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nuclear reprogramming

pluripotent cell → unipotent cell

decrease in developmental potential
Nuclear Reprogramming

Developmental potential

Unipotent

Nuclear Transfer

Somatic cell

Enucleated oocyte

ES cells

Cell Fusion

Somatic cell

ES cells

Pluripotent 4N hybrid cells

Goals of reprogramming:

- understand mechanisms
- apply to human system for therapy

Direct reprogramming would potentially have less limitations.
Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

Fibroblasts

Fbx15-Neo\(^R\)

OFF

+ Retroviral infection

Oct4
Sox2
c-Myc
Klf4

iPS cells
(induced pluripotent stem cells)

ON

Fbx15-Neo\(^R\)

embryoid body formation

teratoma formation

contribution to chimera

Test for ES cell qualities

Limitations…

Takahashi & Yamanaka, Cell (2006)
Fbx15 iPS cells are different from ES cells

1. True pluripotent state reached?
Lack of endogenous pluripotency gene expression

2. Incomplete reprogramming of gene expression
Transcriptional profile in between that of fibroblasts and ES cells

3. Incomplete epigenetic reprogramming
Incomplete promoter demethylation of essential pluripotency genes Oct4 and Nanog

4. Limited developmental potency
Low degree chimeras, no viable pups recovered
Questions

Can iPS cells be generated that are more similar to ES cells?

Why can iPS cells be induced to differentiate despite that four TFs are constitutively expressed?

Does the pluripotent state of iPS cells depend on continuous expression of exogenous factors?

To what extent can four transcription factors reset the epigenetic landscape of a fibroblast into that of a pluripotent cell?
Could selection for expression of an essential gene in ES cells result in better reprogrammed cells?

Oct4 and Nanog are genes essential for the maintenance of the pluripotent state of ES cells and are only expressed in pluripotent cells.
Hypothesis

Selection for an *essential* ES cell gene gives rise to “better” iPS cells.

Fibroblasts + Retroviral infection → iPS cells (induced pluripotent stem cells)

- **Nanog-Puro**
  - OFF
- **Oct4-Neo**
  - OFF
- **Oct4-Neo**
  - ON
- **Oct4-Puro**
  - OFF
- **Nanog-Puro**
  - ON
Nanog or Oct4 selection

Oct4-Neo<sup>R</sup>

- Oct4 promoter
- Oct4
- GFP_Neo

or

Nanog-Puro<sup>R</sup>

- Nanog promoter
- Nanog
- Puro ires GFP

fibroblasts
**Assays**

iPS cells

- Nanog-Puro\textsuperscript{R}
- Oct4-Neo\textsuperscript{R}

OR

ON

- General characterization
- Endogenous vs. viral gene expression
  - Epigenetic analysis
    - Gene-specific
    - Chromosome-wide
    - Genome-wide
  - Transcriptional profiling
  - Chimera contribution
Assays

General characterization
Characterization of Nanog-selectable iPS cells

- ES cell-like morphology
- Nanog-GFP expression

… Do iPS cells possess functional attributes of ES cells?
Dominant reprogramming activity of iPS cells in cell fusion

**Strategy**

Nanog-Puro iPS cells

Oct4-Neo hygroR fibroblasts

Cell fusion; puro/hygro selection

puro/G418 selection for somatic Oct4 reactivation

Pluripotent hybrids

DNA content

Cell hybrids

Nanog-GFP

iPS

MEFs

hybrids

Cell count
Nanog-selected iPS cells are pluripotent (teratoma)

i) epithelial structures
ii) cartilage with surrounding muscle
iii) glandular structures
iv) neural tissues
Assays

Endogenous vs. viral gene expression
Retrovirally induced iPS cells don’t have persistent viral gene expression (and therefore can differentiate)
Can iPS cells be generated without retroviral transduction?

Postnatal Fibroblasts

+ Sox2 c-Myc Klf4 +dox

Retroviral infection

iPS cells

- dox

Oct4 transgene is incorporated downstream of the Col1A locus
Fibroblast with Oct4 selectable allele and dox-inducible Oct4 transgene

Oct4 locus off

endogenous Oct4 loci

transgenic Oct4 downstream of Col1a

Oct4 transgene off

Sox2 Klf4 c-myc* + dox

G418 selection

iPS cell

endogenous Oct4 loci
iPS cells are stable and pluripotent in the absence of transgenic Oct4

Dox-independent self-renewal

+dox  -dox

Pluripotency (teratoma formation)
Assays

Epigenetic analysis
  Gene-specific
  Chromosome-wide
  Genome-wide
Gene-specific epigenetic reprogramming?
DNA within promoters of pluripotency genes are demethylated in iPS cells

