

Changing fibroblasts into neurons: Direct lineage conversion

Journal Club

Uli Herrmann

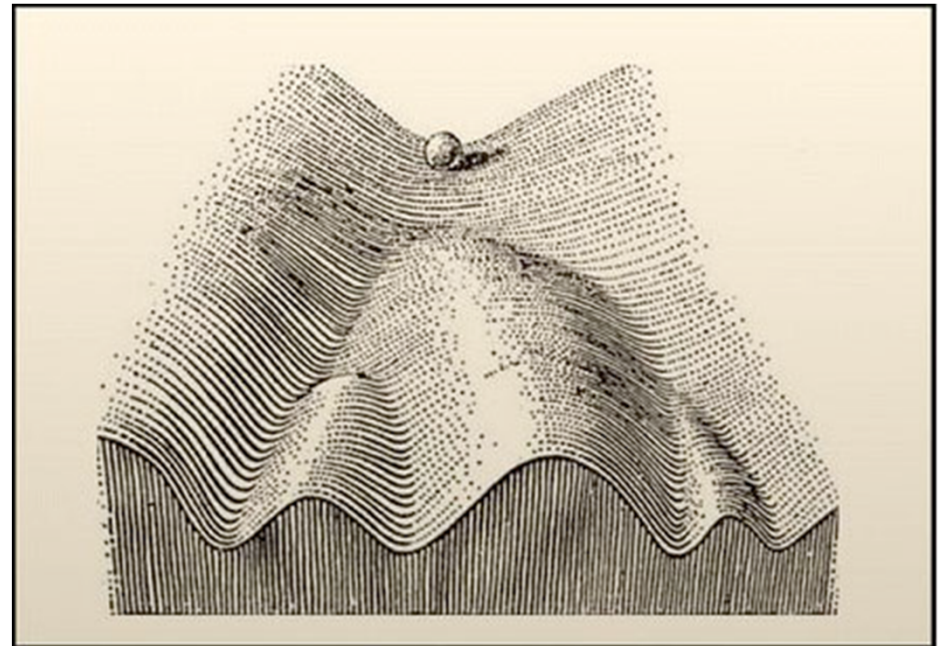
25.6.13

Outline

- Epigenetic landscape model
- Reprogramming cells
- Induced pluripotent stem cells
- Three papers:
 - Direct conversion of fibroblasts to functional neurons by defined factors. *Vierbuchen 2010 Nature*
 - Generation of induced neurons via direct conversion in vivo. *Torper 2013 PNAS*
 - Generation of oligodendroglial cells by direct lineage conversion. *Yang 2013 Nature Biotechnology*
- Outlook

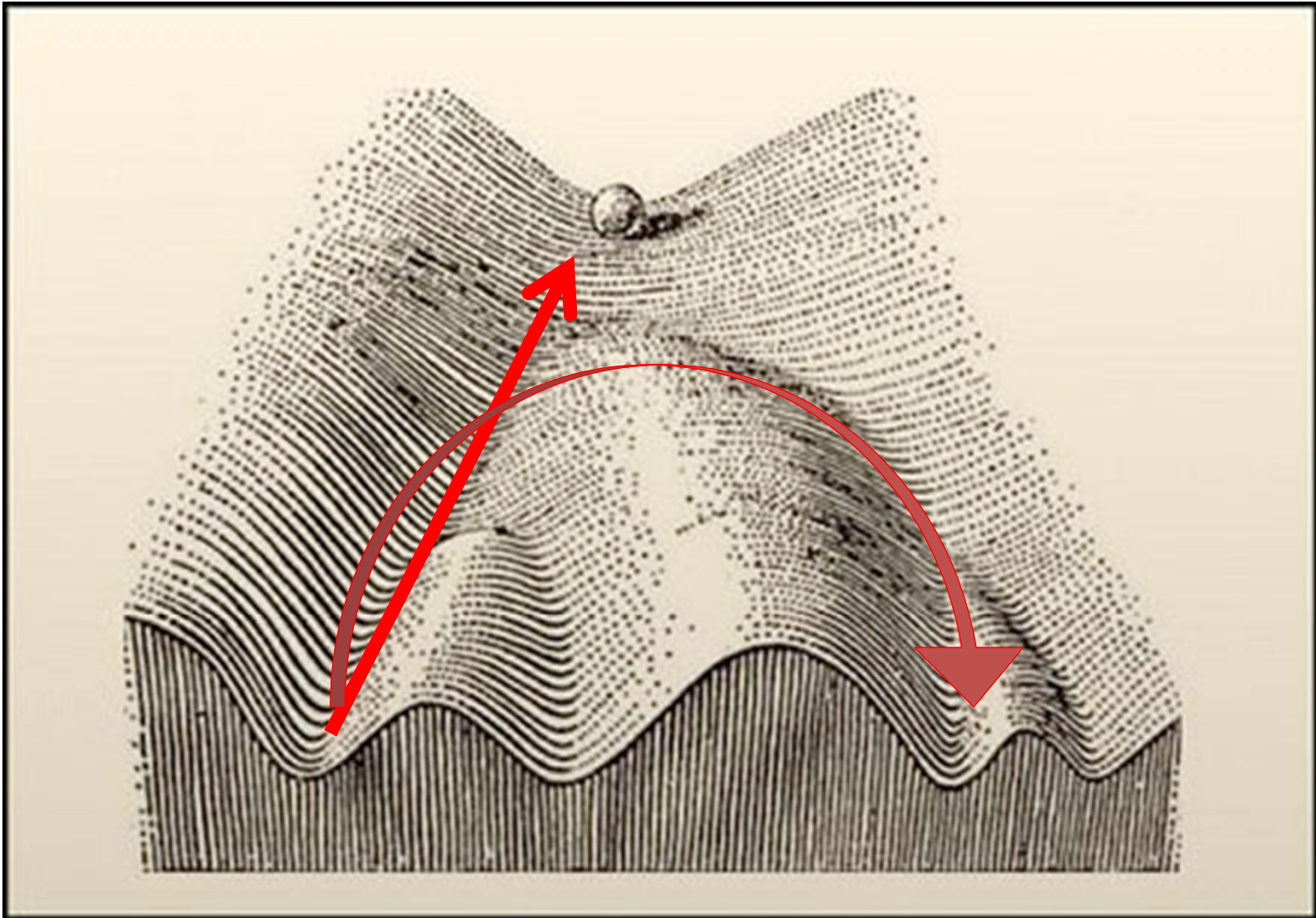
Epigenetic landscape model

- ball, representing a stem cell, rolling down a hill marked by uneven slopes and valleys
- hill represents the cell differentiation process
- genes shape the features on that hill
- slopes and valleys ultimately channel the ball towards a favored position at the bottom of the hill

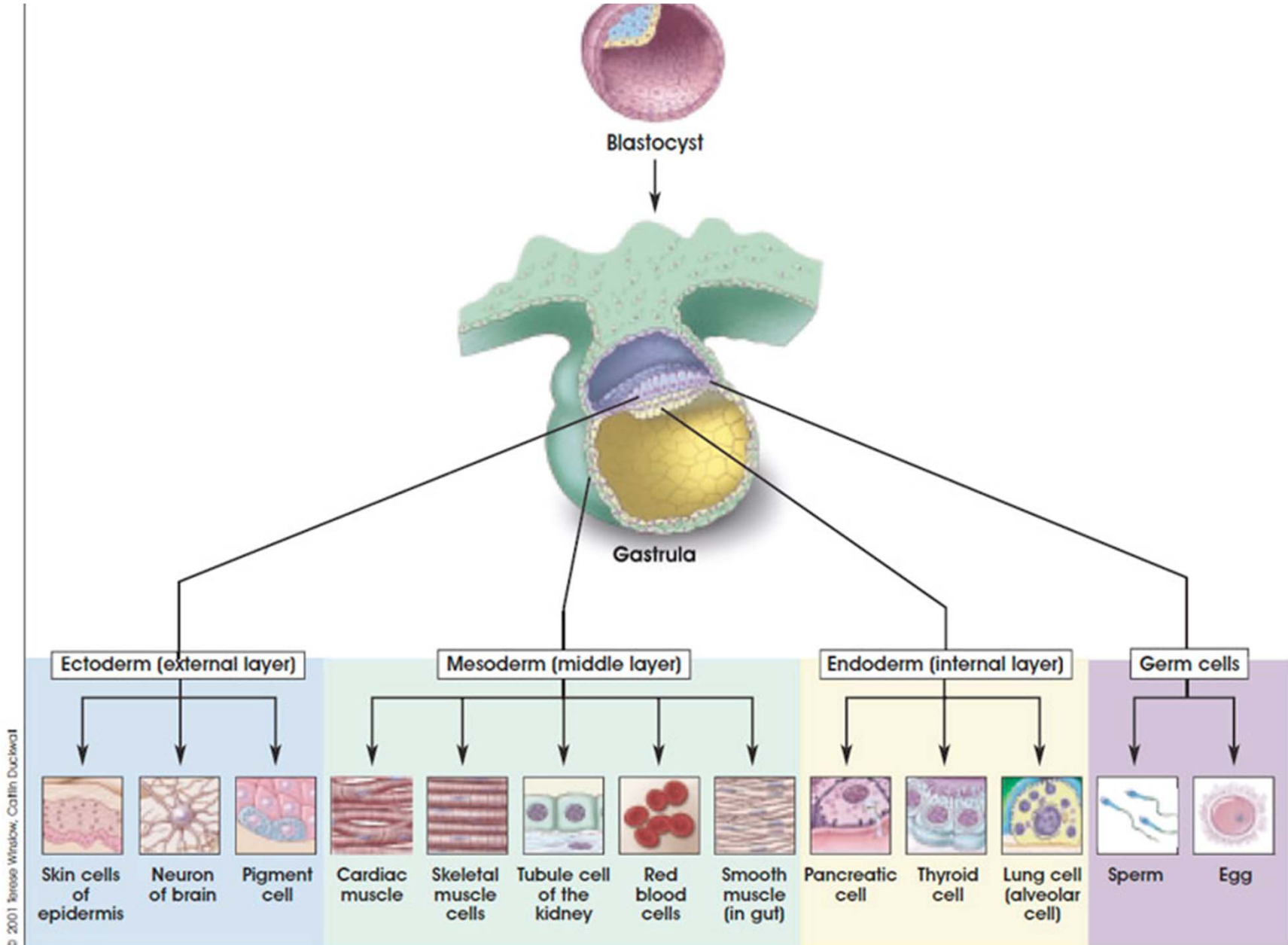


Waddington, CH. The Strategy of the Genes. A Discussion of Some Aspects of Theoretical Biology (Allen & Unwin, 1957)

Basic Idea: Reprogramming cells



The different germ layers

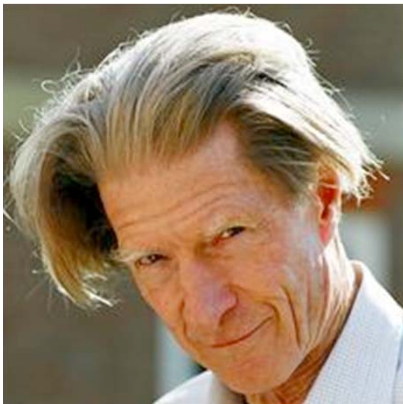
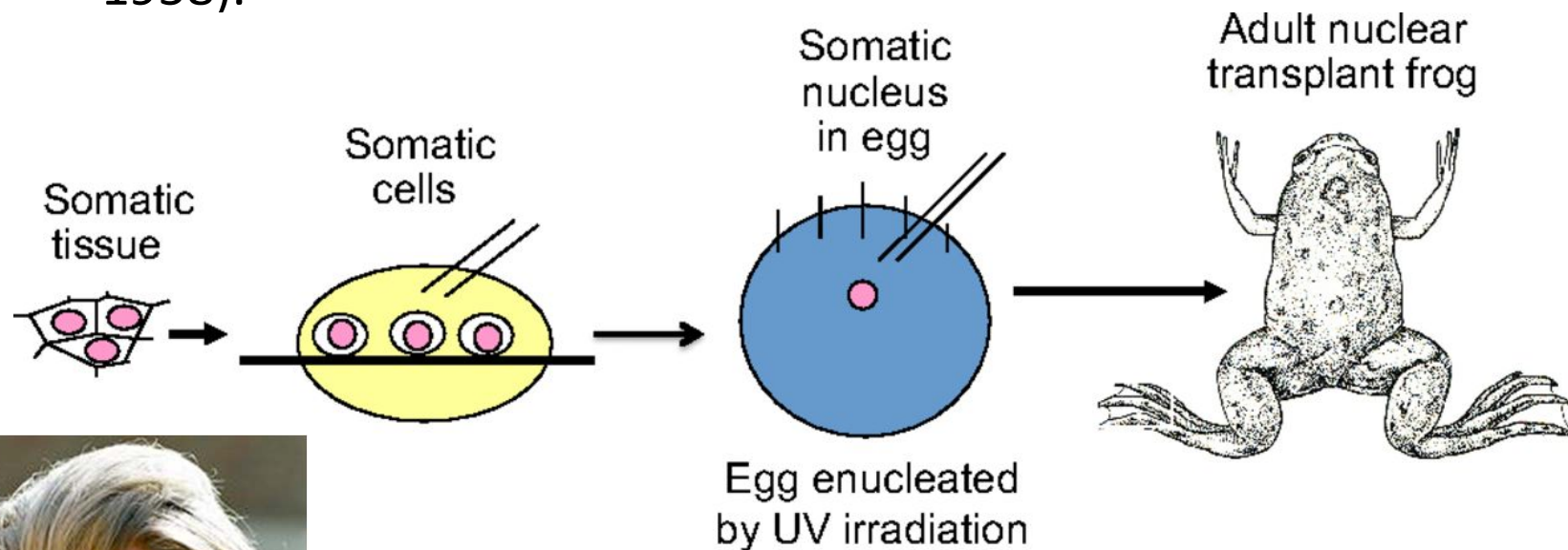


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http://pnp.med.unsw.edu.au/embryology/images/a/ab/inner_cell_mass_cartoon.jpg

Somatic cell nuclear transfer experiment using unfertilised eggs

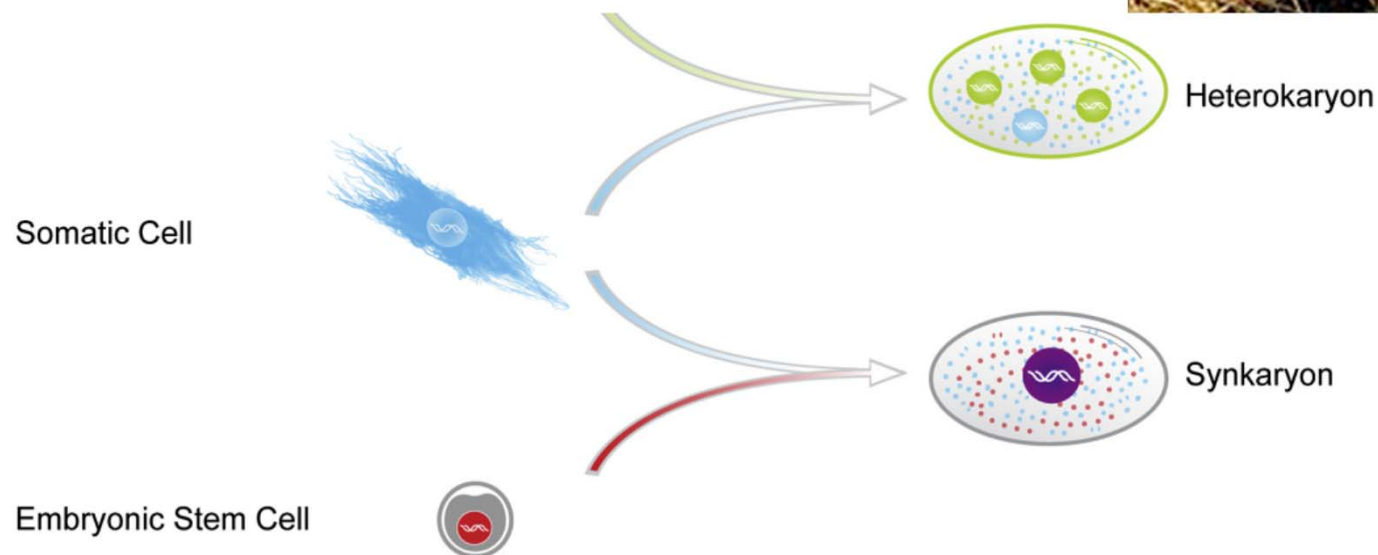
- Design of a somatic cell nuclear transfer experiment using unfertilised eggs as first designed by Briggs and King (Briggs and King, 1952).
- Enucleation in *Xenopus* by ultraviolet light irradiation (Gurdon, 1958).



Gurdon JB, 1958 Nature; Gurdon J B Development 2013;140:2449-2456

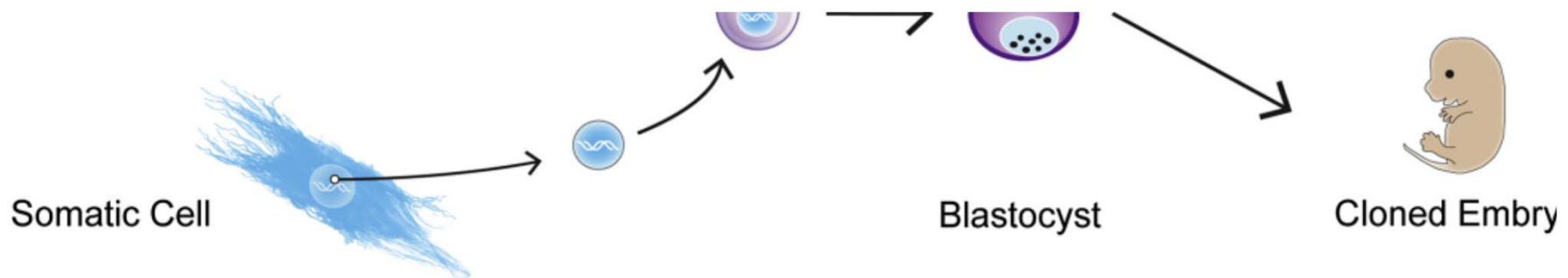
Somatic cell nuclear Transfer

- In 1996 Campbell, Wilmut and colleagues successfully generated live offspring from the nucleus of a mammalian somatic cell



Reprogramming by cell fusion

- use of embryonic stem cells as an alternative to oocytes for reprogramming human somatic nuclei.
- Human embryonic stem (hES) cells fused with human fibroblasts, resulting in hybrid cells that maintain a stable tetraploid DNA content and have morphology, growth rate, and antigen expression patterns characteristic of hES cells.
- hES cells can reprogram the transcriptional state of somatic nuclei.
Cowan 2005 Science



Cell Fusion

Intra-germ layer fate conversion within the mesoderm from fibroblasts to myoblasts

Cell, Vol. 17, 771–779, August 1979, Copyright © 1979 by MIT

Multiple New Phenotypes Induced in 10T $\frac{1}{2}$ and 3T3 Cells Treated with 5–Azacytidine

Shirley M. Taylor* and Peter A. Jones*†

- Treatment with 5-azacytidine (inhibitor of DNA methylation) caused fibroblasts to spontaneously differentiate into myocytes, chondrocytes and adipocytes
- → DNA methylation is important for preventing expression of genes that regulate differentiation into alternative lineages they express one or more genes capable of inducing myogenic differentiation

Intra-germ layer fate conversion within the mesoderm from fibroblasts to myoblasts

Cell, Vol. 51, 987-1000, December 24, 1987, Copyright © 1987 by Cell Press

Expression of a Single Transfected cDNA Converts Fibroblasts to Myoblasts

**Robert L. Davis,*† Harold Weintraub,*
and Andrew B. Lassar***

- **The transcription factor MyoD is sufficient** to convert fibroblasts into contracting myocytes
- MyoD turned out to be a strong inducer of myogenic genes not only in this particular cell line but also in various other cell types
- expression of MyoD in endodermal and ectodermal cells resulted in cellular phenotypes with atypical morphology

Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

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DOI 10.1016/j.cell.2006.07.024

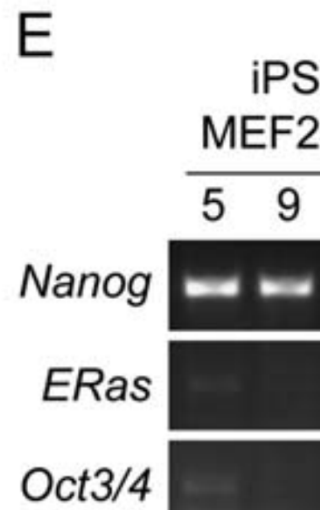
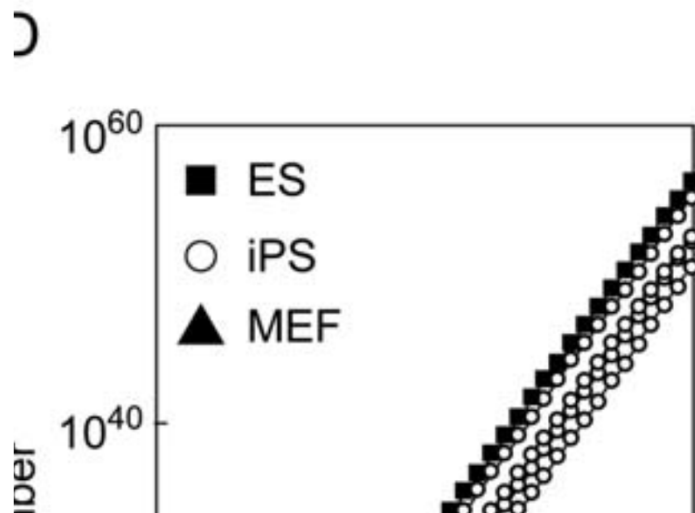
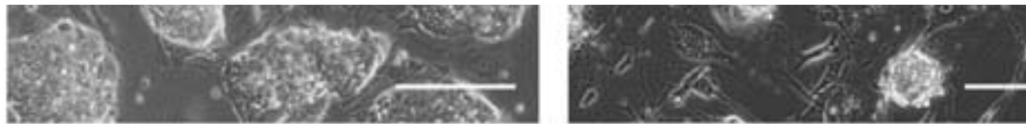
Cell

- Pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few factors



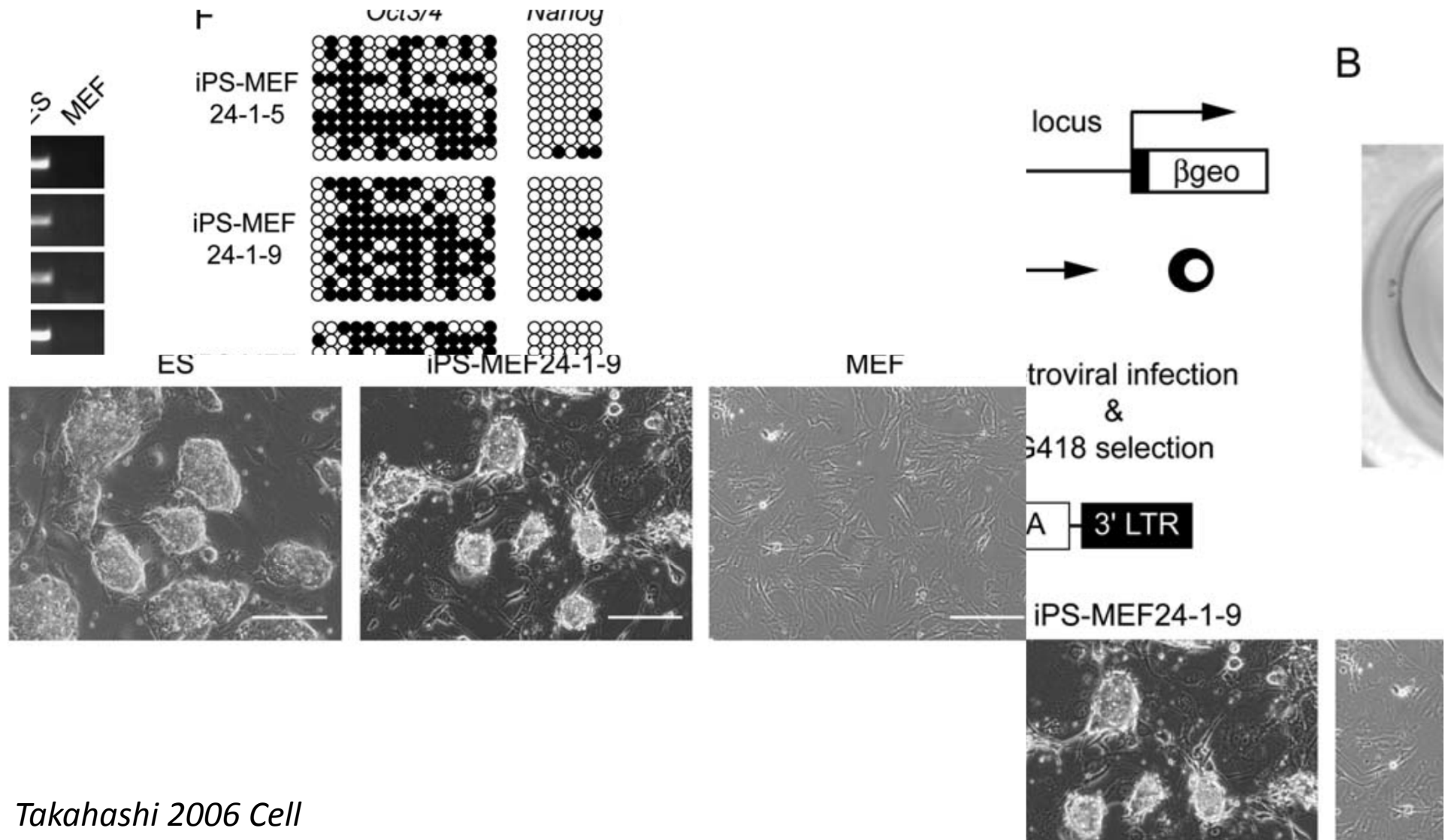
Strategy

- Selection of 24 genes that play pivotal roles in the maintenance of ES cell identity → important to induce pluripotency
- pluripotent state could be detected as resistance to G418
- Insertion of betaGeo cassette (fusion of the betagalactosidase and neomycin resistance genes) into mouse Fbx15 gene by homologous recombination
- Fbx15 is a protein expressed in undifferentiated embryonic stem cells



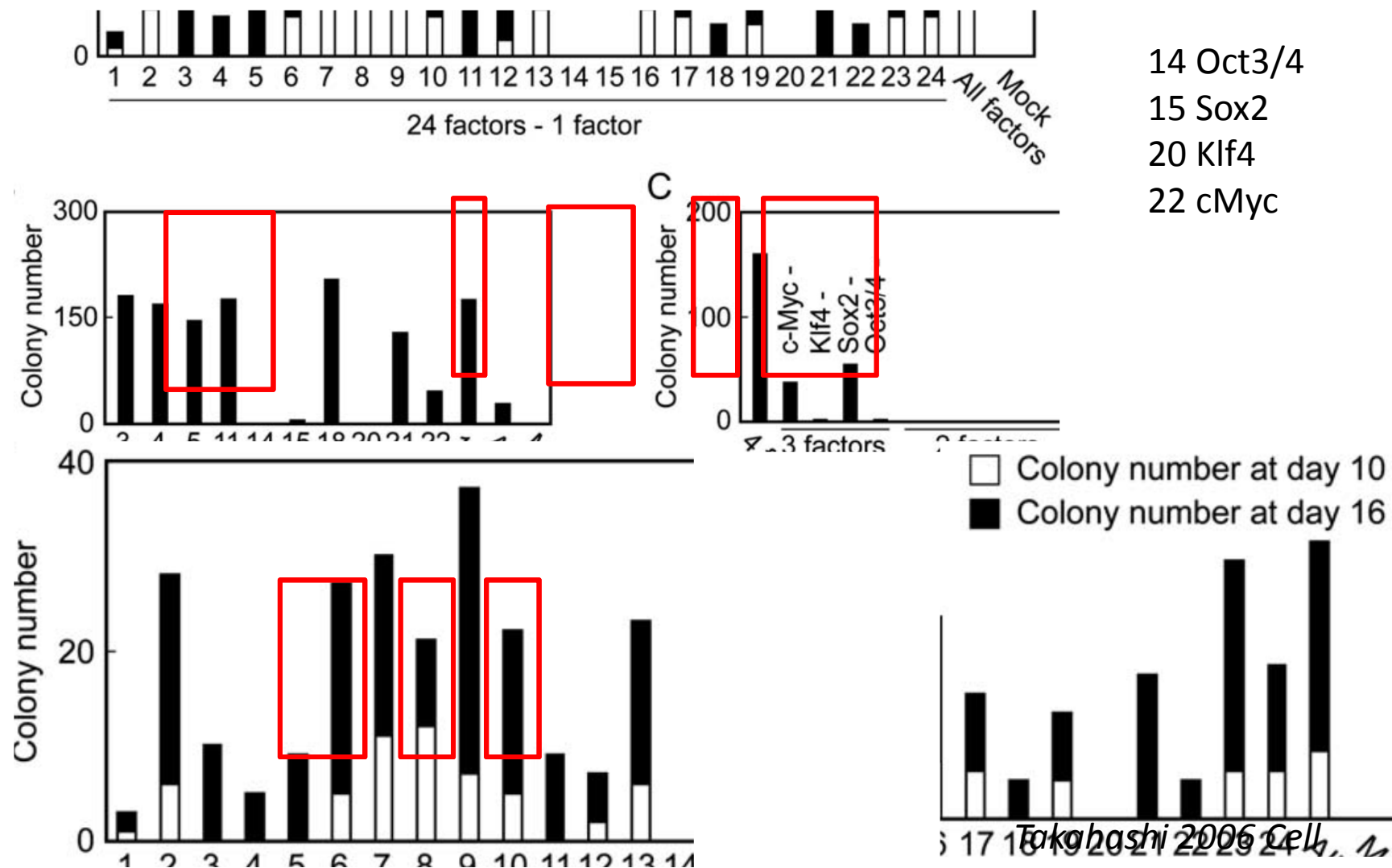
Takahashi 2006 Cell

Generation of iPS from MEF cultures via 24 factors



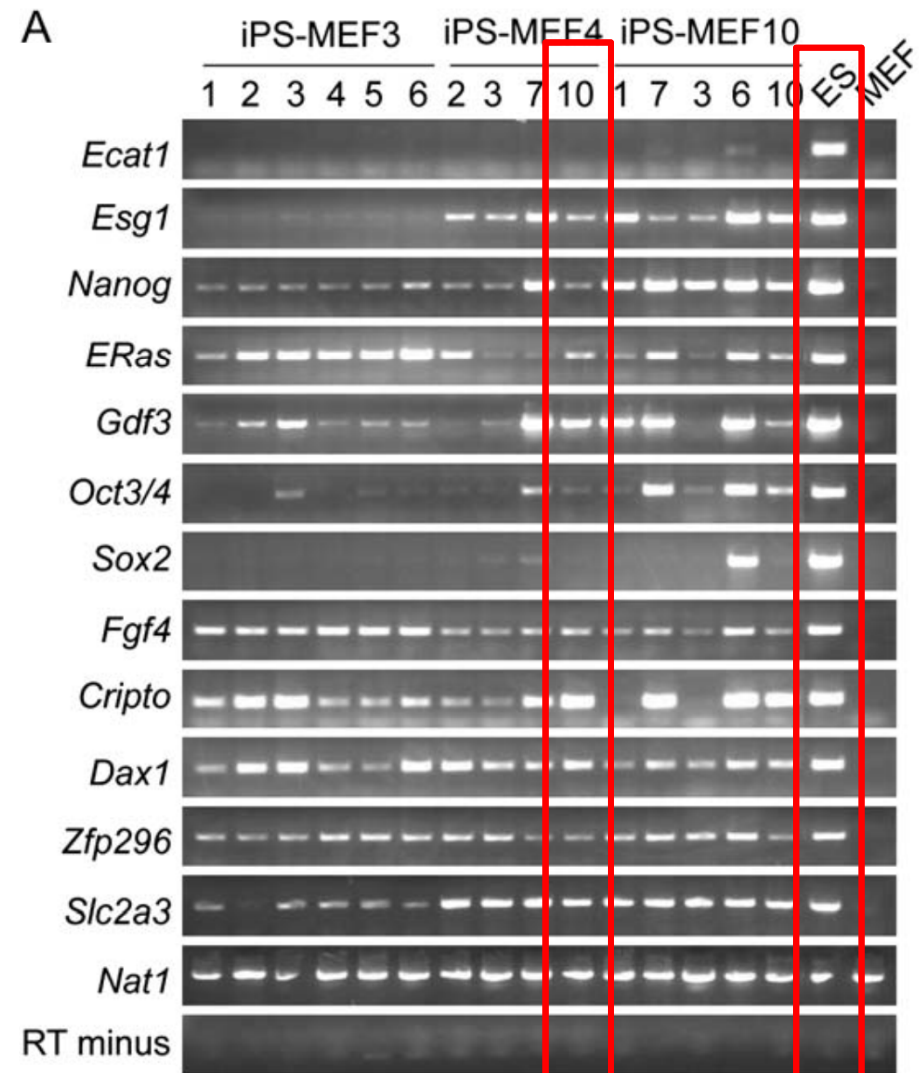
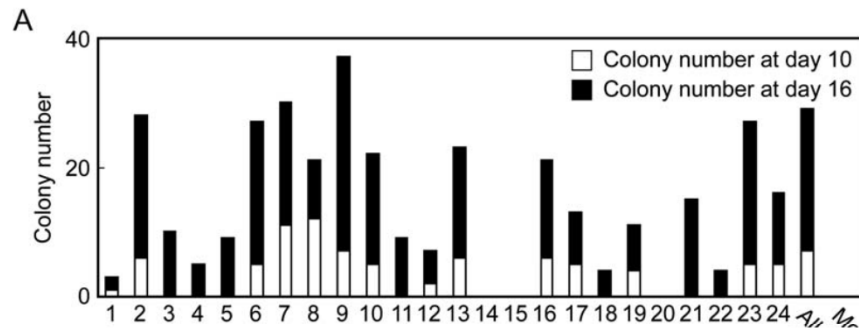
Narrowing down the candidate factors

- Examination of the effect of withdrawal of individual factors from the pool of transduced candidate genes on the formatin of G418-resistant colonies.



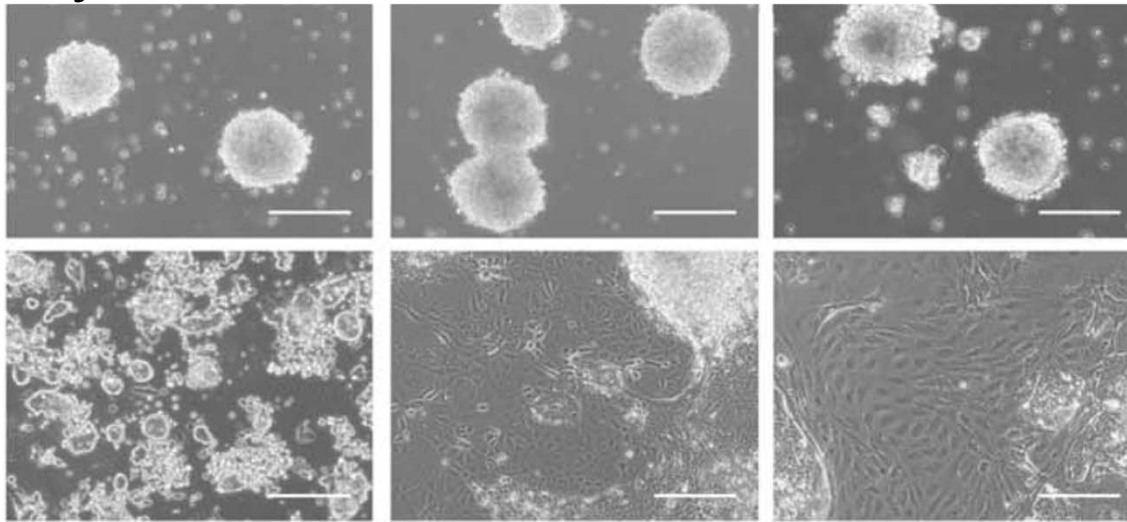
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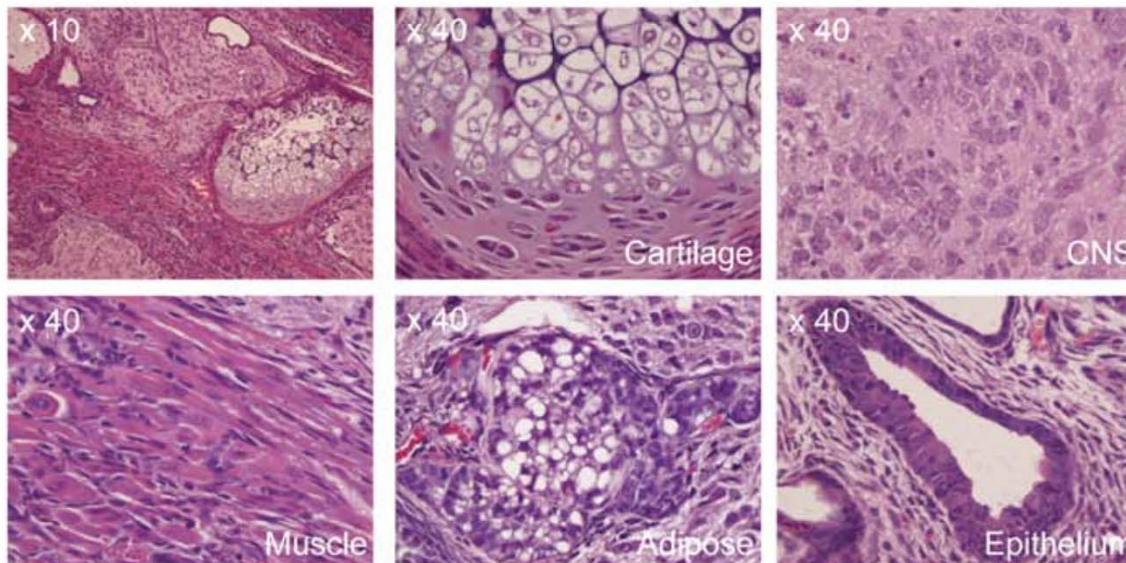


Pluripotency of iPS Cells derived from MEFs

- Injection into nude mice



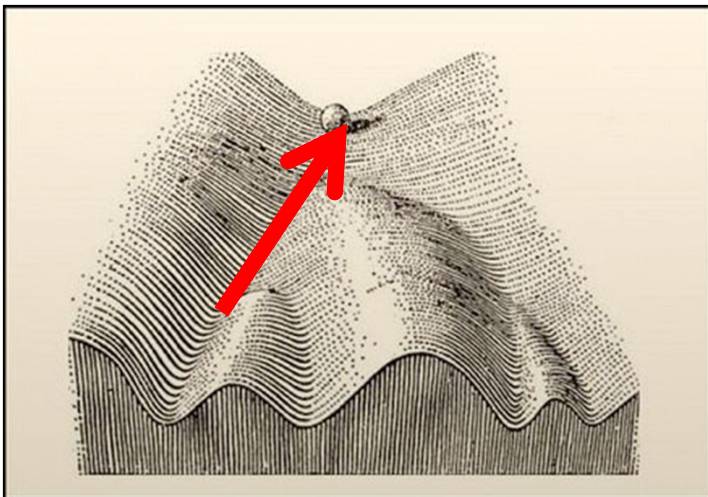
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Takahashi 2006 Cell

Summary

- The OKSM (Oct3/4, Klf4, Sox2, cMyc) could generate induced pluripotent stem (iPS) cells directly from mouse embryonic or adult fibroblast cultures.



Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors

Kazutoshi Takahashi,¹ Koji Tanabe,¹ Mari Ohnuki,¹ Megumi Narita,^{1,2} Tomoko Ichisaka,^{1,2} Kiichiro Tomoda,³ and Shinya Yamanaka^{1,2,3,4,*}

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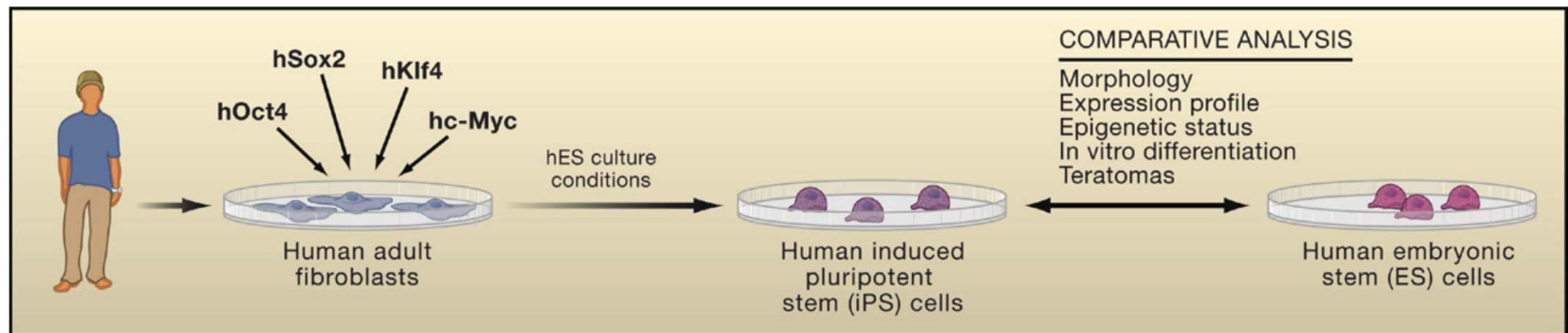
³Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94158, USA

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DOI 10.1016/j.cell.2007.11.019

Cell



Limitations of induced pluripotent stem cells (iPSCs)

- Human embryonic stem cells (HESCs) and iPSCs share basic properties of self-renewal and pluripotency that make them resemble cancer cells.
- When injected into immunodeficient mice they form teratoma
- The OKSM factors are highly expressed in various types of cancer
- iPSC can acquire chromosomal aberrations even more readily than HESCs
- iPSCs are more tumorigenic than HESCs and harbour a risk for teratocarcinomas and possibly somatic tumors

