LECTURES 7-11

BT 636 Tissue Engineering and Regenerative Medicine (3-0-0-6)

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LECTURES 1-2-3

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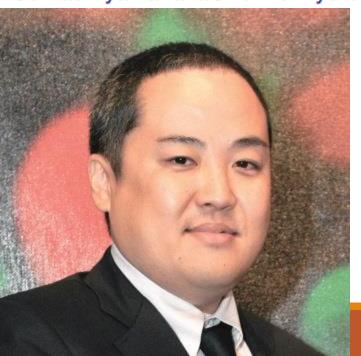
Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

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¹Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

²CREST, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

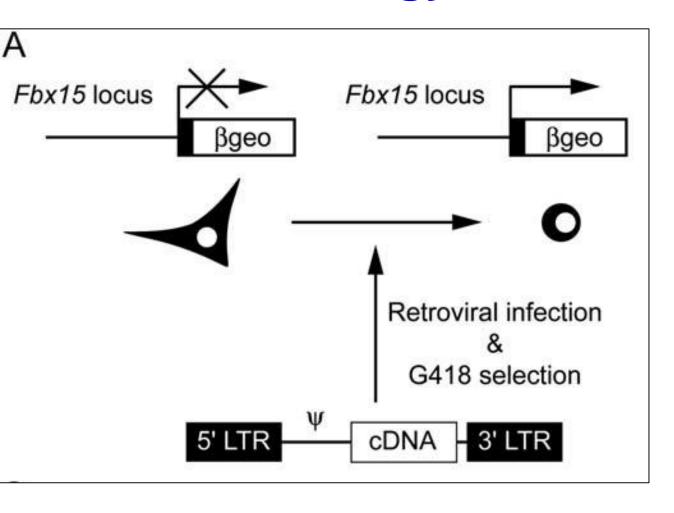
*Contact: yamanaka@frontier.kyoto-u.ac.jp





No	Genes	References
1	Ecat1	Mitsui et al., 2003
2	Dppa5 (Esg1)	Mitsui et al., 2003
3	Fbxo15	Mitsui et al., 2003
4	Nanog	Chambers et al., 2003; Mitsui et al., 2003
5	ERas	Takahashi et al., 2003
6	Dnmt3I	Mitsui et al., 2003
7	Ecat8	Mitsui et al., 2003
8	Gdf3	Mitsui et al., 2003
9	Sox15	Maruyama et al., 2005
10	Dppa4	Mitsui et al., 2003
11	Dppa2	Mitsui et al., 2003
12	Fthl17	Mitsui et al., 2003
13	Sall4	Mitsui et al., 2003
14	Oct3/4 (Pou5f1)	Nichols et al., 1998; Niwa et al., 2000
15	Sox2	Avilion et al., 2003; Maruyama et al., 2005
16	Rex1 (Zfp42)	Rogers et al., 1991
17	Utf1	Okuda et al., 1998
18	Tcl1	Mitsui et al., 2003
19	Dppa3 (Stella)	Mitsui et al., 2003
20	KIf4	Li et al., 2005
21	β-catenin	Kielman et al., 2002; Sato et al., 2004
22	с-Мус	Cartwright eta I., 2005
23	Stat3	Matsuda et al., 1999; Niwa et al., 1998
24	Grb2	Burdon et al., 1999; Cheng et al., 1998; Miyamotot et al., 2004

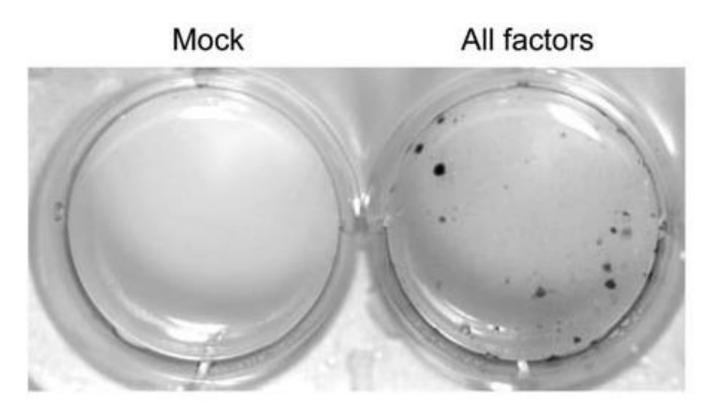
Strategy to test candidate factors



G418, also known as G418 sulfate and Geneticin, is an aminoglycoside antibiotic similar in structure to gentamicin B1, produced by Micromonospora rhodorangea. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred by the Neomycin resistance gene (neo) from Tn5 encoding an aminoglycoside 3'-phosphotransferase, APH 3' II.

β-geo cassette (a fusion of the β-galactosidase and neomycin resistance genes) into the mouse Fbx15 gene by homologous recombination (Tokuzawa et al., 2003). Although specifically expressed in mouse ES cells and early embryos, Fbx15 is dispensable for the maintenance of pluripotency and mouse development.

