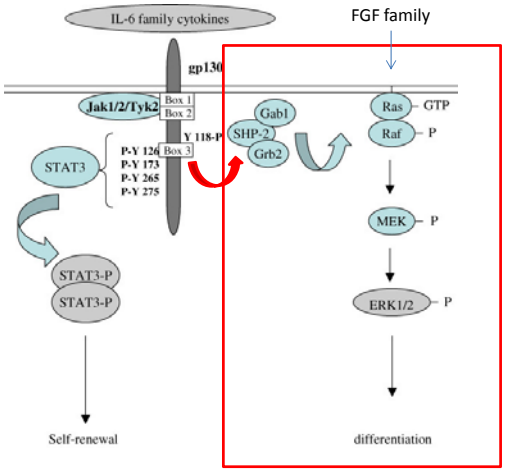
See also https://www.youtube.com/watch?v=waexG16WZrE

„Tankyrase = Poly-ADP-ribosyltransferase drives hESCs into the primed state" (siehe http://www.uniprot.org/uniprot/O95271)

Tankyrase inhibition *with XAV939 (3. i)* promotes a stable human naïve pluripotent state with improved functionality (Zimmerlin et al., Development 2016)

→ 3i + LIF für hESC ausreichend um naiven Zustand zu stabilisieren.

Verbindung zwischen LIF/IL6 Siganling und FGF Signaling

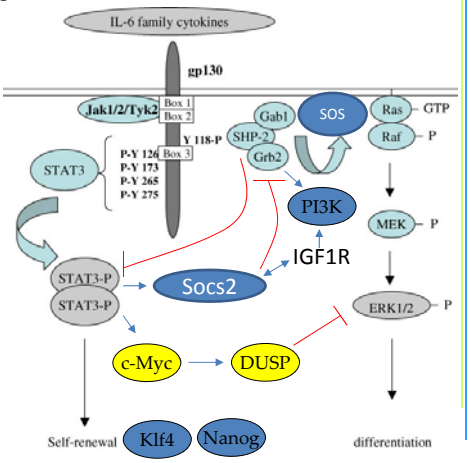


Verbindung zwischen LIF und FGF Signalübertragung

LIF kann auch zu einer Förderung der Differenzierung von ESCs beitragen.

LIF kann auch zu einer Inhibierung der Phopho-STAT3 vermittelten Proliferation von ESC beitragen.

…weil die Phosphatase SHP2, wenn sie an Y118 des gp130 bindet aktiviert wird (durch Phosphorylierung durch JAKs) und so, die stady-state concentration von Phospho-STAT3 reduziert.



Socs2 … Suppressor of cytokine signaling 2
Grb2 … growth factor receptor bound 2
Gab1 … GRB2-Associated Binding Protein 1
SHP-2 … Src-homology 2 domain (SH2)-containing protein (Phosphatase)
DUSP … Dual specific phosphatase

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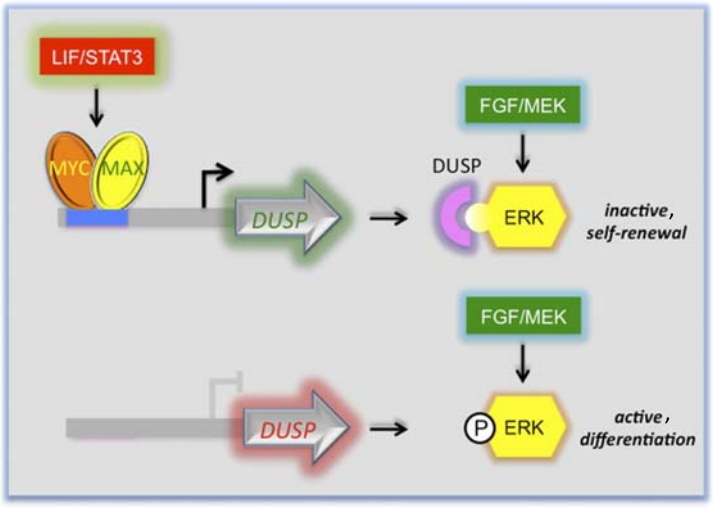
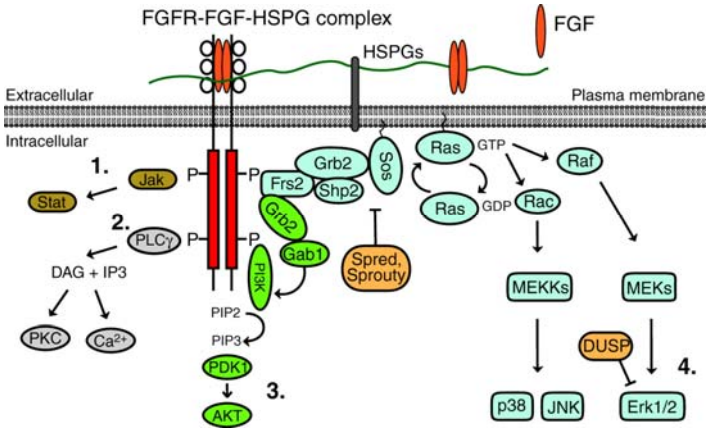
A model for ERK regulation by MYC/MAX complexes in murine pluripotent cells.