-

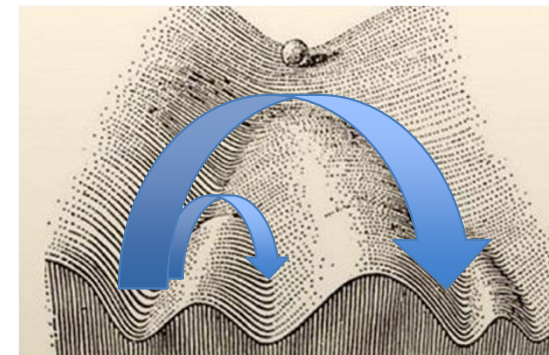
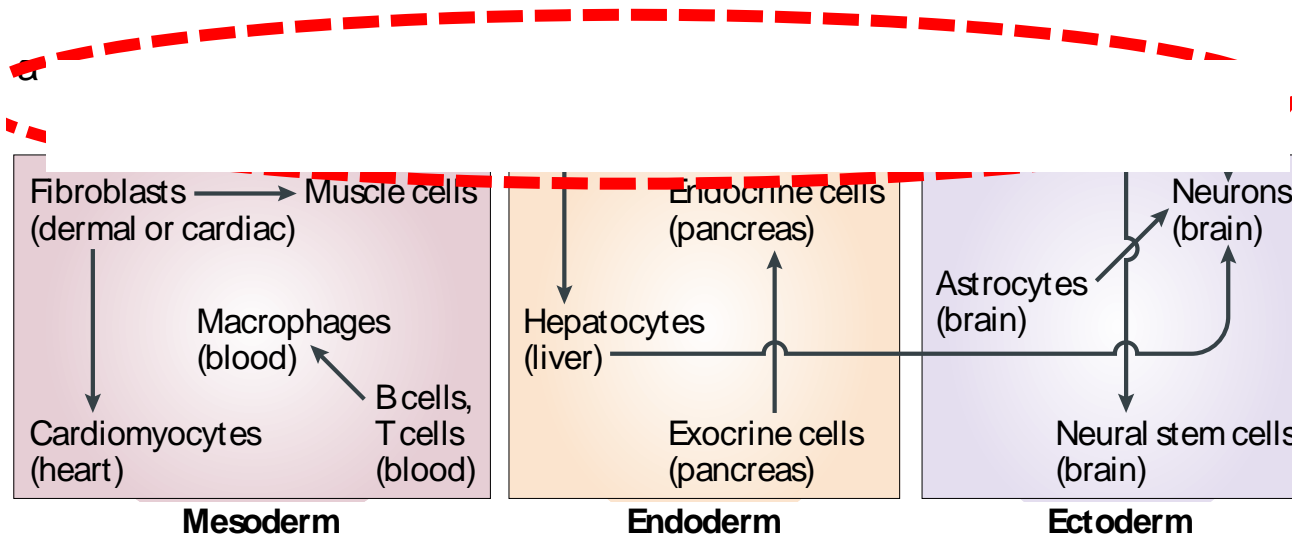
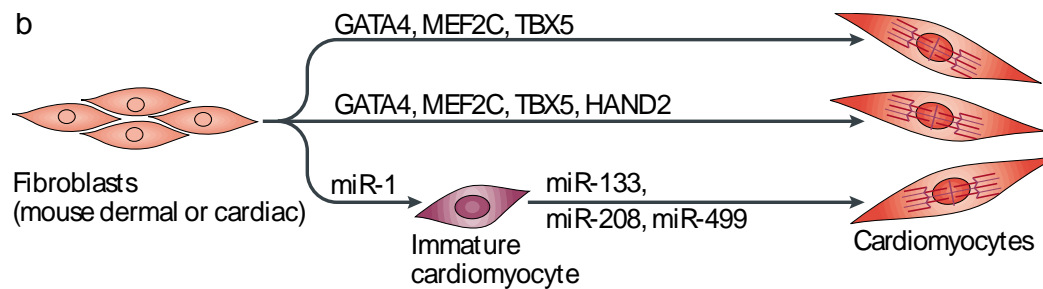
Three general strategies

1. Terminal differentiation or complete elimination of residual pluripotent stem cells from culture
2. Interfering with tumour progression genes to prevent tumour formation
3. Tumour detection and elimination after its initial formation in the patients body

*The tumorigenicity of human embryonic and induced pluripotent stem cells.
Ben-David Nature Reviews Cancer 2011*

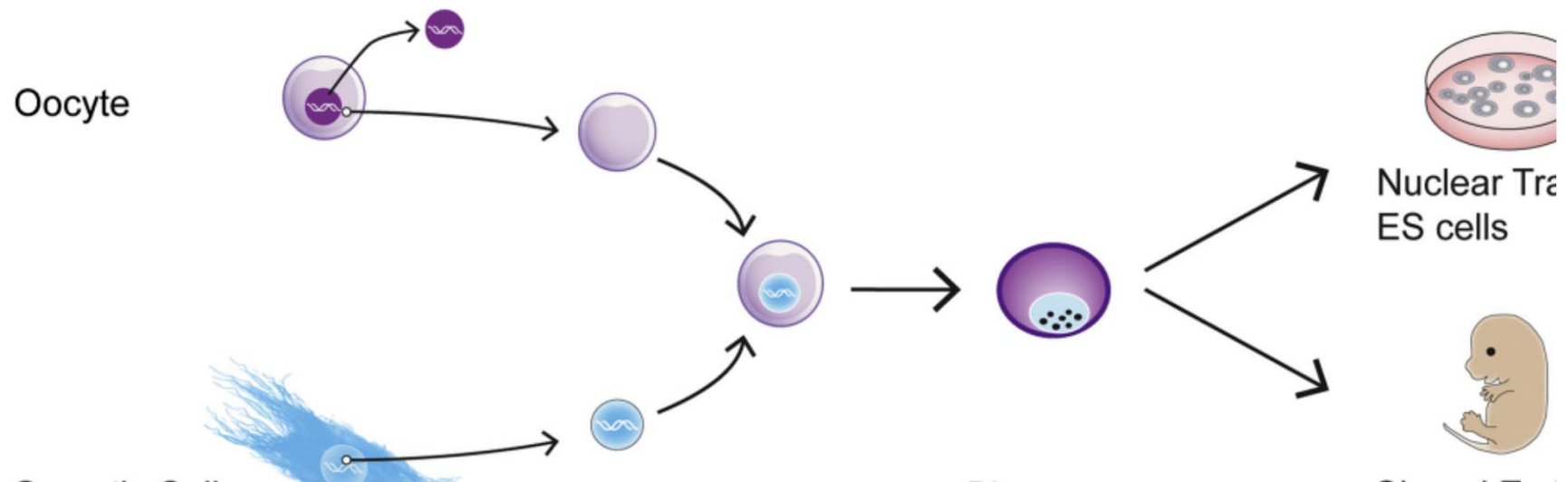
Lineage reprogramming between closely related cell types

- Because they share some epigenetic features as a result of their recent descent from a common progenitor cell



Transcription factor-mediated reprogramming

Somatic Cell Nuclear Transfer



Direct conversion of fibroblasts to functional neurons by defined factors

Thomas Vierbuchen^{1,2}, Austin Ostermeier^{1,2}, Zhiping P. Pang³, Yuko Kokubu¹, Thomas C. Südhof^{3,4} & Marius Wernig^{1,2}

Vol 463 | 25 February 2010 | doi:10.1038/nature08797

nature

- Assuming that multiple transcription factors would probably be required to reprogram fibroblasts to a neuronal fate

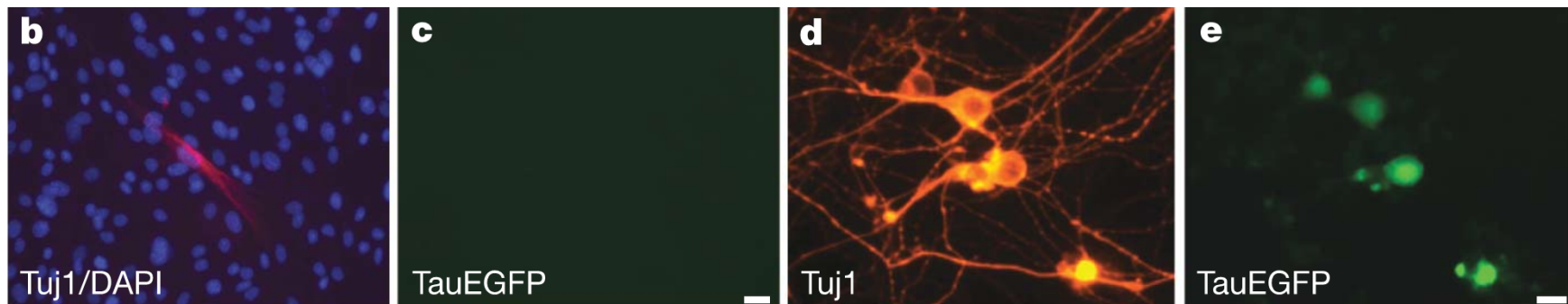
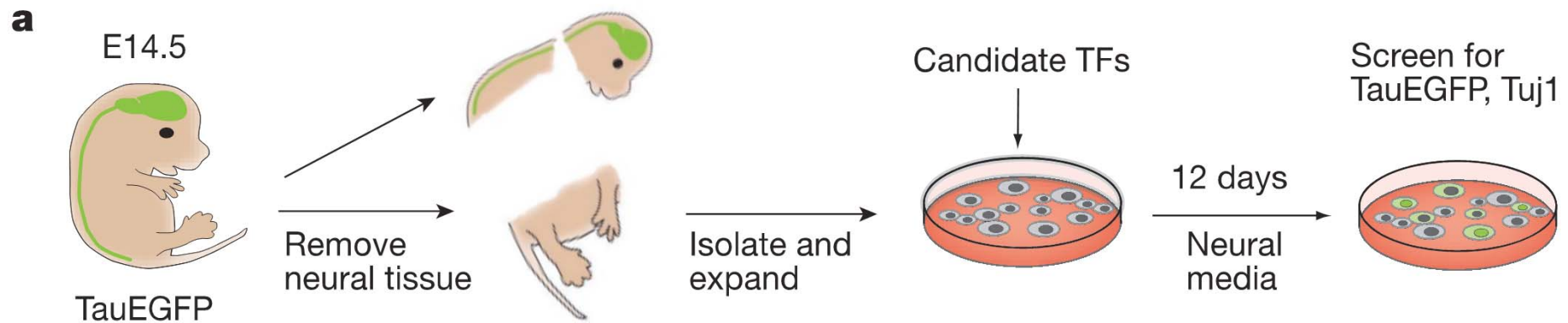
Construction of LV pool with genes implied in differentiation of neural tissue

Gene Name	Gene Bank	
Ascl1	NM_008553	• Cloning of a total of 19 genes, — specifically expressed in neural tissues,
Brn2	NM_008899	
Brn4	NM_008901	
c-myc	NM_010849	— important roles in neural development
Dlx1	NM_010053	— implicated in epigenetic reprogramming
Hes5	NM_010419	
Id1	NM_010495	
Id4	NM_031166	• Pool of lentiviruses containing all 19 genes was prepared (19F) Doxycycline dependent expression
Klf4	NM_010637	
Lhx2	NM_010710	
Mef2c	NM_025282	
Myt1l	NM_001093775	
NeuroD1	NM_010894	
Nhlh1	NM_010916	
Nr2f1	NM_010151	
Olig2	NM_016967	
Pax6	NM_013627	
Sox2	NM_011443	
Zic 1	NM_009573	

Vierbuchen 2010 Nature

Transfection of mouse embryonic fibroblasts (MEFs) from TauEGFP knock-in mice

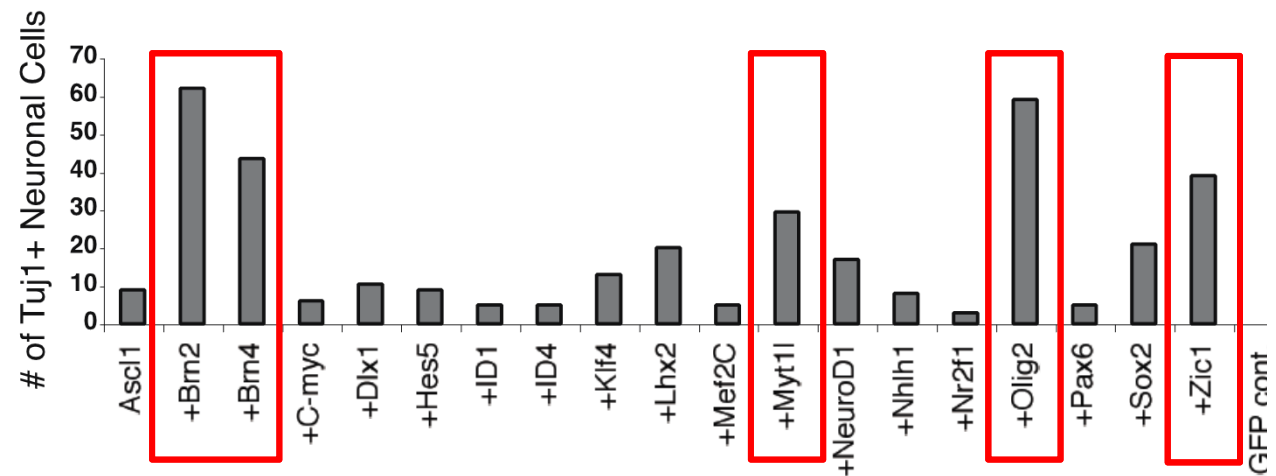
- TauEGFP mice: Only neurons express GFP
- Tuj: Neuron-specific class III beta-tubulin
- 32days post infection



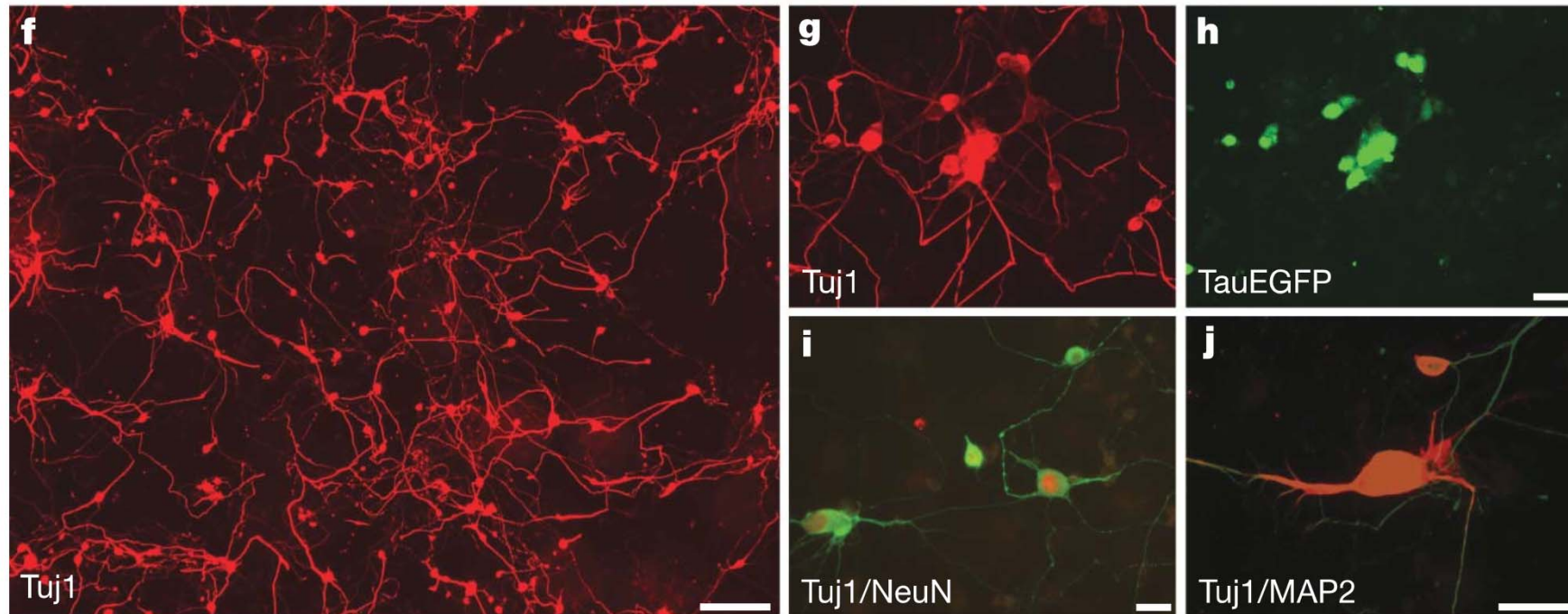
Vierbuchen 2010 Nature

Narrowing down the number of transcription factors

- Individual testing of Ascl1 and Neurod1 (important role in neuronal cell fate determination)
- Ascl1 in combination with the other 18 factors



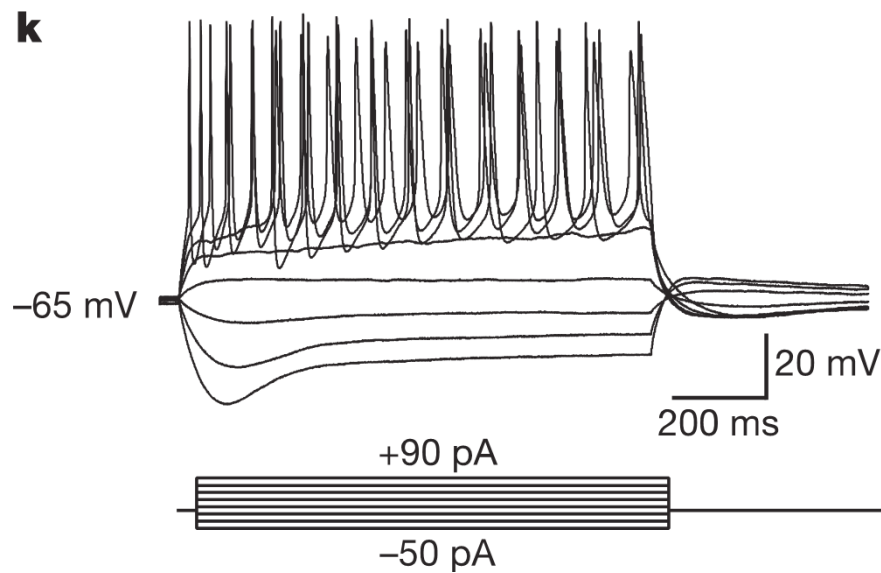
- 5F pools: LV of Ascl1, Brn2, Mtl, Zic1, Olig2