Strategy to test candidate factors



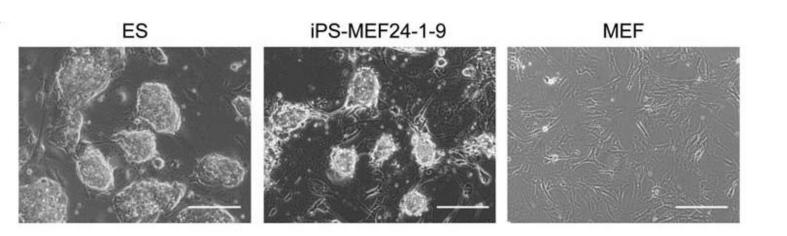
- 1. 22 G418 resistant colonies 12 clones tested 5 clones exhibited ES cell morphology
- 2. 29 G418 resistant colonies 06 clones tested 4 clones exhibited ES cell morphology

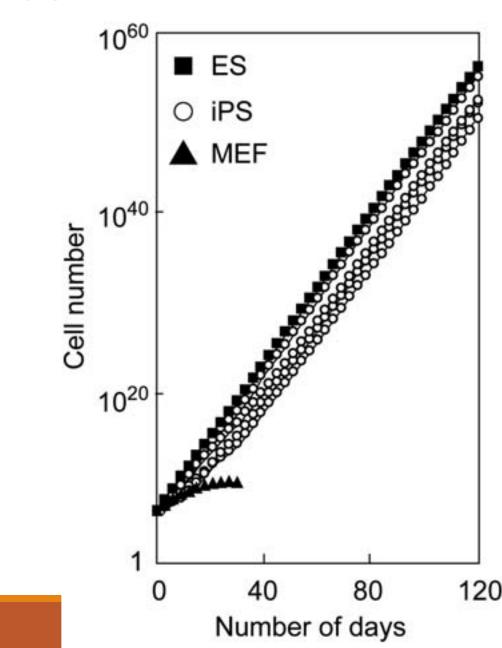
iPS-MEF24

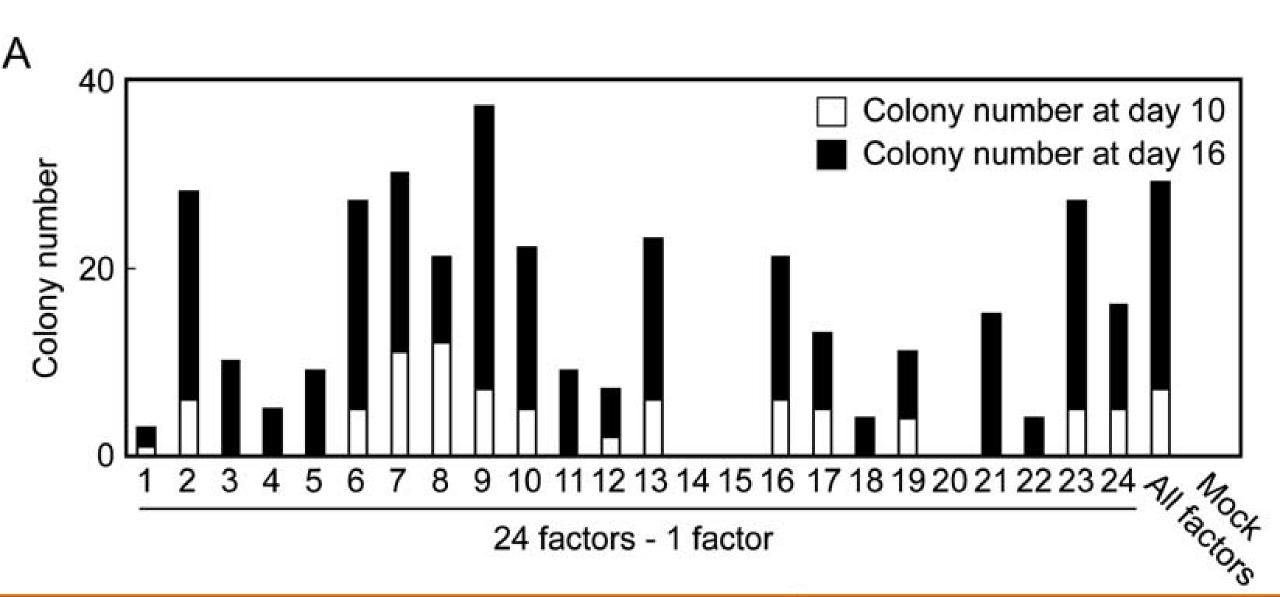
Date	Cells	Transgenes	G418-Resistant	Colonies Picked up	iPS Clones Established
			Colonies		
7/19/2005	MEF	Mock	0		
	1×10^{5}	Ecat1	0		
		Esg1	0		
		Fbx15	0		
		Nanog	0		
		ERas	0		
		Dnmt31	0		
		Ecat8	0		
		Gdf3	0		
		Sox15	0		
		Dppa4	0		
		Dppa2	0		
		Fth117	0		
		Sal14	0		
		Oct3/4	0		
		Sox2	0		
		Rex1	0		
		Utf1	0		
		Tc11	0		
		Dppa3	0		
		K1f4	0		
		β-catenin S33Y	0		
		Myc T58A	0		
		Stat3-C	0		
		Grb2 ΔSH2	0		
		24 factors	22	12	5

Date	Cells	Transgenes	G418-Resistant	Colonies Picked up	iPS Clones Established
			Colonies		
7/19/2005	MEF	Mock	0		
	1 x 10 ⁵	Ecat1	0		
		Esg1	0		
		Fbx15	0		
		Nanog	0		
		ERas	0		
		Dnmt31	0		
		Ecat8	0		
		Gdf3	0		
		Sox15	0		
		Dppa4	0		
		Dppa2	0		
		Fth117	0		
		Sal14	0		
		Oct3/4	0		
		Sox2	0		
		Rex1	0		
		Utf1	0		
		Tc11	0		
		Dppa3	0		
		K1f4	0		
		β-catenin S33Y	0		
		Myc T58A	0		
		Stat3-C	0		
		Grb2 ΔSH2	0		
		24 factors	22	12	5
8/15/2005	1	Mock	0		
	1×10^{5}	24 factors	29	6	4
					MEF24-2-1~4