Bisulfite sequencing of Oct4 and Nanog promoter regions

- methylated CpG
- demethylated CpG
Chromosome wide epigenetic reprogramming?
X inactivation as an example of chromosome-wide silencing

Embryonic stem cells

Differentiated cells
X-inactivation is regulated by a non-coding RNA

**Xist RNA:**
- non-coding, 17.5 kb in length, spliced, and polyadenylated
- encoded by an X-linked gene
- stable expression only from the inactive X-chromosome
- “coats” the inactive X chromosome in female cells

Xi = inactive X chromosome
Xa = active X chromosome
*Xist* RNA is required for initiation of X chromosome silencing

**Epigenetic mark designates active X**

*Xist* RNA spreads in cis on the unmarked X chromosome and initiates silencing.

Stable propagation of the Xi and *Xist* RNA coating through all subsequent cell divisions.
Do female iPS cells reactivate the inactive X chromosome?

**Embryonic stem cell**

- **Tsix** (antisense transcript to Xist)
- **Xist** (expressed at very low levels as repressed by Tsix)
- **Pgk-1** (X-linked gene transcript)

**Differentiated cell**

- **Xist** (high level Xist expression and coating of the Xi)
- **Pgk-1** (X-linked gene transcript)
Embryonic stem cell (two Xa)

Differentiated cell (one Xi and one Xa)

iPS reprogramming?

Xist
Tsix
Pgk-1

Tsix and Xist
Pgk-1

Tsix

Xist
Chromatin modifications accumulate on the Xi

- **days of ES cell differentiation**
  - day 0
  - day 2.5
  - day 4.5

- **initiation**
- **establishment**
- **maintenance**

- **Xist RNA coating**
- **transcriptional silencing**

- **H3-K27 methylation by PRC2**

- **global hypo-histone acetylation**
- **novel histones**
- **DNA methylation**
Do female iPS cells change the chromatin state on the X?
iPS cells undergo X-inactivation

Is X-inactivation, like in ES cells, random?
Proof of random X inactivation in female iPS cells

X\textsuperscript{GFP}/X
postnatal fibroblasts

FACS sort to isolate \(X_i\text{GFP}X_a\) cells

Oct4 Sox2 c-MYC Klf4

differentiate

\(X_i\text{GFP}X_a\)

X chromosome reactivation

Random X inactivation

Erasure of epigenetic memory for previously inactive X chromosome
Genome-wide epigenetic reprogramming?

The two main components of the epigenetic code

DNA methylation
Methyl marks added to certain DNA bases repress gene activity.

Histone modification
A combination of different molecules can attach to the ‘tails’ of proteins called histones. These alter the activity of the DNA wrapped around them.
One-scale location analysis of histone modifications with the following steps:

1. Crosslinking of proteins to DNA-binding sites in ES cells.
2. Harvesting of cells and DNA fragmentation.
3. Enrichment of DNA fragments x-linked to modified histones with antibodies.
4. Differential labeling of total DNA and Chip-enriched DNA.
5. Hybridization to microarrays and comparison of intensity ratios.
6. Hybridization to microarrays and comparison of intensity ratios.
Binding data at high resolution

mouse arrays (Agilent)

60mer oligonucleotide probes: ~ 3 probes/kb

covering the region from -8kb to +2kb relative to the transcript start sites for 15,742 annotated mouse genes
Global epigenetic reprogramming in iPS cells

**Approach**
Genome-wide ChIP-chip analysis of K4/K27 trimethylation (16,500 promoters)

**Findings**
iPS and ES cells are indistinguishable

Reprogramming mainly associated with changes in repressive methylation (K27)
Assays

Transcriptional profiling
Reprogramming of transcriptome in iPS cells

Analysis of differentially expressed genes between ES cells and MEFs

Transcriptional profiles are indistinguishable
Assays

Chimera contribution
In vivo differentiation potential of iPS cells

Live-born chimeras (MEF-derived)

Germline contribution

High degree of somatic contribution
The diagram illustrates a model of developmental potential and epigenetic state in relation to cell differentiation.

- **Developmental potential**
  - Unipotent
  - Multipotent
  - Pluripotent

- **Epigenetic state**
  - Ground state
  - Intermediate state
  - Committed state

- **Differentiated cell**
  - Oct4
  - Sox2
  - Klf4
  - c-MYC

- **Selection**
  - Fbx15 selection
  - Nanog/Oct4 selection

The diagram shows the transition from ground state to committed state, and ultimately to differentiated cell types, influenced by epigenetic factors and selection processes involving specific genes.
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