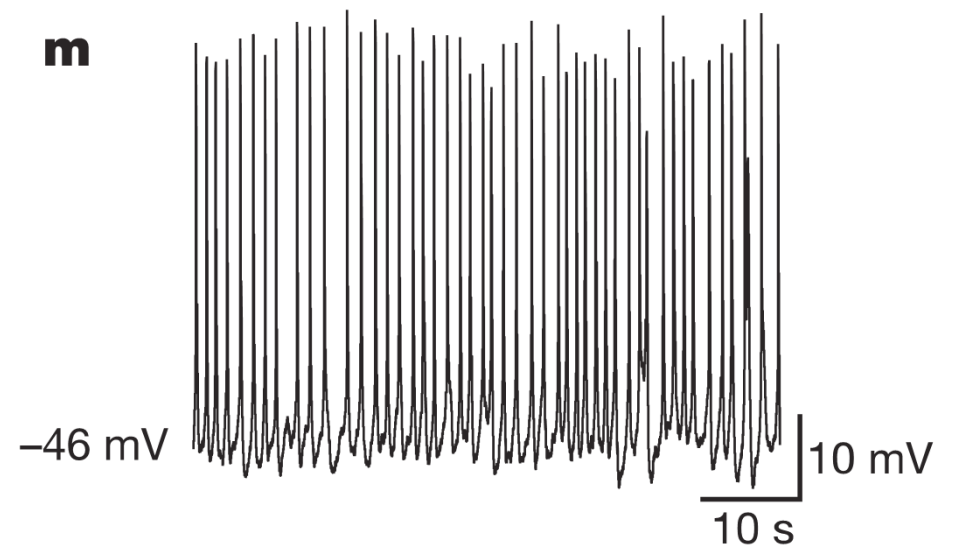
Vierbuchen 2010 Nature

iN cells have functional membrane properties similar to neurons

Membrane potential responding to step depolarization by current injection

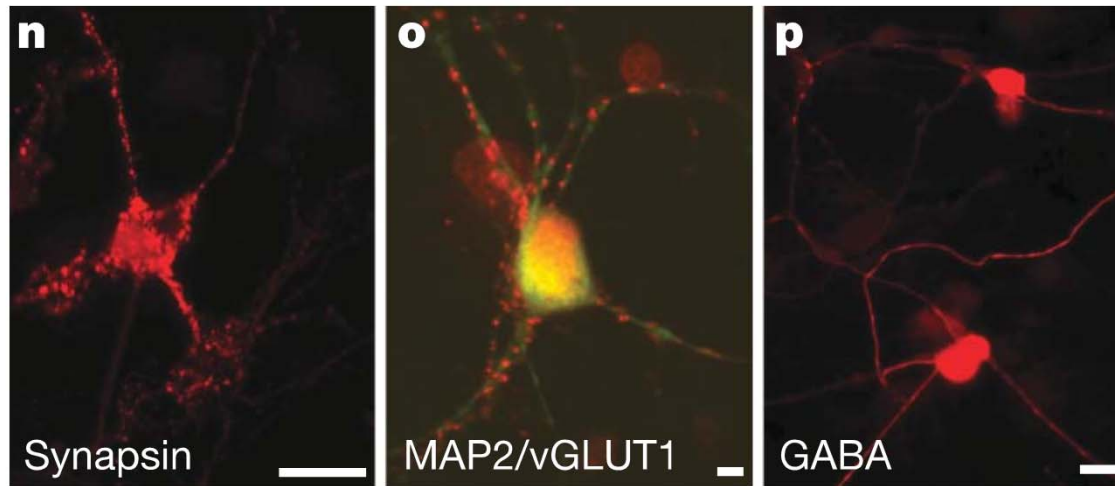


Spontaneous action potentials



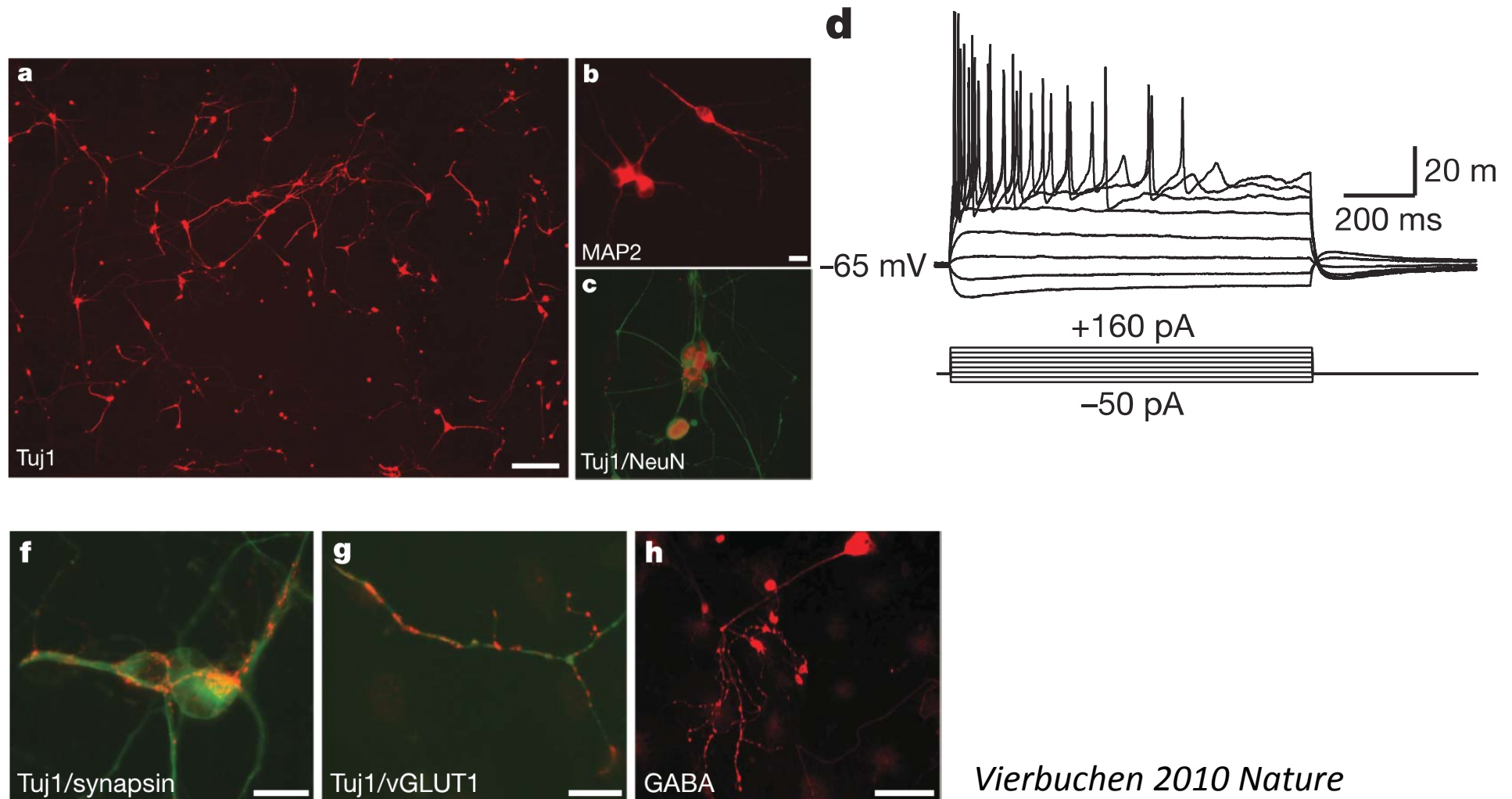
Neurotransmitter phenotype of iN cells

Twenty two days after infection 5F MEF iN cells expressed synapsin and vesicular glutamate transporter 1 (vGLut1) or GABA



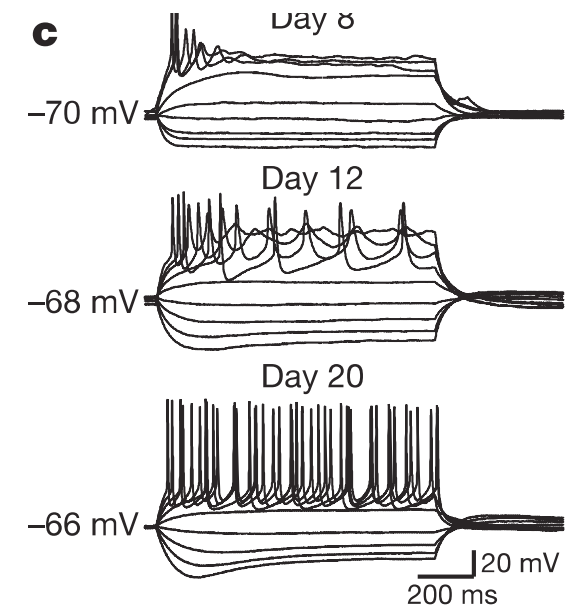
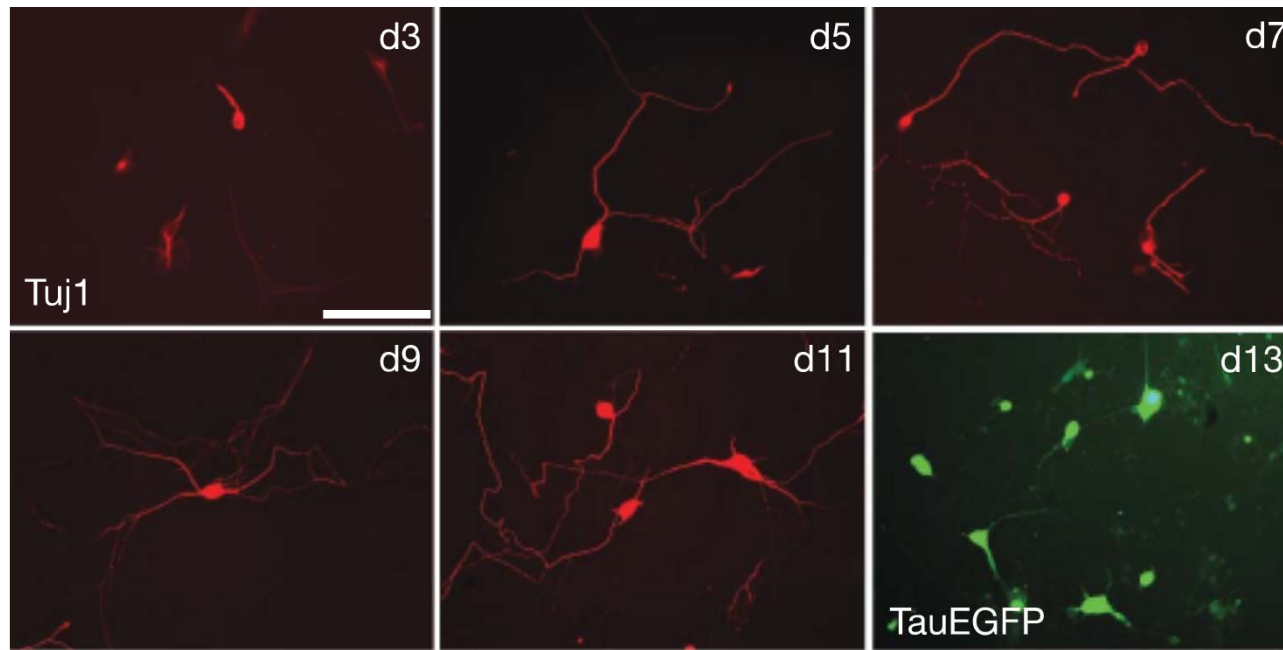
Functional neurons from tail fibroblasts

- Isolation of tail-tip fibroblasts (TTFs) from 3day old Tau eGFP mice and transfection with 5F pool



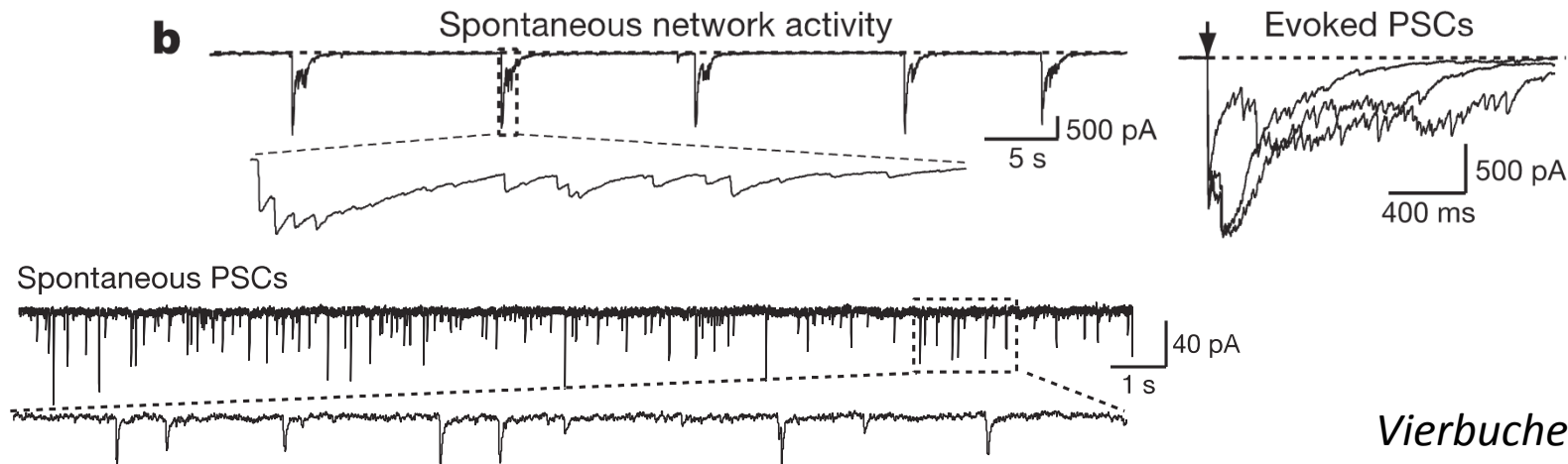
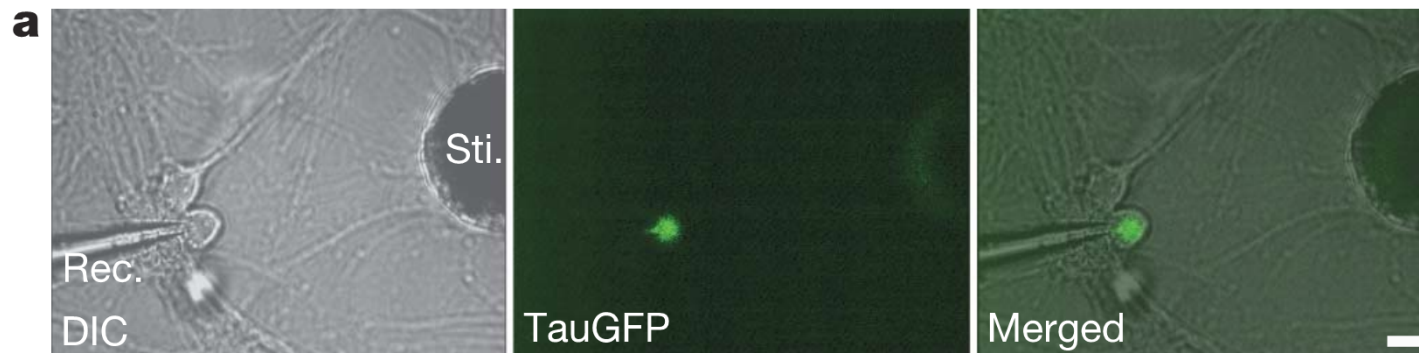
Vierbuchen 2010 Nature

The 5F pool induced conversion is rapid and efficient



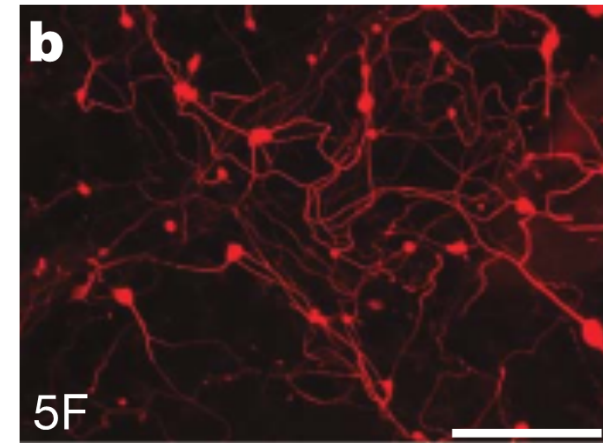
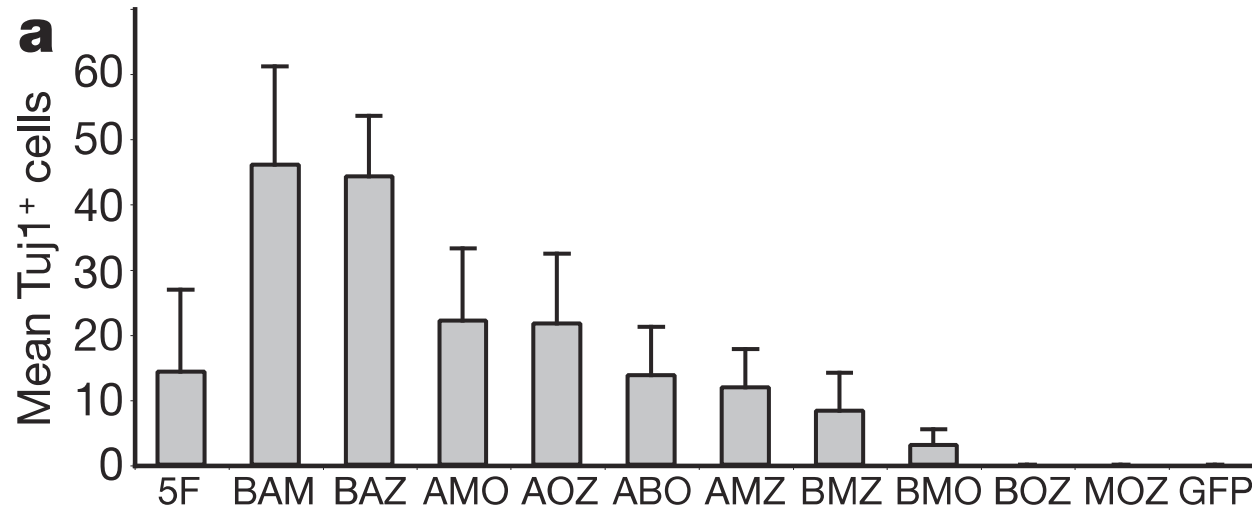
iN cells form functional synapses

- Plating of purified 5F iN cells onto neonatal cortical neurons. Patch clamp recordings from Tau EGFP positive iN cells and detection of spontaneous and rhythmic network activity typical of cortical neurons in culture
- Plating of FACS sorted TauEGFP-positive, MEF derived 5FiN cells onto a monolayer of astrocytes



Genes sufficient for neuronal conversion

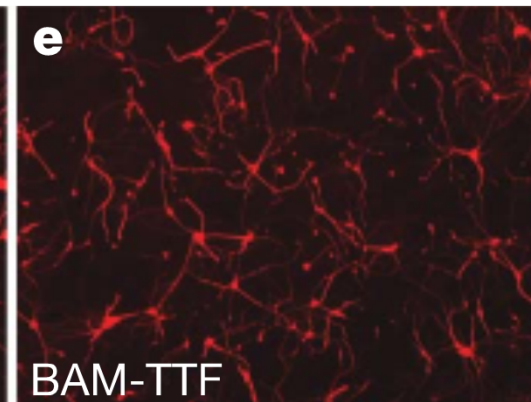
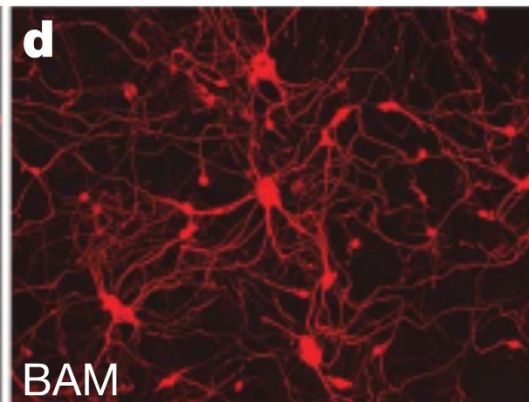
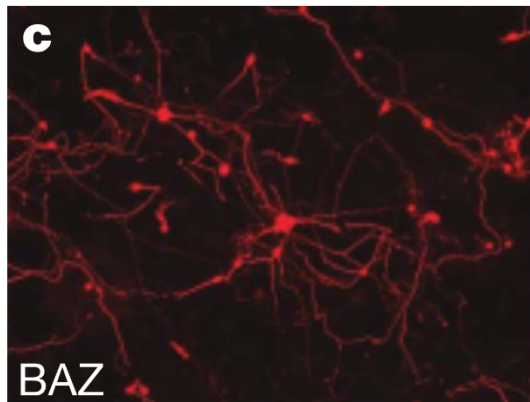
- Only the omission of *Ascl1* had a marked effect on induction efficiency



Ascl1, *Brn2*, *Zic1*

Ascl1, *Brn2*, *Myt1l*

achaete-scute complex like 1, brain-2, myelin transcription factor-like 1



Vierbuchen 2010 Nature

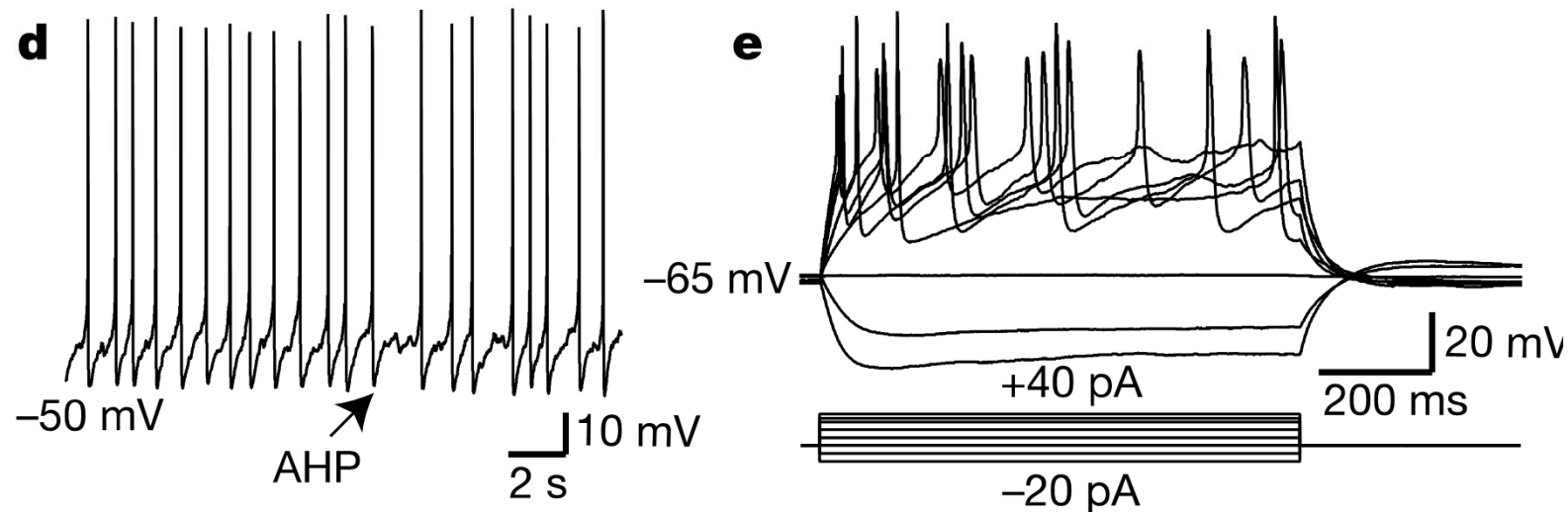
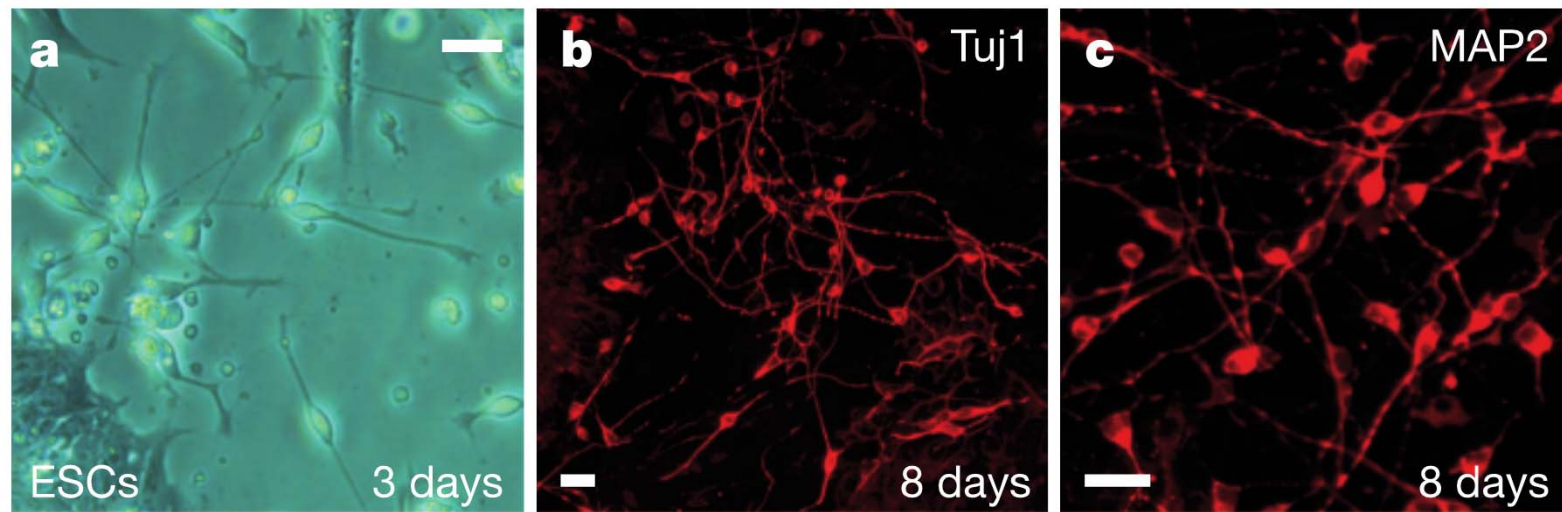
Summary Vierbuchen et al 2010