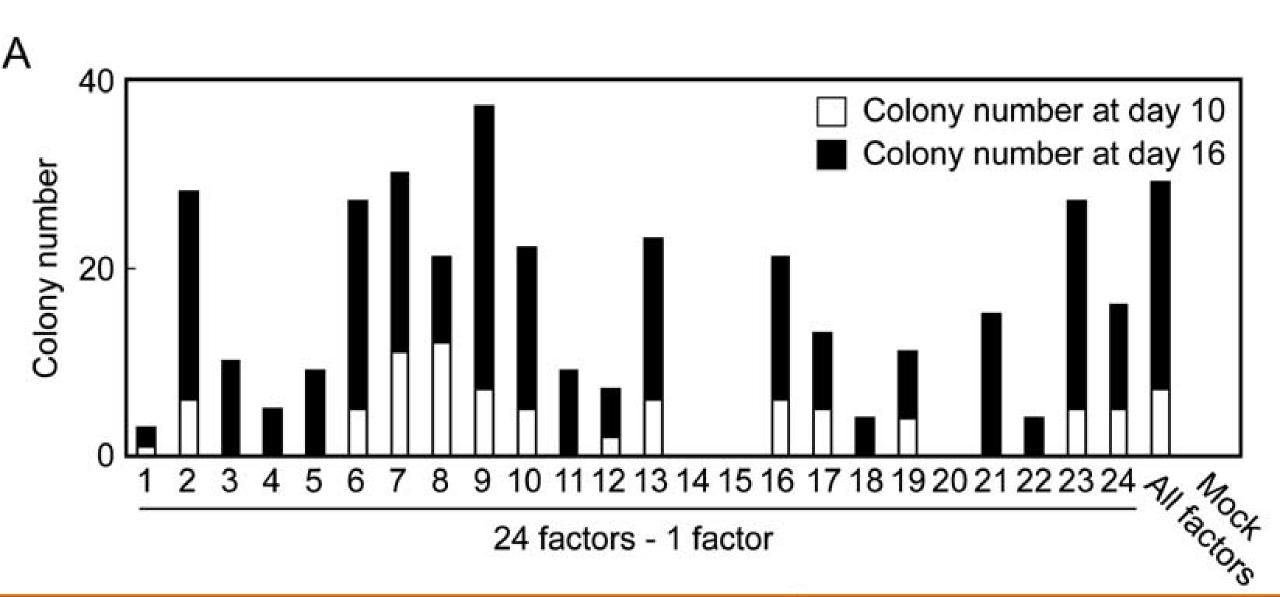
iPS-MEF24 clones





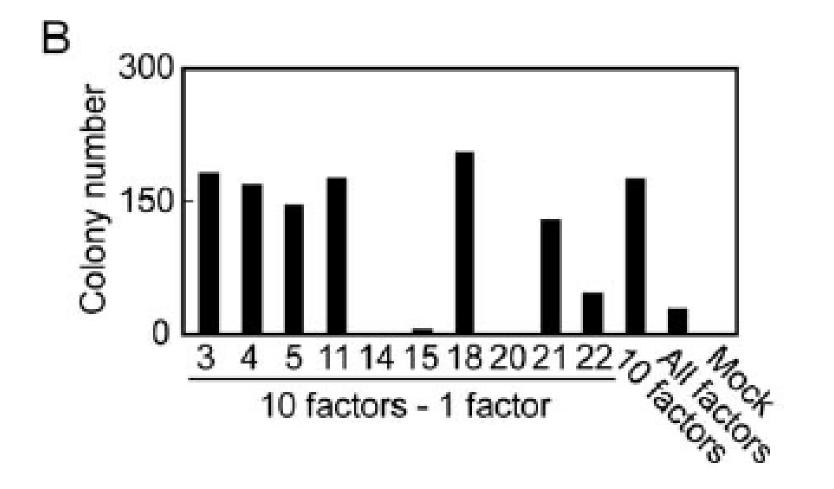


No	Genes	References
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7	Ecat8	Mitsui et al., 2003
8	Gdf3	Mitsui et al., 2003
9	Sox15	Maruyama et al., 2005
10	Dppa4	Mitsui et al., 2003
11	Dppa2	Mitsui et al., 2003
12	Fthl17	Mitsui et al., 2003
13	Sall4	Mitsui et al., 2003
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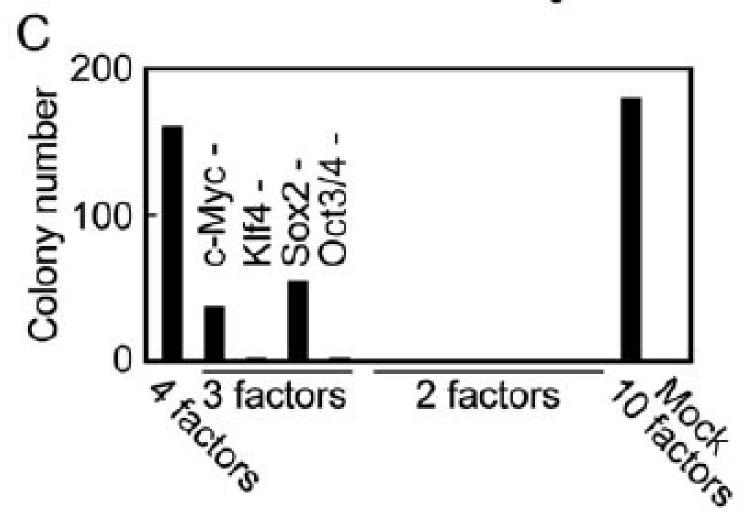
Date	Cells	Transgenes	G418-Resistant	Colonies Picked up	iPS Clones Established
			Colonies		
8/29/2005	MEF	Mock	0		
	1 x 10 ⁵	24 factors - Ecat1	2	1	frozen in 24-well plate
		24 factors – Esg1	22	1	
		24 factors - Fbx15	10	1	
		24 factors – Nanog	5	1	
		24 factors – ERas	9	1	
		24 factors - Dnmt31	22	1	
		24 factors - Ecat8	19	1	
		24 factors - Gdf3	9	1	
		24 factors - Sox15	30	1	
		24 factors - Dppa4	17	1	1
1		24 factors - Dppa2	9	1	
		24 factors - Fth117	5	1	
		24 factors - Sall4	17	1	
		24 factors - Oct3/4	0		
		24 factors - Sox2	0		
		24 factors - Rex1	15	1	frozen in 24-well plate
		24 factors - Utfl	8	1	
		24 factors - Tc11	4	1	
		24 factors - Dppa3	7	1	
		24 factors – K1f4	0		
		24 factors – β-catenin S33Y	15	1	frozen in 24-well plate
		24 factors - Myc T58A	4	1	
		24 factors - Stat3-C	22	1	
		24 factors – Grb2 ΔSH2	11	1	
		24 factors	22	1	