James Chappell et al. Genes Dev. 2013;27:725-733

FGF signaling in stem cells

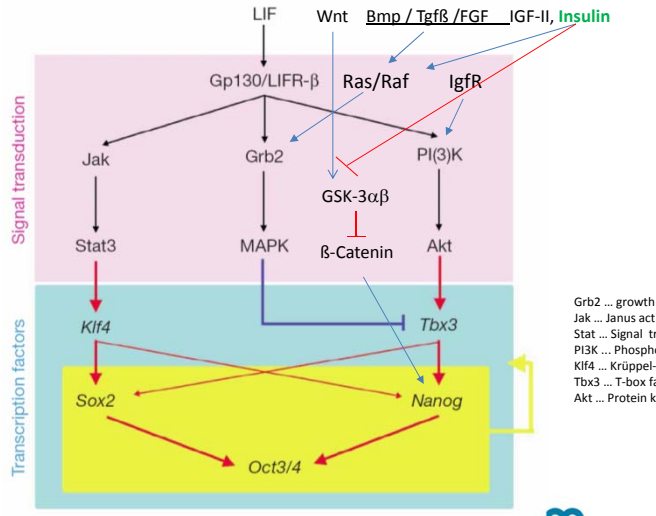


http://dev.biologists.org/content/137/20/3351

A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells and hESCs

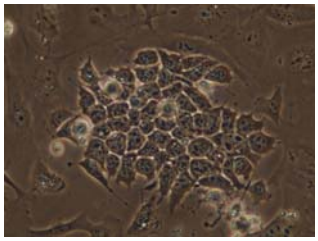
an extremely redundant system

Hitoshi Niwa[1,2,3], Kazuya Ogawa[1], Daisuke Shimosato[1,2] & Kenjiro Adachi[1]
nature Vol 460|2 July 2009|doi:10.1038/nature08113



Grb2 … growth factor receptor binding protein 2
Jak … Janus activated kinase
Stat … Signal transducer and activator of transcription
PI3K … Phosphoinositid-3-Kinasen Phoshpatydyl inositol
Klf4 … Krüppel-like factor 4
Tbx3 … T-box factor 3
Akt … Protein kinase B (PKB)

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-Xenotransplanation)

2.2.4.5. Embryoid body formation and Organoid cultures

http://www.brighamandwomens.org/Research/depts/Medicine/Rheumatology/Lists/Kanasaki/default.aspx

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The most widely used selection agents for transfected mammalian cells carrying the resistance genes Neo^R, Hyg^R and $Puro^R$/Pac respectively.

G418 Sulfate: An aminoglycoside antibiotic similar to gentamycin. Toxic to bacteria, yeast, higher plant and mammalian cells in addition to protozoans and helminths.
neo^R: **aminoglycoside 3'-phosphotransferase**

Hygromycin B: An aminoglycoside antibiotic that inhibits protein synthesis in bacteria, fungi and higher eukaryotes.
hyg^R: **aminocyclitol phosphotransferase**

Puromycin dihydrochloride: A broad spectrum antibiotic that inhibits protein synthesis in both prokaryotic and eukaryotic organisms.
$puro^R$: **puromycin-N-acetyltransferase**

Reporter Konstrukte für transgene Mäuse um die Expression eines Genes in Mäusen zu lokalisieren: LacZ Gen und eFGP, mCherry, etc. Minigene.