- Expression of 3 transcription factors (Ascl1, Brn2, Myt1l) can rapidly and efficiently convert mouse fibroblasts into functional neurons (iN cells)
- Efficiencies up to 19.5%
- Majority of these neurons express markers of cortical identity.
- Low proportion of iN cells expressed markers of GABAergic neurons, but no other neurotransmitter phenotype detected

Induction of human neuronal cells by defined transcription factors

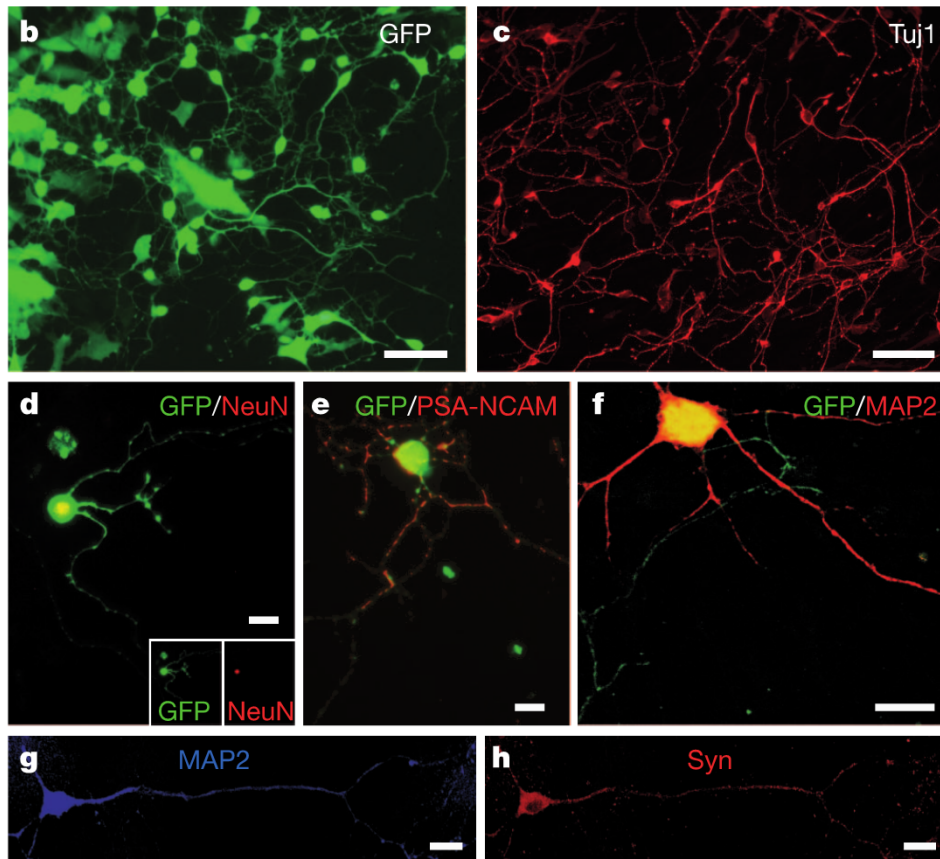
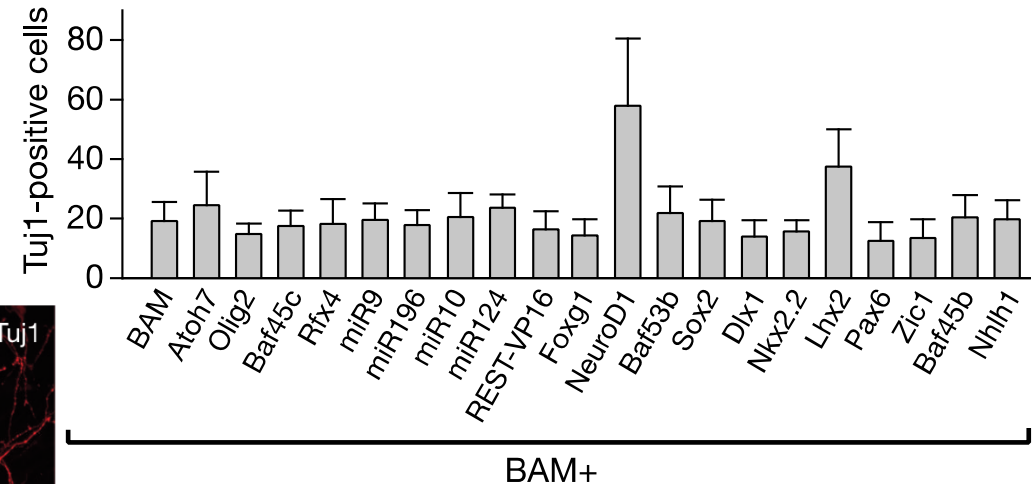
Zhiping P. Pang^{1*}, Nan Yang^{2*}, Thomas Vierbuchen^{2,3*}, Austin Ostermeier^{2,3}, Daniel R. Fuentes², Troy Q. Yang², Ami Citri⁴, Vittorio Sebastiano², Samuele Marro², Thomas C. Südhof^{1,5} & Marius Wernig^{2,3}

Rapid generation of functional neurons from human embryonic stem cells



Pang 2011 Nature

NeuroD1 increases reprogramming efficiency in primary human fibroblasts



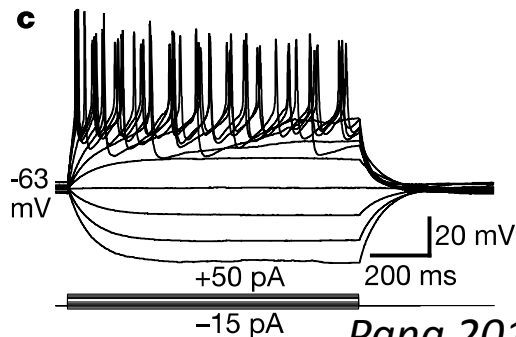
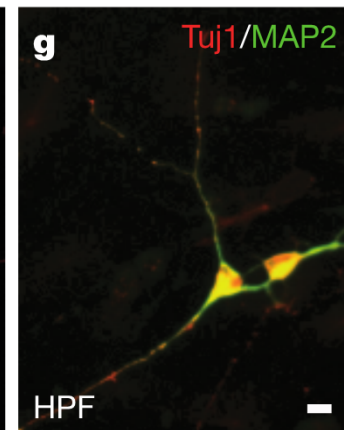
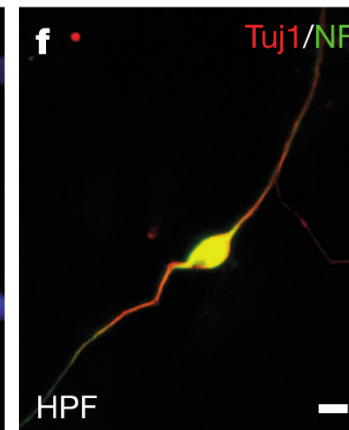
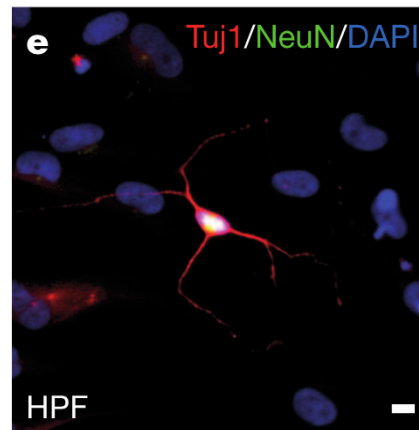
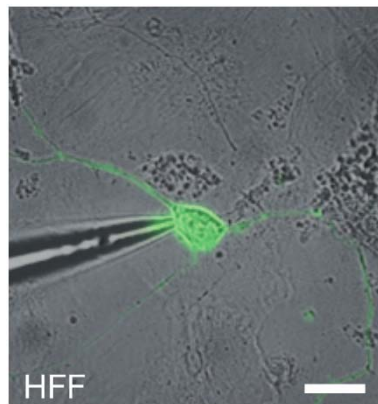
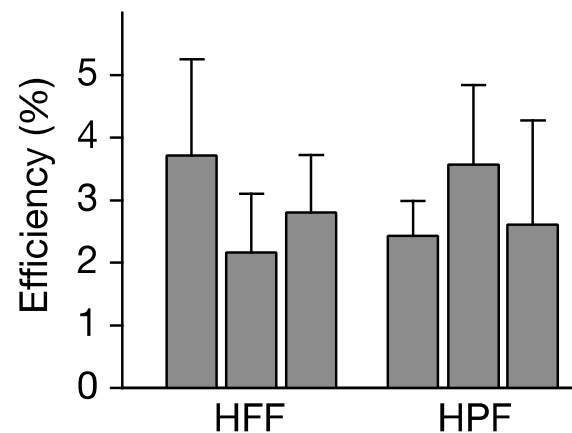
Microtubule-associated protein 2:
neuron-specific cytoskeletal protein
Polysialylated Neural Cell Adhesion
Molecule PSA-NCAM
Synaptophysin

Pang 2011 Nature

Membrane properties of fibroblast iN cells

- HPF: Primary human postnatal fibroblasts (HPF)
- HFF: primary human fetal fibroblasts

a



Pang 2011 Nature

Generation of induced neurons via direct conversion in vivo

Olof Torper^{a,b}, Ulrich Pfisterer^{a,b,1}, Daniel A. Wolf^{a,b,1}, Maria Pereira^{a,b}, Shong Lau^{a,b}, Johan Jakobsson^{a,b}, Anders Björklund^{a,2}, Shane Grealish^{a,b}, and Malin Parmar^{a,b,2}

^aDepartment of Experimental Medical Science, Wallenberg Neuroscience Center, and ^bLund Stem Cell Center, Lund University, 221 84 Lund, Sweden

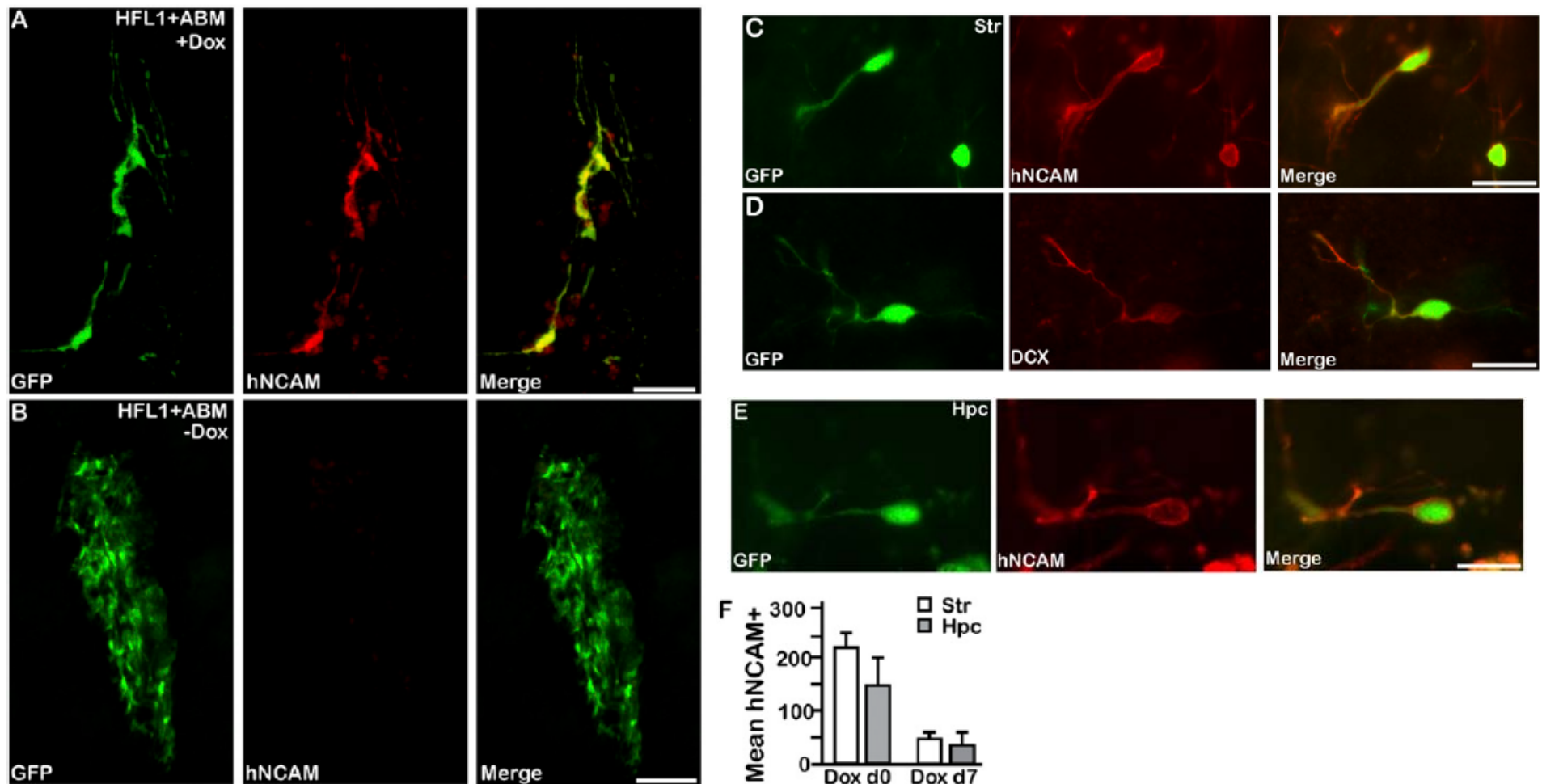
- Direct in vivo conversion has already been successful in organs such as pancreas and heart. Zhou 2008 Nature; Qian 2012 Nature

Experimental setup

- Doxycycline-regulated LVs to deliver the neural conversion genes Brn2, Ascl1, Myt1l (brain-2, achaete-scute complex like 1, myelin transcription factor-like 1)
- GFP-labeled human fetal lung fibroblast (HFL) cells were transduced but never exposed to Doxycycline
- Engraftment of the HFL cells in the striatum and hippocampus of adult rats.
- Recipient animals:
 - 1st group: pretreated with Doxycycline
 - 2nd group: Doxycycline applied at 1 week after engraftment
 - 3rd group: no Doxycycline
 - 4th group: GFP only labeled cells and Doxycycline

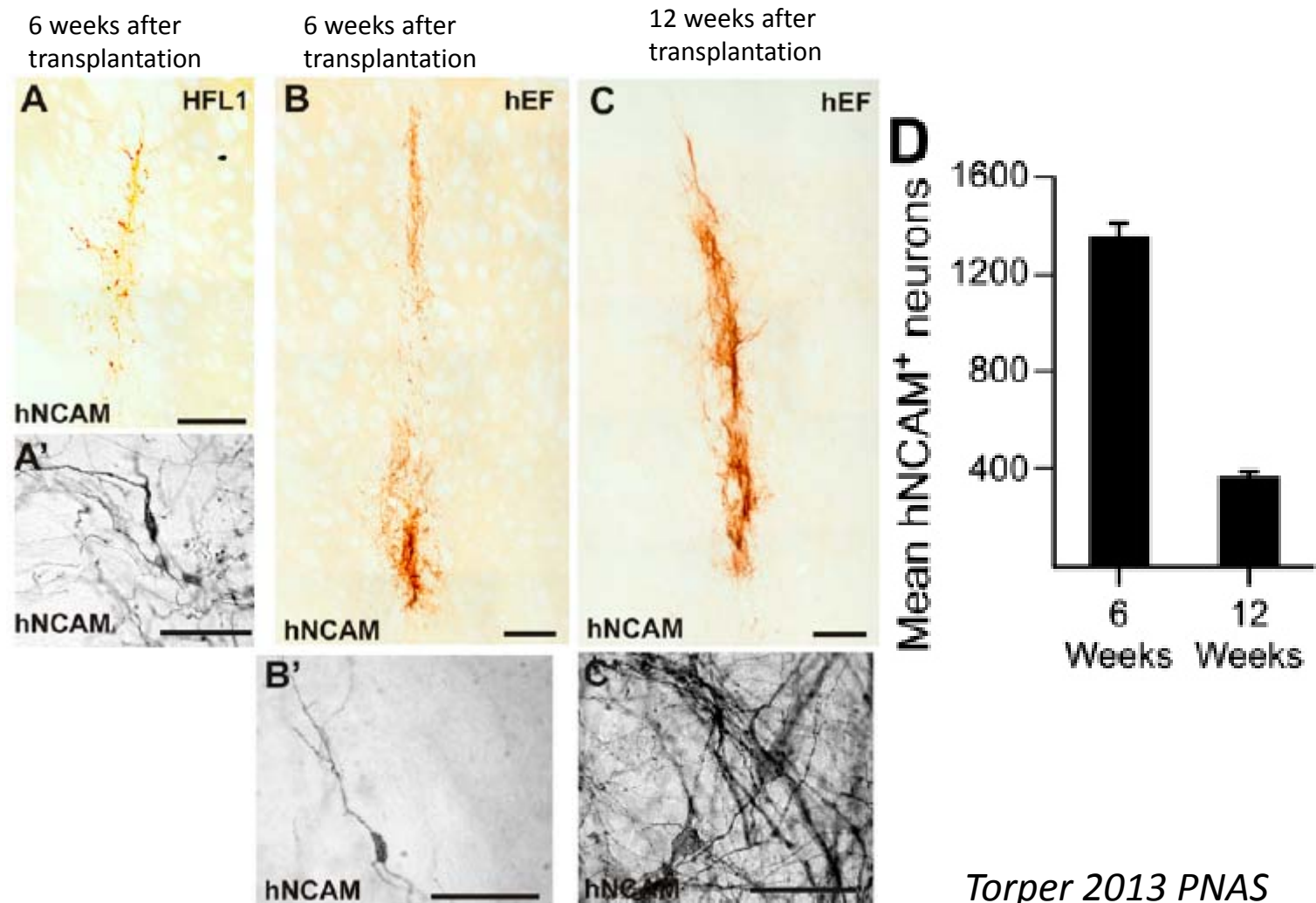
Direct neural conversion from human fibroblasts takes place in vivo

- hNCAM: human-specific neural cell adhesion molecule
- Dxc: Doublecortin Neuronal migration protein double



Long-term survival and stability of iN cells

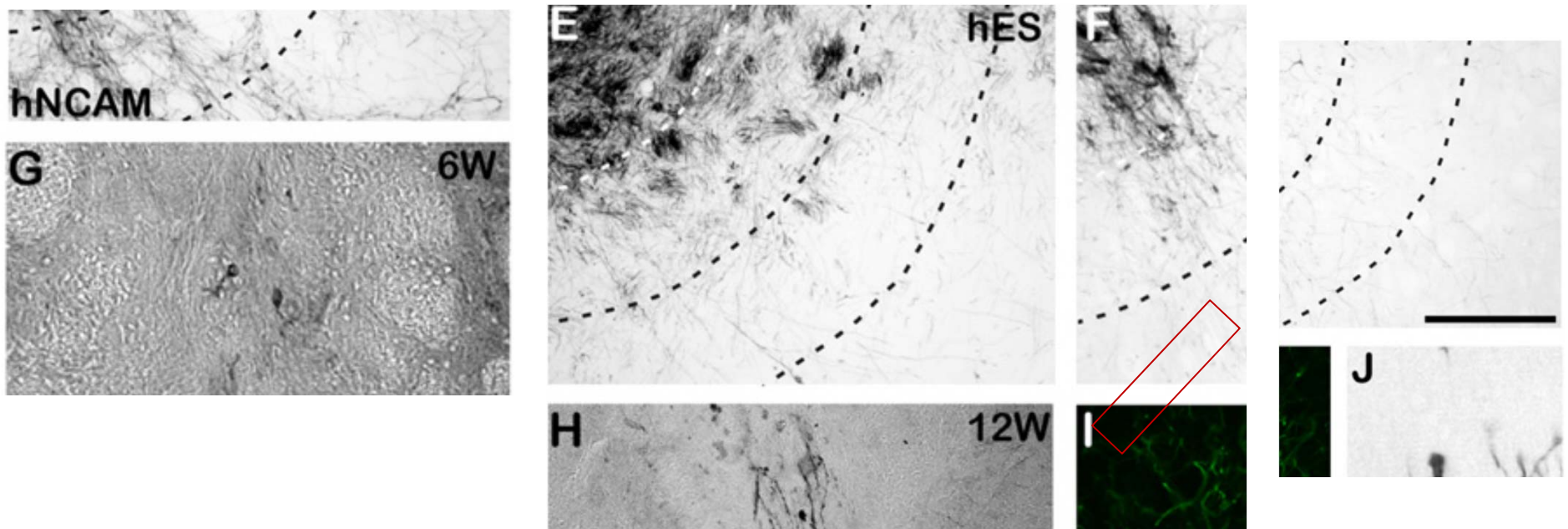
- Engraftment of transduced human embryonic fibroblasts (hEF) or human Fetal Lung Fibroblasts (HFL1) in striatum of Doxycycline pretreated rats. Analysis after 6, 12 week (6 weeks without Doxycycline Tx)