No	Genes	References
3	Fbxo15	Mitsui et al., 2003
4	Nanog	Chambers et al., 2003; Mitsui et al., 2003
5	ERas	Takahashi et al., 2003
11	Dppa2	Mitsui et al., 2003
14	Oct3/4 (Pou5f1)	Nichols et al., 1998; Niwa et al., 2000
15	Sox2	Avilion et al., 2003; Maruyama et al., 2005
18	TcI1	Mitsui et al., 2003
20	Klf4	Li et al., 2005
21	β-catenin	Kielman et al., 2002; Sato et al., 2004
22	с-Мус	Cartwright eta I., 2005



Date	Cells	Transgenes	G418-Resistant	Colonies Picked up	iPS Clones Established
			Colonies		
9/12/2005	MEF	Mock	0		
	8 x 10 ⁵	10 factors – Fbx15	183	2	frozen in 24-well plate
		10 factors - Nanog	170	2	
		10 factors – ERas	146	2	
		10 factors - Dppa2	177	2	
		10 factors – Oct3/4	0		
		10 factors – Sox2	5	2	frozen in 24-well plate
		10 factors – Tcl1	206	2	
		10 factors – Klf4	0		
		10 factors –β-catenin S33Y	129	2	frozen in 24-well plate
		10 factors -	46	2	
		Myc T58A			
		10 factors	176	2	
		24 factors	28	2	

No	Genes	References
1		
2		
3	Fbxo15	Mitsui et al., 2003
4	Nanog	Chambers et al., 2003; Mitsui et al., 2003
5	ERas	Takahashi et al., 2003
6		
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16		
17		
18	Tcl1	Mitsui et al., 2003
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21	β-catenin	Kielman et al., 2002; Sato et al., 2004
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24		

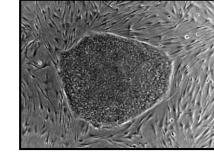


Date	Cells	Transgenes	G418-Resistant	Colonies Picked up	iPS Clones Established
			Colonies		
9/26/2005	MEF	Mock	0		
	8 x 10 ⁵	4 factors	160	12	3
					MEF4-2, MEF4-3,
					MEF4-7, MEF4-10
		4 factors -	36	6	
		Myc T58A			
		4 factors - Klf4	1	1	
		4 factors - Sox2	54	6	6
					MEF3-1~6
		4 factors - Oct3/4	1	1	
		Oct3/4 + Sox2	0		
		Oct3/4 + Klf4	0		
		Oct3/4 + Myc T58A	0		
		Sox2 + Klf4	0		
		Sox2 + Myc T58A	0		
		Klf4 + Myc T58A	0		
		10 factors	179	12	5
					MEF10-1, MEF10-3,
					MEF10-6, MEF10-7,
					MEF10-10
	+	O+C+K = 36	G418 resistant coloni		+

O+S+K = 36 G418 resistant colonies O+K+M = 54 G418 resistant colonies



How will you identify whether a cell is a pluripotent stem cell?



Human ES cells

Mouse ES cells

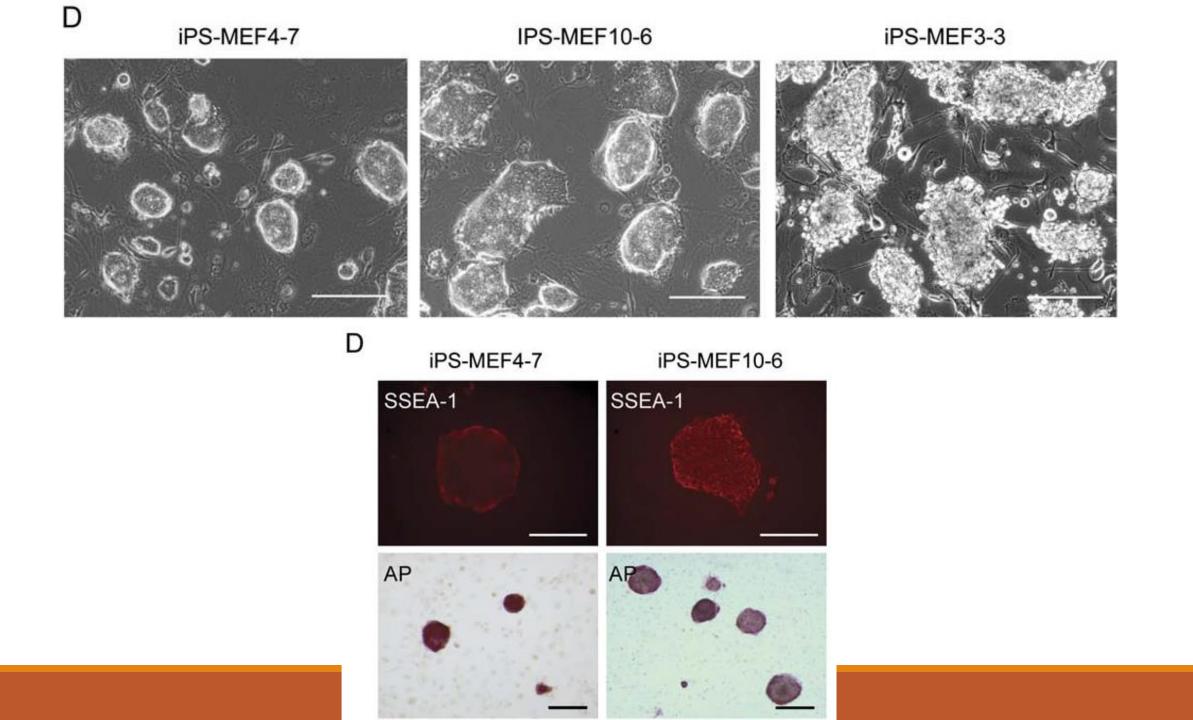
Normal Karyotype (46, XX or 46, XY)