pMSG für die Superovulation bei Mäusen — Ethisch problematisch

Teratoma assay — Ethisch problematisch

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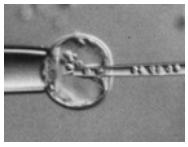
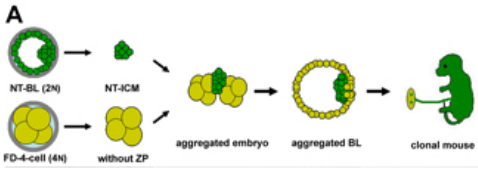
2.2.4.1. Blastozyst ESC Injection and Generation of transgenic and knockout mice



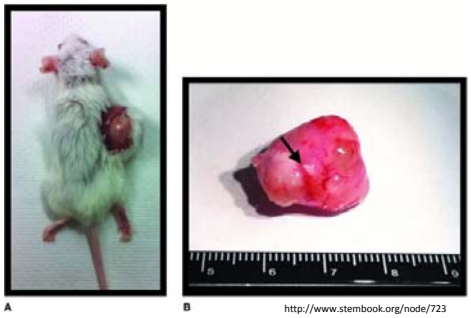
https://research.uci.edu/facilities-services/tmf/services/es-cell.html

2.2.4.2. Tetraploidaggregation



http://english.sibs.cas.cn/ns/es/201104/t20110411_67535.html

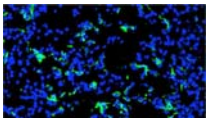
2.2.4.3. Teratomaformation



http://www.stembook.org/node/723

2.2.4.4. Chimeraformation

Human – pig / cow / rabbit embryos



http://dx.doi.org/10.1016/j.cell.2016.12.036

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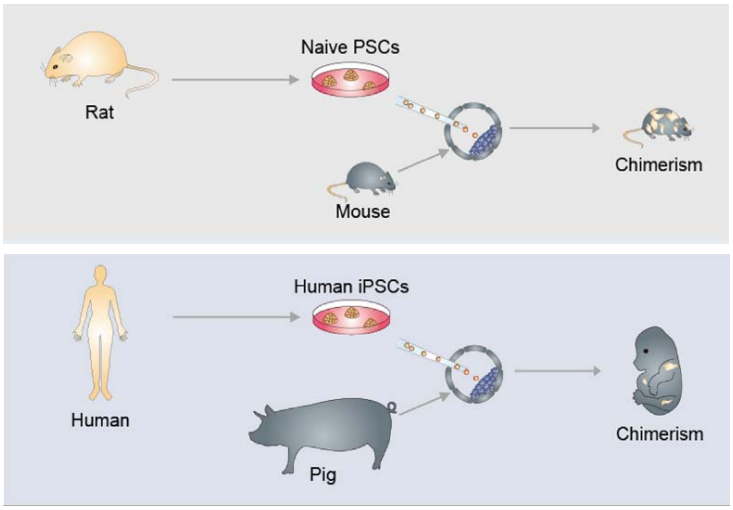
Interspecies Chimerism with Mammalian Pluripotent Stem Cells

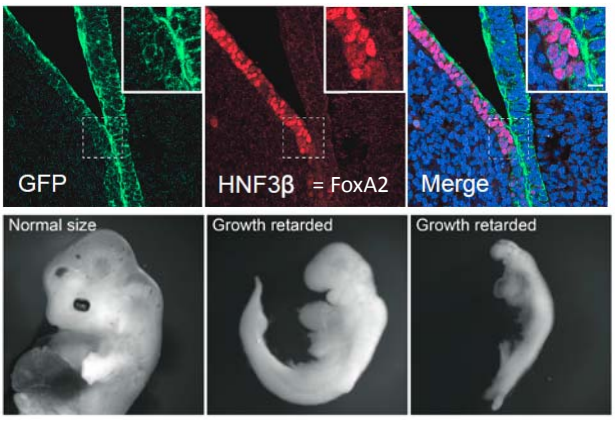
Cell Jänner 2017

Nachtrag zu 2.2.4.4.



http://dx.doi.org/10.1016/j.cell.2016.12.036

Schwein-Mensch Chimera E28



Cell. 2017 Jan 26;168(3):473-486.e15. doi: 10.1016/j.cell.2016.12.036.

Bis wann läßt man diese Embryonen am Leben? Ethisch problematisch