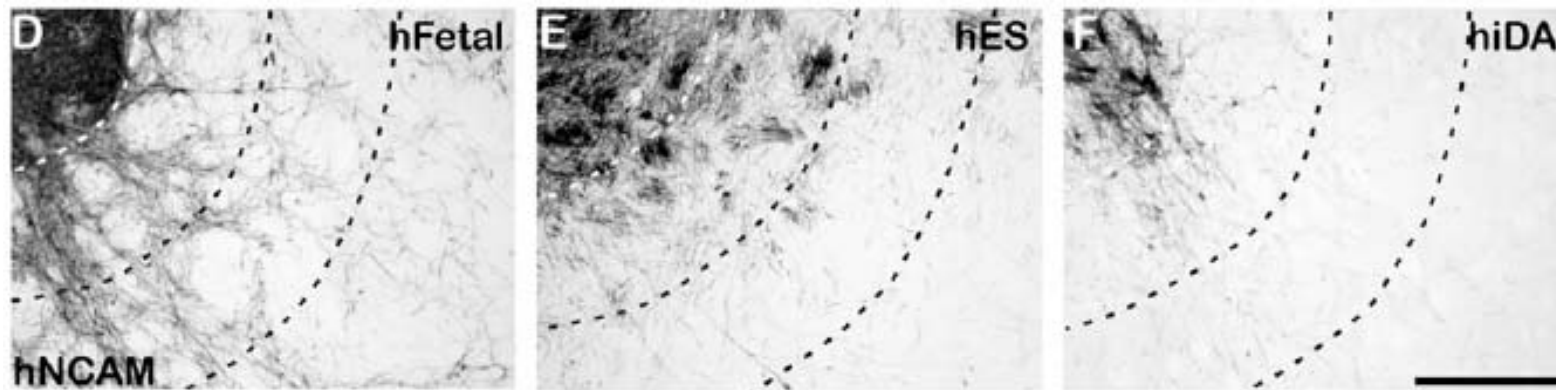
Torper 2013 PNAS

Generation of dopaminergic iN cells

- neural conversion genes are combined with DA fate determinants. Minimal combination Lmx1a and FoxA2
- Lenti DA: FoxA2, Lmx1a, Lmx1b, Otx2, Ngn2, Pax2, Pax5, Nurr1, En1, and Gli1
- LaFo: Lmx1a and FoxA2; already found in earlier studies

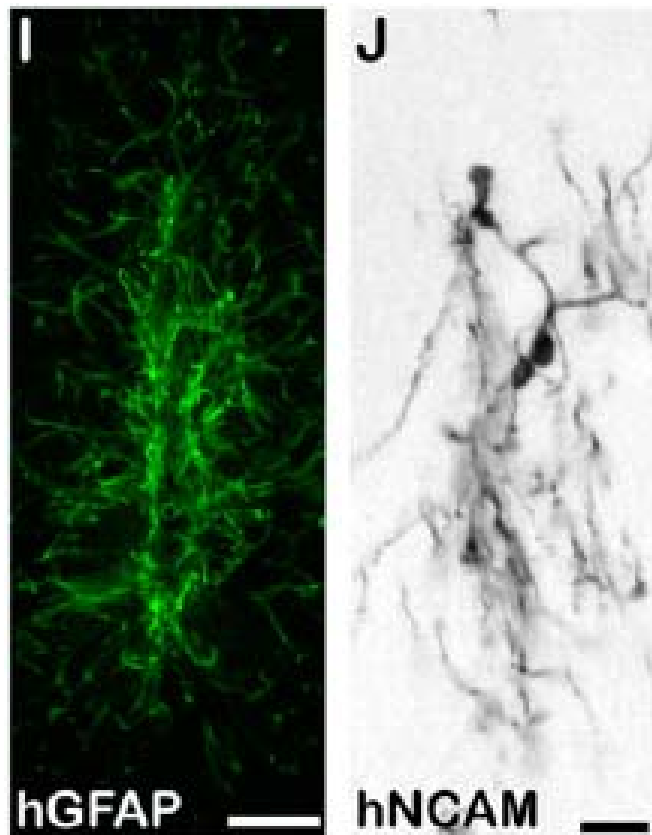


Innervation of the striatum compared to human fetal cells, human embryonic stem cells and DA-iN



In vivo conversion of human cortical astrocytes

- Astrocytes expressing: hGFAP, brain lipid binding protein (BLBP, but not hNCAM, beta3tubulin or sex determining region Y box 2 (Sox2)
- Transfection of the astrocytes, transplantation and Doxycycline for 6 weeks

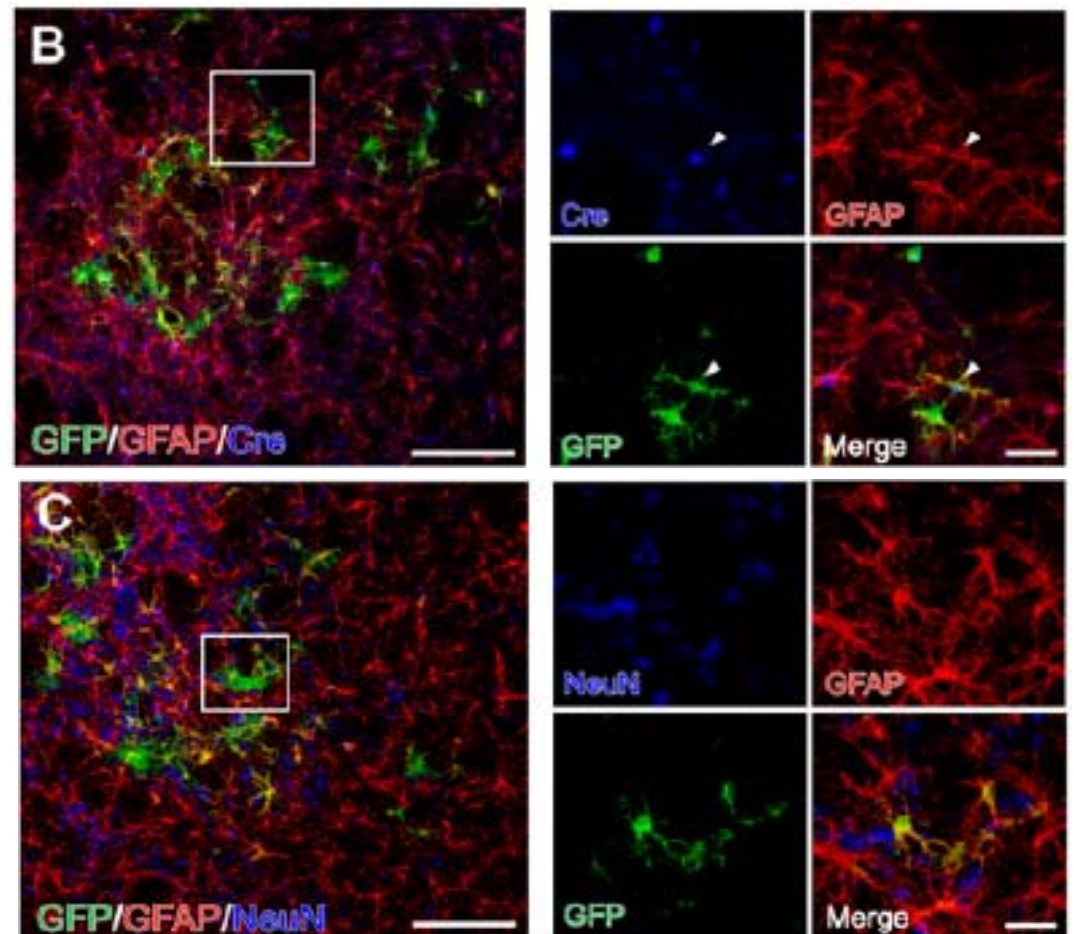
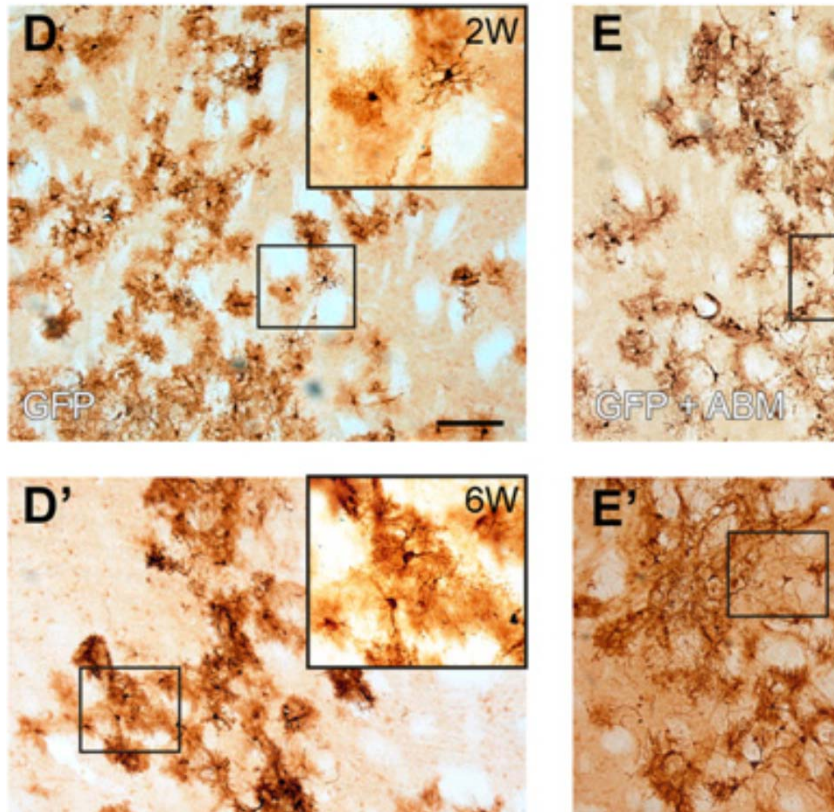


Human-specific neural cell adhesion
molecule (hNCAM)

Torper 2013 PNAS

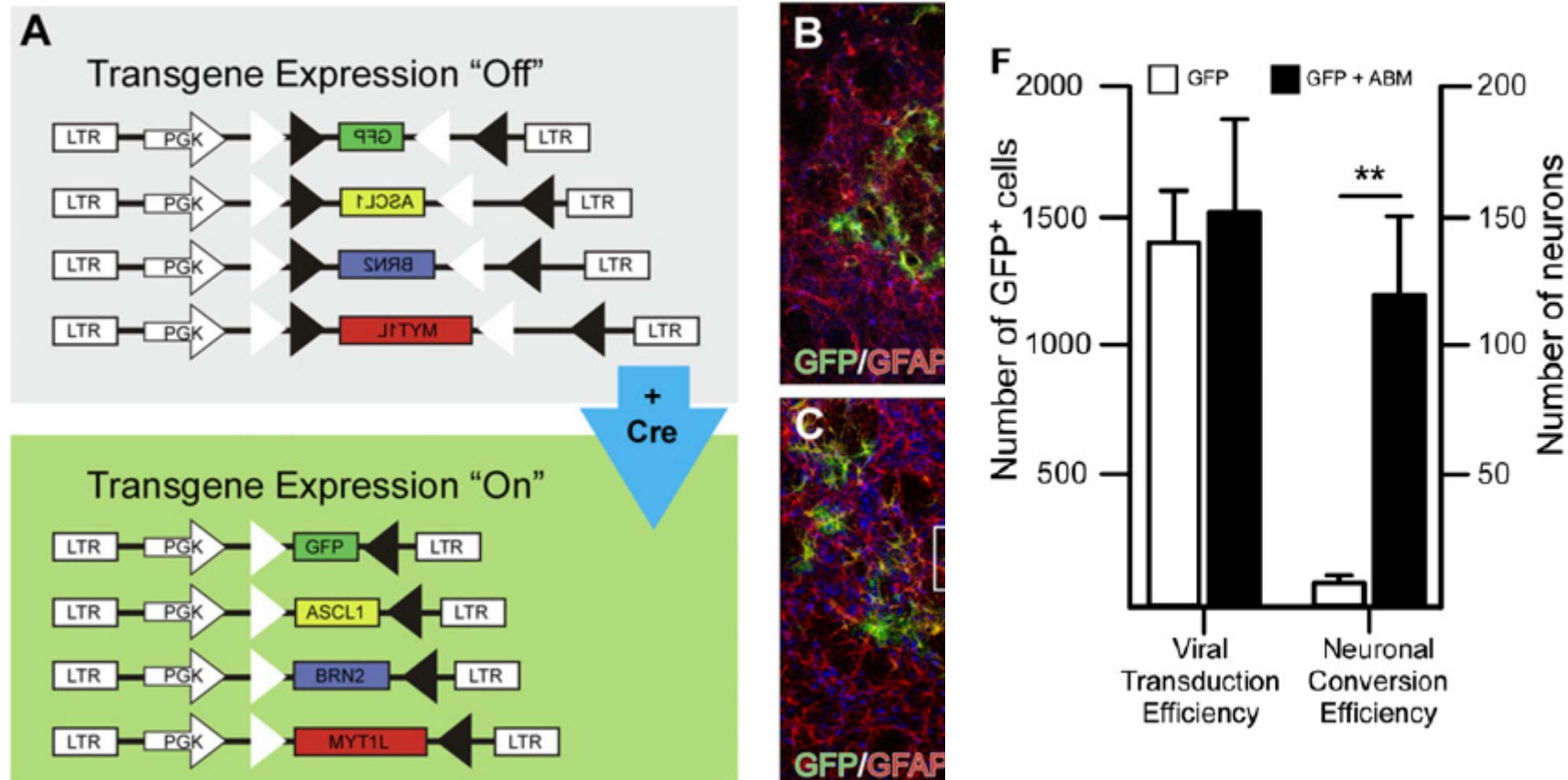
In vivo conversion from resident glia cells

- Cre inducible LVs that contain GFP reporter and reprogramming genes (BAM)
- Stereotactic injection of the LVs into the striatum of GFAP-cre heterozygous mice

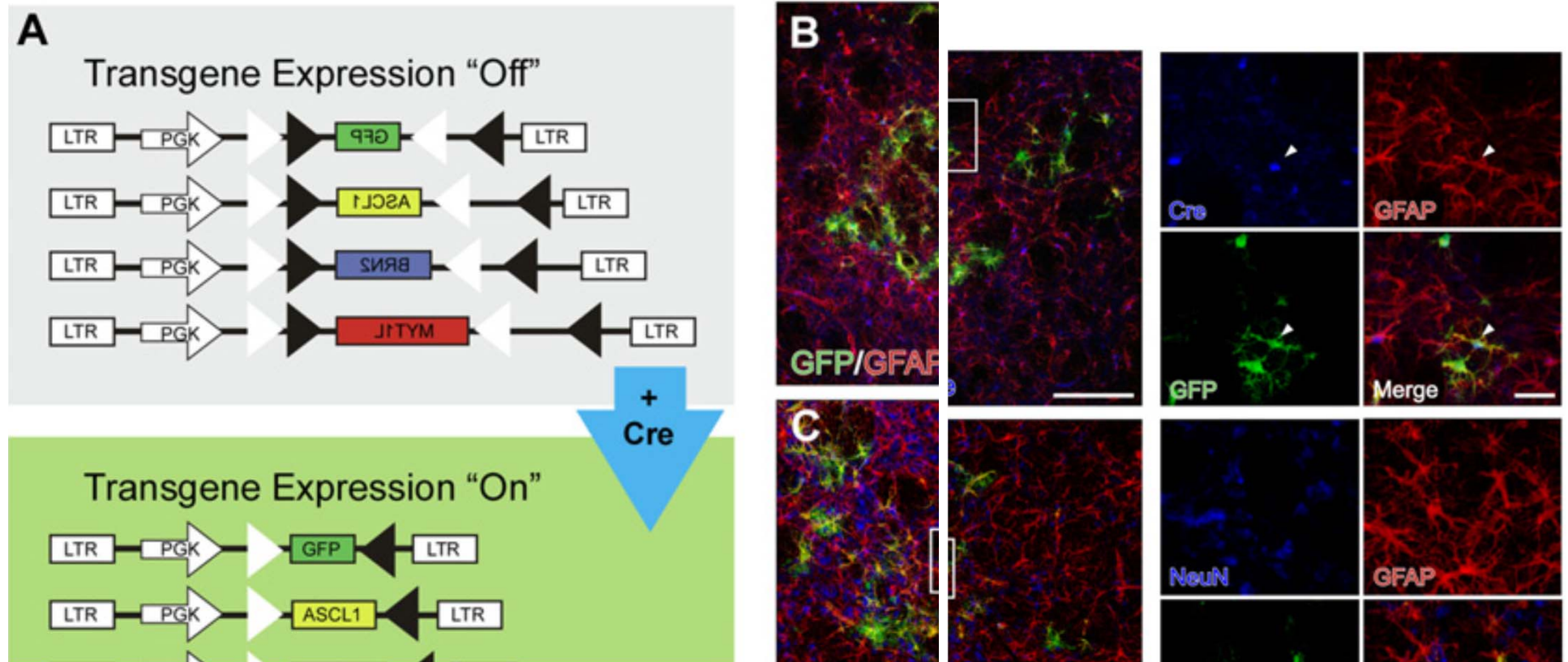


Torper 2013 PNAS

Detection of GFP expressing cells with neuronal morphology can be detected 2 and 6w after injection



Confirmation of neuronal identity of iNs generated via in vivo conversion



Summary Torper et al 2013

- Transplanted human embryonic fibroblasts, human fetal lung fibroblasts and human astrocytes expressing BAM can be converted into neurons while residing in the adult brain.
- The resulting neurons are stably reprogrammed, survive, mature, while not forming tumors or neural overgrowths
- Adding dopamine fate determinants to the reprogramming procedure tyrosine hydroxylase (TH) expressing neurons can be obtained.

Generation of oligodendroglial cells by direct lineage conversion

Nan Yang^{1,2}, J Bradley Zuchero³, Henrik Ahlenius^{1,2}, Samuele Marro^{1,2}, Yi Han Ng^{1,2}, Thomas Vierbuchen^{1,2}, John S Hawkins^{1,2}, Richard Geissler², Ben A Barres³ & Marius Wernig^{1,2}

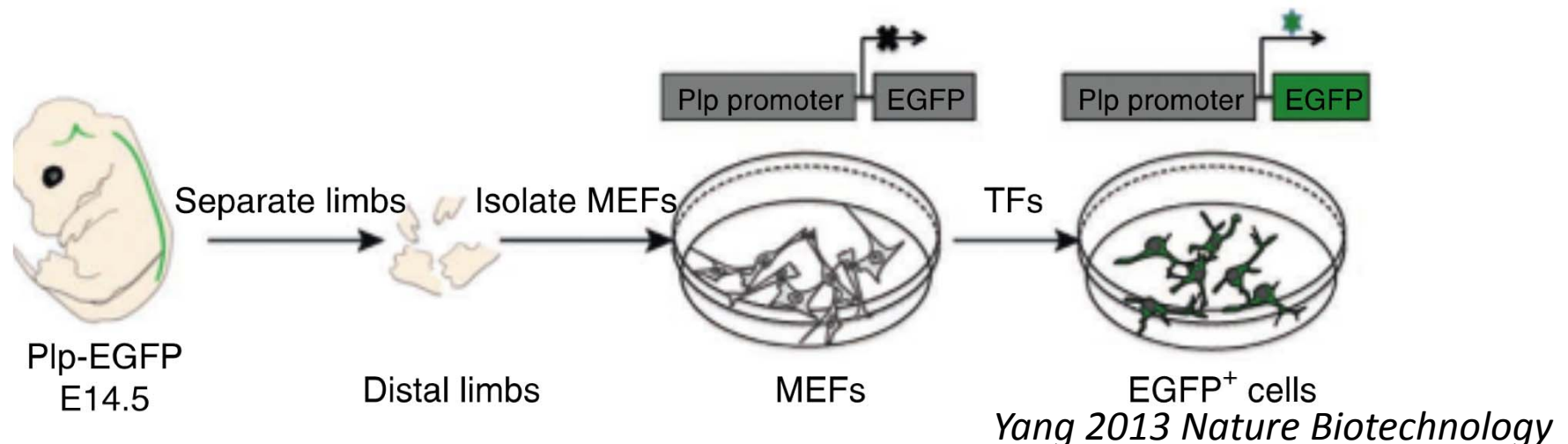
ARTICLES

**nature
biotechnology**

- Oligodendrocyte precursor cells (OPCs)
- It is assumed that myelination is accomplished by OPCs rather than mature oligodendrocytes
- OPCs are a promising cell population for therapeutic approaches in dysmyelinating and demyelinating diseases.