• in vitro

- Colony Morphology analysis
- AP staining
- Telomerase expression
- Expression of stem cell markers (RNA and protein levels)
- Normal in vitro differentiation to all germ layers

• in vivo

- Teratoma formation after injection into immunocompromised mice
- Chimera formation through blastocyst injection or morula aggregation
- Tetraploid complementation



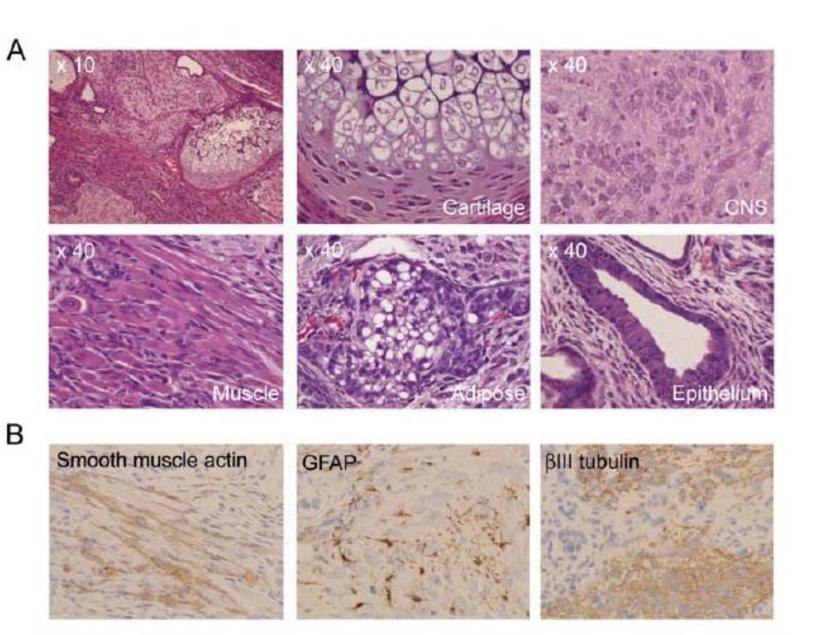
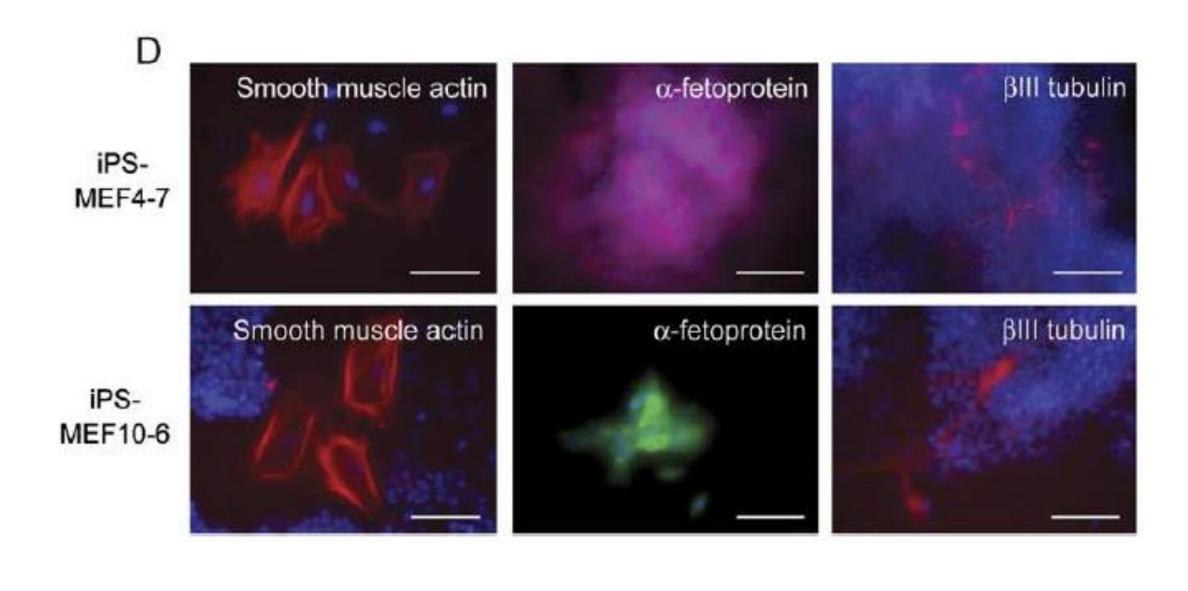


Figure 5. Pluripotency of iPS Cells Derived from MEFs

- (A) Various tissues present in teratomas derived from iPS-MEF4-7 cells. Histology of other teratomas is shown in Figure S3 and Table S6.
- (B) Immunostaining confirming differentiation into neural tissues and muscles in teratomas derived from iPS-MEF4-7.
- (C) In vitro embryoid body formation (upper row) and differentiation (lower row). Scale bars = 200 μm.
- (D) Immunostaining confirming in vitro differentiation into all three germ layers. Scale bars = $100 \mu m$. Secondary antibodies were labeled with Cy3 (red), except for α -fetoprotein in iPS-MEF10-6, with which Alexa 488 (green) was used.



			Colonies		
9/26/2005	MEF	Mock	0		
	8 x 10 ⁵	4 factors	160	12	3
					MEF4-2, MEF4-3,
					MEF4-7, MEF4-10
		4 factors -	36	6	
		Myc T58A			
		4 factors - Klf4	1	1	
		4 factors - Sox2	54	6	6
					MEF3-1~6
		4 factors - Oct3/4	1	1	
		Oct3/4 + Sox2	0		
		Oct3/4 + Klf4	0		
		Oct3/4 + Myc T58A	0		
		Sox2 + Klf4	0		
		Sox2 + Myc T58A	0		
		Klf4 + Myc T58A	0		
		10 factors	179	12	5
					MEF10-1, MEF10-3,
					MEF10-6 MEF10-7,
					MEF10-10
	+			O MEEO .I.	

G418-Resistant

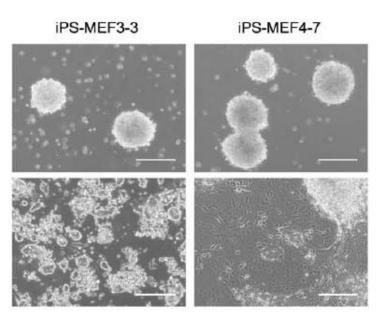
Colonies Picked up

iPS Clones Established

Date

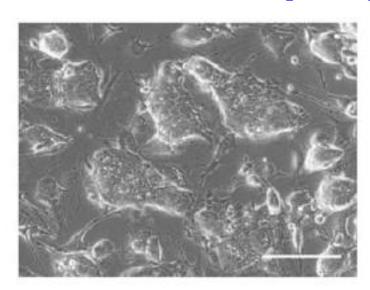
Cells

Transgenes

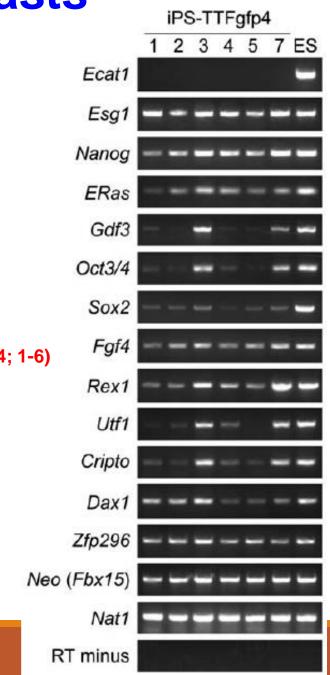


In contrast, all tumors derived from iPS-MEF3 clones were composed entirely of undifferentiated cells

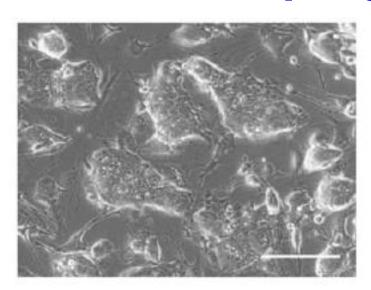
Reprogramming Tail-tip fibroblasts

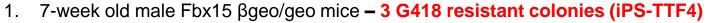


- 1. 7-week old male Fbx15 βgeo/geo mice 3 G418 resistant colonies (iPS-TTF4)
- 2. 12-week-old female Fbx15 βgeo/geo mice with constitutive GFP 13 G418 resistant colonies (iPS-TTFGFP4; 1-6)