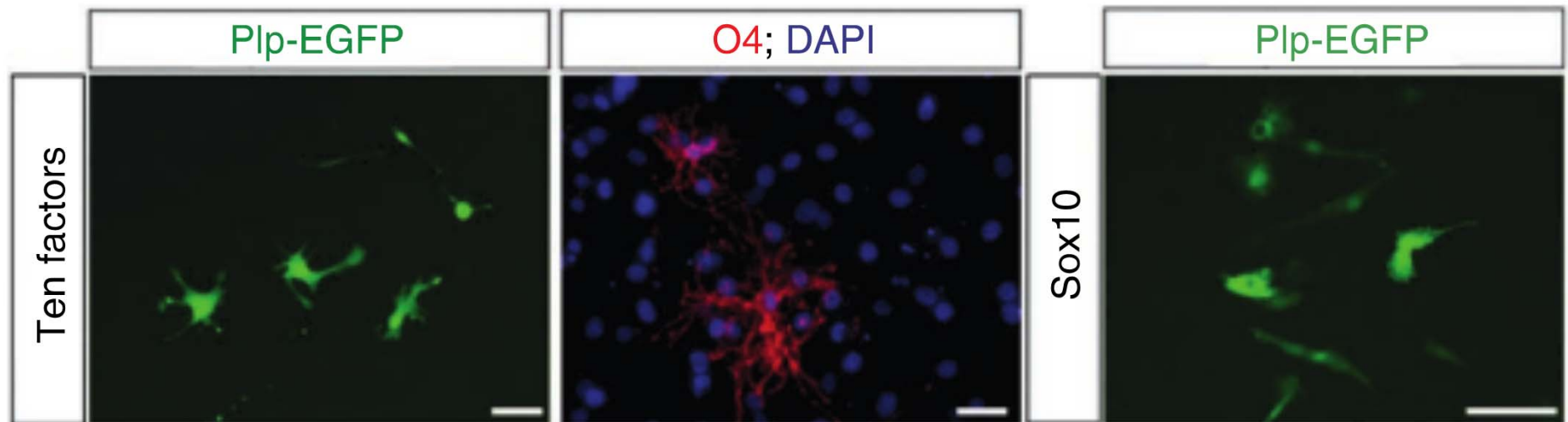
Screen for OPC reprogramming factors

- Selection of 10 factors that participate in various stages of OPC specification and when mutated cause severe developmental oligodendroglia-related defects :
 - Ascl1, GM98, Myt, Nkx2.2, Nkx6.2, Olig1, Olig2, Sox10 and Zfp36
- Proteloid protein (Plp) – EGFP transgenic mouse strain expresses GFP in both OPCs and mature oligodendrocytes as a reporter for oligodendrocytic cells



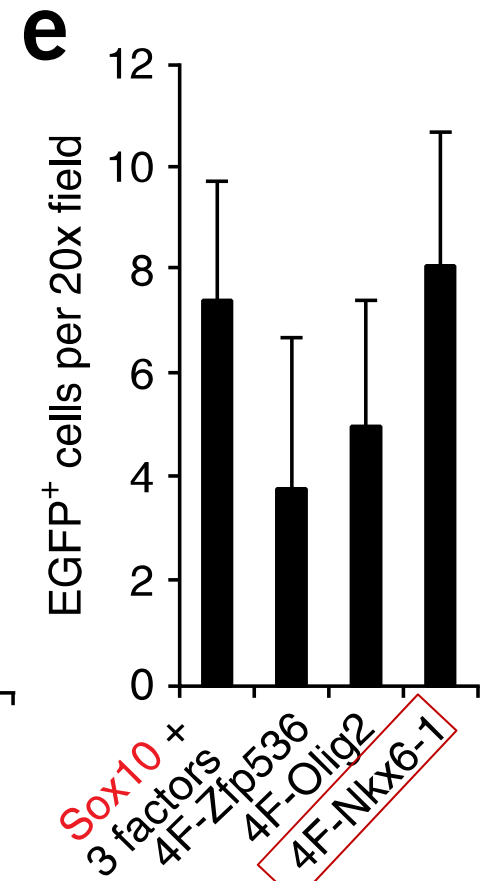
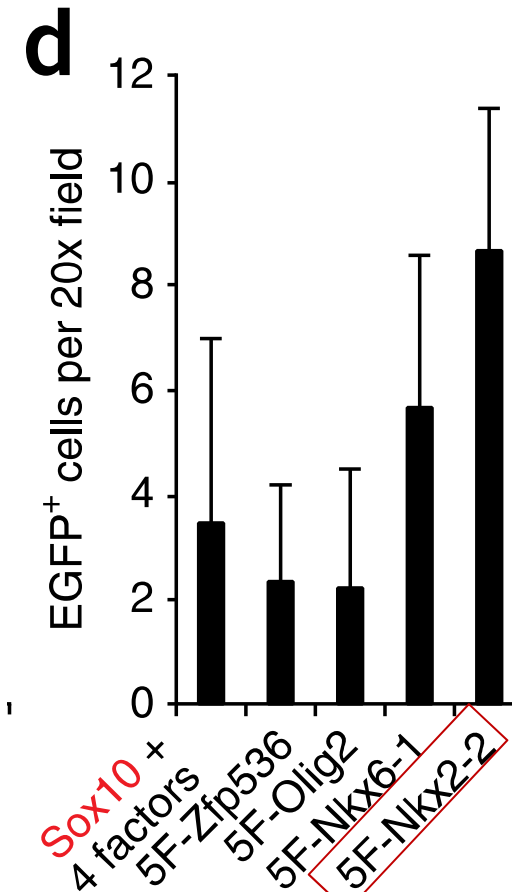
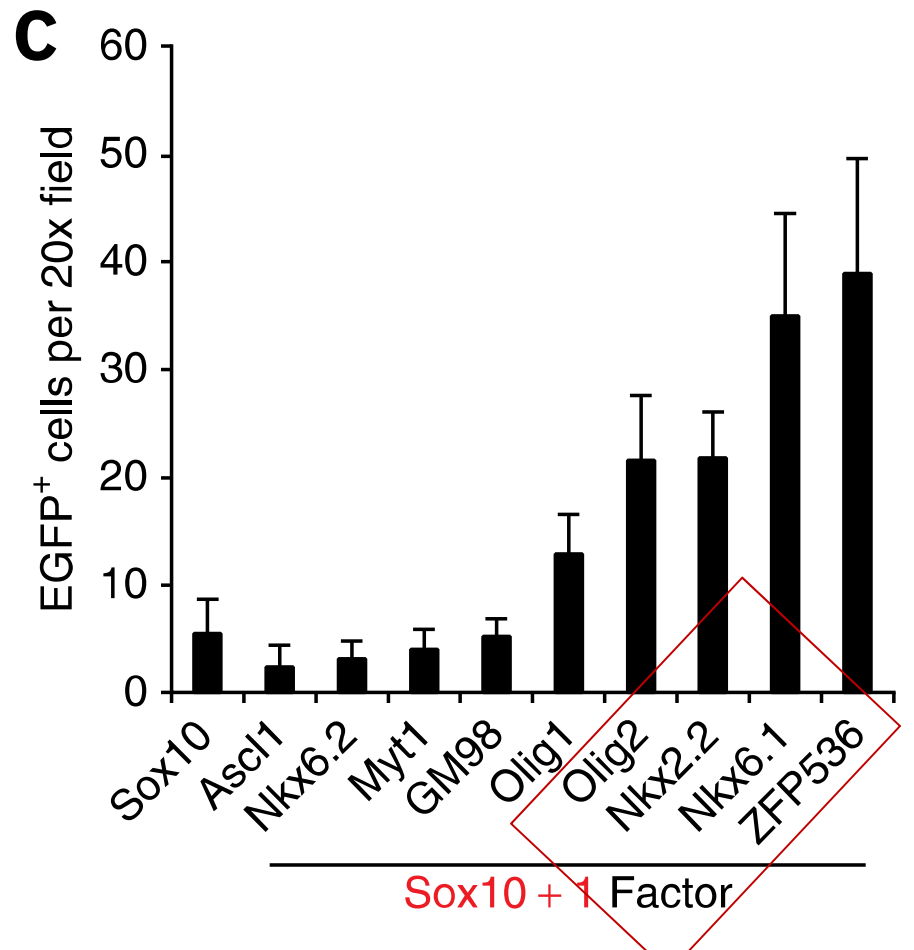
Transfection of the MEFs with the 10factor pool

- O4 antibodies: specifically mark oligodendrocytes as well as late stage OPCs



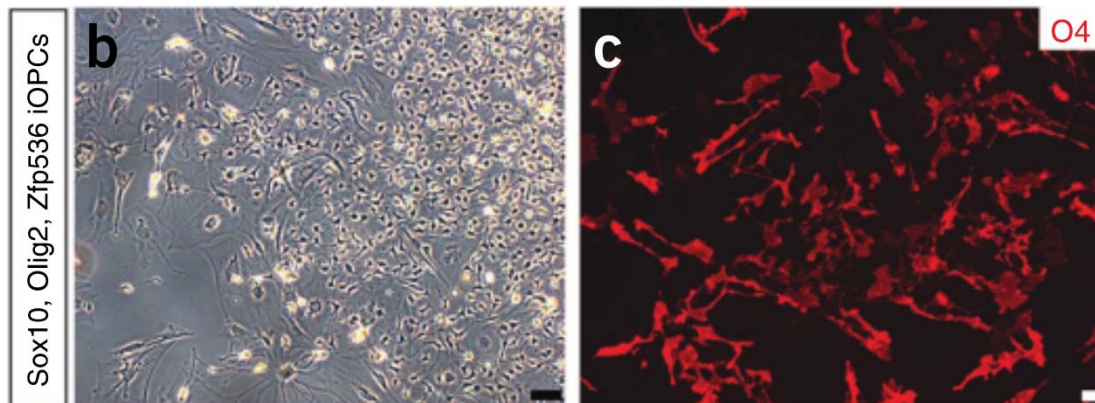
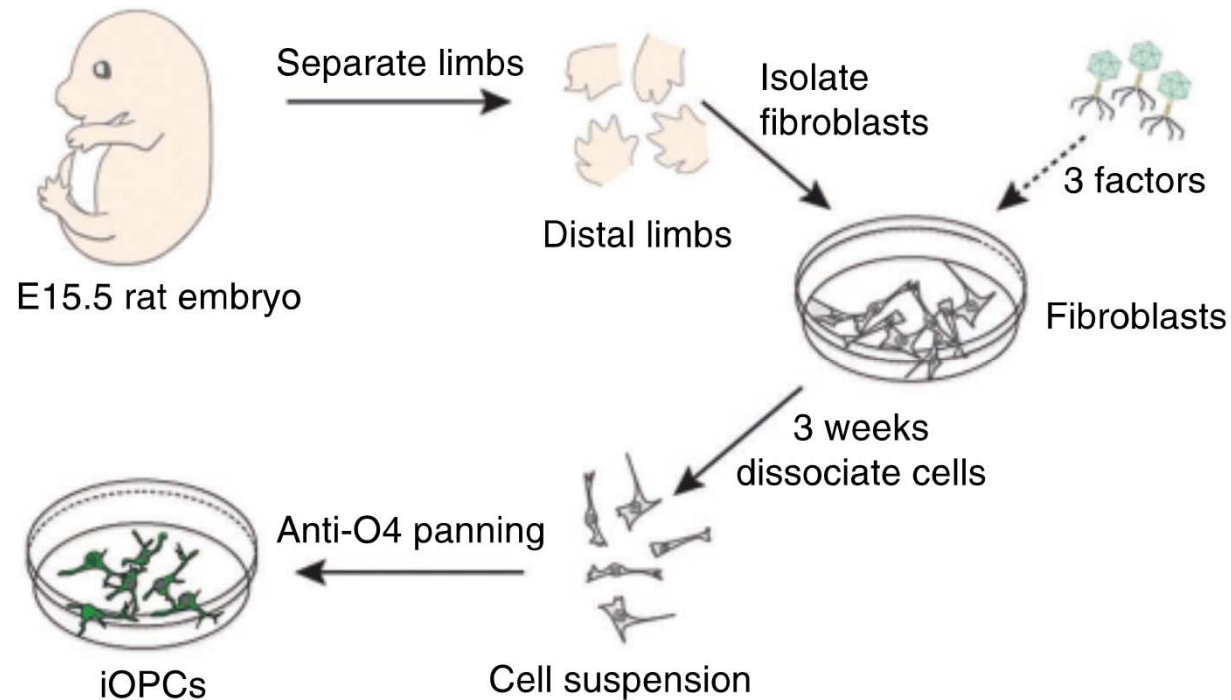
Identification of the minimal amount of required transcription factors

- Testing of the single factors. Only Sox10 able to induce as single factor.
- Sox10, Olig2 and Zfp536

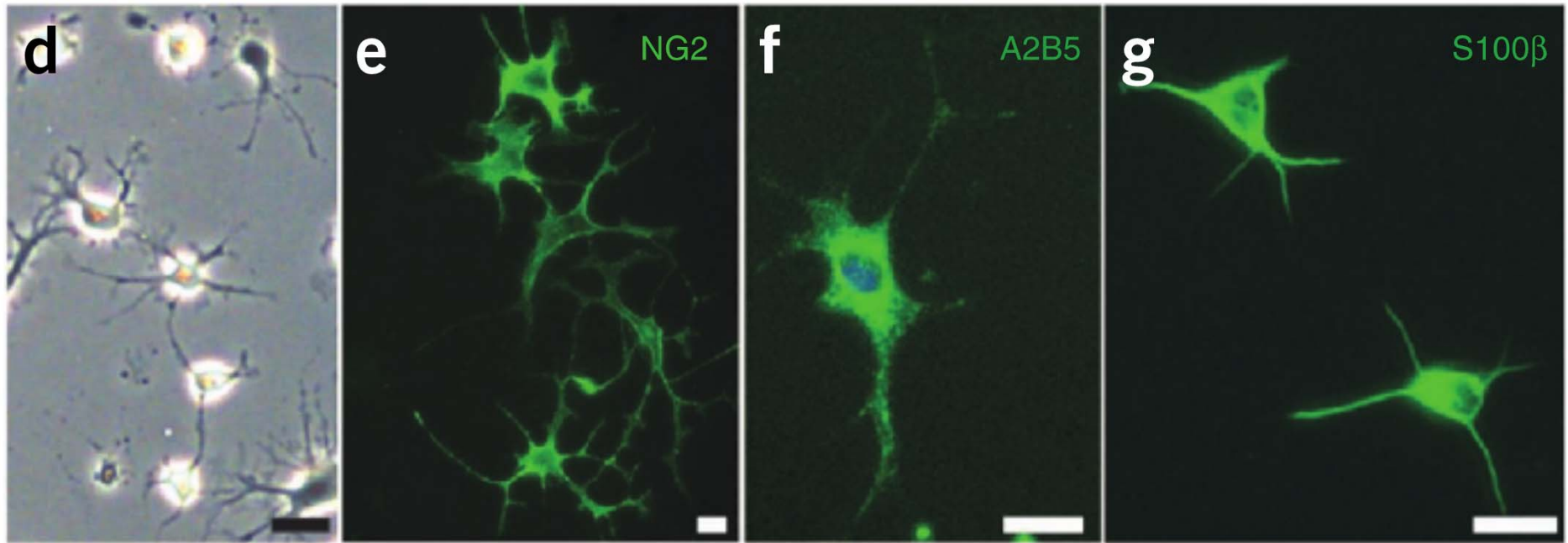


Yang 2013 Nature Biotechnology

Induction of OPC like cells from rat fibroblasts



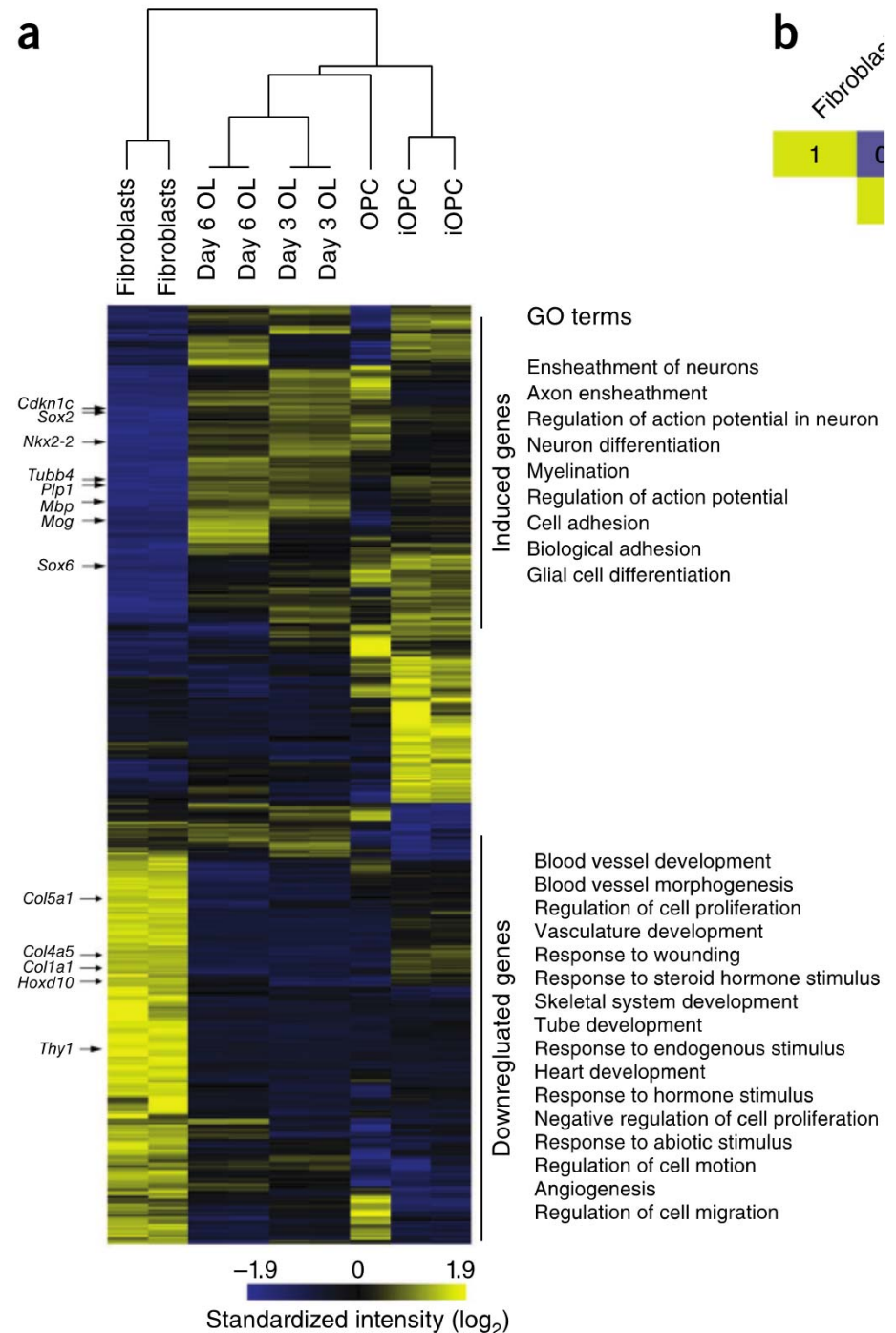
Induction of OPC like cells from rat fibroblasts



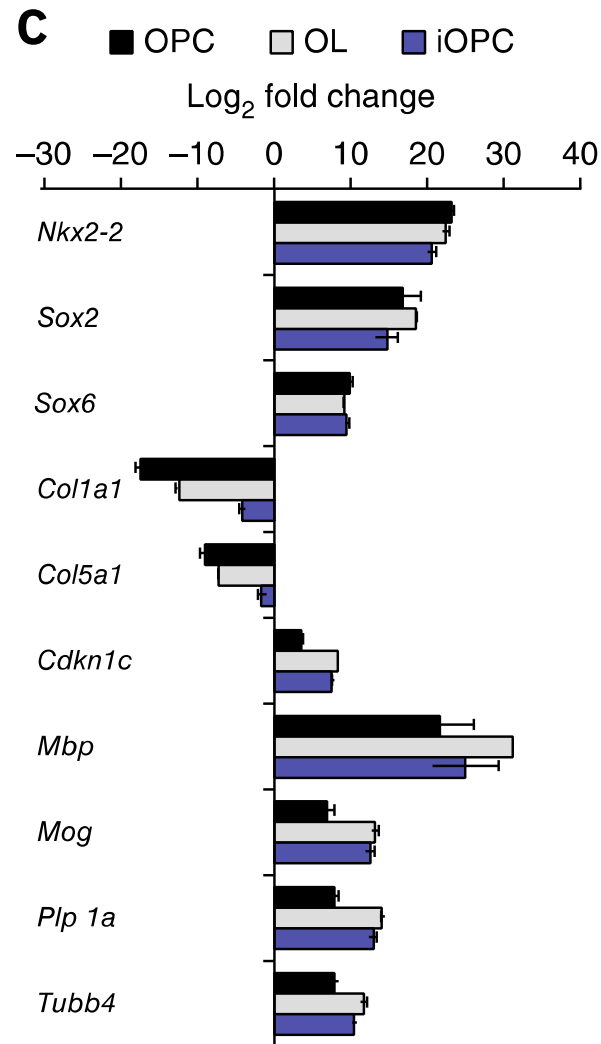
Three additional OPC markers
NG2, A2B5, S100 β

Global transcription remodeling

- OL: differentiated OPCs
- GO terms associated with oligodendroglial biology upregulated in iOPCs
- Fibroblast genes with mesodermal functions were globally downregulated in iOPCs.