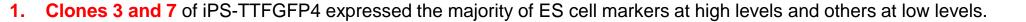


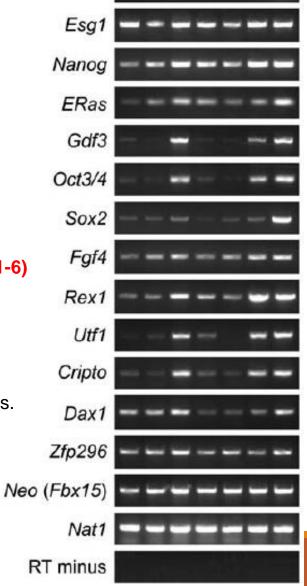
Reprogramming Tail-tip fibroblasts











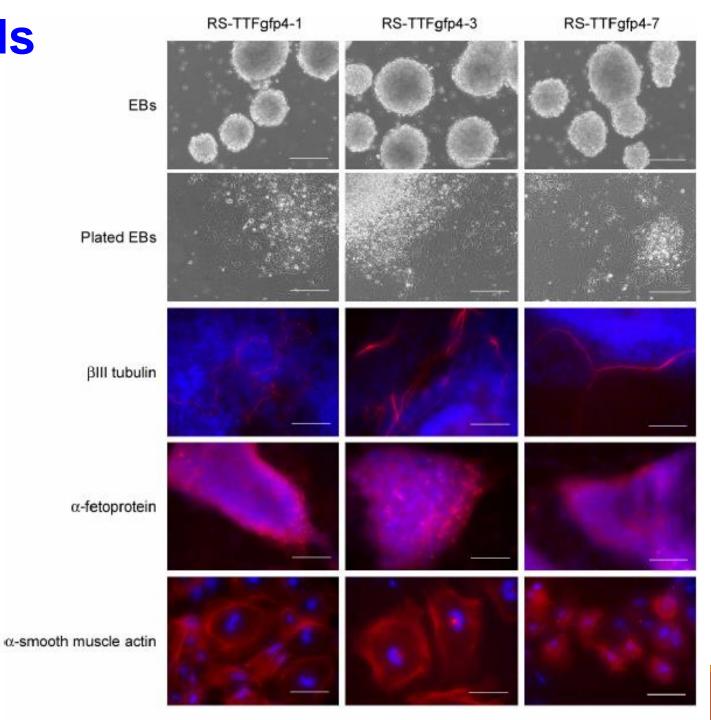
iPS-TTFgfp4

Ecat1

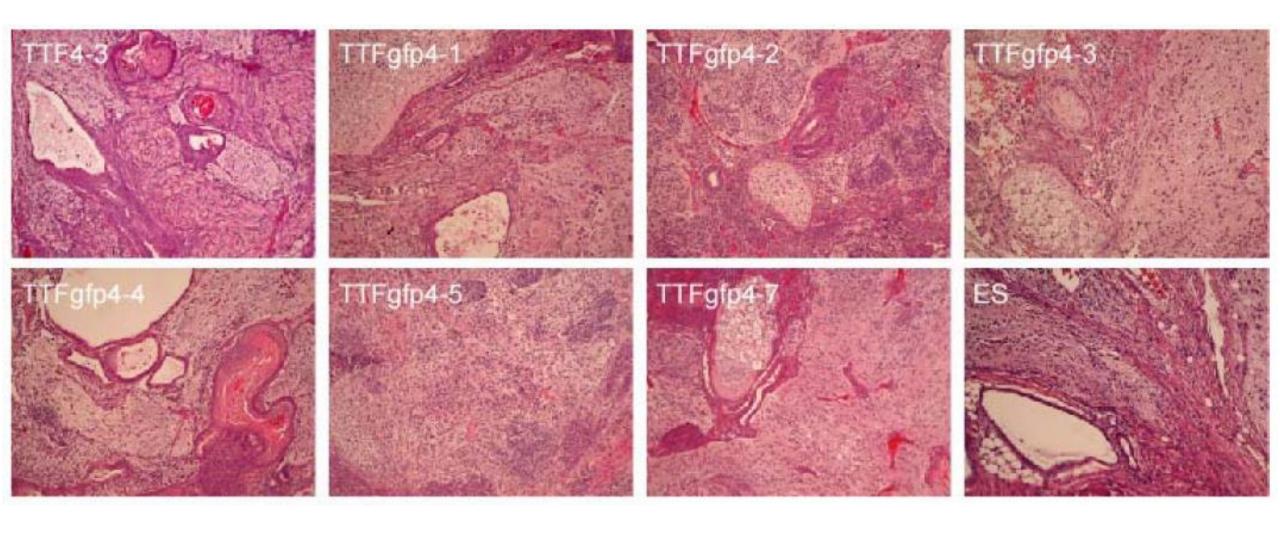
Differentiation of iPS cells from tail-tip fibroblasts

iPS-TTFGFP4-3/7

Clones 3 and 7 of iPS-TTFGFP4 expressed the majority of ES cell markers at high levels and others at low levels.



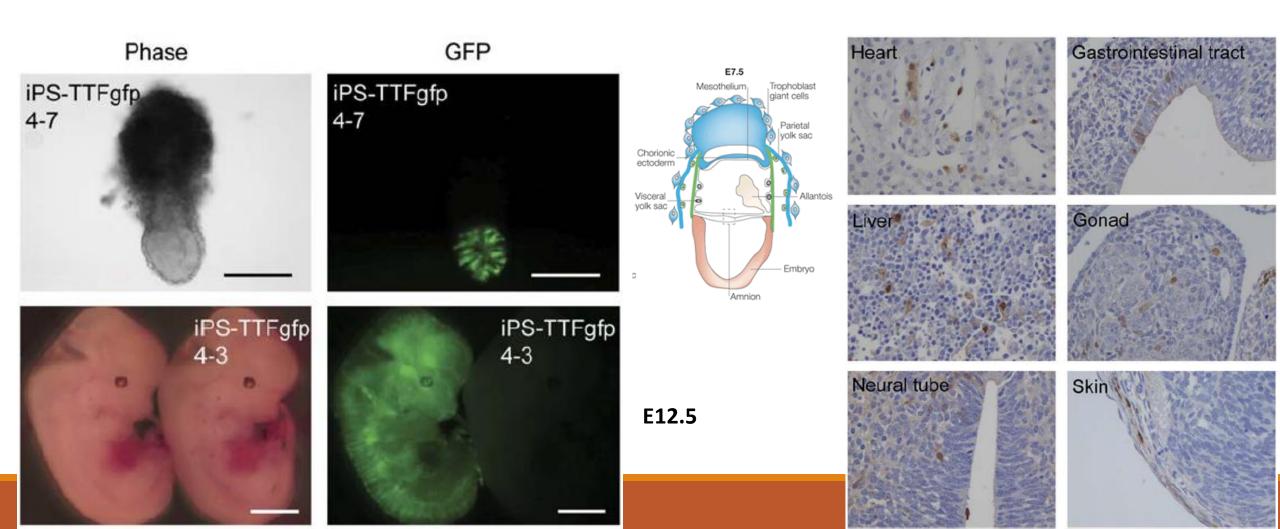
Pluripotency determination of iPS cells using teratoma assay