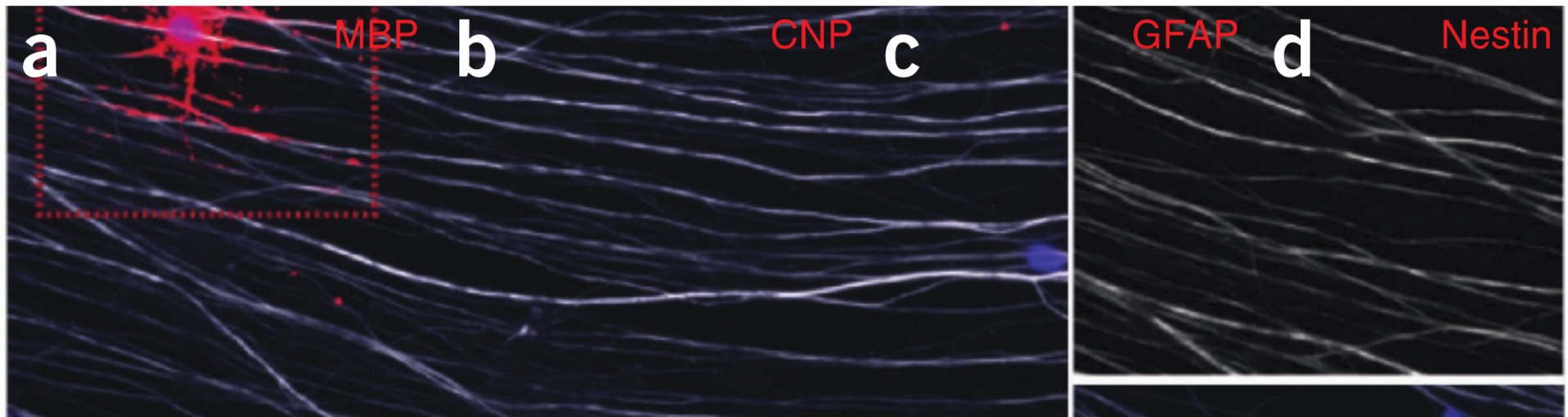
qRT-PCR analysis of the expression levels of characteristic OPC, fibroblast and oligodendrocyte marker genes



OL: differentiated OPCs

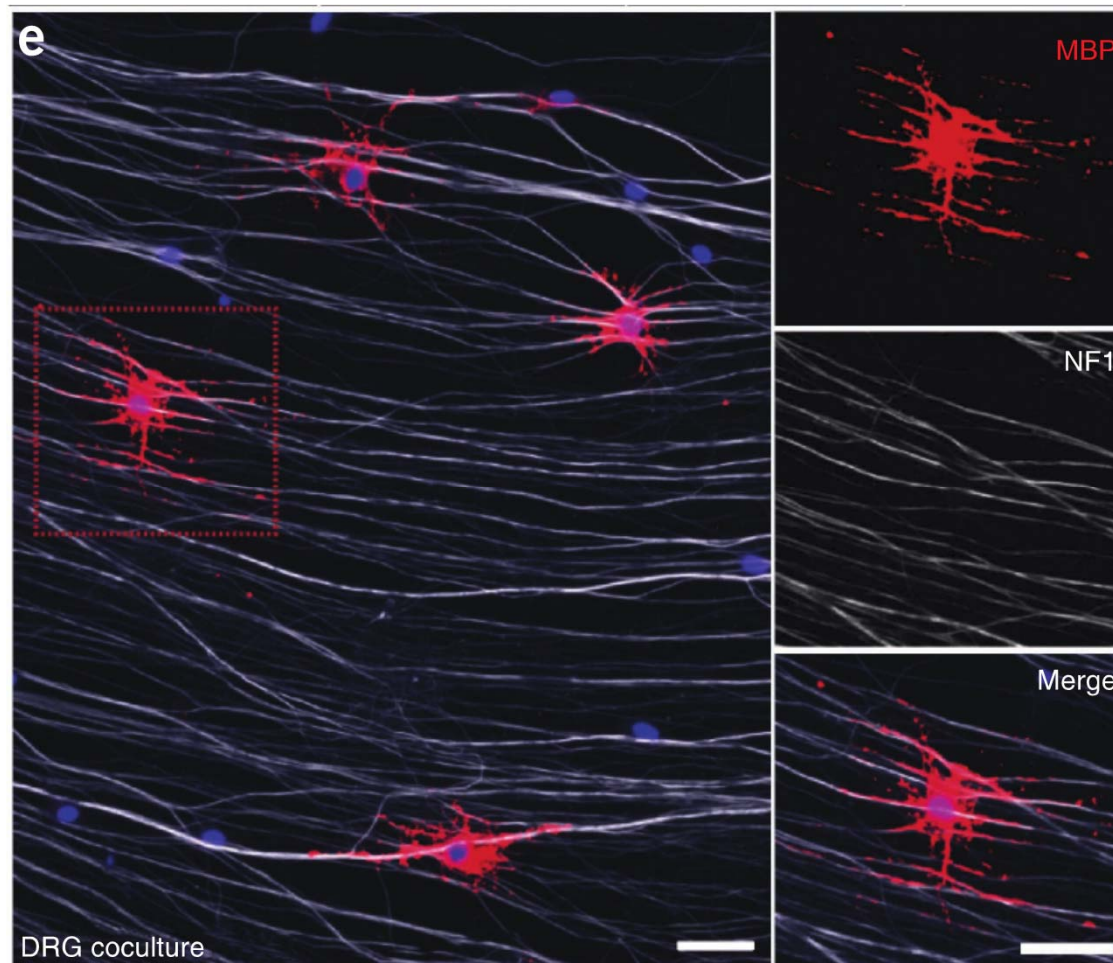
iOPCs differentiate into myelinating oligodendrocytes in vitro

- PDGF withdrawal O4pos cells stop dividing and differentiated into cells that expressed major basic protein (MBP) and CNPase
- CNP: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase also known as CNPase (myelin associated)
- Known that OPC can give rise to astrocytes in vitro
- Reactive astrocytes express GFAP and Nestin (type VI intermediate filament)



iOPC can myelinate dorsal root ganglion neurons in vitro

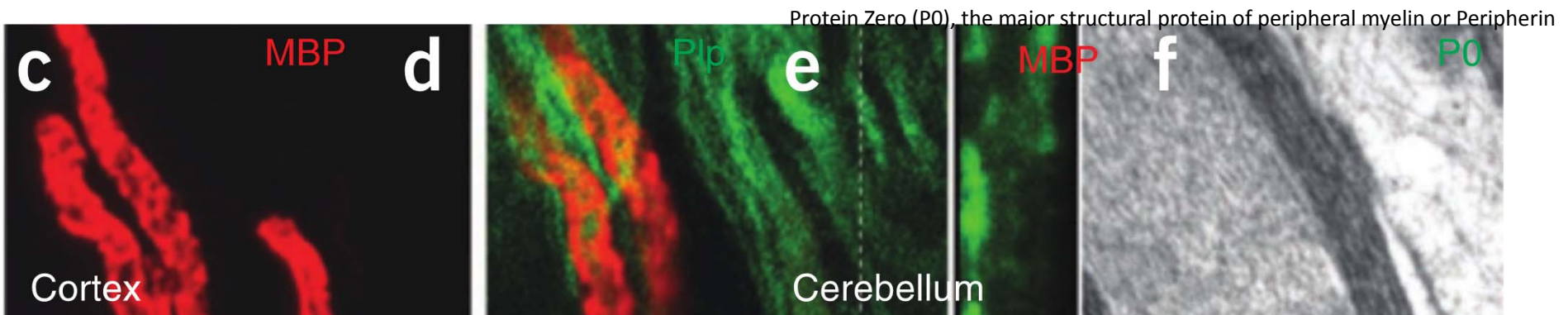
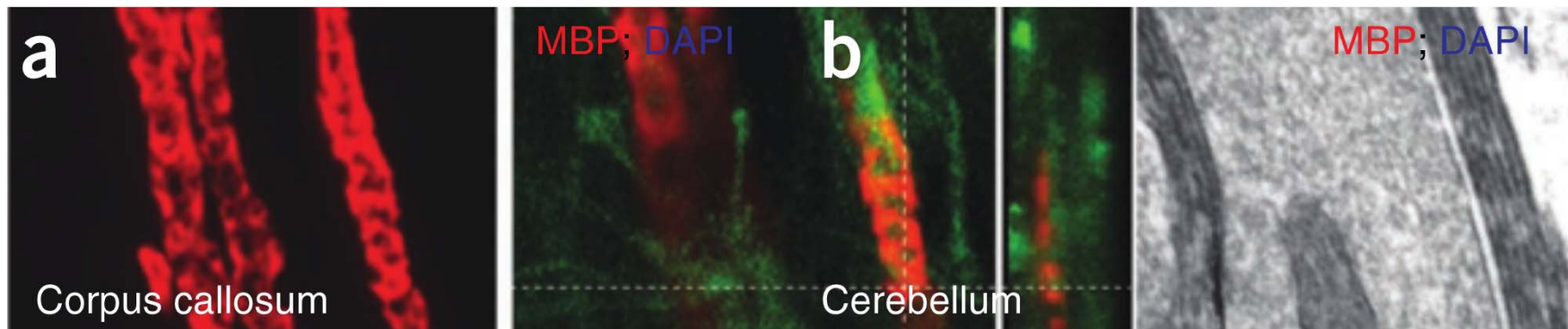
- iOPC were cocultured with preestablished dorsal root ganglion neurons



- Mature oligodendrocytes identified with MBP
- Myelinating iOPC-derived oligodendrocytes extended multiple distinctive smooth tubes, which were aligned with axons marked by neurofilament

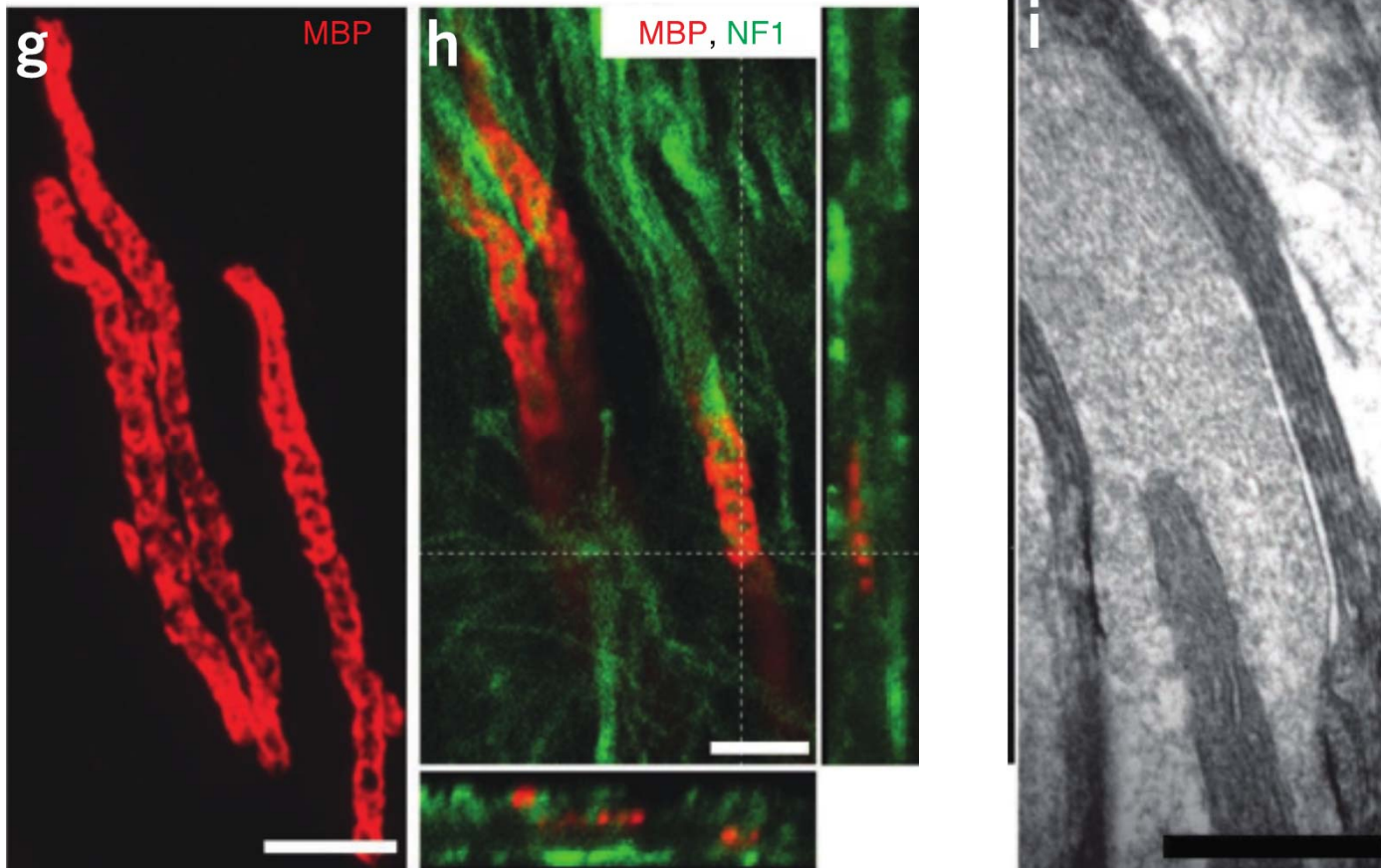
iOPCs are myelinogenic in vivo

- shiverer mice: lack portions of the Mbp gene and have dysmyelinated axons throughout the central nervous system
- Injection of iOPCs into corpus callosum and cerebellum of neonatal mice, Doxycycline in drinking water of nursing female mouse
- Euthanasia at 12 weeks of age
- In all 12 injection sites of the three grafted brains, small scattered groups of MBP pos cells forming tube like structures were detected in the cortex, corpus callosum, white matter of cerebellum



iOPCs are myelinogenic in vivo

- Close association of nerve fibers and MBP+ cells

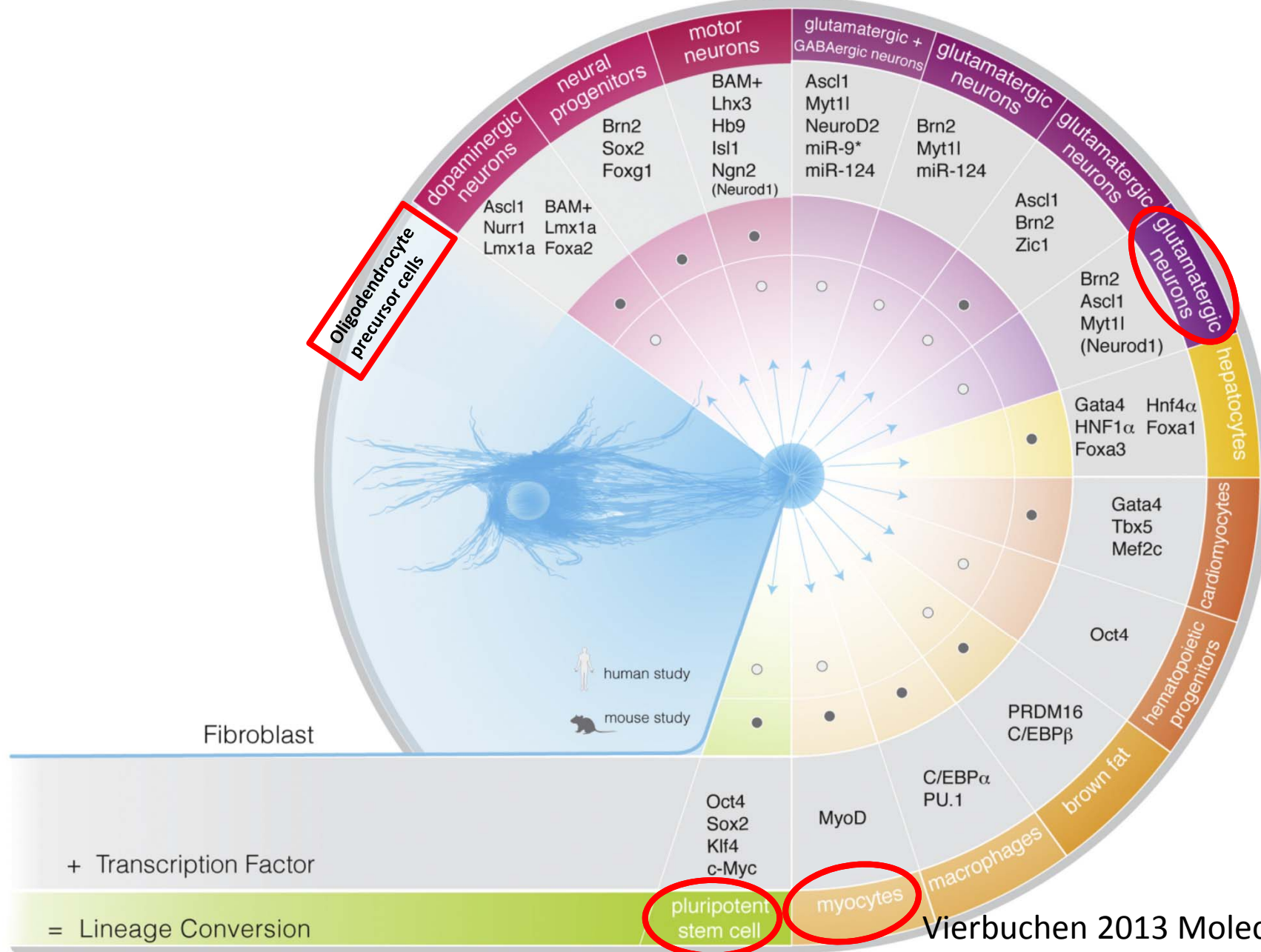


Yang 2013 Nature Biotechnology

Summary Yang et al 2013

- Fibroblasts can be directly converted into iOPCs by forced expression of three defined expression factors (Sox10, Olig2 and Zfp536)
- They give rise to mature oligodendrocytes, that can myelinate host axons in vivo

Transcription Factor-Mediated Conversion of Fibroblasts into Diverse Cellular Lineages



Comparison of the different reprogramming strategies

Reprogramming strategy	Time frame	Efficiencies	Differentiation potential	Requirement for cell proliferation	Expandability	Risk for teratoma	Mechanism
SCNT	Hours–days	Moderate	Pluripotent	None	Yes	High	De-differentiation
iPSC	Weeks–months	Very low	Pluripotent	Yes	Yes	High	De-differentiation
Direct lineage conversion	Hours–days	High	Unipotent	None	No	Low	Transdifferentiation

Outlook

- Directed conversion of Alzheimer's disease patient skin fibroblast into functional neurons
- Offers potential not only for
 - personalized medicine approaches based on cell transplantation, but also
 - For disease modeling and
 - In vivo programming contributing to endogenous regeneration

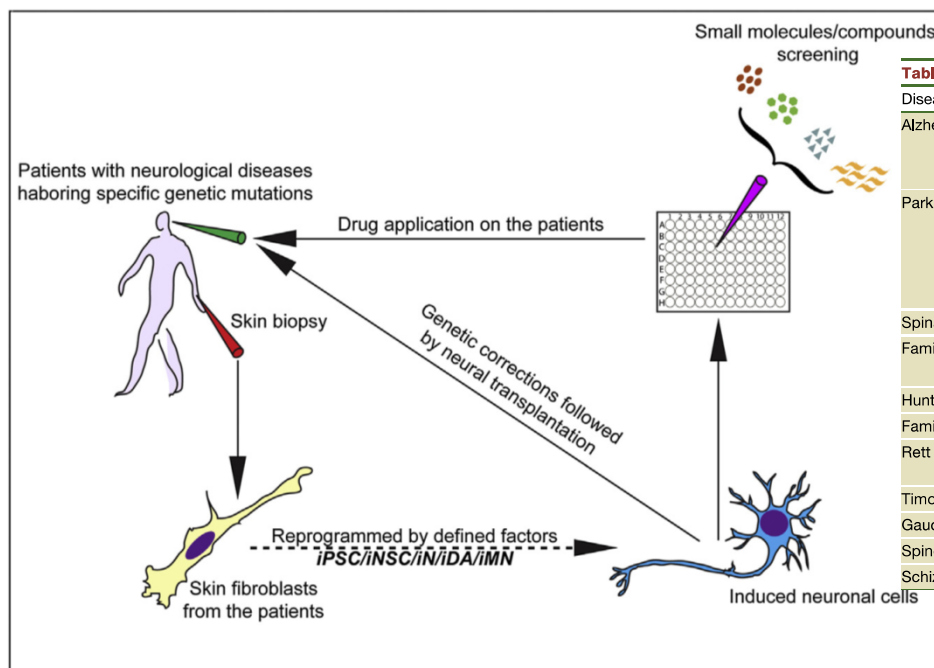


Table 3. Human Somatic Cell Reprogramming-Based Neuronal Models of Disease

Disease	Genetic Defect	Drug Testing	Reference
Alzheimer's disease	Duplication of <i>APP</i> ; Sporadic	no	Israel et al., 2012
	<i>PSEN1</i> A246E; <i>PSEN2</i> N141I	yes	Yagi et al., 2011
	<i>APPE</i> 693Δ; <i>APPV</i> 717L; Sporadic	no	Kondo et al., 2013
Parkinson's disease	<i>α-synuclein</i> A53T	no	Soldner et al., 2011
	<i>LRRK2</i> G2019S	yes	Cooper et al., 2012; Nguyen et al., 2011; Reinhardt et al., 2013; Sánchez-Danés et al., 2012
	<i>PINK1</i> Q456X; V170G	yes	Cooper et al., 2012; Seibler et al., 2011
Spinal muscular atrophy	<i>SMN</i> exon7 deletion	yes ^a	Corti et al., 2012; Ebert et al., 2009
Familial amyotrophic lateral sclerosis	<i>SOD1</i> L144F	no	Dimos et al., 2008
	<i>TDP-43</i> Q343R; M337V; G298S	yes	Egawa et al., 2012
Huntington disease	<i>Htt</i> 72 CAG repeats	no	An et al., 2012; Zhang et al., 2010
Familial dysautonomia	<i>IKBKAP</i> exon20 skipping	yes	Lee and Studer, 2011
Rett syndrome	<i>MeCP2</i> mutations	no	Kim et al., 2011d; Marchetto et al., 2010; Xia et al., 2012
Timothy syndrome	<i>CACNA1C</i> G406R	yes	Paşca et al., 2011
Gaucher disease	<i>GCCase</i> N370S/84GG insertion	no	Mazzulli et al., 2011
Spinocerebellar Ataxia Type 2	<i>ATXN2</i> (CAG) expansion	no	Xia et al., 2012
Schizophrenia	Sporadic	yes	Brennand et al., 2011

Thank you for your interest!