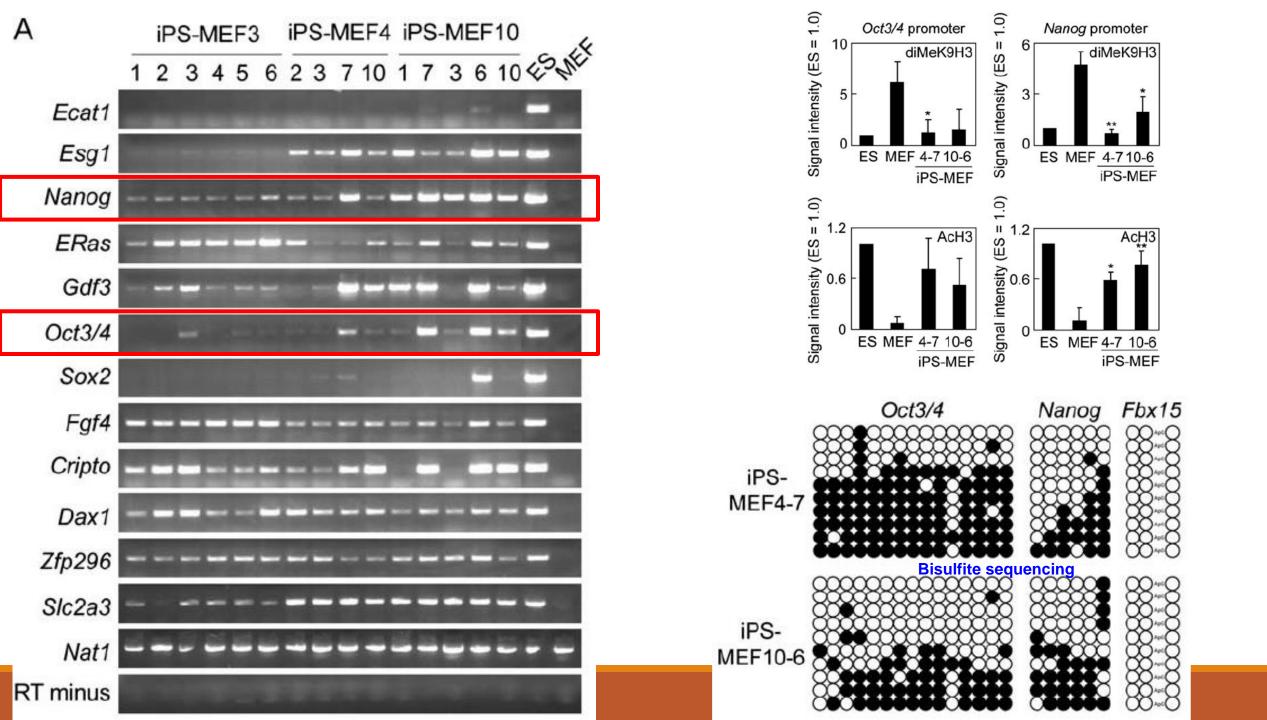


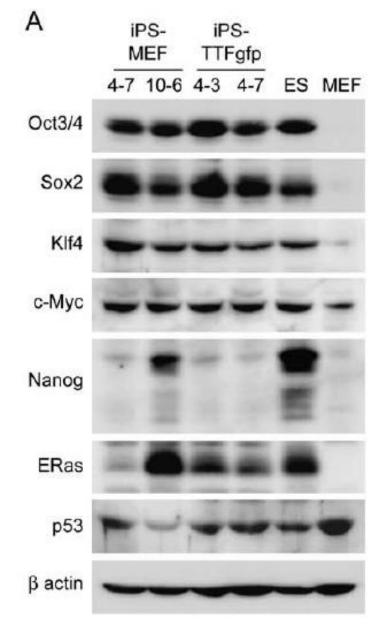
Pluripotency determination of iPS cells – iPSTTFGFP4-3

constitutively expressed green fluorescent protein (GFP) from the CAG promoter

Introduction of 2 clones of iPS-TTFgfp4 cells (clones 3 and 7) into C57/BL6 blastocysts by microinjection. With iPS-TTFgfp4-3, they obtained 18 embryos at E13.5, 2 of which showed contribution of GFP-positive iPS cells







Western blot analyses of the four factors and other proteins in iPS cells (MEF4-7, MEF10-6, TTFgfp4-3, and TTFgfp4-7), ES cells, and MEFs.

Conclusions

- Among the three (O/S/N), Oct3/4 and Sox2 are essential for the generation of iPS cells. Surprisingly, Nanog is dispensable.
- c-Myc and Klf4 as essential factors. These two tumor-related factors could not be replaced by other oncogenes including E-Ras, Tcl1, b-catenin, and Stat3.
- The c-Myc protein has many downstream targets that enhance proliferation and transformation (Adhikary and Eilers, 2005), many of which may have roles in the generation of iPS cells.
- Within the mammalian genome, there may be up to 25,000 c-Myc binding sites (Cawley et al., 2004), many more than the predicted number of Oct3/4 and Sox2 binding sites (Boyer et al., 2005; Loh et al., 2006). c-Myc protein may induce global histone acetylation (Fernandez et al., 2003), thus allowing Oct3/4 and Sox2 to bind to their specific target loci.
- Klf4 might contribute to activation of Nanog and other ES cell-specific genes through p53 repression. Alternatively, Klf4 might function as an inhibitor of Myc-induced apoptosis through the repression of p53 in our system.
- On the other hand, Klf4 activates p21CIP1, thereby suppressing cell proliferation (Zhang et al., 2000). This antiproliferation function of Klf4 might be inhibited by c-Myc, which suppresses the expression of p21CIP1 (Seoane et al., 2002).
- The balance between c-Myc and Klf4 may be important for the generation of iPS cells.
- Low reprogramming efficiency the levels of the four factors required for generation of pluripotent cells may have narrow ranges, and only a small portion of cells expressing all four of the factors at the right levels can acquire ES cell-like properties.
- No multipotent stem cells contamination